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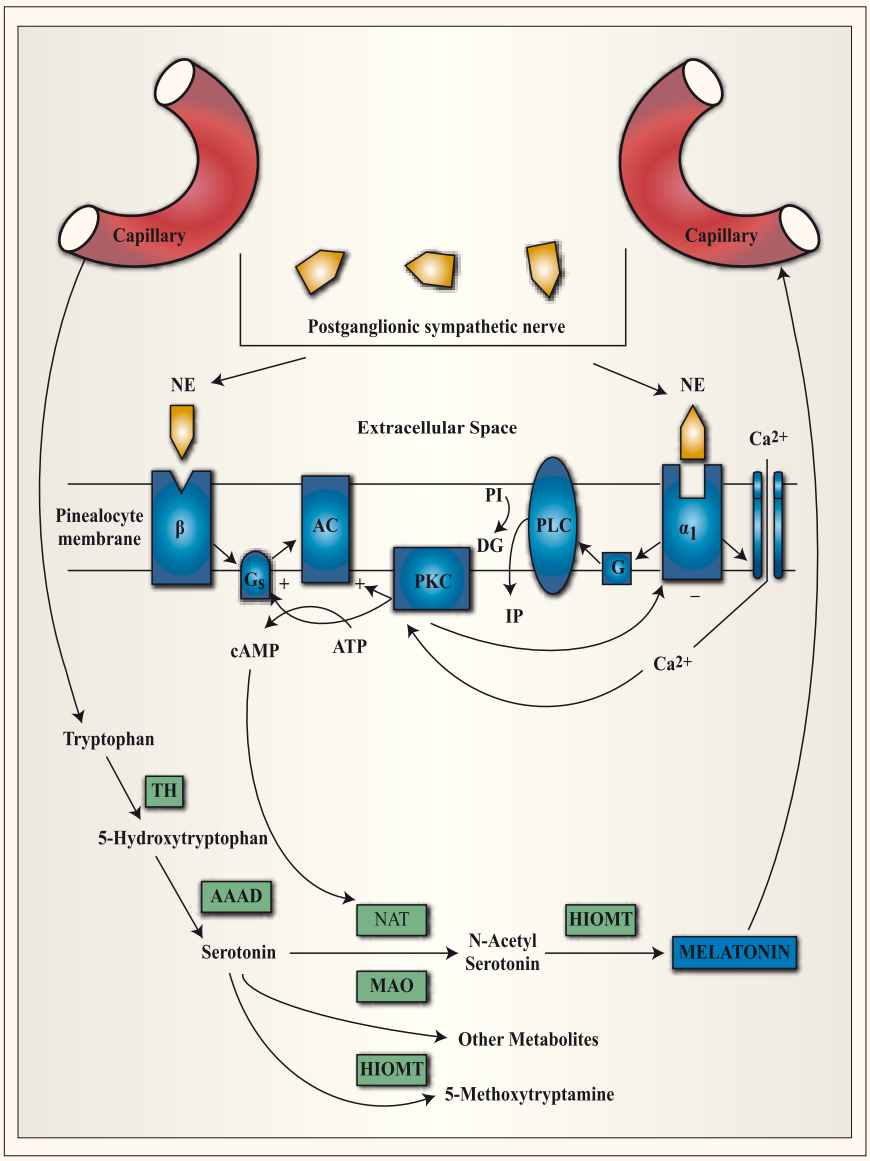

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Cover Illustration: Interactions of the postganglionic sympathetic innervation with the pinealocyte (the major cell in the pineal gland). During darkness these nerve endings release norepinephrine (NE) which acts primarily on β-adrenergic receptors (β) and to a lesser degree on alpha1-adrenergic receptors (α1) to promote the nocturnal synthesis of melatonin. Melatonin is formed from the amino acid tryptophan which is taken up from the blood into the pinealocyte. Four enzymes, i.e., tryptophan hydroxylase (TH), aromatic acid decarboxylase (AAAD), N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT), are responsible for converting tryptophan to melatonin (N-acetyl-5-methoxy-tryptamine). Once produced, melatonin is quickly released into the adjacent capillaries and possibly into the third ventricle of the brain. Hormones from endocrine glands throughout the body do little to perturb the circadian production of melatonin and have only a minor influence on the total amount of melatonin synthesized; Reiter RJ, Tan D-X, Manchester LC, Pilar Terron M, Flores LJ, Koppisepi S. Medical implications of melatonin: receptor-mediated and receptor-independent actions, Advances in Medical Sciences, 2007; 52: 11-28.

Reflections on the future of gastroenterology – unmet needs

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Introduction

To state that the future of gastroenterology (GE) is bright is readily understandable because this speciality is indeed the largest in the internal medical arena. The discipline entails the largest organ with appendices such as liver and pancreas, contains the largest number of endocrine, immune, smooth muscle and nerve cells, carries the largest cancer load, and acute and chronic inflammatory conditions and is marred with the largest number of pathologic conditions, mainy still to be fully characterized. Yet this statement is currently in my view in need of some qualification as progress in GE seems occasionally slower and more incomplete that originally anticipated. This overview will therefore also draw attention to some unmet needs to stimulate professional enthusiasm for the challenges ahead, realising that it is easy to be an armchair critic and that predictions about the future are frought with error.

Key words: ulcer disease, GERD, dyspepsia, IBS, IBD, gastrointestinal oncology, endoscopy, EUS.

Ulcer disease

The greatest impact of *H. pylori* (re)discovery and cure is obviously the surprisingly rapid decrease, if not disappearance of *H. pylori*-associated peptic ulcer disease, not only in the developed, but also in the emerging world. In contrast with this phenomenon is the continuation if not rise in drug-induced (aspirin/NSAID-induced) ulcer formation. Indeed, the expectations are that drug-induced injury will remain a major health problem, particularly now that widespread use of COX-2 selective inhibitors remains uncertain. Prophylaxis, especially with proton pomp inhibitors (PPIs) against the deleterious effects of non-selective COX-antagonists is at best mediocre, if prophylaxis is given at all. In view of the high complication rate, such injury signifies a major unmet need and urgently requires further pharmacological improvement and novel prophylactic approaches.

Gastroesophageal reflux disease (GERD)

The prevalence of GERD will continue to rise also in the emerging world. Overweight/obesity, sedentary lifestyle, dietary habits, *H. pylori* disappearance etc. all contribute to this increase. More attention will also be given to the extra-esophageal manifestations of the disease (*Fig. 1*). PPIs will continue to be the standard of therapy. The problem of the discrepancy between the excellent symptom relief and healing in controlled trials and the rising patient dissatisfaction (especially in the USA) in practice needs to be solved. Also the problem of nocturnal reflux and interference with sleep quality needs to be solved. We need to learn when and how so-called rescue or adjuvant medication is to be used, particularly alginate/antacids, which partition in the acid pocket of the cardia. How to interfere with weakly acid, non-acid (biliary) reflux remains puzzling. The results of GABA-B agonists or metabotropic glutamate antagonists are eagerly awaited.

Functional disorders – dyspepsia

Despite all the recent research and trials, there remains a large unmet need in our understanding of the pathophysiology of (functional/idiopathic) dyspepsia. The real causes of fundic

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Figure 1. **The MONTREAL definition and classification of GERD** [Vakil et al., Am J Gastroenterol, 2006]

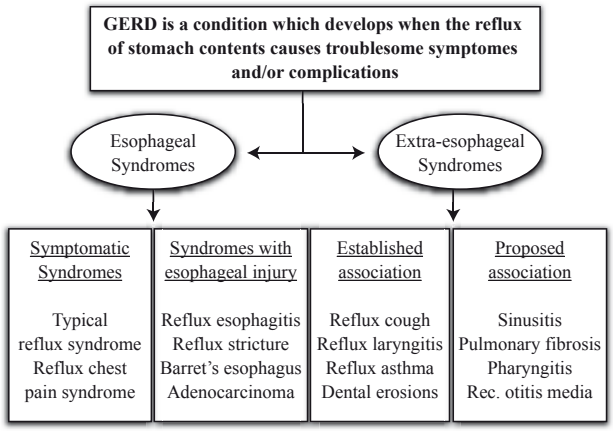


Figure 2. **Dyspepsia therapy**

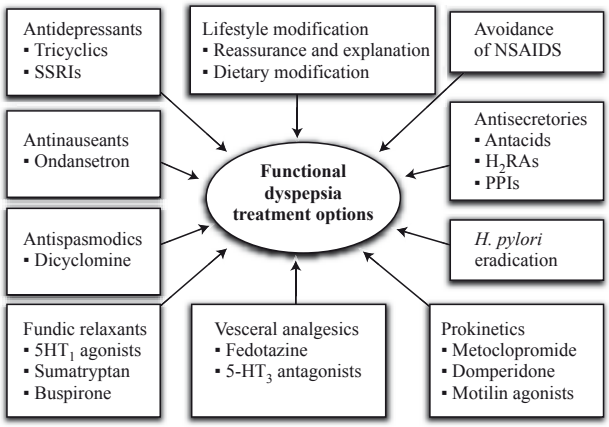


Figure 3. **IBS therapy**

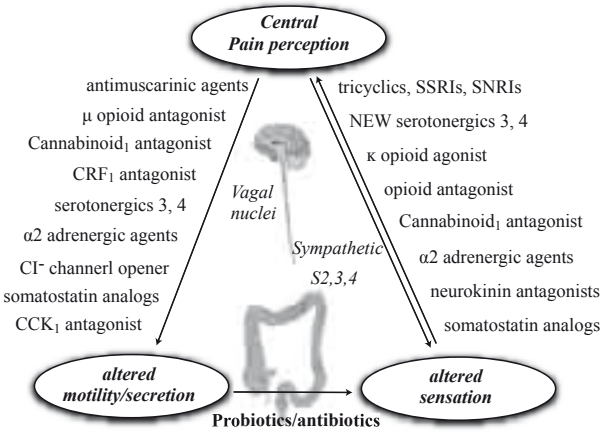
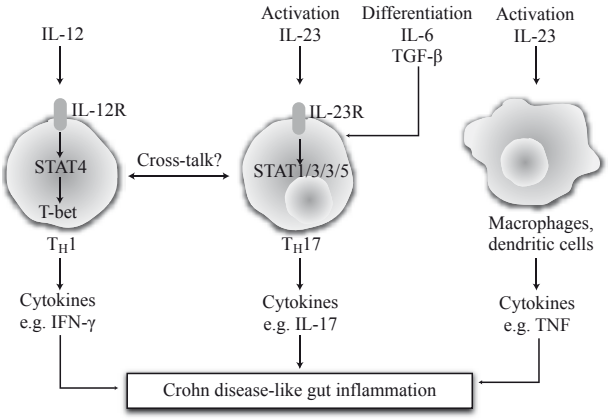


Figure 4. **CD pathogenesis**



dysaccomodation, visceral hypersensitivity, dysmotility and emptying abnormalities etc. remain enigmatic. Equally disappointing is the lack of efficacious pharmacotherapy to truly and reproducibly correct the functional aberrations. The plethora of current therapeutic possibilities (*Fig. 2*) is misleading as the efficacy of all avenues is low/mediocre at best, if present at all. New eager and bright researchers, with genuine interest in GE functional disorders, should tackle these challenging problems with a fresh open mind, willing to explore new paths and avenues, using uncontaminated well selected patient material, and applying the most advanced sophisticated technology.

Irritable bowel syndrome (IBS)

Progress in our understanding of the pathophysiology and therapy of IBS, the most common aberation in Gastroenterology has been disappointingly slow. All current attention is focused on so-called post-infectious IBS but to what extent this will really enhance our understanding remains uncertain. We ultimately need to know what the dominant pathophysiologic abnormalities are, where they are located along the circuit from bowel, afferent nerve, spinal cord, ascending and descending nerve tract. Moreover, we need to find out the proper therapy for these patients. Again, the plethora of drugs being considered (*Fig. 3*) is somewhat misleading as current pharmacotherapeutic possibilities are mediocre at best.

Inflammatory bowel disease – Crohn disease (CD) – ulcerative colitis (UC)

Inflammatory bowel disease (IBD) should definitively remain the territory of the interested and experienced gastroenterologist in view of the complexity and difficulty of this condition. Vast experience is mandatory for optimal coaching and councelling such patients. The pathophysiologic paradigms are constantly changing and switching and that will probably remain so in the years to come. Currently the leading hypothesis focuses on an overly reactive immune system, responsible for driving the lymphocytes in a T_H1 direction in CD and (perhaps) in a somewhat modified T_H2 phenotype in UC but the emphasis is switching from Il-12 to Il-23 as the dominant cytokine driving pathway in CD (*Fig. 4*). We should, however, be aware that other investigators feel that the opposite viewpoint is more relevant, claiming a failing inflammatory/immune response as

the cause of CD as the consequence of inadequate clearing of the bowel from antigenic/bacterial influx.

Therapy has obviously progressed over the past decade and many are bewildered by the occasional rapid mucosal healing with biological therapy to the point that a top-down therapeutic approach is advocated instead of the traditional step-up approach. The enthousiasm of other investigators is more restraint or they realize that ultimately less than half the patients are in true remission at the end of one year, whichever biological scheme has been applied. Moreover, safety concerns are rising as infections and malignancy become non-negligeable. Future progress again demands novel thinking, outside the traditional box, in order to advance the field. Whichever hypothesis is generated, it has to explain all features of these diseases, including the patchiness of CD and the segmental distribution of UC. The ultimate dream is to really find the cause of IBD with the possibility of permanent cure.

Oncology

GE is worldwide responsible for the largest cancer load. The gastroenterologist should become the central player in the multidisciplinary approach to digestive cancer, responsible for all aspects of diagnosis, therapy and care. He should be the permanent direct contact person for the patient, fully capable of per-endoscopic or endosonographically-guided interventions and standard chemotherapy. He should, for example, be well trained in endoscopic resection of early cancer, endosonographically-guided injection of oncolytic viruses, autologous transfected dendritic cells or other modalities for immune therapy, celiac plexus neurolysis etc.

We need to understand the intriguing rise of esophageal adenocarcinoma, whether it is reflux-, obesity- or therapy-related, or explained by the nitrate-nitrite-NO and nitrosating species pathway, responsible for DNA mutution and damage in the columnar metaplastic mucosa. We need to refine the population at true risk for neoplasia to bring the screening/surveillance cost-benefit ratio in balance in parallel with intensified attempts at chemoprophylaxis.

Gastric cancer is largely *H. pylori*-related in its early phases of development. Particularly for high incidence areas, *H. pylori* eradication should be considered but antimicrobial therapy should be carried out early in the evolution before advanced atrophy, achlorhydria and intestinal metaplasia has developed. The results of large scale, well-designed trials are eagerly awaited. If positive plans need to be developed for mass vaccination or early eradication in high gastric cancer areas. For the time being oportunistic screening and screening of individuals with a family history seems sensible in low incidence areas.

Pancreatic cancer will remain the most difficult and dismal cancer. Detection of early malignancy, amenable to cure is rare. Symptomatic cancer usually signifies incurability. Screening, preferably with endosonography should be offered to genetic/familial conditions with increased risk. Otherwise the development of sensitive and specific proteomics marker has to be awaited.

Colorectal cancer is dominating in many areas of the world and is rising in the far east. Population screening for precancerous polyps and (early curable) cancer should be designed and set-up in all countries. The screening modality (FOBT, virtual CT/MR, sigmoidoscopy, colonoscopy) will be largely determined by local resources and facilities. Colonoscopy, if chosen, should be of highest quality and patient acceptability with minimum missed lesions and minimum miss rate in detection of (flat)polyps and cancer: Improved technology and intense teaching will be necessary to reach that goal. Wherever screening modality is ultimately chosen, it will not only cost-effective but also cost-saving in view of the rapidly rising costs for chemotherapy for advanced metastasized colorectal cancer (*Fig. 5*).

Figure 5.

DIGESTETIVE ONCOLOGY

- Multimodality therapy of advanced cancer
  - organ preserving surgery (largerly minimally invasive and robotic)
  - superselectively targeted conformational radiotherapy
  - in vitro selected chemotherapeutic coctail (mutational microarrays)
  - intense and prolonged application of biologicals addressing all aspects of cancer growth

Figure 6.

Possibilities to improve resolution and analysis of surface microarchitecture and vascularity

- high resolution, high magnification CCD-endoscopy
- chromoscopy
- accentuation of vascular pattern (filters, index, hemoglobin)
- narrow band imaging

Figure 7.

Possibilities for molecular characterisation of tissue (bio-endoscopy)

- use of fluorescent monoclonal antibodies
- application of molecular beacons
- detection of cellular chromosomal changes/mutations with FISH

Figure 8.

Therapeutic EUS-indications

- Tissue sampling
  - mass lesions
  - lymph nodes
- Injection therapy
  - celiac plexus block
  - (gene therapy)
- Interventional therapy
- EUS guided (pseudo)cyst drainage

Figure 9.

NOTES

- Retroperitoneal
  - pseudocyst drainage
  - necrosectomy of pancreatic necrosis
- Intraperitoneal
  - surgical procedures
  - gynaecological procedures
  - others…

Diagnostic – therapeutic endoscopy – endosonography

Technology for both diagnostic and therapeutic endoscopy and endosonography will continue to expand and improve (*Fig. 6-9*). High-resolution-high-magnification endoscopy, chromoscopy, autofluorescence endoscopy, narrow-band imaging will become the standard for diagnostic investigation, although competition will increase from standard radiology, CT and

Figure 10.

Future Threats

- GI-specialisation in Primary Care
  - GERD-IBD – Functional Disorders
  - transnasal endoscopy-screening
  - proctology

(Eur. Soc. Prim. Care Gastroent.)

- Nurse-practitioners; Nurse-endoscopist
  - colorectal cancer screening
  - home follow-up malignancy etc.

MRI. Especially the therapeutic dimension of endoscopy and endosonography will remain the territory of the well-trained gastroenterologist. Hemostasis, polypectomy, sphincterotomy, endoscopic resection, pseudocyst/abscess drainage and necrosectomy, NOTES (natural orifice transluminal endoscopic surgery) etc. is and will remain in the hands of the experienced talented therapeutic endoscopist.

Potential future threats

Gastroenterologists should be aware of potential future threats. Specialisation is starting at the primary care level (*Fig. 10*). Indeed, the primary care-gastroenterologist is on the horizon, claiming competence in the treatment of GERD, IBD, proctology, transnasal endoscopic screening etc. Also nurse practitioners, nurse assitants are on the rise, involved in colonoscopic screening, home care of cancer patients etc etc. Integration of all such developments in our discipline will demand substantial creative thinking.

However, the most important threat to our specialty is the lack of pharmacotherapeutic success. For over a decade no blockbuster has been developed and nothing is in the pipeline for the forseeable future. This ultimately translates in loss of attractiveness of the discipline and shrinkage of finantial resources for research and training!

Figure 11.

Optimal GI-Training

- 2 years of general internal medicine training (including general ward care, intensive care, cardiology, pulmonology)
- 4 years specific GI-Training
- 3 years basic GI-Training including endoscopy & preferably (endo)ultrasound
- 1 year advanced GI-specialisation
  advanced therapeutic endoscopy, hepatology, oncology etc.

Figure 12.

THE WAY FORWARD

- RAISING ENTOHOUSIASM AND NURTURE GLOBAL TALENT FOR G-E
- RAISING BUDGETS FOR G-E BASIC AND CLINICAL RESEARCH
- WGO-SUMMIT WITH BMI – AND GI-LEADERSHIP TO ANALYSE FAILURES
- STREAMLINE RESEARCH PRIORITIES, BASIC, CLINICAL, TECHNOLOGIC

The way forward

The future of gastroenterology will largely depend upon the quality of its specialists. A proposal for a uniform training program is give in *Fig 11*. Only optimal diagnostic and therapeutic competence and experience of the GE membership will guarantee progress and expansion. For that we need to enhance enthousiasm and to mobilize and nurture top global talent (*Fig. 12*). Budgets and financial resources need to increase to facilitate training and to activate basic and clinical research. Streamlining research priorities is mandary for optimal effectiveness and minimal waste of resources. The World Gastroenterology Organisation/WGO) should take the lead in bringing the scientific leadership of the Biomedical Industry and the GE profession together to analyse the cause of failure in the past, to discuss in depth the unmet needs, to design priorities and to create a platform for future evaluation and interaction. Hopefully, if all this can be realised, we will truly foster the development of our specialty, to the benefit of the patients we care for.

Medical implications of melatonin: receptor-mediated and receptor-independent actions

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Abstract

The functional versatility and diversity of melatonin has exceeded everyone's expectations. The evidence is substantial that melatonin has multiple receptor-mediated and receptor--independent actions. Considering the unexpectedly widespread distribution of cellular membrane receptors as well as the existence of nuclear binding sites/receptors and the fact that some of melatonin's actions are receptor-independent means that melatonin likely functions in every cell with which it comes in contact. This is highlighted by the fact that there are no morphophysiological barriers to melatonin, e.g., the blood-brain barrier. In addition to its widespread actions, melatonin synthesis occurs in widely diverse tissues with its production not being relegated to the pineal gland. This should not be unexpected given that it is present throughout the animal kingdom including species that lack a pineal gland, e.g., insects, and in single cell organisms. In this review, only a few of melatonin's effects that involve the interaction of the indoleamine with receptors are described. These functions include the control of seasonal reproduction, modulation of sleep processes and influences on bone growth and osteoporosis. Among the actions of melatonin that are likely receptor independent and that are reviewed herein include its ability to neutralize free radicals which leads to a reduction in cataract formation, reducing oxidative stress due to exposure to hyperbaric hyperoxia, ameliorating hyperthyroidism and abating the toxicity of sepsis and septic shock. These actions alone speak to the diversity of beneficial effects of melatonin; however, the review is no way near exhaustive in terms of what melatonin is capable of doing. Because of its ubiquitous benefits, the pharmaceutical industry is developing melatonin analogues which interact with melatonin receptors. Clearly, the intent of the drugs is to take advantage of some of melatonin's numerous beneficial effects.

Key words: antioxidant, cataracts, free radicals, hyperbaric hyperoxia, hyperthyroidism, osteoporosis, sepsis, sleep.

Introduction

Melatonin is often referred to as a hormone. By conventional definition, a hormone is defined as a molecule that is synthesized in an organ, released into a bodily fluid from where it travels to another cell or group of cells were it acts via specific receptors to mediate its effects. Melatonin does not always act in this manner. Sometimes it carries out its actions without the intervention of a receptor, e.g., when it directly scavengers free radicals [1]. In other circumstances, it is released from a cell and acts on another cell in the immediate vicinity, i.e., it functions as a paracoid. Because of this diversity of actions, melatonin is not, in the strictest sense, a hormone. Rather it is a tissue factor, a paracoid, an autocoid, an antioxidant and sometimes a hormone depending on the physiological situation [2].

Also, typically hormones are regulated, either positively or negatively, by other hormones. Thus, hypothalamic releasing/ /inhibiting hormones act at the level of the anterior pituitary to release or inhibit, respectively, other hormonal products present in the adenohypophysis. In turn, these hypophyseal hormones act on peripheral endocrine organs to induce the synthesis and release of yet another set of hormones which then exert either positive or negative feedback effects on the anterior pituitary and/or brain. By comparison the control of pineal melatonin synthesis and release is primarily under control of the sympathetic innervation to the gland (*Fig. 1*) [3,4] and conventional hormones are without a marked influence on its production [5].

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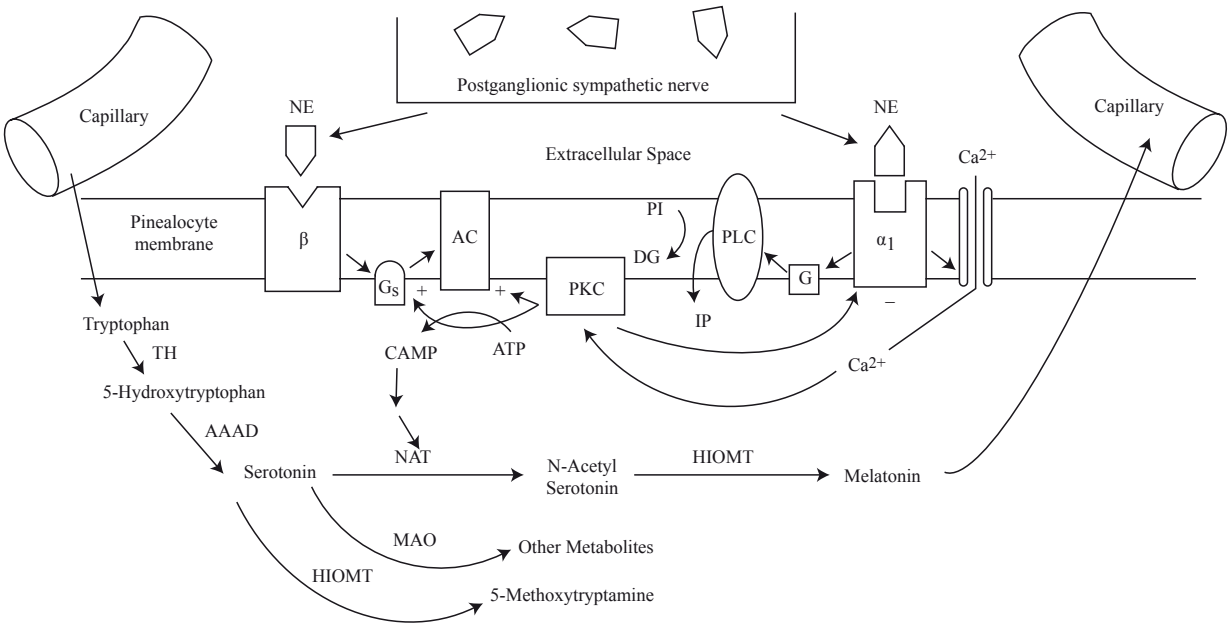
Figure 1. **Interactions of the postganglionic sympathetic innervation with the pinealocyte (the major cell in the pineal gland). During darkness these nerve endings release norepinephrine (NE) which acts primarily on β-adrenergic receptors (β) and to a lesser degree on alpha1-adrenergic receptors (α1) to promote the nocturnal synthesis of melatonin. Melatonin is formed from the amino acid tryptophan which is taken up from the blood into the pinealocyte. Four enzymes, i.e., tryptophan hydroxylase (TH), aromatic acid decarboxylase (AAAD), N-acetyltransferase (NAT) and hydroxyindole-O-methyltransferase (HIOMT), are responsible for converting tryptophan to melatonin (N-acetyl-5-methoxy-tryptamine). Once produced, melatonin is quickly released into the adjacent capillaries and possibly into the third ventricle of the brain. Hormones from endocrine glands throughout the body do little to perturb the circadian production of melatonin and have only a minor influence on the total amount of melatonin synthesized**



Thus, the pineal gland itself is also not a conventional endocrine organ; rather it is probably best described as a neuroendocrine transducer.

Another feature that characterizes melatonin is its widespread distribution in animals and its uncommonly widely diverse functions. Melatonin exists possibly in every species of the animal kingdom [6,7] and even in some plants [8,9], perhaps all plants. It seems possible that melatonin exists in all living organisms. Also, while the pineal gland of vertebrates is the best known site of melatonin synthesis, it is by no means relegated to this organ given that invertebrates and unicells produce melatonin but are devoid of a pineal organ. Indeed, unicells have no organs whatsoever. Given these observations, it should also be no surprise that even in vertebrates, including mammals, the production of melatonin is not restricted to the pineal gland, e.g., it synthesis reportedly occurs in the retinas [10], the gastrointestinal tract [11], the bone marrow [12,13], and a number of other organs [14-17]. When the mRNAs for the two enzymes that generate melatonin from serotonin are localized, they are found in many tissues that are not yet known to produce the indoleamine [18].

Receptor-mediated actions

In mammals, melatonin signals intracellular processes via activation of two high-affinity G-protein-coupled receptors designated MT1 and MT2 [19-21]. These receptors are distinguishable on the basis of their molecular structures [22], their pharmacological characteristics [23] and their chromosomal localization [24]. The MT1 and MT2 receptors signal by coupling to heterotrimeric Gi proteins. Activation of these receptors causes dissociation of G-proteins into α and a βγ dimer which then interact with various effector molecules related to transferring the signal [25]. The effector systems involved in MT1 and MT2 receptor signaling through G-protein coupling include adenyl cyclase, phospholipase C, phospholipase A2, potassium channels and possibly guanylyl cylase and calcium channels [26-28].

The following paragraphs describe conditions where melatonin receptors are involved in mediating physiological changes. In these cases either the MT1 or MT2 receptors, or both, are likely involved. Although these actions of melatonin are described as being receptor-mediated, the specific signal transduction and effector mechanisms are not always clearly defined.

Seasonal reproductive physiology. The first action of melatonin to be well substantiated is its ability to mediate seasonal changes in reproductive competence in photoperiodically-dependent seasonal breeders [29,30]. These studies were initiated by observations made in the mid-1960s which showed that short day exposure (winter-type photoperiods) of Syrian hamsters markedly depressed reproductive function in both male [31-33] and female [34,35] Syrian hamsters and that these reproductive degenerative changes were prevented by either surgical removal of the pineal gland [31,32,35,36] or by superior

cervical ganglionectomy [3], which interrupts the major nerve supply to the pineal gland and renders it non-functional. While the ability of short days to depress reproductive physiology in long day-breeding Syrian hamsters was first documented under stable laboratory conditions, soon thereafter it was also shown that, in animals maintained under natural photoperiod and temperature conditions, pineal removal allowed the hamsters to maintain large functional gonads during the winter months [37]. Furthermore, the pinealectomized animals were capable of successful reproduction during the winter [38], a time at which they would have normally been incapable of doing so. In most cases, delivery of young during the winter months is not conducive to survival of the offspring due to the reduced environmental temperature and the shortage of food supplies for both mother and newborns.

The importance of these findings is that the results document that the circannual rhythm in reproductive competence in photoperiodic species is mediated by seasonally-changing day lengths. Moreover, it is the pineal gland, via the elevated nocturnal secretion of melatonin, the duration of which is proportional to the daily dark period [39,40], that is the critical determinant of the seasonal reproductive cycle. Indeed, in a cleverly designed series of studies, Carter and Goldman [41] confirmed, using melatonin infusion, that long duration daily melatonin elevations (typically of those that occur during the short days of the winter) in pinealectomized Djungarian hamsters caused reproductive collapse while shorter duration (typical of those that occur during the long days of the summer) daily infusions did not. Moreover, Stetson and Tay [42] documented that late afternoon melatonin administration, which then synergizes with nocturnally produced endogenous nighttime melatonin to prolong the duration of elevated melatonin, induced gonadal quiescence even in long day-exposed animals. Collectively, the data summarized above provide compelling evidence that melatonin, and specifically the changing duration of elevated nocturnal melatonin level over the seasons is the impeller of seasonal reproductive breeding. The observations, originally derived from studies on the Syrian hamster, have been shown to also exist in many other long day-breeding species, e.g., the Djungarian hamster [43], vole [44], white-footed mouse [45], ferret [46], etc. This information is now so commonplace that it is considered textbook material.

At the time of these observations, melatonin was generally referred to as an antigonadal [47] or antigonadotropic [48] agent since long duration elevated melatonin levels were associated with reproductive involution. However, the use of these terms was premature and an incorrect designation. Melatonin seems not per se to be directly inhibitory to the reproductive system of seasonally breeding animals inasmuch as many species breed at a time of the year (the winter) when maximal duration melatonin levels exist. These are what are referred to as short day-breeders and include some strains of sheep [49,50], white-tailed deer [51], etc. Clearly, in these species melatonin does not inhibit reproduction and the associated hormone levels. In humans, although melatonin was initially thought to be inhibitory to reproductive development and physiology and was tested (in combination with progestin) as a contraceptive agent [52], it is now generally accepted that melatonin has a minor or no influence on the reproductive physiology of the human. While many individuals take melatonin on a regular basis, there have been no reports of suppressed reproductive function.

The site at which melatonin acts to synchronize seasonal reproductive capability has not been unequivocally proven. The evidence, however, is very strong that the neuroendocrine-hypophyseal axis is the site of this interaction. Hence, the medial basal hypothalamus [53-55] and the anterior pituitary gland itself [56,57] have received a great deal of attention. These cites certainly contain melatonin receptors which are capable of modifying cellular events that would alter reproductive physiology [21,58].

Sleep. Circadian rhythms play an important role in determining optimal functioning of organs and physiological processes in all animals. The sleep/wake cycle in man is internally synchronized with the body temperature rhythm and with the 24 hour blood melatonin cycle [59]. These three rhythms normally have a stable phase relationship with maximal sleepiness coinciding with highest melatonin levels and minimal core body temperature. In normally-entrained individuals, major sleep occurs during the night with the late evening rise in melatonin preceding slightly the propensity to sleep [60]. When humans enter darkness at 22:00-23:00 h, circulating melatonin levels reach their peak 3 to 5 hours thereafter and then begin to drop as the time of awakening approaches [61]. Core body temperature usually reaches its peak in the early evening and decreases to a nadir between 03:00 and 06:00 hours [62] with sleep onset usually occurring 5 to 6 hours before the lowest core body temperature [63].

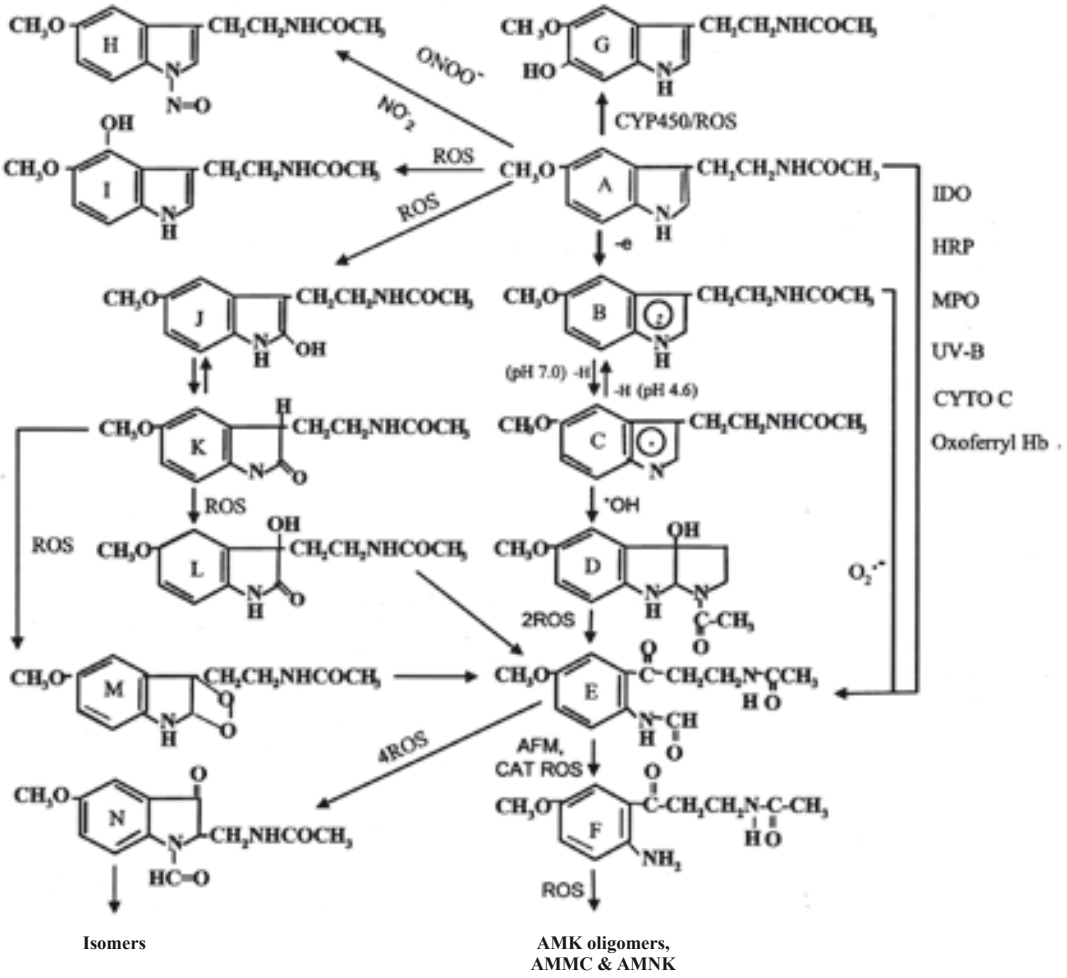
Given the obvious association between sleep propensity and rising melatonin levels, investigations into the effects of exogenously-administered melatonin on sleep induction would be expected. Many studies in the last two decades have suggested an association between sleep an elevated induced melatonin levels [64] although there are contrary reports [65].

Exogenously-administered melatonin functions as a non-photic Zeitgeber when its administration is appropriately timed. As a result it can phase advance or phase delay the circadian system including sleep onset when it is given during the proper interval. The human phase response curves for melatonin have been defined [66,67]; when exogenous melatonin is given in the late afternoon/early evening, the endogenous melatonin rise is phase advanced. When melatonin is given in the morning, a delay in the melatonin rise is seen in the evening of the same day, i.e., it is phase delayed [68].

Some chronic sleep disorders are a consequence of disturbances of the relationship between components of the circadian system. Besides sleep disorders, difficulties in alertness, fatigue, etc., in shift workers and during jet lag have a similar dyssynchronization of their circadian cycles. The ability of melatonin to correct or partially alleviate these conditions has been examined.

Individuals with delayed sleep phase syndrome (DSPS) experience difficulty in falling asleep at their desired bedtime and an inability to wake spontaneously in the morning [69]. Exogenous melatonin has been given to individuals with DSPS with the intent of correcting this problem. Because of different doses of melatonin given to patients at different times, it

***Figure 2*. Melatonin (A), during the process of scavenging toxic reactants, generates a number of metabolites that are equally effective or better scavengers than is the parent molecule. Some of the most important of these are cyclic 3-hydroxymelatonin (D), N1-acetyl-N2--formyl-5-methoxykynuramine (E), and N-acetyl-5-methoxykynuramine (F). 6-hydroxymelatonin (G), is the major hepatic enzymatic metabolite of melatonin**



is difficult to compare the results of the studies that have been published. In general, however, evening melatonin administration to DSPS patients often phase advances sleep onset [70-72], improves sleep and results in less daytime fatigue. Furthermore, evening melatonin treatment combined with early morning bright light therapy induces even a greater phase advance suggesting that these two treatments have additive effects and that it may be the best treatment to improve sleep in individuals with severe DSPS [73].

Sleep disorders in children have also been successfully treated with melatonin. Melatonin therapy in children with neurodevelopmental disorders (NDD) has yielded a 70-90% response rate in terms of sleep improvement [74,75]. DSPS in children, as in adults, as well as other circadian rhythm sleep disorders can usually be improved or corrected with melatonin. On the other hand, early morning awakening, which is common in children with NDD, is more difficult to treat with evening melatonin. This may relate to the rapid metabolism of melatonin after it is given. Thus, it can reduce sleep onset latency but early morning awakening still occurs [76,77].

The dose of melatonin required to induce sleep seems to be highly variable among individuals and some patients show no sleep improvement whatsoever in response to the indoleamine. Once the therapeutic threshold is established for sleep promotion in an individual, higher doses generally do not result in additional sleep promoting benefits [78]. Also, there are some individuals who exhibit a rapid response to melatonin treatment while others are slow responders [77]. Thus, some patients exhibit sleep improvement essentially the first night while in others melatonin must be given for weeks or months before sleep improvement becomes apparent.

Insomnia is a prevalent problem in elderly individuals with up to 50% of that population exhibiting inefficient and/or non-restorative sleep despite ample opportunities to sleep [79]. Given that endogenous melatonin wanes with increasing age and because of the proposed association of melatonin with sleep improvement, it has often been surmised that insomnia in the elderly is related to the diminished melatonin levels. The soporific effect of melatonin has been tested in elderly subjects with varying degrees of success [80,81]. The use of melatonin

to treat insomnia in older people is supported by the findings of Zisapel [82] who reports that it is effective in promoting earlier onset and more restful sleep in the elderly and, although no specific melatonin formulation has been approved for sleep, its efficacy and high safety profile provide a rational for its use.

One novel approach to improve nighttime sleep and daytime activity in elderly institutionalized subjects was to give them melatonin-rich milk [83]. In this case, milk containing 10-40 mg melatonin per liter was given as a drink with meals. The amount of milk consumed by each subject was, on average, 0.5 liters daily. In this study, there was some subjective improvement of sleep quality as judged by the caregivers and a more noticeable improvement in daytime activity. The authors suggested that even ultra-low doses of melatonin, which do not measurably change circulating melatonin values, may nevertheless have some beneficial effects in elderly humans, particularly in relation to increased daytime activity.

Due to the very wide variety of factors that lead to sleep disturbances, it is not surprising that the use of melatonin to improve these problems has not been uniformly successful. While many investigators conclude that melatonin is efficious for improving sleep, the most effective doses and the ideal time of administration may vary among individuals, making generalizations about treatment difficult. Despite a significant amount of data to the contrary, as mentioned above, there are some clinicians/scientists who contend that melatonin is not beneficial in terms of sleep promotion [65]. This brief resume is certainly not exhaustive in discussing all the reports on melatonin in relationship to sleep and the interested reader can consult other reviews on this subject. Also, when melatonin does influence sleep processes the authors of the reports usually assumed that this result is a consequence of melatonin's interaction with neural membrane receptors, probably in the suprachiasmatic nuclei.

Osteoporosis. In 1992, despite the absence of any compelling data indicating an association between melatonin and bone metabolism, Sandyk et al. [84] made the suggestion that perhaps the indoleamine would be beneficial in reducing the severity of postmenopausal osteoporosis. The idea is interesting inasmuch as osteoporosis becomes most obviously manifested after menopause/andropause when melatonin levels, along with a variety of other hormonal agents, wane. While individuals have subsequently pointed out the temporal relationship between the age-related reduction in melatonin and the progression of bone loss in the elderly, neither directly suggested melatonin be used to treat this condition [85,86]. Considering the data from recent publications, melatonin administration may yet prove to be a feasible treatment to improve bone health in the elderly. In 2003, Cardinali and co-workers [87] reviewed the world's published literature on this subject and defined the rational for the potential use of melatonin therapy to augmented bone mass in diseases characterized by low bone density and increased fragility of osseous tissue.

Circulating levels of melatonin can be reduced in young animals by surgical removal of the pineal gland. When this procedure is performed in chickens, the development of scoliosis is a common finding, a change consistent with the loss of bone mass and deterioration of skeletal microarchitecture [88-92]. While pineal ablation does not by itself result in scoliosis in the rat, this may relate to the fact that rats do not walk upright like chickens and, therefore, the amount of pressure on the spine of the rat is greatly relieved.

Theorizing that being bipedal was potentially a requirement for the development of scoliosis after pinealectomy, Machida et al. [93] published a series of studies indicating this is the case. To create bipedal rats, this group surgically removed the forelegs and the tail from pups shortly after birth. These animals then learned to walk upright, i.e., they became bipedal, by using their hind legs only. When these rats were subsequently pinealectomized, they too developed spinal malformations similar to those in chickens lacking their pineal gland. Importantly, treating pinealectomized bipedal rats with a subcutaneous melatonin pellet prevented the deterioration of the vertebrae and the development of idiopathic scoliosis. Thus, the authors concluded that postural processes along with a melatonin deficient state are critical factors in the development of a weakened vertebral column in pinealectomized rats [93].

Machida and co-workers [94] have now extended these studies to another rodent, the C57BL/6J mouse. This strain of mouse is an ideal model in which to examine the role of melatonin in preventing bone loss given that it is genetically deficient in melatonin [95,96]. As in their studies using rats, Machida et al. [94] surgically-induced bipedalness and some of the animals were supplemented with an intraperitoneal injection of melatonin daily (8 mg/kg). After 5 months the mice were killed and the vertebral column was examined by spine X-ray and 3 dimensional computerized tomography. Scoliosis and rib humps developed in 29 or 30 genetically melatonin-deficient bipedal mice; interestingly, even 5 of 20 quadrupedal mice that had genetically-depressed melatonin levels exhibited spinal curvature. When melatonin was given as a daily supplement, no mice developed scoliosis.

A recent study from the same laboratory extended these findings using this unique strain of mouse [97]. Again, the animal selected for their studies was the C57BL/6J mouse because of its melatonin deficiency [95,96]. These mice were again rendered bipedal by surgical removal of the forelimbs. These animals, even though they were not pinealectomized (but they were melatonin deficient) developed scoliosis in a large percentage of the cases, i.e., 7 of 9 mice. In another strain of mice, the C3H/HeJ, which is not deficient in melatonin, bipedal ambulation by itself caused 25% of the animals to develop spinal curvature, this was increased to 70% when bipedal C3H/HeJ mice were additionally pinealectomized. The conclusion of these studies is that spinal deformations occur as a result of a melatonin deficiency combined with bipedal ambulation.

These findings attracted the interest of clinical researchers who surmised that bipedal primates, e.g., humans, may develop scoliosis and/or osteoporosis in later years since at this time endogenous melatonin levels are depressed. To test this they selected the rhesus monkey [98], 18 of which were pinealectomized when they were 8-11 months of age; the completeness of pineal removal was assessed by the minimal levels of a major melatonin metabolite, 6-hydroxymelatonin sulfate, in the urine. Following the surgical procedure, the follow-up interval varied from 10-41 months during which bone structure was analyzed

by means of monthly radiographs. Of the 10 monkeys that had complete pineal excision, none developed scoliosis (average follow-up was 29 months).

Given the apparent negative outcome of this study, Cheung et al. [98] reasoned that the observations made in bipedal rodents regarding melatonin deficiency, bipedalness and weakened bone structure cannot be extrapolated to bipedal primates or to the human. They surmised that the difference may be possible etiological factors that contribute to the development of idiopathic scoliosis in different animal groups. There are, however, several factors to consider. Firstly, whereas the rhesus monkey is classified as a bipedial primate, rarely does it walk upright. Also, in this case the animals were likely maintained in cages that greatly limited their mobility and stress on the vertebral column; if these animals would have been in their natural setting with free movement perhaps the outcome of the studies may have been different. Thus, it would seem premature to conclude that melatonin is without effects of bone growth, remodeling or deterioration in primates including man.

Ladizesky et al. [99,100] used two different animal models to test whether melatonin is influential in terms of bone formation and resorption. Ovariectomy in rats is accompanied by changes in bone metabolism and demineralization. To monitor these changes, they assessed urinary deoxypyridinoline (a marker of bone resorption) and calcium excretion as well as blood levels of calcium, phosphorus and bone alkaline phosphatase (a marker of bone formation); additionally, they evaluated bone mineral density (BMD), bone mineral content (BMC) and bone area (BA) of the entire skeleton at 60 days after ovary removal. Half of the rats had melatonin added to their drinking water (250 μg/ml). By 30 days after ovariectomy, urinary deoxypyridinoline increased by 50%, a change that did not occur in the ovariectomized rats that were ingesting melatonin in their drinking fluid. At 15 days after surgery, a significant rise in serum phosphorous and bone alkaline phosphatase was apparent in rats lacking their ovaries but receiving melatonin. The BMD, BMC and BA, although reduced after ovariectomy was not modified by melatonin in the drinking fluid. While not all parameters of bone remodeling were preserved by melatonin treatment, the authors were confident in concluding that melatonin does modify bone remodeling following surgical removal of ovaries, but for maximal benefit some estrogen may also have to be available [100]. Castration in male rats causes similar changes in indices of bone loss which are reduced when melatonin is given [101,102].

In a second study, Ladizesky et al. [103] treated male rats for 10 weeks with either melatonin or methylprednisolone or both agents. While each molecule independently had positive effects on indices of bond health, when given in combination the benefit was the greatest. The indices that exhibited positive effects included BMC, BMD, and BA and, during a femoral biomechanical test the combination of the glucocorticoid and the indoleamine produced the highest values of work to failure. Clearly, both methylprednisolone and melatonin independently reduced bone resorption and had bone protective effects.

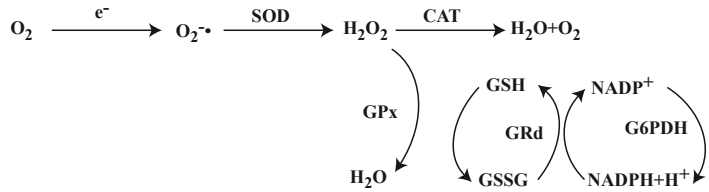
The consequences of melatonin on the proliferation of osteoblasts differ slightly among the findings that are reported in the literature. Roth et al. [104], using two rodent osteoblastic cell lines claimed that melatonin was either without effect or slightly depressed osteoblastic cell proliferation. This group also reported, however, that melatonin promotes osteoblast differentiation and enhances bone formation. In contrast, Nakade et al. [105] found that, in the presence of melatonin, human osteoblast cell proliferation was improved.

The most complete examination of the association of melatonin and bone growth is that recently published by Satomura et al. [106]. The intent of their studies was to test whether melatonin could be effectively used as a pharmacological agent to shorten the treatment period of bone fracture, osteotomies and bone distraction. For their *in vitro* studies they used human osteoblasts and for the *in vivo* experiments the mouse was the animal of choice. In terms of its effects on human osteoblasts, melatonin dose-dependently stimulated cell proliferation and alkaline phosphatase activity. Additionally, the indoleamine intensified gene expression of type 1 collagen osteopontin, bone sialoprotein and osteocalcin. As with cell proliferation, the degree of heightened gene expressions were related to the concentrations of melatonin used. Also, importantly the intraperitoneal administration of melatonin (100 mg/kg for 21 days) increased the volume of newly formed cortical bone in the femurs of mice. Finally, reverse transcription-polymerase chain reaction and Western blot analysis showed that human osteoblasts express the melatonin 1a receptor.

That melatonin influences the differentiation of progenitor cells toward osteoblasts was recently suggested by the studies of Sanchez-Hidalgo et al. [107]. In mammalian bone marrow, two of the major cell types that occur develop from a common precursor cell, the multipotent bone marrow-derived cell, or the mesenchymal stem cell [108]. Melatonin may be able to direct these undifferentiated cells toward the osteoblast line rather than to the adipocyte [104]. Using the ROS17/2.8 cell line which was also used by Roth and co-workers [104], Sanchez-Hidalgo et al. [107] found that melatonin inhibited oleic acid uptake by these cells reducing the formation of adipocytes and directing cell differentiation toward the osteoblast. They also found, with the aid of the melatonin receptor antagonists, luzindole and S20928, that melatonin inhibited triglyceride uptake by these cells via a receptor-mediated mechanism, although the signaling events were not identified. These findings were interpreted in light of the reduced melatonin production that occurs in the elderly. Normally, bone marrow cell differentiation with increased aging shifts toward the adipocyte line of cell development at the expense of osteoblast formation. This may contribute to osteoporosis which is common in aged individuals. These changes occur coincident with reduced endogenous melatonin production during aging [109-111]. The findings of Sanchez-Hidalgo et al. ([107] indicate that supplemental melatonin administration in the elderly may preserve bone strength and decrease fat cell accumulation in the bone marrow, a feature common to the marrow of aged individuals. The outcome of these *in vitro* findings are consistent with the observations summarized above related to melatonin's ability to prevent bone deterioration in melatonin-deficient bipedal rodents.

The structural integrity of the skeletal system relies on the persistent remodeling processes carried out by bone-resorbing osteoclasts and bone-forming osteoblasts. Melatonin may

***Figure 3*. Under elevated oxidative stress conditions melatonin either upregulates and/or prevents the loss of the activities of important antioxidative enzymes. The enzymes that melatonin protects include the superoxide dismutases (SOD, both the cytosol and the mitochondrial forms, i.e., CuZnSOD and MnSOD, respectively) and glutathione peroxidase (GPx) and glutathione reductase (GRd). Although less evidence is available, glucose-6-phosphate dehydrogenase (G6PDH) and catalase (CAT) may also be stimulated by melatonin. CAT and GPx act to metabolize H_2O_2 to harmless molecules thereby reducing the formation of the hydroxyl radical, which is highly destructive**



enhance bone formation by suppressing osteoclasts as suggested by Suzuki and Hattori [112] and Koyama et al. [113] via its free radical scavenging properties and actions on RANKL and/or by promoting osteoblastic activity [105,114] by mechanisms that involve membrane melatonin receptors on these cells [115,116]. The down-stream signaling mechanisms whereby melatonin enhances the activity of osteoblasts theoretically involve a number of mechanisms none of which have much support [117].

Non-receptor mediated actions

The ability of melatonin and its metabolites to expunge free radicals and related reactants possibly involves all of the following actions: a) direct detoxification of radicals and radical products, b) stimulation of the activities of several antioxidative enzymes, c) inhibition of the activities of prooxidative enzymes, d) promotion of the synthesis of glutathione, another essential antioxidant, e) synergistic actions with other antioxidants, and f) mitochondrial actions of melatonin that reduce free radical generation. Of these actions, some clearly require no specific receptor (are non-hormonal) while others may well be receptor-mediated (are hormonal). For the purposes of this presentation, however, they are listed under the non-hormonal category of actions.

While melatonin has the capability of donating one or more electrons to free radicals resulting in their detoxification [118--120], the metabolites that are formed during this process, i.e., cyclic 3-hydroxymelatonin (3-OHMel), N-acetyl-N-formyl--5-methoxykynuramine (AFMK) and N-acetyl-5-methoxykynuramine (AMK) [121-126] also have similar capabilities. This progressive annihilation of radicals and their products by melatonin and its metabolites is referred to the antioxidant cascade and is graphically depicted in figure 2 [1].

The activities of antioxidative enzymes may exhibit increases or decreases depending on the duration and severity of oxidative stress and when they are measured during the stress response. Initially, early in the oxidative stress response the activities of antioxidative enzymes may show a compensatory rise to overcome massive free radical generation. As the oxidative stress response is prolonged, free radicals either directly damage the enzyme molecule or its upstream processes leading to a reduction in enzyme activities. Thus, melatonin's apparent actions in terms of these enzyme responses may be at least two-fold, i.e., it prevents the upregulation of the enzymes presumably due to the fact that as melatonin and its metabolites scavenge radicals the oxidative stress environment is reduced and upregulation of antioxidative enzyme activity is less necessary. Secondly, melatonin due to its combined scavenging actions with that of its metabolites may reduce the likelihood that the enzyme itself or its upstream processes are damaged, thereby preventing a reduction in the activities of the antioxidative enzymes. These reported effects of melatonin have been summarized in several reviews [127-130].

The antioxidative enzymes that are influenced by melatonin include the superoxide dismutases (both the mitochondrial and cytosolic isoforms), glutathione peroxidase and glutathione reductase (*Fig. 3*) [129,130]. The antioxidative enzyme, catalase, has been less extensively investigated in terms of the effects of melatonin on its activity.

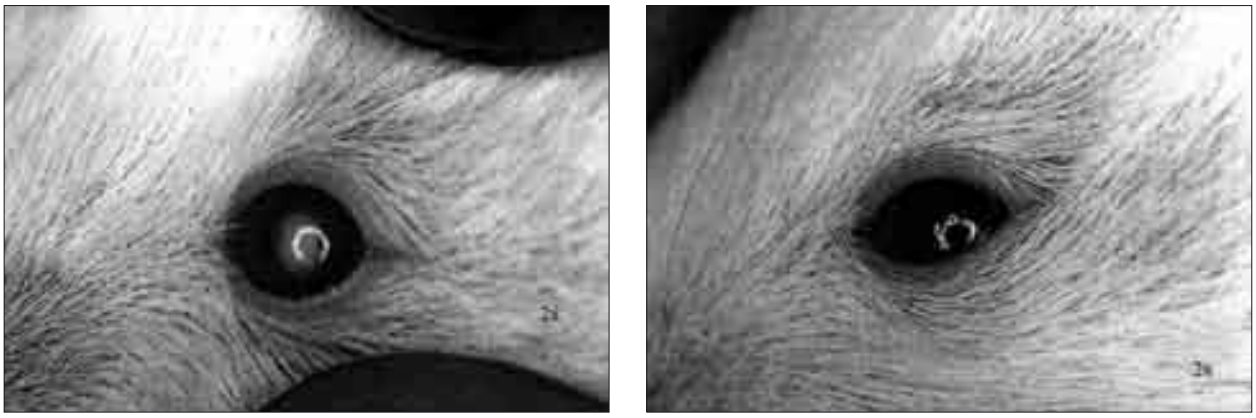
There are a number of prooxidative enzymes in multicellular organisms which generate free radicals. Examples include nitric oxide synthase which generates NO• and the lipoxygenases which result in the formation of the superoxide anion (O2•-). Whereas NO• is not a powerfully damaging free radical, when it couples with O2•- it forms the peroxynitrite anion (ONOO-) which is potently reactive and damaging. Melatonin inhibits lipoxygenase [120], while AMK reduces the activity of the enzyme that catalyze the formation of NO•, nitric oxide synthase (NOS) [131]. As of result, free radical and/or toxic reactant generation is alleviated.

The concentration of the intracellular antioxidant, glutathione, is very high in many cells. During high oxidative stress conditions total glutathione levels can be reduced. One action of melatonin seems to be to ensure that glutathione levels do not drop significantly. This may be achieved by melatonin's ability to stimulate the enzyme, gamma-glutamyl-cysteine synthase, the proposed rate limiting enzyme in glutathione production [132,133].

At least in *in vitro* experiments, melatonin has been shown to synergize with vitamins C and E and others to reduce free radical damage [134,135]. It is not uncommon for antioxidants to couple their actions such that the beneficial effect is greater than when the individual antioxidants act independently.

Prevention of free radical generation may be an important activity which allows melatonin to limit oxidative stress. A number of studies have documented that the fumbling of

***Figure 4*. Cataracts in the lens of 17-day-old rat (left) treated with L-buthionine-S,R-sulfoximine (BSO) at day 1 after birth to deplete intracellular levels of the antioxidant, glutathione. Prominent "cloudiness" of the lens was apparent at 17 days after birth. Melatonin, given as a daily intraperitoneal injection, prevented the formation of cataracts (right)**



electrons during their transfer though the electron transport chain may be reduced by melatonin [136,137]. When the escape of electrons from the chain is diminished, the number of radicals that are formed in the mitochondria is likewise reduced. Melatonin's ability to limit free radical generation at the mitochondrial level is readily apparent when cells subjected to high oxidative stress conditions are treated with melatonin [138].

In the following examples where melatonin has been used to reduce oxidative mutilation, one or more of the processes outlined above may have been operative. Indeed, it is impossible to determine what percentage of the protection is afforded by an individual action of a free radical scavenger/antioxidant. Also, the following list includes only a few of the experimental situations in which melatonin has been shown to attenuate free radical damage and improve tissue function.

Cataracts. Oxidative stress is identified as a major cause of cataracts [139,140]. In humans, cataracts usually develop late in life and are more common in individuals who have spent much of their time working outdoors where they were exposed to high levels of ultraviolet radiation. The lenticular damage that occurs is mainly in the epithelium and cortex [141].

A frequently-used experimental model to investigate processes associated with cataractogenesis and the means of inhibiting their development includes the newborn rat treated with L-buthionine-S, R-sulfoximine (BSO); this drug inhibits gamma-glutamyl-cysteine synthase, the rate limiting enzyme in glutathione production. Hence, the drug depletes tissues, including the lens, of the important antioxidant glutathione. This depletion leads to exaggerated oxidative stress, molecular damage and the formation of cataracts [142,143]. Typically, BSO is given on the day rat or mice pups are born and the cataracts are grossly apparent by the time the palpebral fissures open at 10-12 days after birth.

Abe and co-workers [144] used this model to induce cataracts and half of the newborn rats treated with BSO were given a daily intraperitoneal injection of melatonin (4 mg/kg BW) for the duration of the study period which ended 17 days after birth. They anticipated that melatonin would reduce cataract formation since it had been shown to be a free radical scavenger [145] and lenticular opacification is related to oxidative stress [146]. Moreover, melatonin had been identified in the fluid of the anterior chamber of the eye [147] and, therefore, it would likely have ready access to the lens. In this model, Abe et al. [144] indeed found that melatonin readily substituted for glutathione and almost totally prevented cataractogenesis (*Fig. 4*). In BSO-treated rats given only diluent, 18 of 18 pups had visually apparent cataracts while only 1 or 15 BSO-injected pups given melatonin on a daily basis had obvious bilateral cataracts. Since all rats treated with the glutathione-depleting drug had very significantly depressed (>90%) lenticular glutathione levels, the authors surmised that the protection against oxidative stress provided by melatonin was a consequence of its antioxidative actions.

Li and colleagues [148] used the same newborn rat model to study the efficacy of melatonin in reducing lenticular opacification and lipid peroxidation. In this case, the lenses were examined on both day 9 and day 17 after birth of the pups. When the data from the two days were combined, again virtually all pups (16 of 18) given BSO plus diluent developed cataracts while 3 or 18 of those that had the benefit of melatonin injections had cataracts. The levels of lipid peroxidation were elevated in many organs of pups given BSO with the amounts of oxidized lipid being reduced after daily melatonin treatment.

Bardak et al. [149] approached the problem of cataractogenesis and the protective effects of melatonin differently. This group specifically exposed rat lenses to ultraviolet light to induce cataracts and, again, melatonin reduced their incidence.

Of interest in relation to the ability of melatonin to limit the frequency of cataracts is that it was subsequently reported that the rat lens itself produces melatonin, at least after adulthood. This was initially reported by Abe et al. [150,151] and has been recently confirmed by Itoh and co-workers [17]. Abe and colleagues [150,151] detected melatonin and the serotonin acetylating enzyme, AANAT, in the rabbit and rat lens and in the latter species they described a circadian rhythm of AANAT activity. Most recently, Itoh et al. [17] documented that the mRNAs for both AANAT and HIOMT exist in the adult rat lens; immunocytochemical localization showed that AANAT is localized in the lenticular cortical fiber cells. The locally-produced melatonin may help to protect the lens from oxidative

stress and the formation of cataracts. Melatonin produced in the lens along with that synthesized in the ciliary body [152], which probably accounts for the concentrations of the indoleamine in the fluid of the anterior chamber of the eye [147], likely aids in protecting the lens from free radical-mediated oxidative damage.

Melatonin receptors are not required when the indole functions as a direct free radical scavenger. In the ocular tissues including in the lenticular cortical fiber cells of *Xenopus laevis*, however, receptors for melatonin are present [153]. This suggests that these lenticular cells are direct targets for melatonin and they could assist in reducing oxidative stress by mediating the effects of melatonin in stimulating antioxidative enzymes [130]. Whether any cells in the mammalian ocular lens possess melatonin receptors has not been determined.

Recently, Siu and co-workers [154] summarized the data related to the protective actions of melatonin against a variety of free radical-related ocular conditions/diseases. Besides cataracts, oxidative stress contributes to retinopathy of prematurity, retinoblastoma, age-related macular degeneration, retinitis pigmentosa, glaucoma, photokeratitis and ischemia/reperfusion injury in the orbital globe. Given the association of each of these conditions with excessive free radical damage, melatonin may help to attenuate the severity of these conditions as well.

Hyperbaric hyperoxia. Hyperbaric oxygenation (HBO) involves exposure to 100% oxygen at a pressure typically higher than atmospheric pressure. This treatment modality is a commonly-used procedure for a variety of disorders and has been successfully implemented in clinical situations involving ischemia and/or hypoxia [155]. The rational for HBO therapy is that it elevates pO_2 levels in the blood and tissues, especially those deficient in O_2. The higher concentration of O_2 administered and the higher pressure increases the level of dissolved oxygen entering the blood [156]. The down side to oxygen therapy, however, is the elevated levels of toxic oxygen-based free radicals and related products that are generated under elevated oxygen conditions [157,158]. Given the involvement of free radical-mediated molecular damage with this treatment paradigm, several studies examined whether melatonin would reduce oxidative damage to tissues of rats exposed either acutely or chronically to 100% oxygen.

In the first study in this series, Pablos and colleagues [159] exposed adult rats to 100% oxygen at 4 atmospheres for 90 minutes in a plexiglas chamber. Half of the rats were given a single intraperitoneal injection of 10 mg/kg melatonin before the onset of the exposure. As a result of the HBO, levels of lipid peroxidation products were elevated in the lungs, liver and brain; the rises in lipid degradation were prevented, in all organs, in the animals that had received melatonin. The antioxidative enzymes, GPx and GRd, were reduced as a result of hyperbaric oxygen exposure, changes also reversed by melatonin. Based on the indices measured, melatonin was highly effective in preventing oxidative damage resulting from hyperbaric oxygen exposure.

In the other two acute studies [160,161], adult rats were placed in a stainless steel oxygen chamber which was flushed with 100% oxygen with the chamber pressure being increased to 2.5 atmospheres pressure. Following the exposure session, the chamber was decompressed to normbaric air gradually over a 5 minute period. Immediately following the exposure, tissues were collected to evaluate the degree of oxidative stress.

As in the study Pablos et al. [159], both Topal and colleagues [160] and Dunbar et al. [161] reported that exogenously administered melatonin (10 mg/kg) before HBO exposure reduced oxidative damage in both the lungs and brain. Perhaps of greater importance is that both groups also showed that performing the oxygen exposures a night produced less severe effects in terms of free radical damage than when the exposures were done during the day. These authors, therefore, concluded that the nocturnal rise in endogenous melatonin provides some antioxidative protection against HBO.

In the only chronic exposure study, the investigators also examined the effect of both exogenously-administered pharmacological melatonin levels and endogenously-produced physiological melatonin concentrations on oxidative damage in rats after repeated hyperbaric oxygen exposure [162]. In this case, rats were given 10 consecutive daily HBO sessions of 1 hour duration; the parameters of exposure were 100% oxygen at 2.5 atmospheres. Immediately prior to each session, half of the animals were given an intraperitoneal injection of 5 mg/kg melatonin. The HBO treatment caused rises in protein carbonyls in both the lungs and brain and a compensatory increase in SOD activity in the same tissues. The changes in both protein carbonyls and SOD activity were blocked by daily pharmacological melatonin treatment.

To test the potential protective effect of physiological melatonin levels, the hyperbaric oxygen exposures were performed for 10 consecutive days at night when circulating melatonin levels are elevated [162]. The study was carried out with the aid of a dim red light which does not depress endogenous melatonin levels [163]. While not as effective as exogenously-administered pharmacological melatonin, when the hyperbaric oxygen exposures were performed at night in the presence of elevated endogenous melatonin concentrations, the damage to proteins was in part prevented.

From these studies, it is apparent that melatonin would be a worthy adjunct therapy to be given in combination with hyperbaric oxygen treatment. Undoubtedly, this HBO exposure is limited by the toxicity of oxygen and the use of melatonin may allow more prolonged or frequent treatment periods which may benefit recovery and avoid the associated molecular damage.

Hyperthyroidism. The hypermetabolic state associated with hyperthyroidism generates an excess of free radicals in the heart and other tissues [164,165]. During hyperthyroidism, the heart undergoes hypertrophy [166]. Due to the increased metabolic rate imposed by elevated thyroid hormone, additional free radicals are produced which damage the heart (and other tissues); the dysfunctional heart then undergoes a compensatory growth response to counterbalance the altered cardiac function.

Ghosh et al. [167] tested whether melatonin would overcome cardiac hypertrophy and oxidative alterations associated with triiodothyronine (T3) administration for 15 days. Additionally, they examined whether depressed gene expression for GLUT4 and the reduced glucose uptake by cardiomyocytes would be

changed by melatonin treatment. T3 (8 μg/100 g) was given via an intraperitoneal injection as was melatonin (2 mg/100 g); melatonin was always given 1 hour in advance of T3.

Melatonin proved to be protective against the cardiac enlargement that resulted from T3 treatment and, furthermore, it reduced •OH generation in the myocardium. Melatonin also reversed the marked drop in CuZnSOD in the T3-treated rat heart as well as the reduction in glutathione levels. Gene expression for GLUT4 was lowered as a consequence of T3 administration, an effect also prevented by melatonin. Finally, the 50% reduction in insulin-stimulated glucose uptake by hyperthyroid-induced hypertrophic cardiomyocytes was restored by melatonin. GLUT4 normally mediates facilitative transport of glucose into cells. Thus, in this study it was theorized that the down regulation of the GLUT4 gene compromised glucose uptake by the cardiomyocytes. Given that glucose transport/utilization is obviously very important for the optimal functioning of the heart, restoration of these processes by melatonin is important as is the reduction in oxidative damage to the organ.

Sepsis and septic shock. Sepsis is a common cause of mortality in both children and adults in intensive care units [168,169] and is a consequence of the host response to a microbial invasion. Multiple organ failure is frequently associated with sepsis and is characterized by severe hypotension and hyperactivity of blood vessels to vasoconstrictor agents. There are a variety of highly negative consequences of sepsis including respiratory distress syndrome, acute renal failure, disseminated intravascular coagulation and central nervous system dysfunction. The predominant agent responsible for sepsis is lipopolysaccharide (LPS), a component of the cell walls of Gram-negative bacteria [170,171].

In addition to the deaths related to septic shock, this condition has considerable economic cost. An estimated 700,000 patients develop severe sepsis annually in the United States [172,173]. The cost of treatment for each of these individuals is about $24,000. Unfortunately, there is no specific treatment for this condition, possibly related to the lack of definition of the underlying mechanisms of sepsis [174]. Given that sepsis is an extremely high proinflammatory state and a condition in which multiple free radicals and related reactants are produced [175], attempts have been made to combat the toxic reactions associated with this deadly disease in both experimented animals and in humans. The initial animal experiments, Sewerynek and coworkers [176,177] reported that the injection of melatonin into rats that had been treated with LPS reduced the associated peroxidation of lipids in hepatic membranes and also limited the leucocytosis in this organ. Also using the rat model of sepsis, Crespo et al. [178] found that melatonin attenuated the degree of lipid peroxidation and the exaggerated nitric oxide production that accompanies LPS toxicity in both the liver and lungs. Melatonin, in a dose-response manner, lowered nitric oxide by inhibiting inducible nitric oxide synthase, a well known action of this indoleamine which is actually mediated by a melatonin metabolite, AMK [131].

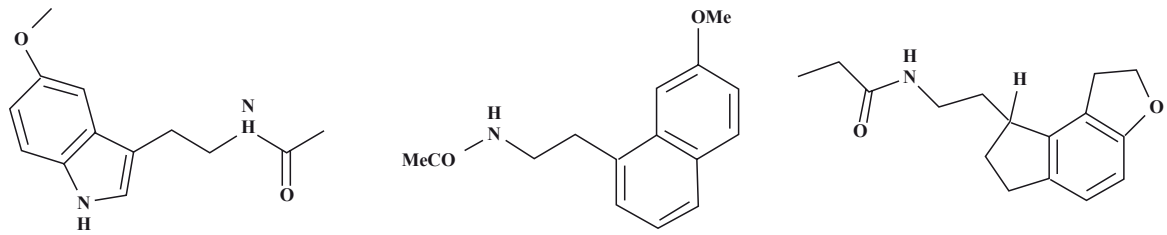
Besides reducing molecular damage resulting from LPS toxicity, melatonin also has been shown to lessen the death rate of endotoxemic rats [178,179]. Melatonin probably protects against death by improving the hemodynamics of these animals since the indoleamine was shown to counteract the release of TNF-α (and possibly other cytokines) into the plasma and reduce O2•- production by the aorta [180]. Moreover, melatonin also limits ONOO- formation and the activation of poly (ADP ribose) synthase [181], curtails the breakdown of lipids and replenishes GSH levels in a variety of organs [182,183] during experimental sepsis. As mentioned above, during sepsis melatonin prevents the recruitment of leucocytes to the affected organs [176,177]. When leucocytes infiltrate organs the enzyme myloperoxidase (MPO) is elevated resulting in the formation to a toxic chlorine-based species, hypochlorous acid. Since melatonin inhibits MPO activity and scavenges hypochlorous acid [184] even the reduced number of leucocytes within tissues are neutralized in terms of inflicting damage because of the presence of melatonin.

Sepsis is associated with marked changes in the physiology of mitochondria and these alterations obviously contribute significantly to the malfunction and death that septic animals/ /humans experience. Escames et al. [175,185] have examined melatonin's protective actions at the level of the mitochondria in LPS-treated rats. This group showed that melatonin administration reduces mitochondrial oxidative damage and inhibits mtNOS protein expression and enzyme activity [186] in both rat lungs and liver following LPS exposure. These observations may well explain some of the beneficial actions of melatonin during the septic response. This group has recently extended these findings by showing that melatonin similarly improves mitochondrial function in mice rendered septic by cecal ligation and intestinal puncture to induce severe peritonitis [187]. Importantly, this group showed that in addition to reducing oxidative/nitrosative damage at the mitochondrial level, melatonin also restored ATP production.

Given that animal studies have repeatedly confirmed the beneficial actions of melatonin in preventing sepsis-mediated molecular damage and death in animals [178,179,186,188], it is not surprising that Gitto and colleagues [189] utilized melatonin to treat premature newborn humans suffering from sepsis. The results of their study documented the high efficacy of melatonin in humans with septic shock. Ten neonates with sepsis were treated with melatonin in addition to receiving conventional therapy while ten infants received conventional therapy only. The melatonin dose was 20 mg given orally in two equal doses separated by 1 hour within the first 12 hours of the diagnose of sepsis. These neonates exhibited early beneficial signs of the melatonin therapy with levels of lipid peroxidation products in the blood being already reduced 1 hour after being given melatonin. Furthermore, melatonin caused a marked drop in circulating C-reactive protein (an inflammatory indicator) levels. Most important, however, was the prevention of death in the septic neonates treated with melatonin. It is common for up to 50% of these infants to die [189]. Of the 10 septic neonates who did not receive melatonin in the study of Gitto et al. [190], three infants died; conversely, none of the septic neonates treated with melatonin died.

These findings are compelling given the serious nature of sepsis and septic shock and the lack of an adequate therapy for this often fatal condition. Research related to use of melatonin as a treatment for sepsis should be aggressively pursued; along

***Figure 5*. Structure of melatonin in comparison to the two melatonin mimetics developed by the pharmaceutical industry. Ramelteon is currently being marketed while agomelatine is in phase III trials. Ramelteon is prescribed as a sleep aid whereas agomelatine will be sold to reduce depression**



these lines Buonocore and Groenendaal [191] have suggested that melatonin be thoroughly tested in clinical trials to overcome oxidative/nitrosative toxicity in clinical conditions such as bacterial sepsis.

Patented melatonin minetics

Since the molecule melatonin is not *per se* patentable, the pharmaceutical industry has pursued the development of melatonin analogues which can be patented. One of these drugs, ramelteon (Roserum™) developed by Takeda Pharmaceuticals has been issued a US patent (US6034239) [192] and is currently being marketed. The second drug, agomelatine (commercial name Valdoxan™, US5318994) [193] is currently in phase III trials and is expected to be on the market soon. This drug will be sold by Servier and Norvatis Pharmaceuticals. The structures of ramelteon and agomelatine relative to that of melatonin are shown in *Fig. 5*.

Melatonin itself has been widely reported to have sleep promoting activity [77,194-196] although this has also been disputed in recent years, at least in the case of some sleep disorders [65]. It is of interest that among mammals, melatonin is uniquely elevated during the night, yet many species are night active and actually sleep during the day when melatonin levels are at their lowest. This implies that melatonin is not a direct hypnotic or soporific but rather influences sleep propensity via other means.

Ramelteon. Ramelteon, which is marketed as a sleep aid, is an indenofuran derivative of melatonin which binds to MT1 and MT2 melatonin receptors [197]. The major interest seems to be its binding to the melatonin receptors in the biological clock, the suprachiasmatic nuclei (SCN); these nuclei are believed to be involved in mediating the effects of melatonin on circadian rhythms and on sleep.

Ramelteon inhibits forskolin-stimulated cAMP production in neonatal rat pituitary glands with an IC_{50} of 20.8 pM suggesting it is a potent agonist of the MT1 receptor [198]. In reference to its MT2 receptor activity, ramelteon binds to Chinese hamster ovarian cells with a binding affinity is roughly 3-fold lower than for the MT1 receptor (Ki=45pm vs Ki=14pm, respectively). Compared to melatonin, the binding affinity of ramelteon for the MT3 hamster brain binding sites is weak. Ramelteon does not bind to a variety of other ligand binding sites; some of those that have been tested include benzodiazepines, dopamine, ion channels and transporters and opiates [199,200]. This melatonin mimetic has a longer half life than melatonin [201,202] and promotes sleep. Chronobiologically, the drug accelerates re-entrainment of running wheel activity in rats [203] to the same degree as does melatonin.

Ramelteon was initially designed as a drug to treat patients with insomnia and related circadian rhythm disorders. In particular, the drug is proported to advance sleep onset in individuals who have difficulty falling asleep, i.e., to reduce sleep latency, but it does not have generalized depressive actions on the electrical activity of the central nervous system [81,204,205]. Ramelteon has not exhibited evidence for abuse or psychological or physical dependency [206]. When used as a sleep aid, the usual recommended dose is 8 mg orally at 30 minutes before desired sleep onset [204].

Agomelatine. Agomelatine was synthesized by replacing the indole scaffold of melatonin with a naphthalene ring [198]. Agomelatine is an agonist of the MT1 and MT2 melatonin receptor [207]. The affinity of agomelatine for cloned human MT1 and MT2 receptors (Ki=6.15 x 10^{-11} and 2.68 x 10^{-10} M respectively) is similar to the binding affinities of melatonin to the same receptor subtypes which are Ki=8.52 x 10^{-11} and 2.63 x 10^{-10} M, respectively.

Agomelatine is designed as a once-daily treatment of major depressive disorders particularly when anxiety and sleep problems are features of the condition [208]. Its efficacy in reference for depression was originally tested in rats and mice and in transgenic murine models [209]. Under these conditions, agomelatine at a dose of 10 mg/kg was as effective as imipramine or fluoxetine at the same doses; the latter two drugs are commonly used antidepressant molecules.

In clinical trials, agomelatine was found to clinically relief depressive symptoms and to be well tolerated [210,211]. It has been found effective in a range of mild to severely depressed patients with its efficacy in relieving symptoms increasing in more severely depressed subjects [210].

Agomelatine has fewer side effects than the other classes of antidepressive drugs, i.e., the selective serotonin reuptake inhibitors and the serotonin and norepinephrine reuptake inhib-

itors [210,212]. Additionally, it has been reported to relief some sleep disturbances and improved daytime alertness [213]. After discontinuing the use of agomelatine, no symptoms associated with ending the treatment have been observed [214]. For antidepressant efficacy, agomelatine is recommended at a dose of 25 mg once daily in the evening.

Concluding remarks

Functionally, melatonin is a remarkably diverse molecule that has been identified in species throughout the animal kingdom and, in the last decade, it has also been found in plants as well. It seems possible that melatonin exists in all life forms.

For decades it was assumed that melatonin's effects at the cellular level are mediated exclusively via membrane receptors on the target cells. These receptors were first definitively identified slightly over a decade ago. A feature of the membrane melatonin receptors which was probably a surprise to most scientists is the distribution of these receptors. Whereas they were initially found in and thought to be confined to a small number of nuclear groups in the brain (especially in the suprachrasmatic nuclei of the hypothalamus) they have subsequently been uncovered not only in many parts of the central nervous system but in numerous sites throughout multicellular organisms. This widespread distribution of melatonin membrane receptors portends numerous functions for melatonin, certainly more diverse actions than originally envisaged.

In addition to the typical membrane receptors that have been identified for melatonin, nuclear binding sites/receptors also have been unveiled [215] and functions have been attributed to them. These receptors belong to the RZR/ROR subfamily and include the products of three genes, i.e., splicing variants of RORα1, RORα2, RORα3, RZRα), which differ in the N-terminal domain, and RZRβ and RORγ [216]. How widespread the nuclear receptors are in the body remains to be established. Thusfar these receptors have been linked to melatonin's actions on the immune system [217-220] and possibly to the stimulation of antioxidative enzymes [130]. In the latter case, they may cooperate with membrane melatonin receptors in promoting the activities of the radical detoxifying enzymes. Finally, some of melatonin's actions involve its binding to intracellular calmodulin [221].

Melatonin's ability to vanquish free radicals and related reactants, an action that is independent of any receptor or binding site, is a rather recent discovery [118]. When this function was uncovered, a dynamic new field of investigation was launched, i.e., melatonin as an antioxidant. This area of study has flourished within the last decade and melatonin has been shown, in more than a thousand reports, to reduce oxidative damage due to the direct free radical scavenging capacity of not only melatonin itself [118,222,223], but also of its metabolites [120,122,126,224]. Free radicals and related reactants create a highly inhopsital environment with cells; to preserve the functional integrity of essential molecules and to protect them from the destruction of toxic reactants, cells utilized a large number of antioxidants including melatonin.

This review summarizes only a very few of the areas where melatonin's receptor-mediated and its receptor-independent actions have been described. The reader should be reminded that, although they are categorized according to the presumed modes of action of the indole, it also seems likely that melatonin may, in many circumstances use both processes to achieve its effects. For example, while melatonin may work via membrane and/or nuclear receptors, when it is in a cell it will also always function of a free radical scavenger. This only requires that melatonin be in the vicinity of the radical when it is generated.

Some of the most critical actions of melatonin may be those in the mitochondria were the indole has been shown to improve the transfer of electrons through the electron transport chain [137]. While doing so, melatonin limits electron leakage and the subsequent formation of free radicals; this has been referred to as melatonin's ability to avoid free radical generation [120], which supplements its ability to scavenge radicals that are formed. Also in the mitochondria, melatonin enhances ATP production [225,226]. The generation of energy in the form of ATP is critical to optimal cell function, including aiding in repairing any cellular damage that has occurred, and in improving survivability of the cell, of the tissue and of the organism.

Many important areas where melatonin may have clinical applicability are not discussed in this brief review. For example, melatonin's ability to prevent initiation [227-229] and progression [230-233] of many cancer cell types have been well documented. Also, in models of Alzheimer's [234-237] and Parkinson`s disease [238-241] the indole has proven beneficial effects. Likewise, free radical mediated ischemia/reperfusion injury has yielded to melatonin therapy; benefits of melatonin for this condition have been shown for every organ where it has been tested including in models of heart attack [242-244] and stroke [245-247]. Melatonin has been found to reduce the toxicity of a variety of prescription drugs [248-250] and the damaging effects of heavy metals [251-253]. Also, hyperglycemia associated with animal models of diabetes is highly destructive, usually via free radical processes, to the cardiovascular system as well as to other organs; this damage has been shown to be attenuated by melatonin treatment [254-256].

Clearly, the pharmaceutical industry has been attentive to the potential utility of melatonin in clinical medicine. In lieu of using melatonin, however, which is inexpensive and nonpatentable, they have developed patentable melatonin receptor agonists that are either currently on the market or soon will be. At this point the data are incomplete in terms of whether these analogues will be more or be less efficacious than melatonin in their specific use paradigm. Likewise, their long-term safety is unknown.

Overall, melatonin would appear to be a highly beneficial molecule with unexploited clinical potential. It could prove to be an important drug, especially in countries where financial resources are limited. Even in the well-developed countries, melatonin should be considered as a treatment for a number of conditions because of its multiple beneficial actions and uncommonly low toxicity.

Considering the increased life span of humans in many countries, a condition typically associated with increased prescription drug usage, the availability of an inexpensive agent such as melatonin could potentially save individuals and government agencies millions of dollars annually.

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Molecular therapy and prevention of liver diseases

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Abstract

Molecular analyses have become an integral part of biomedical research as well as clinical medicine. The definition of the molecular and genetic basis of many human diseases has led to a better understanding of their pathogenesis and has in addition offered new perspectives for their diagnosis, therapy and prevention. Genetically, liver diseases can be classified as hereditary monogenic, acquired monogenic, complex genetic and diseases. Based on this classification, gene therapy is based on six concepts: gene repair, gene substitution, cell therapy, block of gene expression or function, DNA vaccination as well as gene augmentation. While recent developments are promising, various delivery, targeting and safety issues need to be addressed before gene therapy will enter clinical practice. In the future, molecular diagnosis and therapy liver diseases will be part of our patient management and complement existing diagnostic, therapeutic and preventive strategies.

Key words: gene repair, gene replacement, gene augmentation, block of gene expression or function, ribozymes, antisense oligonucleotides, small interfering RNA, interfering peptides or proteins, suicide genes, cytokine genes, antiangiogenesis genes, immunization, cytolytic viruses, immune therapy, DNA vaccination.

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Introduction

Molecular biology and recombinant DNA technology increasingly contribute to the diagnosis, therapy and prevention of human diseases. Molecular methods allow the early and/or specific detection of inherited, infectious and malignant liver diseases. In addition, such analyses increasingly lead to a better understanding of the pathogenesis of the various liver diseases which in turn had an impact on patient management, including the presymptomatic identification of patients at risk, the correct staging of the disease and the follow-up of patients undergoing therapy. Thus, molecular biology is increasingly becoming an integral part of basic as well as clinical hepatology. In the following we will briefly review current concepts and potential applications of gene therapy for the treatment or prevention of various liver diseases.

Genetic classification of liver diseases

Genetically, human diseases can be classified into three major categories [1-4]: (1) Hereditary monogenic diseases that are caused by a single gene defect and inherited by the classical Mendelian rules. There are more than 4,000 monogenic diseases described. For an increasing number of these diseases the genetic basis is being identified; (2) Acquired monogenic diseases are infections as well as malignancies that are caused by the mutation or epigenetic modification of a single gene; (3) Complex genetic diseases are associated with mutations of several genes that are acquired and frequently accumulated during life-time. Several common human diseases belong to this category, such as most malignancies.

Gene therapy is defined as the introduction of genetic material into human cells with a therapeutic or preventive benefit. In a broader definition, cell or organ transplantation are included. In the following we will discuss the basic concepts of gene therapy [1-7] as well as some therapeutic and preventive applications for liver diseases.

Table 1. **Concepts of gene therapy**

Diseases	Gene Therapy
Hereditary monogenic diseases	Gene repair
	Gene replacement
	Cell therapy or organ transplantation
Acquired monogenic diseases	Block of gene expression
	DNA vaccination
Complex genetic diseases	Gene augmentation
	DNA vaccination

Table 2. **Hereditary monogenic liver diseases (selection)**

Gene	Disease
UDP-glucuronosyl transferase	Crigler Najjar syndrome type I
Alpha-1-antitrypsin	Liver cirrhosis, emphysema
CF transmembrane regulator	Mucoviscidosis, cystic fibrosis
Factor VIII	Hemophilia A
Factor IX	Hemophilia B
Fumarylacetoacetate hydrolase	Tyrosinemia type 1
LDL receptor	Familial hypercholesterolemia
Ornithine transcarbamylase	Hyperammonemia

Molecular therapy of liver diseases

Based on the genetic classification of diseases detailed above, the principle of gene therapy involves 6 therapeutic concepts (*Tab. 1*): gene repair, gene substitution and cell therapy for hereditary monogenic diseases, block of gene expression and DNA vaccination for acquired monogenic diseases and gene augmentation and DNA vaccination for complex genetic diseases. For clinical applications, gene therapy is explored with the aim to either provide novel therapeutic strategies for diseases for which there is no treatment available or to replace and in some cases complement existing treatment modalities, thereby increasing therapeutic efficacy and/or reduce adverse events.

Gene repair

An increasing number liver diseases has been molecularly defined as a defect of a single gene (*Tab. 2*). In this context, one therapeutic concept is the *in vitro* or *in vivo* repair of the defective gene. Indeed, in the Gunn rat model of the Crigler-Najjar syndrome type I Kren et al. [8] were able to partially correct the genetic defect underlying the UDP-glucuronosyl transferase deficiency by the intravenous injection of a cyclic normal/wild-type chimeric oligonucleotide. While these findings have not been independently confirmed or extended to other hereditary monogenic (liver) diseases, the data suggest that it is in principle possible to repair a gene defect *in vivo*. Further, it has been shown that cellular RNA species can be modified by trans-splicing group I ribozymes. Such ribozymes may in principle allow to treat a variety of inherited diseases at the RNA level [9-11].

Gene substitution

The targeted substitution of a defective cellular gene by the normal/wild-type homologue with production of the physiological gene product is another approach to correct a hereditary or acquired monogenic gene defect. Indeed, in an animal model of hereditary tyrosinemia type 1 (HT1), a liver disease caused by a deficiency of fumarylacetoacetate hydrolase (FAH), multiple injections of a retroviral vector carrying the FAH gene resulted in a gene transfer efficiency of >90% of hepatocytes and the restoration of a normal liver function [12,13]. In patients, examples for gene substitution are the partial correction of severe hemophilia A by the *ex vivo* transduction of autologous skin fibroblasts with the normal/wild-type factor VIII gene, followed by laparoscopic implantation of the genetically modified fibroblasts into the omentum majus [14] or of hemophilia B by adenovirus-associated vector (AAV)-based gene transfer [15].

In rare situations in which a hepatocelluar carcinoma (HCC) is caused by the mutation of a tumor suppressor gene, e.g., the p53 gene, the substitution of the mutated by the normal/wild-type gene *in vitro* can reduce the number of tumor cell colonies and restore cisplatin sensitivity [16,17].

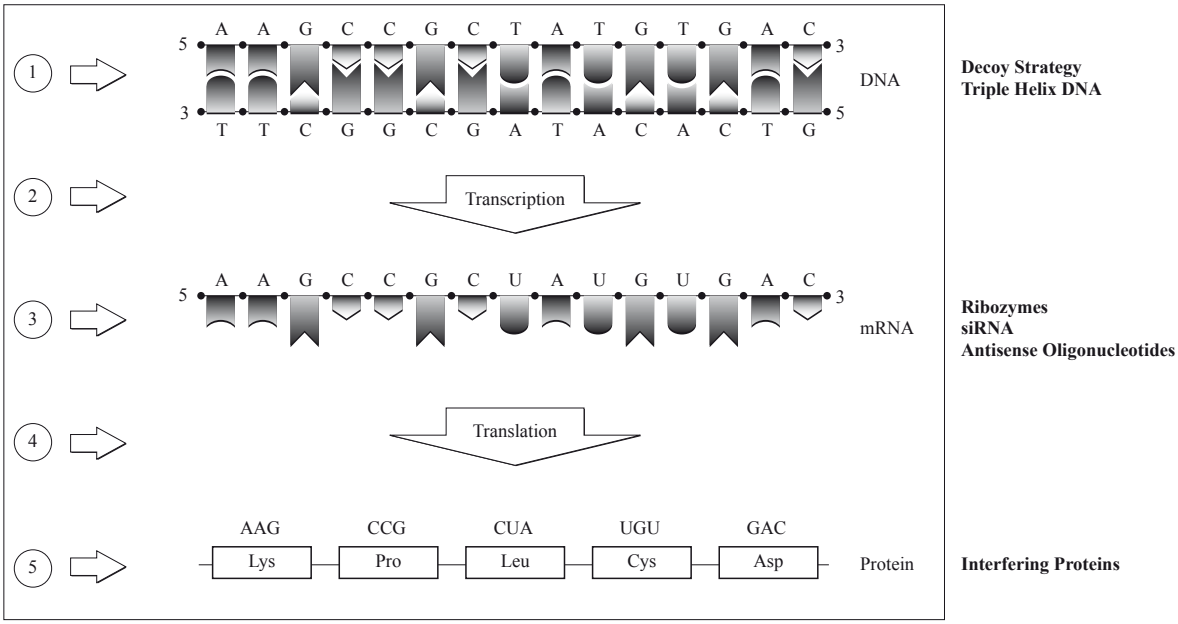
Cell therapy

Allogeneic or *ex vivo* genetically modified autologous hepatocyte transplantation is a promising strategy to treat hereditary monogenic liver diseases. In patients with familial hypercholesterolemia (FH) that is caused by various mutations in the low density lipoprotein (LDL) receptor gene [18], apart from orthotopic liver transplantation [19,20], liver-directed gene therapy has been performed in a pilot study in five patients [21,22]. Autologous liver cells, prepared from a surgical biopsy, were transduced *ex vivo* with a recombinant retrovirus expressing the normal LDL receptor. These *ex vivo* genetically modified hepatocytes were transplanted by portal infusion and resulted in significant and prolonged reductions in LDL cholesterol in 3/5 patients for at least four months, demonstrating the feasibility of engrafting a limited number of *ex vivo* transduced hepatocytes. Also, allogeneic hepatocyte transplantation has been successfully used in patients to partially correct Crigler-Najjar syndrome type I [23] and glycogen storage disease type I [24].

Block of gene expression or function

For diseases caused by the expression of an acquired gene or the overexpression of an endogenous gene, blocking gene expression can be an effective therapeutic approach. Several strategies can be employed (*Fig. 1*): interference with the transcription of genes by binding of transcription factors to nucleic acids introduced into or synthesized in the cells (decoy

Figure 1. **Strategies aimed at blocking gene expression**



strategy) [25,26], by binding of single-stranded nucleic acids to double-stranded DNA, forming a triple helix structure [25,26], hybridization of RNA molecules possessing endonuclease activity (ribozymes) to RNA, resulting in its sequence-specific cleavage [27,28], RNA interference (RNAi) by small inhibiting RNA (siRNA) or microRNA (miRNA) [29-32], block of translation by antisense oligonucleotides [25,26,33,34] and the intracellular synthesis of peptides or proteins, interfering with their normal counterpart, termed dominant negative (DN) mutant strategy [35]. These different strategies have been applied to a number of malignant and infectious diseases. In particular ribozymes, siRNAs, antisense oligonucleotides and DN mutants have been experimentally explored to treat hepatitis B virus (HBV) and hepatitis C virus (HCV) infections.

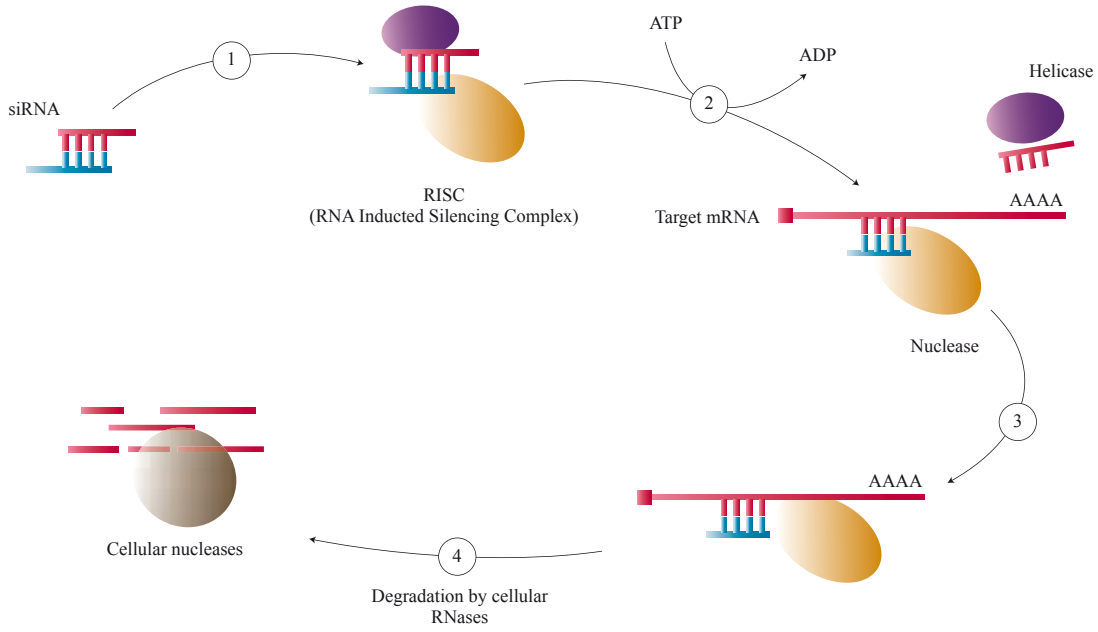
Ribozymes. Ribozymes ('ribonucleic acid enzymes') were originally discovered as naturally occurring RNA molecules that catalyze the sequence-specific cleavage of RNA and RNA splicing reactions [27,28]. This catalytic activity is the major attraction of the ribozyme concept since one ribozyme can cleave many target RNAs. Ribozymes that cleave RNA are being developed as inhibitors of gene expression and viral replication. Several studies have clearly demonstrated that hammerhead ribozymes can specifically cleave HBV RNA [36,37] or HCV RNA [38,39] *in vitro*. *In vivo*, however, an efficient ribozyme-mediated cleavage of HBV RNA could not be demonstrated to date. For HCV infection, the elimination of HCV RNA in infected hepatocytes by ribozymes has also been reported [38,40].

Small interfering RNA. RNAi is a recently discovered basic intracellular mechanism [29-32] that has been explored also for the inhibition of HBV and HCV infection. For HBV, inhibition of viral gene expression and replication has been shown *in vitro* [41-43] and in different mouse models *in vivo* [44-48]. For HCV, inhibition of viral gene expression and replication has been shown *in vitro* in the replicon system [49-51]. While effective in blocking viral gene expression and replication, *in vivo* oversaturation of celluar miRNA/short hairpin RNA (shRNA) pathways can result in lethal hepatotoxicity [48]. For future RNAi-based strategies in animals or humans, these findings indicate that the control of intracellular shRNA expression levels through optimizing shRNA dose and sequence will be key to reduce the risk of oversaturating endogenous small RNA pathways.

Antisense oligonucleotides. Antisense nucleic acids are designed to specifically bind to RNA or mRNA, resulting in the formation of RNA-DNA (antisense oligodeoxynucleotides) or RNA-RNA hybrids (antisense oligoribonucleotides) with an arrest of RNA replication, reverse transcription or mRNA translation [25,26,33,34,52]. Antisense effects can be potentiated by degradation of RNA in RNA-DNA hybrids by cellular RNases H. While conceptually simple, it is clear now that not all desired as well as undesired effects are caused by the target sequence specific antisense action of the oligonucleotides or the cellular enzymes mentioned above [53,54].

The antisense strategy has been successfully applied *in vitro* to HBV infection, [55-58] as demonstrated in *Fig. 2*, and to HCV infection [59-64]. In addition, studies in nude mice[65], in the duck hepatitis B virus (DHBV) [66] and the woodchuck hepatitis virus (WHV) model of HBV infection [67] demonstrated the *in vivo* applicability of this approach. While no toxic effects have been observed in these experiments, the contribution of non-antisense effects to the inhibition of viral replication or gene expression has not been systematically assessed in most studies. Independent of the antisense or non-antisense mechanism of the biological effects, an *in vitro* screening procedure for the identification of functionally active oligonucleotides

***Figure 2*. Principle of RNA interference [31]**



[53,68] should greatly facilitate the design of oligonucleotide based antiviral therapies.

Interfering peptides or proteins. The intracellular synthesis of interfering peptides or proteins, including single chain or whole non-secreted antibodies, is aimed at the specific interference with the assembly or function of viral structural or non-structural proteins and represents a type of intracellular immunization [69]. This approach has been shown for block mammalian and avian hepadnavirus gene expression and replication *in vitro*. For example, the fusion of different polypeptides of various lengths to the carboxy-terminus of the viral core protein yields DN mutants [70-73]. These DN mutants are species-specific and suppress viral replication by at least 90% at an effector to target ratio of 1:10. Moreover, the non-secretory form of the hepatitis B e antigen (HBeAg) was shown to effectively inhibit viral replication and may indeed act as a natural regulator of HBV propagation [74-76]. The potential advantage of DN mutants over ribozymes or antisense oligonucleotides is their relative independence from viral sequence variations, minimizing the risk of selecting or accumulating 'therapy escape' mutants.

DNA vaccination

A novel approach is DNA vaccination resulting in the manipulation of the immune system by introduction of expression vectors into muscle cells or dendritic cells and long lasting cellular and humoral immune responses.The direct gene transfer into muscle [77] represents an exciting new development and elegant application of gene therapy [78,79]. The therapeutic DNA vaccine acts by the intracellular plasmid-derived synthesis of a viral protein which enters the cell's MHC class I pathway [78]. Only proteins that originate within the cell can be processed by MHC class I molecules that carry fragments of the protein to the cell surface. There they stimulate CD8+ cytotoxic T cells, resulting in cell-mediated immunity. In principle, this strategy is applicable to the treatment of acquired genetic diseases, associated with the expression of disease-specific antigens serving as targets for CD8+ cytotoxic T cells.

Therapeutic DNA vaccination has been experimentally explored for HBV [80-84] as well as HCV infection [85,86] and holds great promise as an effective molecular therapy for these viral diseases. In this context, the coexpression of HBsAg and interleukin-2 was shown to greatly increase humoral as well as cellular immune response [87].

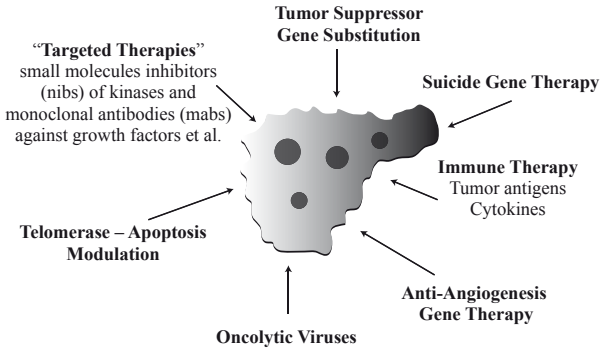
Further, DNA-based tumor vaccination against HCC may be possible, for example, by intramuscular introduction of a plasmid expressing HCC-specific antigens or antigens that are highly overexpressed in HCC cells, such as AF-20 antigen, insulin receptor substrate-1 [88], alpha-fetoprotein [89], aspartyl asparaginyl hydroxylase, mutated p53 protein and others. Potential limitations of this strategy include the regulation of the immune response as well as the low level expression of the targeted antigen in non-malignant cells [90], rendering them susceptible to immune mediated elimination as well.

Gene augmentation

Complex genetic diseases are among the most prevalent clinical problems. In this situation, gene augmentation is aimed at the local expression of a therapeutic gene product that is physiologically not expressed or expressed at therapeutically insufficient levels. This strategy is explored among others for the treatment of HCC.

Suicide gene therapy. An interesting strategy to treat HCCs is genetic prodrug activation therapy *via* the introduction of a 'suicide gene' into malignant cells followed by the admini-

***Figure 3*. HCC treatment: experimental strategies, incl. gene therapy**



stration of the prodrug. This concept has been experimentally explored in HCC cells *in vitro* and *in vivo*, e.g., for the HSV-tk gene [91-95], the gene encoding cytosine deaminase (CD) that converts the prodrug 5-fluorocytosine to 5-fluorouracil which inhibits RNA and DNA synthesis during the S-phase of the cell cycle [96], the gene encoding purine nucleoside phosphorylase that converts purine analogs into freely diffusible toxic metabolites [97,98] as well as the gene encoding cytochrome p450 4B1 [99]. A significant bystander effect of cell killing caused by suicide gene expression could be demonstrated *in vitro* and *in vivo*, based on cell-cell contact rather than release of cytotoxic substances from the transduced cells [100]. At the same time, the bystander effect may also affect non-malignant dividing cells in the target tissue, potentially limiting the application of this strategy.

Immune therapy. In the process of malignant transformation new antigenic surface proteins can be expressed (tumor antigens) or oncofetal proteins can be re-expressed, e.g., alpha fetoprotein (AFP).

AFP-specific immune therapy has been explored in mice and humans. Vaccination with an AFP-expressing DNA construct resulted in tumor rejection and prolonged survival in a mouse model [89]. Also in patients AFP-specific T cells could be detected [101,102]. Since AFP is not only expressed by tumor cells but also by regenerating liver cells and in liver cirrhosis immunization against AFP carries the risk of autoimmune hepatitis, as has been experimentally shown in mice [90].

Immune therapy with antigen presenting cells (APC) is another strategy that has been explored using dendritic cells (DC) exposed to tumor lysates, peptides or *ex vivo* transduced with tumor antigen expressing DNA constructs. While this strategy is conceptually very interesting, to date there are no data available that demonstrate its clinical efficacy [103].

Cytokine gene therapy has been explored using tumor necrosis factor (TNF)-alpha, GM-CSF, interferon-alpha or interferon-gamma, interleukin (IL)-2, -4, -6, -7, -12 and -18, B7-1 as well as CD40 ligand. Complete regression of a HCC was demonstrated *in vivo* by TNF-alpha [104], IL-2 [105], IL-12 [106] and an activatable interferon regulatory factor-1 in mice [107]. Gene transfer was achieved *in vivo* by delivering retroviral [104] or adenoviral vectors [105] systemically, directly into the tumor or into the peritoneal cavity. A pilot study in patients with gastrointestinal tumors exploring the intratumoral injection of an adenoviral IL-12 expression construct showed only marginal efficacy, however [108].

Antiangiogenic gene therapy. This concept has been experimentally explored in a HCC mouse model using the angiostatin gene. Angiostatin gene transfer resulted in reduced tumor volume and vascular density [109].

Oncolytic viruses. This new and elegant approach uses p53 mutations for selective, adenovirus-mediated lysis of tumor cells. Thus, an adenovirus mutant was engineered that replicates selectively in p53-deficient human tumor cells [110-112]. Other examples are the adenoviral introduction of Smac that antagonizes the inhibitor of apoptosis proteins in HCC tumor cells and enhances tumor cell death [113] and tumor-specific replication-restricted adenoviral vectors [114]. Further, the intravascular administration of a replication-competent genetically engineered herpes simplex virus (HSV)-1 resulted in oncolysis of a diffuse HCC [115]. More efficient HSV-1-based vectors have been developed [116].

Molecular prevention of liver diseases

DNA-based prophylactic vaccination against HBV infection, for example, is possible by intramuscular introduction of a plasmid expressing hepatitis B surface antigen (HBsAg). HBsAg is taken up by cells *via* phagocytosis or endocytosis, processed through the major histocompatibility complex (MHC) class II system and primarily stimulates an antibody response through CD4+ helper T cells with the production of anti-HBs [78,79,117-120]. While the DNA-based vaccination against HBV infection induces anti-HBs antibodies and prevents HBV infection, DNA-based vaccination against HCV infection of chimpanzees has been shown not to prevent infection but to result in the resolution of acute HCV infection through an effective vaccine-induced cellular immune response [121,122].

Conclusions

Molecular analyses have become an integral part of biomedical research as well as clinical medicine. The definition of the genetic basis of many human diseases has led to a better understanding of their pathogenesis and has in addition offered new perspectives for their diagnosis, therapy and prevention. Genetically, human diseases can be classified as hereditary monogenic, acquired monogenic and complex genetic diseases. Based on this classification, gene therapy is based on four concepts: gene repair, gene substitution, cell or organ transplanation, block of gene expression or function, gene augmentation and DNA vaccination. While the recent developments in gene therapy for liver diseases are promising, various delivery, targeting and safety issues need to be addressed before these strategies will enter clinical practice. Nevertheless, gene therapy will become part of the management of patients with liver diseases, complementing existing diagnostic, therapeutic and preventive strategies.

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Familial Pancreatic Cancer: a review and latest advances

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Abstract

Familial Pancreatic Cancer (FPC) is the autosomal dominant inheritance of a genetic predisposition to pancreatic ductal adenocarcinoma, penetrance is assumed to be high but not complete. It was first described in 1987 and since then many families have been identified, but the candidate disease gene remains elusive and the very existence of the syndrome is sometimes questioned. FPC identifies a target group for secondary screening. As well as being potentially life saving for the subjects, screening offers researchers the opportunity to elucidate the early pathogenesis of pancreatic cancer. The scientific incentive for screening should not blind us to the challenges facing clinicians in managing high risk patients. Early surgical treatment may dramatically improve the five year survival for pancreatic cancer, but this must be balanced against the risks of false positives, where healthy individuals are subjected to the mortality and morbidity of major pancreatic surgery.

Key words: Familial Pancreatic Cancer, FPC, pancreatic cancer, secondary screening, EUROPAC, Ca19-9, CT, EUS, ERCP, K-ras, p53, p16, BRCA2, anticipation.

Introduction

Familial Pancreatic Cancer (FPC) is the term used to describe the occurrence of multiple cases of pancreatic cancer within families in a pattern consistent with autosomal dominant inheritance. FPC was initially described in 1987 with the first cohort of FPC families presented in 1989 [1]. The emerging evidence for FPC and the potential implications for research into the pathogenesis of pancreatic cancer prompted the establishment of FPC registries around the world. The authors are closely associated with the European Registry of Hereditary Pancreatitis and Familial Pancreatic Cancer (EUROPAC), which was established in 1997.

The definition of FPC has gradually been strengthened by the registries to exclude families that belong to other cancer syndromes, which carry a predisposition to pancreatic cancer (e.g. breast-ovarian syndrome), or hereditary illnesses such as hereditary pancreatitis (HP), which carry an increased pancreatic cancer risk [2]. The exact proportion of pancreatic cancer deaths that are linked to inherited genetic factors remains uncertain although it has been estimated as 10% [3,4].

Early symptoms are subtle and non-specific and by the time the disease presents clinically, the vast majority of pancreatic cancers can only be treated with palliative intent. For patients diagnosed in the UK between 1996 and 1999, five year survival was between 1.7 and 3.5% [5]. There is evidence that this survival rate is improving, based on better treatment modalities for the minority of patients that have resectable disease [6]; Jemal *et al*. estimated the number of new cases of pancreatic cancer in the US in 2007 to be 37,000 [7] with only 33,000 deaths.

Segregation analysis of FPC families suggests a rare major gene conferring predisposition [8], whilst other studies claim an autosomal dominant transmission [9]. Autosomal dominant transmission remains controversial, but mechanistically this is the most likely form of transmission given a single major gene. Inherited predisposition for cancer is usually the result of a heterozygous defect in a tumour suppressor, loss of the second copy of the tumour suppressor being the second "hit". Genetic instability is part of the ageing process and so the second hit will be inevitable if an individual lives long enough.

In the post-genomic world, a genetic syndrome will only truly be accepted once a mutation segregating with the disease

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is identified. In 20% of FPC families a mutation in the BRCA2 gene has been shown to segregate with the disease [49], but for the majority of FPC families, no disease gene has been identified. It is quite possible to have more than one case of pancreatic cancer in a family without any particular genetic predisposition. Thus selection of families retrospectively, on the basis of multiple cases of cancer, could give a false appearance of an autosomal dominant disease.

Potential causes of artefactual familial clustering of pancreatic cancer

To qualify as an FPC kindred on the EUROPAC registry, families need to have at least two proven cases of pancreatic cancer that are consistent with high penetrant autosomal dominant inheritance. For example, a family with pancreatic cancer in both a father and son could be classified as FPC if at least one paternal grandparent died at a reasonably young age (EUROPAC classify this as below the age of 75), but if both paternal grandparents were over the age of 75, then the family would not be classed as FPC. The unavoidable consequence of this is that a kindred with two cancers can be classified as FPC on the basis of an incomplete pedigree, only to be reclassified, as grandparents and great grandparents are added to the family tree. The overall lifetime risk of developing pancreatic cancer for the general population is 0.5-1% [10]. By definition, the chance of developing pancreatic cancer in an FPC kindred approaches 50%. Even so, it is not inconceivable that a large family could have two cases by chance alone.

Selection Bias

Pancreatic cancer patients in the USA were asked to report any other cases in first degree relatives. Approximately one in ten were able to do so [11,12]. The chance of identifying two cases will be determined by the number and ages of individuals in the kindred, furthermore, in these studies all the families will include at least one pancreatic cancer case (the proband), so the chance of two cases in one of these families is roughly equivalent to the chance of finding a single case in an unselected kindred. Case-control studies are desirable, although of necessity more difficult to carry out. In a study of individuals under the age of 75 admitted to Ospedale Magiore [13], the relative risk of a pancreatic cancer patient reporting a first degree relative with pancreatic cancer was 3.3 fold that of controls (95% CI 1.42-2.44). This was based on 14 cancers in 362 families with the proband suffering from cancer compared to 15 cancers in 1408 control families. A subsequent American study indicated very similar relative risks (3.2, 95% CI: 1.8-5.6) [3] comparing the families of 484 pancreatic cancer cases with the families of 2099 controls. Familial studies like these are open to criticism as they are affected by the size and closeness of relationships within the family kindreds and there is no easy way to control for this.

Genetic Factors

The relative influence of genes and environment is a notoriously difficult area, people who share common genetic backgrounds often have similar diets, occupations and customs. Pancreatic cancer has been shown to be more common in black than white Americans [14]; this could be due to low penetrance or multigene susceptibility, or simply that black Americans lead a lifestyle that is more “high risk”. In support of an environmental rather than genetic link, migration studies show that pancreatic cancer risk amongst Japanese migrants moving to the US increases and overtakes the level of cancer risk of white Americans [15]. The most likely cause of this is the Japanese adopting the “Western” high meat, high fat diet. However, a direct link between Western diet and pancreatic cancer has not been shown despite large cohort studies [16]. An indirect link via obesity and diabetes (see below) cannot be ruled out, but neither is there any evidence that it explains the migration studies.

Gender

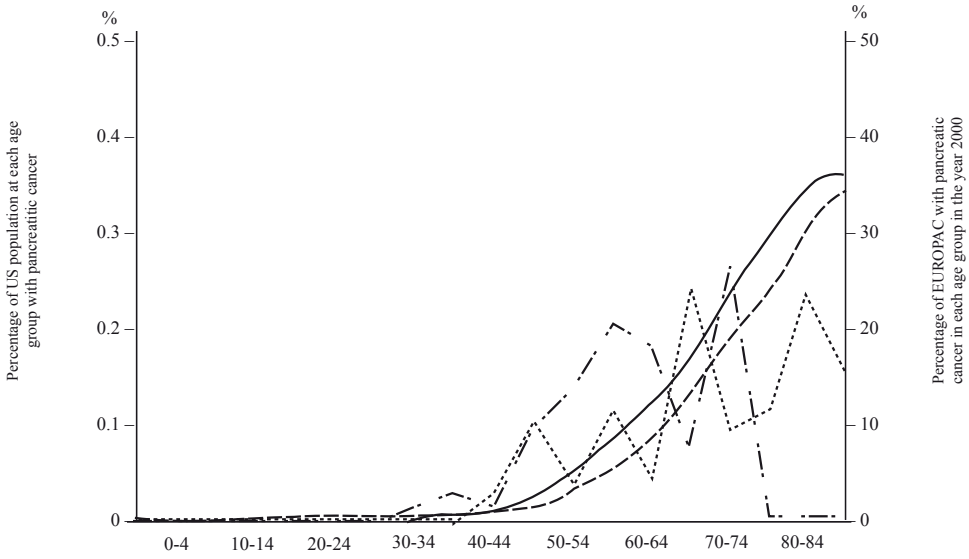
Analysis of the Surveillance Epidemiology and End Results (SEER) data [10] shows a slightly greater incidence of pancreatic cancer in men than women (see *Fig. 1a*). The SEER data is cross-sectional, while familial data, such as that held by familial pancreatic cancer registries is, by definition, longitudinal. To compare the two sets of data it is either necessary to model longitudinal data using the SEER figures or to take a date for a cross-sectional study of the registry data. A comparison has been carried out in *Fig. 1a* with data from the EUROPAC registry, taking individuals alive in the year 2000 and using a five year window for occurrence of pancreatic cancer. The SEER data shows a clear higher incidence of pancreatic cancer for men in all age groups, the data is far less clear cut for the EUROPAC data, although this could be because of the small numbers of at risk individuals in each age group. Overall, in the EUROPAC families death from pancreatic cancer does occur slightly earlier in males, this is shown in the Kaplan-Meier curve in *Fig. 1b*. However, the final lifetime risk for men and women is roughly equivalent in this population (approximately 50%). This is in stark contrast to the situation in sporadic disease.

Environmental and Lifestyle Factors

The best evidence for a link between an environmental risk factor and incidence of pancreatic cancer exists for tobacco smoking [17]. Overall, smoking increases the risk of pancreatic cancer by two-fold [18], with some evidence for a dose-response relationship [19]. The risk posed by passive smoking remains unproven, thus clustering of pancreatic cancer within families is more likely to be related to a common habit shared by family members, than contamination of the family home by a single heavy smoker. Analysis of the EUROPAC database has shown no direct evidence for smoking as the cause of familial clusters of pancreatic cancer [9].

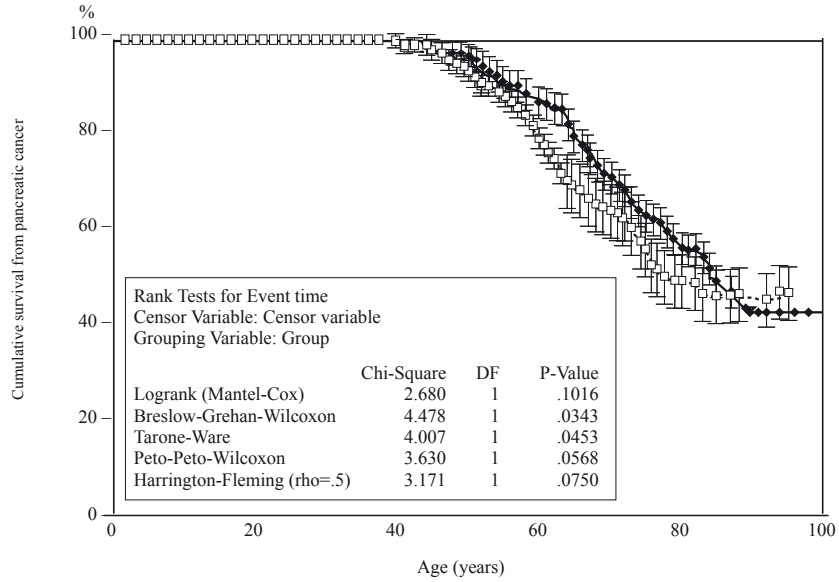
***Figure 1*. Gender and risk of pancreatic cancer in FPC families and sporadic disease.**
In 1a the incidence of pancreatic cancer for each gender is plotted for 5 year age groups using the SEER data, more men develop pancreatic cancer than women in each age group. This is compared to data from the EUROPAC database using a separate scale, taking the age of individuals alive in 2000 and following for pancreatic cancer until 2005. The number of individuals taken for the analysis are given below the graph (E=EUROPAC). There is a trend for a higher percentage of men to develop pancreatic cancer in the earlier age groups, but the small number in each group makes comparison difficult. In 1b a survival curve is plotted for the EUROPAC data, women develop pancreatic cancer significantly later, but overall lifetime risk is equivalent (data as used in McFaul et al. [9])

1a



	Number of at risk individuals in each age range (m=million)								
Female E	36	54	60	72	63	73	42	36	17
Male E	30	53	69	68	61	58	28	23	1
Female SEER	1.4m	1.5m	1.4m	1.5m	1.7m	1.4m	0.8m	0.7m	0.5m
Male SEER	1.5m	1.6m	1.5m	1.6m	1.7m	1.3m	0.8m	0.6m	0.3m

1b



At risk					
Male	437	374	269	135	23
Female	456	383	278	148	36

A link has been shown between pancreatic cancer and obesity [20]. Obesity shows familial clustering, thought to be due to shared behaviours, so this may contribute to some cases classified as FPC.

There has been particular emphasis on searching for a link between pancreatic cancer and occupations that lead to contact with chlorinated hydrocarbons, especially dichlorodiphenyltrichloroethane (DDT), though no definite link has been established [21,22].

Associated Medical Conditions

There are two major illnesses linked to pancreatic cancer; diabetes mellitus and chronic pancreatitis. Some 80% of pancreatic cancer patients have impaired glucose metabolism. Tumours can induce production of diabetogenic peptides which result in insulin resistance reminiscent of type 2 diabetes [23], this can often be alleviated by resection of the tumour [24]. In sporadic disease development of higher baseline fasting glucose levels appears to be a very early symptom of pancreatic cancer [25] but this has not been shown in familial pancreatic cancer patients. It is also possible that diabetes is a risk factor, as well as a symptom, of pancreatic cancer but this remains unproven [24]. Diabetes shows familial clustering and is a feature of Family X, one of the best characterised of all FPC families [26]. It is possible that diabetes could explain some cases classified as FPC on the EUROPAC database, but an analysis has failed to show an increased incidence of diabetes mellitus, above that expected as a symptom of pancreatic cancer.

A second possible cause of familial clusters could be multiple cases of chronic pancreatitis within a family. This could be caused by a shared tendency to heavy alcohol intake or the rare genetic syndrome, hereditary pancreatitis. Chronic pancreatitis has been shown to lead to a 15% lifetime risk of pancreatic cancer [27] and the cumulative lifetime risk increases to 35-40% in hereditary pancreatitis families [28,29].

Interaction of Genetic and Environmental Factors

It is conceivable that multiple cases of pancreatic cancer in a family could be caused by genetic variations other than the elusive FPC mutation that could possibly increase the impact of environmental factors. Such variations, although inherited, would not justify the description of FPC, as the link is indirect and the elevation in risk should not give the prospective appearance of autosomal dominant inheritance of pancreatic cancer. Genetic polymorphisms have already been linked to the development of pancreatic cancer. Pancreatic adenocarcinoma was shown to be associated with the UGT1A7*3 allele of UDP-glucuronosyltransferase, an enzyme known to be involved in detoxifying tobacco carcinogens [30]. Both thymidylate synthase and methylenetetrahydrofolate reductase promoters have a direct association with occurrence of pancreatic cancer [31], a surprising observation as interest in these genes was based on the assumption that they would influence response to chemotherapeutics rather than incidence. Developments in SNP-based array technology and a more empirical approach will allow further predisposing polymorphisms to be identified. However, as over 90% of the population has a very small risk of pancreatic cancer, it is unlikely that any commonly occurring polymorphism would cause a sufficient increase in risk to account for FPC and a rare combination of multiple unlinked polymorphisms should not lead to a family history of pancreatic cancer covering more than one generation.

Cystic Fibrosis (CF) affects multiple systems by causing obstruction of ducts; one organ affected is the pancreas. Two early onset cases of pancreatic cancer were identified in 28,000 cases of cystic fibrosis (odds ratio 31.5 vs control group) [32]. CF is a recessive disease but there are a number of clinical implications for heterozygotes with mutations in the cystic fibrosis transmembrane receptor (CFTR) gene; amongst these is a greatly increased risk of chronic pancreatitis [33,34] and so it is at least conceivable that a similar autosomal dominant inheritance of pancreatic cancer risk may be observed under certain circumstances, or with specific CFTR mutations. A study of 166 early onset pancreatic cancer patients (under the age of 60) found 14 carriers of disease related CFTR mutations (8.4%) compared to 4.1% in controls (odds ratio 2.18, 95% CI: 1.24-3.29) [35]. None of the 14 cancer patients had a family history of pancreatic cancer, which is unsurprising given the fairly modest increased risk.

Autosomal dominant inheritance of a predisposition to other forms of cancer is well known, for example colorectal cancer in hereditary non polyposis colorectal cancer or breast cancer in breast ovarian syndrome. In many cases the disease mutations have been identified by linkage and sequencing of candidate genes, furthermore these mutations have been shown to be common to many families. These inherited syndromes often have a spectrum of cancer sites; some of them include an elevated risk of pancreatic cancer. Therefore, it is conceivable that by chance a family with a more general syndrome will present with pancreatic cancer cases in the absence of other tumours. It should also be understood that although FPC is defined specifically in terms of pancreatic ductal adenocarcinoma, it is possible that ampullary tumours, extrahepatic cholangiocarcinomas, acinar cell tumours and even pancreatic neuroendocrine tumours may have been included due to misdiagnosis when defining a family, accounting for part or all of a familial cluster. It is even possible that misdiagnosis, or misreporting of colorectal or gastric tumours may explain part of a cluster.

Registries such as EUROPAC, the National Familial Pancreas Tumor Registry (NFPTR) and the German National Case Collection for Familial Pancreatic Cancer (FaPaCa) require reliable evidence of pancreatic ductal adenocarcinoma before registering a family. This means that highly penetrant syndromes with known disease mutations are unlikely to be confused with FPC. For example mutations in the VHL gene which cause von Hippel-Lindau syndrome are associated with pancreatic neuroendocrine tumours [36], only occasional pancreatic ductal adenocarcinoma have been reported in these families [37]. Li-Fraumeni Syndrome is associated with p53 and CHK2 mutations. At least 24 families have been reported with multiple cases of pancreatic cancer, which superficially would

be consistent with FPC [38]. However, in the same study the families were followed for 10 years and over 200 cases of non-pancreatic cancer were reported [38]. It is unlikely that such an extreme cancer risk would be missed by even the most cursory family analysis and so such families would not be included as FPC by any of the large registries. Another example, is Peutz-Jeghers syndrome (PJS); autosomal dominant inheritance of hamartomatous polyposis. The reported increased risk for pancreatic cancer is very great (132 fold) [39], this is an adequate level to give multiple cases within a family. However, in the largest study of PJS only 6 pancreatic cancer patients were reported. The reason for the small number of cases is the high mortality from other cancers in these families, so as for Li-Fraumeni it is very unlikely that a PJS family will be mistaken for FPC [40,41]. EUROPAC originally had a policy of screening possible FPC families for the STK11 mutations that cause PJS, but no mutations were identified [42].

Similarly, low penetrance cancer syndromes associated with well defined phenotypes other than cancer would be unlikely to be confused with FPC. For example, mutations in the ATM gene cause ataxia-telangiectasia, an autosomal recessive inherited disease characterised by oculocutaneous telangiectasias, cerebellar ataxia and cellular and humoral immune deficiencies. People with ataxia-telangiectasia have increased cancer risk, estimated at 50 to 150-fold, but this would clearly be a recessive risk. Heterozygotes for ATM mutations have an approximately 3-fold increase in risk [43]. The specific risk for pancreatic cancer is at most marginal [44], it is unlikely that such a low increased risk would give many familial clusters of pancreatic cancer and even if this did occur, a familial history of ataxia would be likely. Familial adenomatous polyposis (FAP), which is caused by a mutation of the tumour suppressor gene APC, is characterised by the presence of multiple adenomatous polyps within the gastrointestinal tract. The colon is the most commonly affected site and there is a high incidence of colon cancer. The elevation in risk of pancreatic cancer is relatively small, 4.46 (95% CI: 1.2-11.4) or 21.4 cases per 100 000 person years [45]. Although it is possible that a family would contain multiple cases of pancreatic cancer, due to the numbers of colonic cases, an FAP family would be unlikely to be diagnosed as an FPC kindred.

Although the majority of cancer syndromes are unlikely to be confused with FPC by major registries, there appears be heterogeneity in the phenotype associated with certain mutations, to such an extent that the same mutation may give a well defined syndrome in one family but give a very different phenotype in another. For example, hereditary non-polyposis colorectal cancer (HNPCC) can be divided into two groups (Lynch syndromes I and II). Both syndromes result from mutations in mismatch repair genes but Lynch syndrome I is almost exclusively associated with colorectal cancer whilst Lynch syndrome II features extra-colonic tumours in sites such as the stomach, breasts, uterus, bladder and small bowel; this group shows a clearly elevated risk for pancreatic cancer [46]. Another example is mutation of the BRCA2 gene. This can lead to an autosomal recessive syndrome associated with lymphomas and hepatomas (Fanconi Anaemia), in most cases these families have no noticeable increased risk for pancreatic or breast cancer [47]. In other families BRCA2 mutations are associated with autosomal dominant predisposition for breast and ovarian cancer [48]. Furthermore, other families have an autosomal dominant predisposition for pancreatic cancer without any elevated risk of breast cancer. The latter example includes families that have been defined as FPC [49]. Mutation of the CDKN2A ($INK4a^{p16}$) gene is associated with multiple naevi and cases of melanoma, a syndrome known as Familial Atypical Multiple Mole Melanoma (FAMMM) [50]. In other CDKN2A families there are also one or more cases of pancreatic cancer, this has been described as a separate syndrome (FAMMM-PC, OMIM #606719). To date all FAMMM-PC families have included cases of melanoma, hence the probability of confusion with FPC is low. Testing of genuine FPC families has yet to identify any CDKN2A mutations [51].

The evidence for FPC

Epidemiological evidence

The sheer number of families that are included in registries provides strong evidence pointing towards FPC as a genuine genetically defined syndrome. EUROPAC has registered 250 families with multiple cases of pancreatic cancer, of which 83 are consistent with a specific autosomal dominant predisposition for pancreatic cancer; the remaining families can be explained by the causes of clustering outlined above. Within these 83 families there are no obvious non-genetic risk factors. Although inclusion of some artefactual families cannot be ruled out, the rigorous evidence required to meet the strict inclusion criteria would tend to result in omission of many genuine families, so the incidence of the syndrome may be underestimated. It is likely that the nature of pancreatic cancer in FPC is different from that seen in sporadic cases, but to date no obvious earlier or later onset has been described and differences in molecular biology are still under investigation.

Although, on average, age of onset is similar to that seen in sporadic disease, one phenomenon that has been discovered is “anticipation” [12,52]. In simple terms, the age of onset of pancreatic cancer within FPC families occurs at an increasingly young age in consecutive generations. The fact that average age of onset remains consistent with the sporadic disease is explained by earlier generations having a later age of onset than is normal, compensated for by the younger onset in later generations. This could be explained by various forms of bias, but meticulous statistical analysis suggests that the phenomenon is real [9].

The Genetic Evidence

Identification of the gene responsible for FPC requires a mutation that segregates with the disease. For most genetic syndromes linkage analysis has been used to identify such mutations, but FPC presents particular problems when applying such an approach. Pancreatic cancer is a late onset disease making it difficult to distinguish a carrier who is yet to develop cancer, from a family member who is not carrying the mutation. Ethical and logistical reasons make it impractical to obtain samples from every family member prior to an individual developing the

disease. Once a family member is diagnosed, there is only a very short window of opportunity for research groups to approach patients for DNA, at a time of great stress or denial for those affected. This makes conventional linkage studies very difficult and as a consequence most work has concentrated on candidate genes. Various candidates have been suggested but these have either been found not to be mutated in FPC kindreds, such as STK11 [42], RNasel [53] and various Fanconi anaemia genes [54], or they are only associated with pancreatic cancer as part of more general cancer syndromes, such as CDKN2A [51] and mismatch repair genes [55]. The only exception has been a small number of families which are entirely consistent with FPC which carry BRCA2 mutations [49]. The lack of progress that has been made using candidate genes has prompted a return, despite its problems, to conventional linkage analysis.

To overcome the problem of identifying carriers, Brentnall *at al.* used a surrogate of pancreatic dysplasia for pancreatic cancer. Patients with dysplasia were identified by screening within Family X, a large family characterised by a high incidence of diabetes as well as pancreatic cancer. Using this approach they were able to identify a region at the end of chromosome 4 which gave two point LOD scores of greater than 3, with three point LOD scores reaching a maximum value of 5.36 [26]. The minimum defined area was 4q32-34 and the same group have now provided evidence that the disease mutation for this family lies within the palladin gene [56]. However, recent work from the EUROPAC/FaPaCa study groups [57] and the NFPTR [58] suggest that the 4q32-34 locus is unlikely to account for a significant proportion of families and the palladin mutation has not been identified in other FPC kindreds [59,60].

Work is ongoing in a number of institutions, exploiting novel mathematical models to account for the ambiguity in defining carrier status [9] and new technology, such as SNP arrays, to increase the efficiency of linkage and association studies [61].

Management of risk in FPC

What is the risk?

The definition of FPC as an autosomal dominant condition suggests that risk is equivalent to penetrance, however, this is complicated by the issues of misclassification (as discussed above) and the lack of a recognised disease mutation in most families. It is assumed that penetrance in FPC is high, but not 100%. If penetrance in FPC were 80% to 75 years, then lifetime risk for a mutation carrier would be 80%. The risk to an individual in the same family without a mutation would be that of the general population (0.5-1%) [62]. In the absence of a test for mutation status in most families, the lifetime risk can only be estimated on the basis of some form of probability calculation giving the perceived chance that the individual is a mutation carrier. For example, half of all first degree relatives of pancreatic cancer patients in a genuine FPC family would be mutation carriers; on the basis of 80% penetrance they would therefore be estimated to have a 40% lifetime risk. On the discovery of a disease mutation the estimation of risk for these same individuals would rise to 80% or fall to that of the general population. This does not take into account the possibility that the family only appears to be FPC. An attempt at risk quantification was performed by Klein et al. [63]. A prospective registry-based analysis showed that members of families with one confirmed pancreatic cancer death had a 4.6 fold increase in risk over the general population. If there were two confirmed cases the risk increased to 6.4 fold and was increased 32-fold in families with three affected members. Ignoring low penetrance conditions, this equates to estimation of the likelihood that an individual is a member of an FPC family.

Lifetime risk is a poor measure when considering the possibility of screening to identify cancer cases. As will be discussed below, the benefits of identifying an early cancer must be balanced against the loss of quality of life adjusted years as a result of the morbidity and mortality associated with screening and surgery. In order to make a rational decision on the benefits of screening, the short term risk of cancer is much more relevant. Data from the EUROPAC study group was graphed against the SEER data from the United States of America (*Fig. 2a*). This suggests a constant increased risk for all age groups, approximately equating to a 120 fold increase in normal risk (*Fig. 2b*). Even with a 120 fold increase, risk below the age of 40 is negligible. On this basis, EUROPAC only propose screening after the age of 40, although exceptions are made on the basis of anticipation.

Screening Tools

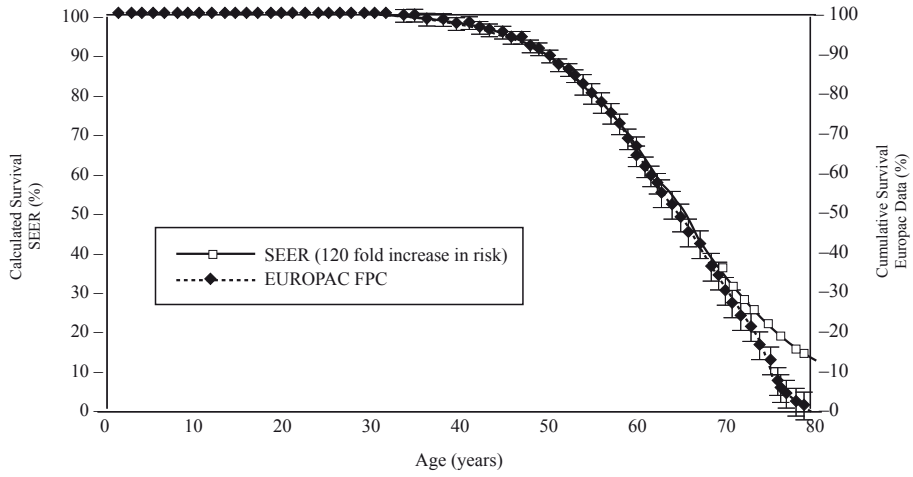
The identification of the high risk group is another way of saying “primary screening”. This is predominantly achieved by careful history taking and confirming causes of death using histological records or cancer registry information. The attempt at diagnosing emerging pancreatic cancers within these groups is “secondary screening”. Members of FPC kindreds are increasingly well informed and generally realise they have an elevated cancer risk, not surprisingly this causes anxiety and a demand for some form of surveillance.

Screening for pancreatic cancer is particularly challenging because the blood testing and imaging available are insufficiently sensitive and specific to detect curable pancreatic tumours without an unacceptably high number of false positives. Unlike many other high risk groups, members of FPC families are generally healthy when they approach clinicians for screening. A positive test could result in major pancreatic surgery, which carries a perioperative mortality rate of approximately 4%, even at the best centres [64]. In addition to the risk of death, any false positives could lead to resection of healthy pancreatic tissue rendering the patient dependent on pancreatic enzyme supplementation and insulin for life.

In addition to the morbidity of the operation there is also morbidity associated with the screening modalities. Ideally the screen should be safe and non-invasive, in practice the closest that is possible to this ideal, is a serum test. Many such tests have been proposed, the most commonly applied diagnostically is serum Ca 19-9. This is a sialylated Lewis antigen produced by patients with digestive tract cancers, particularly those of the pancreas and biliary tree. Estimates of sensitivity and specificity vary depending on the size and stage of the tumours

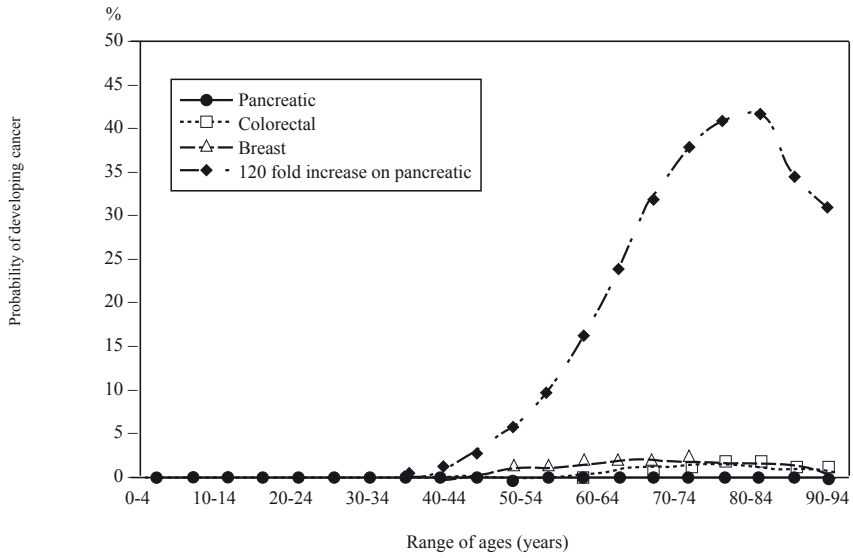
Figure 2. **Age specific risk of pancreatic cancer in families with FPC.**
The Surveillance Epidemiology and End Results (SEER) survey in the USA has provided cross-sectional data on cancer risk in five year ranges. This can be used to model longitudinal cumulative survival, allowing comparison with a Kaplan-Meier survival curve. Approximately 50% of the FPC family members are predicted to be mutation carriers, meaning minimum survival from pancreatic cancer of all potential carriers should be approximately 50%. The model of the SEER data matches with survival from pancreatic cancer produced with EUROPAC data (A) if each 5 year risk is increased by 120 fold (B) (data as used in Greenhalf et al. [103])

2a



At risk (EUROPAC)	2011	1772	1311	689	133
Cumulative events (EUROPAC)	0	0	14	158	359

2b



and the nature of control groups. Estimates of sensitivity in the literature range from 67-92% and specificity ranges from 68-92% [65-68]. These values were all obtained using samples from symptomatic patients, when used as a screening modality these figures would be far worse. Only 50% of cancers <2 cm are associated with a rise in Ca19-9 [69] and it is rarely elevated in the presence of dysplasia [70]. The obvious limitations mean that it cannot be used in isolation in a screening context. In a study of 71,000 patients described as asymptomatic undergoing transabdominal ultrasonography, CA19-9 was found to have a positive predictive value of less than 1% [71]. Other serum blood tests such as Carcinoembryonic antigen (CEA), DU-PAN-2, CA 50, SLX (sialyl difucosyl Lex), ST-439 (sialyl Lex-Tn) and CA 125 are also not applicable as single modality screening tests [72]. The EUROPAC study group take a serum fasting glucose from patients on entry to the screening programme and as part of the screening cycle. Fasting glucose is a known marker for early cancer in sporadic cases, although this has yet to be proven in familial pancreatic cancer [25]. New serum markers are under investigation.

There are a number of imaging tests available. Each has its unique advantages and disadvantages. The simplest method is transabdominal ultrasound (US). It is non-invasive, readily acceptable and involves no ionising radiation. However, the physical distance from the abdominal wall to the pancreas and the number of tissue interfaces involved requires the use of low frequencies, limiting the picture quality. Whilst the sensitivity of transabdominal ultrasound in the detection of pancreatic cancer is 95% in tumours >3 cm, it reduces dramatically with smaller tumours [73,74]. Nevertheless, the advantages of US mean that it has been applied for screening. Periodic US checks were performed by Tanaka *et al*. in a group of high-risk patients. Patients over 35 years old were recruited on the basis of pancreatic duct dilatation, pancreatic cysts and common bile duct dilatation [75]. Serum amylase, elastase-I, alkaline phosphatase, bilirubin, fasting glucose, Ca19-9, CEA and a pancreas-specific US were carried out every three or six months. Any abnormality prompted a CT or ERCP with pancreatic juice collection. Of the 393 patients enrolled, pancreatic cancer was diagnosed in 41 patients. Eighteen patients had a surgical resection, three of which turned out to be false positives. Despite these encouraging figures, screening was not necessarily of benefit to these patients. Only four patients had stage I disease at diagnosis and one of these died within three years despite treatment [75].

Computed Tomography (CT) produces a three dimensional image of the pancreas using a computer to convert information derived by conventional Roentgen principles. There is evidence to support its use in the detection of early pancreatic tumours. One paper described its diagnostic accuracy for this to be as high as 85-90% [76], although other papers have found CT less useful giving a sensitivity of 69-83% and a specificity of 59-93% [77-79]. There is a significant reduction in specificity in the presence of chronic pancreatitis and CT has insufficient resolution to detect PanIN lesions. CT scanning also carries the disadvantage of exposing the patient to 10 mSv of radiation for each abdominal CT performed [80]. With at least some FPC kindreds shown to have a DNA repair defect (BRCA2) [49], the repeated use of ionising radiation to image the pancreas needs to be thought through carefully.

Magnetic Resonance Imaging (MRI) has many of the advantages of CT scanning. It is fast, non-invasive and produces a three dimensional image of the anatomy of the pancreas. MRI has the advantage that it does not involve the use of ionising radiation, but the low resolution and the large number of artefacts produced with movement have in the past limited its use [81]. Recent advances in MRI have improved the imaging of pancreas cancer, the contrast agent mangafodipir trisodium enhances normal pancreatic parenchyma but not neoplasms [82,83]. It has even been reported that T1 weighted spin-echo MRI can be superior to spiral CT imaging for detection of small lesions [83]. The reported sensitivity of MRI ranges from 83--87% and specificity 81-100% [79,84,85].

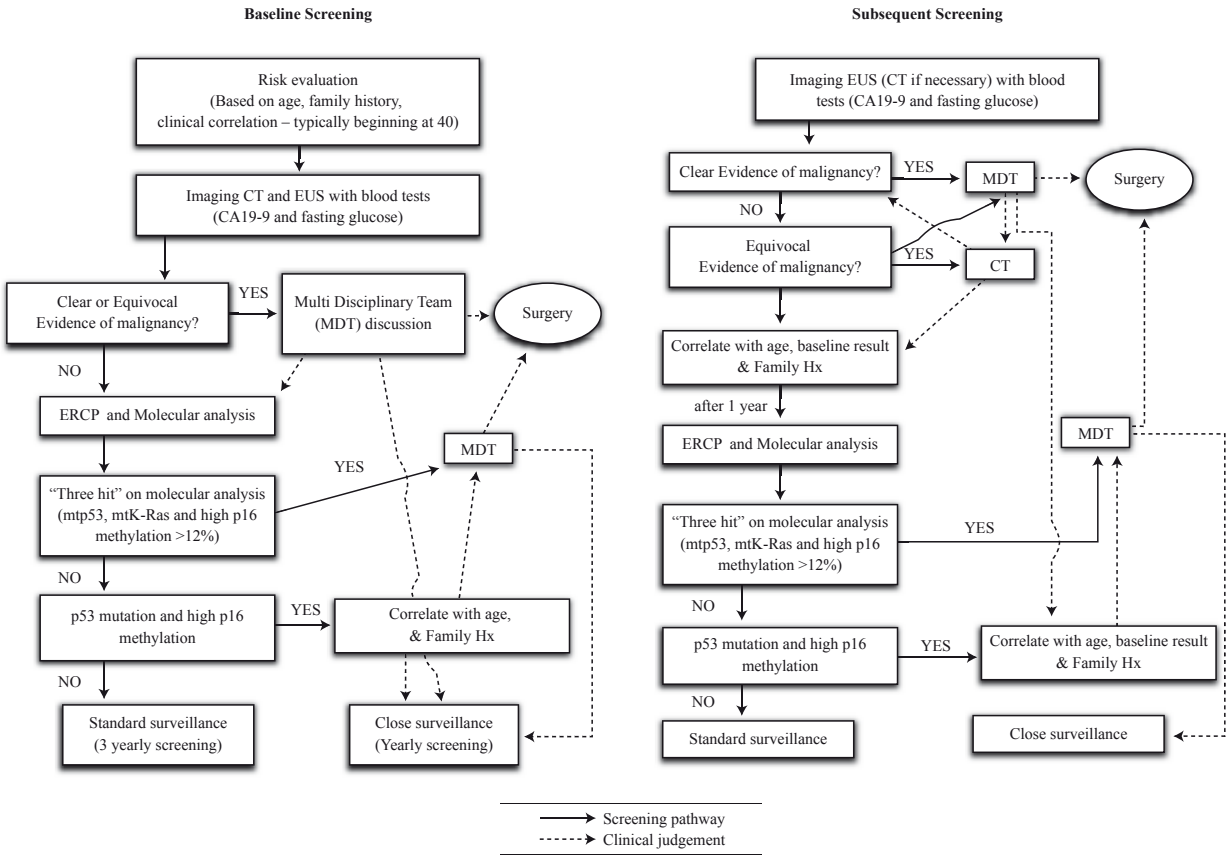
Endoluminal Ultrasound (EUS) is the imaging method of choice in patients with healthy pancreatic tissue. EUS is low risk and has a very high sensitivity (> 90%) for the detection of pancreatic masses, even in patients with very early tumours [83,86,87]. It has been suggested that the changes consistent with precancerous lesions can also be detected, making it ideal for a screening programme in high risk groups. Changes in duct histology and cytology have been observed in patients with tumours and it is widely assumed that there is a progression from normal duct architecture, through various morphological stages leading to carcinoma. These morphological changes are called pancreatic intraepithelial neoplasms (PanINs) which are numbered from 1a to 3 according to increasing abnormality. PanIN 1a lesions are little more than elongation of the ductal cells whilst PanIN 3 lesions have large displaced nuclei and are papillary, often budding off into the lumen of the duct. PanIN lesions may cause parenchymal heterogeneity, which can be visualised using EUS as echogenic foci and hypoechoic nodules. There is also a suggestion that mucin changes resulting from PanINs cause the main pancreatic duct to become hyperechoic [88]. Other lesions commonly assumed to be associated with development of pancreatic tumours are Intraepithelial Mucinous Neoplasms. These can be visualised as cystic masses [89].

EUS is not good at distinguishing between benign lesions and cancers. In a small study (n=85) aimed at distinguishing between chronic pancreatitis and pancreatic cancer, positive predictive value was only 60% based on imaging alone [90]. To improve specificity, EUS has been used to guide fine needle aspiration (FNA) or "Tru-cut" biopsy from pancreatic lesions. A study in Birmingham, Alabama conducted 300 consecutive EUS-FNA procedures on patients referred with a suspicion of cancer. It showed that diagnosis of cancer in the presence of background pancreatitis remains problematic. Of 22 patients with cancer and chronic pancreatitis, EUS-FNA detected only 14 (64%). In the absence of pancreatitis 180 out of 188 (96%) cancers were successfully diagnosed [91]. These data were obtained with symptomatic patients. It is reasonable to assume that figures would have been worse with asymptomatic cancers.

Endoscopic retrograde cholangiopancreatography (ERCP) has traditionally been used for advanced pancreatic disease and has a crucial role in the management of obstructive jaundice, with the potential to obtain cytology or place stents. A tumour causing a marked stricture could normally be visualised by less invasive modalities. For screening, interest has focused on identification of irregular or ectatic ducts with possible sacculations which are said to be associated with PanINs [88]. These changes normally occur in the side branches or in the tail of the pancreas and require an expert radiologist to perform and interpret. An alternative approach is molecular analysis of pancreatic juice obtained at ERCP [92]. Pancreatic juice is the secretion most intimately in contact with tumours and so may contain either tumour cells sloughed from the duct or cell components, including DNA, from necrotic cancer cells. This approach is only suitable for selected patients on a research basis as the potential benefits must be weighed against the risk of inducing acute pancreatitis during ERCP [93].

Magnetic Resonance Cholangiopancreatography (MRCP) is a non-invasive method of imaging the biliary tree and avoids the risks associated with ERCP. In a prospective study of MRCP using 124 patients referred with a suspicion of malignancy (37 of whom went on to develop pancreatic cancer), Adamek *et al.* found a sensitivity of 84% and a specificity of 94% [94]. Some studies have stressed the value of secretin administration in improving pancreatic ductal details in MRCP [95], but whilst

***Figure 3*. The EUROPAC screening protocol**



MRCP is a useful, non-invasive tool in the diagnosis of pancreato-biliary obstruction, it has not been fully evaluated in the context of secondary screening. The limited sensitivity even with symptomatic tumours suggests it has limited use as a modality in this context.

There is no single serum or imaging test that is sensitive and specific enough to be used in isolation for screening. By combining investigations it may be possible to improve sensitivity and specificity of the overall process, but there are also cost and safety issues relating to screening that need to be addressed. One possibility is to further stratify risk, increasing the pre-test incidence and avoiding unnecessary screening of relatively low risk participants. Stratification according to smoking status, diabetes or gender may all contribute to this, but is unlikely to make an adequate difference. More effective stratification can be obtained by monitoring the presence of cancer related nucleic acid or protein changes in pancreatic juice. Modalities for molecular analysis of pancreatic juice have evolved since the early experiments showing that K-Ras mutations can be detected in cellular material obtained at ERCP [96]. K-Ras is almost ubiquitous in pancreatic cancer [98], but unfortunately it was soon established that K-Ras mutations are also common in the pancreatic juice of control patients [99]. Technical difficulties have restricted detection of p53 mutations as a modality for screening, despite a high proportion of p53 mutations in pancreatic tumours and an apparent high specificity for cancer [99]. Various other markers have been investigated including telomerase expression and methylation of specific promoter sequences. Most of these have shown promise, but this has not been sufficient to justify their inclusion as an independent screening modality [100]. EUROPAC has proposed a combination of different molecular tests to phase their screening programme. The technical aspects of the methods were published in 2005 [92], cell free pancreatic juice samples are analysed for presence of K-ras and p53 mutations and quantification of p16 promoter methylation. It was proposed that a combination of results with the three molecular tests could stratify risk between negligible and 90% probability of cancer. Stratification is less marked in patient groups with a background of pancreatitis (approximately 0 to 50%), but molecular analysis may conversely have the most impact in HP patients where the sensitivity and specificity of conventional imaging is limited [90]. The techniques have yet to be proven in a prospective study and currently there is no clear evidence that the molecular markers seen in the juice of sporadic cancer patients are also seen in patients who develop pancreatic cancer as a result of FPC. At present molecular analysis is used by the EUROPAC study group to determine the frequency of imaging.

Screening studies

There are now three groups (Johns Hopkins, the University of Washington and EUROPAC) that have pilot secondary screening programmes. The most recent of which is that proposed by EUROPAC which is outlined in *Fig. 3*. Participants

are recruited to the EUROPAC registry after consulting a clinical geneticist, at the time of recruitment they are advised that a pilot screening programme is available. Participants are then seen in an outpatient clinic by a consultant pancreatologist. If the participant is over 40 and belongs to a confirmed FPC kindred the possibility of screening will be raised; the limitations of the existing technologies are discussed and the potential risks of the screening modalities are explained, particularly post-ERCP pancreatitis and the risk of radiation exposure. The clinician and the participant then decide what elements of the screening programme are appropriate. The full screening programme includes a baseline analysis involving measurement of fasting glucose and CA19-9, with imaging by CT scan and EUS. Where appropriate ERCP is offered for juice analysis rather than imaging. The baseline results will lead to the participant entering either a "standard" or a "close" surveillance pathway. A deciding factor is the presence or absence of pancreatic juice DNA abnormalities. If patients undergo pancreatic juice analysis and there are no DNA changes detected, they enter the standard surveillance cycle, where serum tests, imaging and the juice analysis are repeated on a three yearly staggered basis. Patients that do not have juice analysis are entered onto the close surveillance pathway. This consists of annual follow up, serum tests and imaging. In almost all FPC patients, as they have healthy pancreatic tissue, the screening modality of choice is EUS. If the baseline imaging confirms significant fibrosis (e.g. chronic pancreatitis) CT is used in preference. To date 39 FPC patients have been screened. The programme has yet to discover its first cancer.

The University of Washington group aims to identify patients with histologically confirmed PanIN 3 lesions or very early pancreatic cancers before they progress to incurable disease. Baseline EUS is performed 10 years prior to the earliest age of onset of pancreatic cancer in that family. If the EUS is normal, the patient is offered a repeat EUS in one year. If the EUS indicates an abnormality unrelated to pancreatitis, an ERCP is offered after a discussion of risk and benefit. Patients with an abnormal EUS and a normal ERCP are followed up by EUS in one year. Patients with an abnormal EUS and ERCP are given the option of continuing with surveillance until a mass forms or obtaining a tissue diagnosis. Histology is obtained via laparoscopic resection of the pancreatic tail as needle biopsies would be inadequate to exclude the presence of PanINs. Out of a cohort of 75 patients, 15 high risk patients with abnormal EUS and ERCP have undergone surgery. Twelve patients had a total pancreatectomy, the remaining three had a partial pancreatectomy or "tailectomy" and chose to continue with surveillance. Of the 15 patients operated on so far, five had PanIN 2 lesions and ten had PanIN 3's. None of the pancreata resected were normal or had pancreatic cancer. In the remaining 60 patients that were being followed with annual imaging one patient developed an unresectable pancreatic cancer (personal communication from Dr T. Brentnall, Washington University).

The Johns Hopkins group aims to identify early pancreatic masses when the lesion is either precancerous or a resectable malignancy. The methods used are baseline EUS and CT with imaging repeated at 12 months. High risk individuals (n=78) were either recruited on the basis of FPC (n=72) or Peutz-Jeghers syndrome (n=6). All participants had an EUS; 67 had images that were considered abnormal, 17 of which were consistent with neoplasia. In patients where EUS detected an abnormality, FNA was performed on the pancreatic head, body, and tail. Spiral CT scanning was performed on the 67 high risk patients with an abnormal EUS and 65 accepted the offer of an ERCP, of which 64 were successful. Although ERCP did identify abnormalities (cysts, saccules, dilated ducts or other signs of pancreatitis), the Hopkins group have expressed the opinion that the benefits of ERCP imaging do not justify the risk of post-ERCP pancreatitis; 5 of the 64 participants where the pancreatic duct was cannulated developed pancreatitis as a result of ERCP. There were eight participants with pathologically confirmed neoplasms, 7 participants underwent a subtotal pancreatectomy as a result of screening. None of the participants undergoing surgery as part of the screening programme had confirmed adenocarcinoma, although IPMN and PanIN lesions were common. One participant had a cyst on CT but developed metastatic pancreatic cancer in the interval before returning to clinic [101].

Cost Effectiveness

Risk and benefit cannot only be considered in terms of patient survival and risk of maleficience; cost implications cannot be ignored. Papers have discussed the cost of cancer screening in HP and Peutz-Jeghers syndrome (PJS). Screening of hereditary pancreatitis patients was considered to be prohibitively expensive; it was calculated that it would cost $164,285 per pancreatic cancer detected [77]. In PJS the cost per life saved was estimated at just $50,000, which is economically viable, but only if all other causes of cancer death in PJS could be eliminated. With existing levels of cancer risk in this syndrome, the cost of screening would rise to a prohibitive $297,000. This cost model also assumed use of molecular analysis to phase screening; without this added element costs would rise even further to $373,000 [103]. Potentially, FPC costs would be much lower as the proportion of the screened population that would be expected to develop pancreatic cancer would be much higher. Estimation is complex, but with the use of molecular analysis, the figure would be at, or below, $50,000 per life saved. A better idea of the true cost will be possible once the results from the pilot screening programmes have matured.

Recent advances and future challenges

The most exciting and contentious topic to emerge relating to FPC in recent months is the discovery of a Palladin mutation in Family X. The data were published late in 2006 and initially looked as if they were a definitive breakthrough. However, subsequent work has suggested that Palladin may not be the FPC gene. Work by the EUROPAC/FaPaCa [57] study groups and the NFPTR [58] has shown that the 4q32-34 locus (the site of the Palladin gene) is unlikely to account for a significant proportion of families and the Palladin mutation has not been identified in other FPC kindreds [59,60].

Another contentious issue is anticipation. Rigorous testing suggests that this is a genuine phenomenon, although detractors may still question whether it is due to a statistical artefact [9]. Inclusion of anticipation in models used for linkage analysis may allow successful application of linkage or association studies in the hunt for FPC mutations.

The greatest clinical benefit that research can provide to members of FPC families would be a viable screening programme. Pilot studies have highlighted many ethical and management dilemmas. Hopefully, in the near future, they will begin to provide the solutions. Once this has been achieved, there may be scope for introducing screening in other high risk groups such as late onset diabetics. Demand for screening will surely grow as high risk individuals become more aware of their risk, but until issues of efficacy and safety have been resolved, secondary screening should only proceed in specialist centres. The process requires a collaborative approach between groups developing new screening modalities and those carrying out pilot trials.

Conclusions

Familial pancreatic cancer is a genuine syndrome leading to autosomal dominant inheritance of a predisposition to pancreatic cancer. Individuals from these families recognise they are at high risk and demand screening; but in most cases the lack of a known disease mutation makes identification of those at greatest risk difficult and the lack of a proven screening protocol limits our ability to help family members. Despite the challenges, progress is being made in many directions. It is likely that the FPC gene will soon be identified and in parallel, new screening modalities are being developed and applied in pilot studies. If risk can be adequately defined, screening studies offer the hope, not just of a greater understanding of pancreatic tumorigenesis, but the potential for early diagnosis and cure in high risk groups.

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Supplements of interest for sport-related injury and sources of supplement information among college athletes

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Abstract

Purpose: This study examined incidence of sport-related injury, interest in supplements to treat injury, and sources of supplement information among 145 college athletes (89 males, 56 females).

Materials and methods: A survey was used to assess sport-related injuries, interest in three categories of supplements to treat injury, and sources of supplement information among college athletes who used athletic training room and weight training facilities. Pearson χ^2 was used to evaluate differences in frequency distribution of responses by sex.

Results: Sport-related injuries were experienced by 91% of athletes (93% males, 88% females). Overall, 17% of participants were interested in supplements to improve circulation, 34% for joint and soft tissue repair, and 22% to reduce inflammation. Significant sex differences were not found for any supplements in any categories evaluated. Males were more likely than females to rely on strength coaches (37%, 20%) for supplement information. Athletic trainers (71% of athletes), coaches (60%), and physicians (41%) were the primary professionals, and the internet (79%), magazines (68%), and television (52%) the most popular sources of media for supplement information.

Conclusions: The majority of athletes experience injury during their college athletic career and 17% to 34% express an interest in supplements for injury treatment. Athletes would benefit from scientifically sound guidance to identify appropriate supplements for injury treatment and internet sites for supplement information. Future research should identify if athletes are more likely to increase supplement use when they are injured or if supplement use is more prevalent among athletes who are prone to injury.

Key words: male, female, circulation, soft tissue, inflammation.

Introduction

Supplements are marketed to athletes to improve health and performance and accelerate the body's recovery from exercise, injury, and the healing process [1-3]. Supplements, also referred to as dietary or nutritional supplements, were defined into law by the Food and Drug Administration in the Dietary Supplement Health and Education Act as substances that are taken by mouth, contain a dietary ingredient that is intended to supplement the diet, and are not represented for use as a conventional food or sole item of a meal or diet [4]. Use of vitamin, mineral, and herbal supplements are a popular practice among adults for nonspecific reasons such as "health" [5]. Among athletes, supplements are reportedly taken to improve athletic performance, build muscle, prevent illness, to provide nutrients that are lacking in the diet, and based on recommendations by sports professionals, family members, and friends [6]. Supplement use among college athletes is a reportedly common practice. For example, Burns and colleagues [7] found that 88% of National Collegiate Athletic Association (NCAA) Division I athletes used one or more supplements and Krumbach and colleagues [6] reported 31% to 83% of college athletes taking supplements.

It has been proposed that injury rate is high among young adult athletes (20 to 24 years old), as compared to younger and older athletes, because training and competition are extremely intense at this age [8]. Among children and young adult athletes, 20 to 24 year olds have the greatest incidence of sport

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injuries [8]. For team sports, 46% to 59% of injuries occur during competition, whereas for individual sports, 70% occur during training [8]. Furthermore, overuse injuries are common among highly competitive athletes, such as college athletes, due to a combination of factors [9], including high training volume, high training load, and repetition associated with developing, fine-tuning, or changing techniques. Because many college athletes want to quickly recover from sport-related injuries, supplements that are marketed to treat sport-related injury may be of special interest to them.

The specific objectives of this study were to: (a) identify the incidence of sport-related injuries among college athletes, (b) identify if three categories of supplements related to injury treatment, including those marketed to improve circulation, accelerate joint and soft tissue repair, and reduce inflammation, are of interest among college athletes, (c) identify sex differences for supplements of interest, and (d) determine the primary sources of people and media used by college athletes for supplement information. Adapted from the Food and Drug Administration definition of dietary supplements [4], and the descriptions of supplements of Burke [1] and Juhn [3], supplements are defined in this study as amino acids (e.g., L-arginine), herbs (white willow bark), fatty acids (omega-3 fatty acids), food supplements (flax seed), and products with combinations of these substances (joint support supplements) that are marketed by the supplement industry to treat sport-related injury.

Materials and methods

From mid-January through March, 2006, three trained research assistants recruited NCAA Division I athletes and one institution-sponsored sport program (cheerleading) at a single college from athletic training room and athletic weight training facilities that are used exclusively by college student-athletes. The institution is a state university, located in the Southeastern region of the United States, with an undergraduate enrollment of approximately 18,000 students. Research assistants recruited all student-athletes who entered the athletic training room or athletic weight training facility when the research assistant arrived. To diversify our sample, research assistants varied the time of day between 06:00 and 18:00 hour and days of the week during weekdays to recruit participants. In compliance with the university's Institutional Review Board for Research with Human Subjects, athletes were informed of the study protocol. Written informed consent was obtained and those willing to participate completed the self-administered questionnaire.

Overall, 145 athletes completed the study, which represents a 30% participation rate among all student-athletes at the university, as determined by team rosters. There was a 95% participation rate among student-athletes who were invited to participate. Those who declined participation reported not having enough time or not being interested in completing the survey. All university-sponsored non-club sport teams, with the exception of golf, were represented. Football (24% of participants), swimming and diving (13%), track and cross country (12%), soccer (11%), and basketball (6%) were 3% to 6% over-represented as compared to total athletes competing in their respective sport as a student-athlete at the university. Baseball or softball (9% of participants), tennis (3%), and volleyball (3%) were representative of athletes by sport, whereas cheerleading (3% of total participants) was under-represented; 6% of total-student athletes at this university were cheerleaders. Mean (± SD) age of participants was 20 ± 1.4 years. Participation was fairly evenly distributed between lower- and upper-classmen; 48% were 1st- and 2nd-year classmen, whereas the remainder were 3rd-, 4th-, and 5th-year classmen. Sixty-eight percent of participants were non-Hispanic white, 29% were non-Hispanic black, 2% were Asian, Pacific Islander, or Hispanic, and 1% did not indicate their racial origin.

We designed a questionnaire that assessed demographic information, prevalence of sport-related injuries, and supplements that student-athletes expressed interest (had used, were using, or presently considering using) to treat sport-related injury. The questionnaire was developed by a Registered Dietitian after interviews were conducted with three student-athletes who had used supplements to treat sport-related injuries, and a strength coach, all from the university, and two nutrition store employees. The questionnaire was reviewed for content validity by the head athletic trainer, a strength coach, and two student-athletes, all from the university, and two nutrition store employees. To pilot test the survey, eight college student-athletes from various sports completed the questionnaire. Only minor syntax modifications to the questionnaire were necessary, based on their responses. The supplements listed in the final questionnaire contained three categories for injury treatment; supplements to improve circulation, joint or soft tissue repair, and to reduce inflammation. Three to four supplements were listed for each category. Each of the supplements listed were unique to that category, with the exception of methyl-sulfonyl-methane (MSM), which was listed in joint and soft tissue repair as well as to reduce inflammation categories. MSM had a dual-listing because it is marketed by the supplement industry for both of these purposes. Athletes checked the supplements, by category, that they had an interest. The following are the original main questions that elucidated the "interest in three categories of supplements" as stated above:

The following are supplements and diet changes (grouped into areas) that are promoted as preventing or enhancing recovery of sport injuries.

Put a check by all of the following supplements and diet changes you would consider using, have used, or are currently using to prevent or enhance recovery of sport injuries:

Improve circulation

nitric oxide supplement (NO_2 hemodilator); example – Oxylene

L-arginine supplement

alpha-ketoglutarate (AKG) supplement.

Joint or soft tissue repair

glucosamine, chondroitin, and/or methyl-sulfonyl-methane (MSM) supplement

joint support supplements; examples – Triflex, Trivestin, Lubri-Joint, Therajoint, Phosphoplex

***Table 1*. Sport-related Injury Rate of College Student-Athletes by Sex and Academic Classification**

Academic Class	Males (n=89)		Females (n=56)	
	n	% males by academic class reporting injury	n	% females by academic class reporting injury
Freshmen	19 of 21	90%	13 of 15	87%
Sophomore	22 of 23	96%	8 of 10	80%
Junior	10 of 13	77%	13 of 15	87%
Senior	21 of 21	100%	10 of 11	91%
5th-year senior	10 of 10	100%	4 of 4	100%

Note: Academic class was not reported by one male and one female

***Table 2*. Supplements College Athletes Report an Interest to Improve Circulation**

Variable	Frequency "yes" response (%)	χ^2	p (by sex)
Nitric oxide (NO_2 hemodilator)		2.85	.09
Males[a]	17%		
Females[b]	7%		
L-arginine		0.34	.56
Males[a]	8%		
Females[b]	5%		
α-ketoglutarate		<0.01	.95
Males[a]	3%		
Females[b]	4%		

Note: χ^2 = (1, N=145). [a]n=89; [b]n=56

***Table 3*. Supplements College Athletes Report an Interest to Repair Joint or Soft Tissue**

Variable	Frequency "yes" response (%)	χ^2	p (by sex)
Glucosamine, chondroiton, MSM		0.21	.65
Males[a]	25%		
Females[b]	21%		
	Joint support*	0.84	.36
Males[a]	12%		
Females[b]	18%		
S-adenosyl-methionine (Sam-e)		0.34	.56
Males[a]	3%		
Females[b]	5%		
Shark cartilage		0.06	.81
Males[a]	4%		
Females[b]	5%		

Note: χ^2=(1, N=145). [a]n=89; [b]n=56; *Joint support examples included Triflex, Trivestin, Lubri-Joint, Therajoint, and Phospholex; MSM – methyl-sulfonyl-methane

S-adenosyl-methionine (Sam-e) supplement
shark cartilage supplement.

Reduce inflammation
flax seed supplement
increase omega-3 fatty acids in my diet (eating more fish or flax seeds)
methyl-sulfonyl-methane (MSM) supplement
omega-3 fatty acid supplement
white willow bark supplement.

Analyses were performed using JMP IN® software [10]. Descriptive statistics included means, standard deviations, and frequency distributions. Pearson χ^2 was used to evaluate differences in frequency distribution of responses by sex. An alpha level of .05 was used for all statistical tests.

Results

Regarding the first research question, 93% of male (83 of 89) and 88% of female (49 of 56) participants reported injuries from sport participation, which did not differ significantly by sex, χ^2 (1, N=145)=1.36, p=.24, or by academic classification, χ^2 (4, N=143)=5.56, p=.23. Injury incidence ranged 77% to 100% by sex and academic classification, as reported in *Tab. 1*.

Regarding the second and third research questions, 17% of athletes reported an interest in one or more supplements to improve circulation, 34% for joint or soft tissue repair, and 22% to reduce inflammation. By sex, supplements that participants reported an interest to improve circulation are reported in *Tab. 2*, to repair joint or soft tissue are in *Tab. 3*, and to reduce inflammation are in *Tab. 4*. We did not identify significant differences by sex for any supplements in any categories that were evaluated.

***Table 4*. Supplements College Athletes Report an Interest to Reduce Inflammation**

Variable	Frequency "yes" response (%)	χ^2	p (by sex)
	Flax seed	0.15	.69
Males[a]	9%		
Females[b]	7%		
Methyl-sulfonyl-methane (MSM)		0.41	.52
Males[a]	12%		
Females[b]	9%		
	Omega-3 fatty acids	< 0.01	.99
Males[a]	9%		
Females[b]	9%		
	White willow bark	0.23	.64
Males[a]	2%		
Females[b]	4%		

Note: χ^2 = (1, N=145). [a]n=89; [b]n=56

Finally, regarding the fourth research question, both males and females solicited supplement information most frequently from athletic trainers (73% of males, 70% of females), coaches (62%, 57%), and physicians (40%, 43%). Males (37%) were more likely than females (20%) to rely on strength coaches for supplement information, χ^2 (1, N=145)=4.94, p=.03. The most popular sources of media for supplement information included the internet (84%, 75%), magazines (70%, 67%), and television (50%, 53%). Females (23%) were more likely than males (9%) to rely on texts for supplement information, χ^2 (1, N=145)=5.62, p=.02.

Discussion

As the interest in sports nutrition has increased, so have the sales of supplements to improve performance and promote recovery [11]. We found that the majority of athletes had experienced sport-related injuries, and similar to others [6-7], that use of a variety of supplements is a popular practice among college athletes. Burns [7] reported that among NCAA Division I athletes, 73% used vitamins and minerals, 22% used herbs, 40% used protein supplements, and 31% used creatine. Krumbach and colleagues [6] reported a vitamin and/or mineral supplement use range of 20% to 86% among college athletes. The most popular research regarding supplement use among athletes focus on nutrients that may be lacking in the diet (calcium and iron among females), to meet the demands of exercise (vitamins, minerals), and those that promote muscle anabolism (protein and creatine). This is the first study to investigate supplements that are marketed to treat injury among college athletes. We found that 3% to 17% of college athletes were interested in supplements that are marketed to improve circulation, 3% to 25% for supplements that repair joints or soft tissues, and 2% to 12% for supplements that reduce inflammation. It is of interest that some supplements, such as glucosamine, chondroiton, MSM, and joint support supplements, have a moderate interest among college athletes, considering the finding that college athletes have low perceived benefits for use of supplements to promote healing [7]. Further research should investigate the motivation for use of supplements for injury among competitive, young adult athletes. More specifically, if supplement use is more prevalent among athletes who are frequently injured, have injuries that require an extended recovery time, and feel pressure or desperation to recover quickly or prevent future injury.

Of the three categories that we evaluated, joint and soft tissue repair was the most popular category that both sexes had an interest to supplement. In contrast to Froiland and colleagues [12], we did not identify significantly different sex differences for supplements of interest to improve circulation, repair joint or soft tissue, or reduce inflammation. These findings suggest that sports professionals should refrain from stereotyping supplement use by sex. Additionally, sport professionals should be aware that the supplements promoted to treat injury are of at least moderate interest among college student-athletes. Therefore, efforts should be directed toward educating athletes about legal supplements that may promote injury treatment.

Of concern with the growing interest of supplement use among athletes is that many supplements contain NCAA banned substances [14]. The NCAA reported that the primary substances in positive drug tests have been over-the-counter nutritional supplements that contain ephedrine and androstendione [14]. Bents and Marsh [15] reported that 52% of American college hockey athletes from a single conference reported using stimulants before games or practice sessions, and 49% reported using the NCAA banned drug ephedra on at least one occasion to improve performance. The NCAA banned drug classes include substances generally reported to be performance enhancing and potentially harmful to the health and safety of the student-athlete [16]. Further, the US Food and Drug Administration does not strictly regulate the supplement industry, thus the purity of supplements on the market is not guaranteed [13]. Student-athletes who use impure supplements may present with a positive drug test. Thus, the student-athlete should educate themselves about the supplements they choose to take, as ultimately, it is the individual student-athletes responsibility to have a clean drug test, no matter the complexity of the supplement label they have taken [16].

Burns and colleagues [7] reported that 40% of NCAA Division I athletes use athletic trainers, 24% use strength coaches, and 14% use dietitians as primary sources of nutrition information. Krumbach and colleagues [6] reported that 25% of males and 29% of females reported taking supplements due to recommendations of coaches or trainers. Froiland and colleagues [12] reported family members (used by 32%) and fellow athletes (32%) were the most popular sources of nutrition information in their study, and identified sex differences; females were more likely to obtain nutrition information from family members, whereas males relied on store nutritionists, fellow athletes, friends, and coaches. We reviewed sources of people as well as media, and found that males were more likely to rely on their strength coach, whereas females to use texts for supplement information. Both sexes used a variety of sources for supplement information. The most popular people, in addition to strength coaches, included athletic trainers, coaches, physicians, friends and relatives, and nutrition store personnel. Because supplements pose both benefits and risks to athletes [2], and because many athletes rely on the recommendations of coaches and athletic trainers [6], it is important that sports professionals are fully informed of when taking a supplement may pose an advantage or would be contraindicated for athletes whom they work, particularly among those athletes who are most susceptible to injury. Shifflett, Timm, and Kahanov [17] surveyed athletes, coaches, and athletic trainers at NCAA Division I, II, and III programs and found that athletic trainers scored highest on the knowledge test than any other group. Athletes may benefit from scientifically sound supplement information from professionals who are knowledgeable in this area. Furthermore, the most popular media sources used for supplement information by the college athletes included the internet, magazines, the television, texts, and journals. Further research is needed to identify if student athletes recognize legitimate and scientifically sound sources of nutrition information, and how susceptible they are to use supplements that impair health and/or contain NCAA banned drugs [13].

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Relationship between *Helicobacter pylori* gastritis, gastric cancer and gastric acid secretion

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Abstract

Epidemiological evidence strongly indicates that *Helicobacter pylori* infection is an essential factor for the development of most non-cardia gastric cancer. Furthermore, the identification of an effective animal model and a plausible biological hypothesis provide further compelling evidence for its pathogenic role. Nevertheless, it will be some years before prospective studies in humans are able to confirm cause and effect beyond any doubt. In the meantime sceptics point out that the prevalence of *Helicobacter pylori* in different countries do not always correlate with the incidence of gastric cancer. It is unclear why patients with duodenal ulcer (who are almost invariably infected) are protected from the disease. Cancer often develops in patients from whom *Helicobacter* disappeared from the stomach years previously. This paper discusses the relationship between *Helicobacter pylori* infection, the development of gastritis and its evolution to non-cardia gastric cancer. It also addresses possible reasons why the incidence of gastric cancer does not always mirror the prevalence of *Helicobacter* infection throughout the world and why patients with duodenal ulcer may be protected from developing gastric cancer.

Key words: *Helicobacter pylori*, non-cardia gastric cancer, antral predominant gastritis, corpus predominant gastritis, gastric atrophy, gastric intestinal metaplasia, gastric acid secretion.

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Introduction

It is generally accepted that gastric infection by *Helicobacter pylori* was the underlying cause for around 90% of gastric and duodenal ulcers that affected up to 10% of the population of Europe between the end of the 19th century and the middle of the 20th century. It still remains the most important cause of these diseases in many parts of the world. The causal relationship between *Helicobacter pylori* and gastric cancer, however, has been more difficult to prove. Whereas it was relatively simple to demonstrate that its eradication led to lasting cure of peptic ulcer disease, the length of time it takes for gastric cancer to develop following infection has led to logistic and ethical difficulties in designing prospective studies to show the same effect in this disease. Nevertheless, convincing retrospective epidemiological evidence indicates a relative risk of over 20 for patients who have been infected when compared with controls. Animal models show that infection with this class of organism and with *Helicobacter pylori* in particular leads to the development of gastric cancer. A plausible hypothesis for the pathogenesis of the disease based on experimental and observational work with *Helicobacter pylori* has convinced the majority of gastroenterologists that in its absence non-cardia gastric cancer would be rare, whereas this disease is at present the second commonest cause of malignant death in the world.

In spite of the evidence that *Helicobacter pylori* is responsible for the development of gastric cancer, a number of inconsistencies remain. Sceptics have pointed out that although infection with this organism is widespread in the developing world with a prevalence of around 80%, only a minority develop gastric cancer. More particularly the incidence of the disease varies widely from country to country even when figures are corrected for the age of the population and the numbers infected. Another observation is that the incidence of gastric cancer has fallen rapidly in the developed world. Although some of this change can be attributed to the decline in prevalence of *Helicobacter* infection, the fall in the incidence of cancer seemed to start

earlier and has fallen more rapidly than would have been predicted. Another surprising finding is that patients who develop duodenal ulcer and who are almost invariably infected with *Helicobacter pylori* are unlikely to develop gastric cancer even though they have been infected with *Helicobacter pylori* for many years. Furthermore, although gastric ulcer is known to be associated with gastric cancer, duodenal ulcers never become malignant in spite of the long standing, ulceration and inflammation caused by the organism.

Perhaps it is because of these epidemiological observations that the hypothesis stating that *Helicobacter pylori* is the cause of gastric cancer has met with some scepticism within the medical profession and the public health authorities. There has not been a strong lobby advocating total eradication of *Helicobacter pylori* in any country even in those with a high incidence of gastric cancer. This is in contrast to the overwhelming public and political pressure on governments to introduce colonoscopic screening for the prevention of colonic cancer, a policy which is more expensive and carries higher personal risks than a *Helicobacter pylori* eradication campaign.

This paper addresses the relationship between *Helicobacter pylori* infection, the development of gastritis and its evolution to cause non-cardia gastric cancer. The possible reasons why the incidence of gastric cancer does not always mirror the prevalence of *Helicobacter* infection throughout the world are also addressed.

Helicobacter pylori gastritis

Helicobacter pylori a gram negative, flagellated, curved organism is a parasite of the human gastric mucosa. It is adapted to colonize the mucus that overlies the specialized gastric epithelial cells to which it adheres, gaining nutrition and causing damage. During its evolution *Helicobacter pylori* has acquired a powerful urease that protects it from gastric acid and it has also developed mechanisms that enable it to evade the host immune system [1], so it usually persists within the stomach for many years, often indefinitely. All individuals infected with *Helicobacter pylori* have microscopic gastritis characterized by the infiltration of chronic inflammatory cells, with super added neutrophil leucocytes indicative of continued active inflammation. The gastritis varies in its severity between individuals. The pattern of inflammation may be limited to the antral region of the stomach, or it may affect the whole stomach, or predominantly affect the corpus [2].

Long continued inflammation if sufficiently severe causes destruction of the glandular mucosa of the stomach leading to atrophy and hypochlorhydria, and the gastric epithelial cells become metaplastic taking on the characteristics of intestinal mucosa. These changes are patchy initially, but they may eventually coalesce and progress to extensive intestinal metaplasia.

The association of *Helicobacter pylori* with gastric cancer

It has long been recognised that gastric cancer arises in stomachs that have developed atrophy and intestinal metaplasia [3]. Until *Helicobacter pylori* was discovered the only recognised cause of chronic atrophic gastritis was autoimmune gastritis but this accounted for only a minority of cases of gastric cancer. When it was realised that *Helicobacter pylori* caused chronic gastritis, leading to gastric atrophy and intestinal metaplasia, it seemed a reasonable assumption that this organism was responsible for most gastric cancer. Early epidemiological studies demonstrated a positive relationship between the prevalence of *Helicobacter pylori* in European countries and the incidence of gastric cancer [4]. Based on these data the IARC concluded that *Helicobacter pylori* was a Group 1 carcinogen causing gastric cancer by its destructive effect on the gastric mucosa and the development of intestinal metaplasia [5].

More persuasive data favouring the association has been derived from studies in which serum acquired from normal individuals some years previously for the purposes of long-term epidemiological studies in other diseases were analyzed for *Helicobacter pylori* antibodies. The results showed a strong association between the infection and the subsequent development of gastric cancer [6]. The odds ratio gave a relative risk of 2.36. An interesting observation was that those sera taken ten years previously gave a higher risk than those banked later at 5.9. When *Helicobacter pylori* has been present for many years and the stomach becomes atrophic and hypochlorhydric the intragastric environment is no longer optimal for *Helicobacter pylori*. Competing faecal type organisms are able to colonize the stomach at that stage. Furthermore, *Helicobacter pylori* is able to colonize only normal gastric cells, not those that have differentiated into intestinal metaplasia. For these reasons *Helicobacter pylori* disappears from the stomach when atrophy and intestinal metaplasia supervenes. In its absence the antibody titre declines. Thus in patients who are most likely to develop gastric cancer (those with extensive intestinal metaplasia and atrophy) the serology is often negative. Epidemiological research based on ELISA studies for *Helicobacter* serology have therefore underestimated the relative risk of infection. When studies are done on younger patients with gastric cancer, particularly those with early gastric cancer, the risk ratio is as high as 20 [7].

In a recent paper antibodies to the CagA protein were studied [8]. CagA is an antigen present in most of the *Helicobacter pylori* organisms that are responsible for peptic ulcer and gastric cancer. Unlike the antibodies detected by the standard ELISA techniques those to CagA remain in the serum long after the organism has disappeared. When this technique was used to assess *Helicobacter* infection the risk ratio for infection rose to 20, similar to that found in young people with early cancer. This risk is of an order that suggests *Helicobacter* to be essential for the development of non-cardia gastric cancer in nearly all cases.

Animal models

Helicobacter pylori infection in humans is unusual in that it invariably causes inflammation. This is in contrast to the majority of other vertebrates. Although most are infected with species specific *Helicobacter*. In the main these do not cause inflammation. It was some time before a suitable model could

be identified that mirrored the human condition. The most useful one is the *Mongolian gerbil*. This animal can be infected by *Helicobacter pylori* and it causes an inflammation similar to that in humans [9,10]. It causes gastric ulceration, atrophy and intestinal metaplasia. If the animals are kept alive long enough gastric cancer develops [11]. Eradication of the organism at an early stage prevents progression to cancer when compared with those who are not given treatment [12,13].

How does *Helicobacter pylori* cause cancer?

Considerable experimental work has been undertaken to elucidate the pathogenic mechanism that leads from *Helicobacter* infection to the development of cancer. Inflammation induces a hyperproliferative state suggesting that there may be a greater likelihood of mutation [14]. The inflammatory response also stimulates the production of reactive oxygen species [15] that are known mitogens. Apart from the secondary effects of the inflammatory reaction *Helicobacter pylori* itself subverts cell physiology by activating growth factor receptors, increasing cell proliferation, reducing apoptosis and inducing angiogenesis [16]. Some, or all of these factors, may be relevant in the pathogenesis of gastric cancer, however, on their own they do not fully account for the development of gastric cancer.

Non-cardia gastric cancer is associated with gastric atrophy and intestinal metaplasia, however, the distribution of the epithelial damage is of even more importance. A Japanese study has shown that cancer does not arise in patients with antral gastritis such as is found in duodenal ulcer (patients with duodenal ulcer have long been known to have a surprisingly low incidence of gastric cancer). Cancer develops in individuals who have pan or corpus predominant gastritis where the odds ratio is around 34 [17]. If *Helicobacter pylori* had a direct carcinogenic effect through the mechanisms set out above gastric cancer would also be common in patients with antral predominant gastritis. The severity of inflammation in in these cases is similar or more severe than that which occurs in the corpus. If the cancers that arise in patients with corpus predominant gastritis were usually found in the corpus it could be argued that the corpus mucosa has a greater propensity to become malignant than the antral mucosa, however, in these cases, gastric cancer is usually found in the antrum so this theory does not hold.

It seems that the mechanism responsible for inducing cancer results from the physiological changes that accompany the development of atrophy and intestinal metaplasia within the gastric corpus. This implies that *Helicobacter pylori* itself or the inflammation that it produces cannot be the direct cause of gastric cancer.

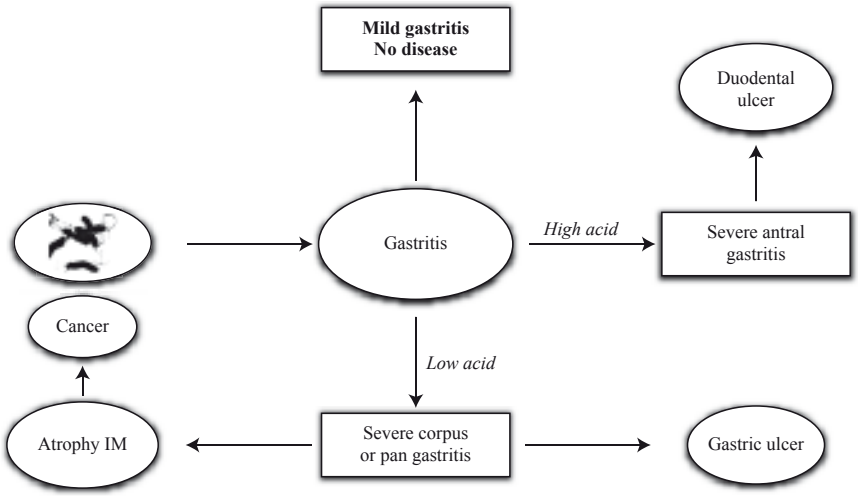
The pattern of gastritis in relation to gastric acid secretion

When individuals are first infected with *Helicobacter pylori* there is an acute pan gastritis associated with achlorhydria [18]. The cause of the achlorhydria is not fully understood, but can persist for months or even years. The acute inflammation may impair the ability of parietal cells to secrete acid by a direct effect. However, the generation of the inflammatory response involves secretion of interleukin-1 beta [19]. This is a pro-inflammatory cytokine but is also an extremely powerful acid inhibitor. This may be responsible for the hypochlorhydria. With the passage of time the acute inflammation gradually settles and is replaced by the typical acute on chronic infection seen in patients with long standing *Helicobacter* infection. With the decline in acute inflammation the stomach again starts to secrete acid. It seems that the amount of acid that the stomach secretes is what determines the pattern of the gastritis, i.e. whether the whole of the stomach will be inflamed (pan gastritis) or whether the inflammation will be limited to the antrum. If there is a high acid secretion the inflammation in the corpus diminishes and the parietal cells secrete more acid. Furthermore, the antral inflammation affects the gastrin and somatostatin secreting cells (those that are responsible for controlling the amount of gastrin secreted) the effect is to hyperstimulate the parietal cells. Thus antral predominant gastritis is associated with a high acid secretion, whilst pan gastritis or corpus predominant gastritis is associated with a low acid secretion. Those with antral gastritis have a propensity to develop duodenal ulcer the others with a corpus gastritis may develop gastric ulcer. The long-term effects of these differences are even more profound because in those with an antral predominant gastritis, atrophy and intestinal metaplasia is limited to the antral region where there are no acid secreting cells. Long standing inflammation in the corpus, however, leads to atrophy, intestinal metaplasia which affects the parietal cells and causes a further reduction in acid secretion [20].

The normal stomach is not colonized with bacteria and in the majority of individuals who are infected with *Helicobacter pylori* the only colonizing species is *Helicobacter pylori*. This is because gastric acid serves as a bacteriocidal barrier ensuring that ingested food containing infecting organisms do not pass into the rest of the gastrointestinal tract. In the absence of gastric acid, however, the stomach rapidly becomes colonized. This happens even in normal individuals who take regular proton pump inhibitors or H2 receptor antagonists [21]. Bacterial colonization is more prominent in patients who have corpus atrophy and hypochlorhydria where a wide range of faecal organisms may colonize the stomach. Many of these are metabolically active and produce a variety of potential carcinogens. In the years before *Helicobacter pylori* had been identified it was hypothesized that the direct cause of gastric cancer was the production of N-nitrosamines by colonizing bacteria within the stomach [22]. Nitrosamines are known carcinogens and they induce gastric cancer in the experimental animal. N-nitrosamines are labile chemicals and difficult to work with. Since *Helicobacter pylori* was found to be associated with gastric cancer, the nitrosamine hypothesis has not been promulgated to the same extent that it was in earlier years. Nevertheless, this remains a possible explanation as to why cancer develops in patients with gastric atrophy and intestinal metaplasia.

Ascorbic acid is believed to protect against a number of carcinogens including reactive oxygen species and nitrosated compounds. Normal individuals have a high concentration of ascorbic acid in their gastric juice, indeed the concentration is

***Figure 1*. Potential outcomes following *H. pylori* infection**



higher than that present in the plasma, it is in effect "secreted" into the stomach. When infected by *Helicobacter pylori* this secretory mechanism disappears, however, in the presence of achlorhydria, not only is there a reduced secretion, but ascorbic acid is rapidly destroyed and is no longer detectable [23].

The presence of faecal organisms within the stomach may be responsible for further damage arising within the mucosa, in particular it may accelerate the development of intestinal metaplasia [24].

Helicobacter pylori may not therefore be directly responsible for gastric cancer. It seems more likely that the organism sets the scene for its development by destroying the ability of the stomach to secrete acid in addition to causing direct injury to the gastric epithelium through inflammation.

Why does the incidence of gastric cancer vary?

There is a positive relationship between the prevalence of *Helicobacter pylori* and the incidence of gastric cancer. However, there are some notable exceptions. The subcontinent of India has a high prevalence of *Helicobacter pylori*, but a modest incidence of cancer. The same applies to parts of Africa, whilst in the Far East where there is a similarly high prevalence of *Helicobacter pylori*, gastric cancer is much more common. The same obtains to a lesser extent in Russia and the Eastern part of Europe. Certain countries in South America have an extremely high cancer rate, but others somewhat lower.

In the Western part of Europe and the United States the incidence of gastric cancer has fallen rapidly over the past century. This has been attributed to the decline in the prevalence of *Helicobacter pylori*. However, the evidence for this is not totally persuasive. It is true that the prevalence of *Helicobacter* has fallen, but the decline is mainly in younger people. When the elderly population is considered (the ones who are currently at risk of gastric cancer) the incidence of cancer has fallen more than would have been anticipated.

Factors other than *Helicobacter pylori* prevalence influence the incidence of gastric cancer. As indicated earlier gastric cancer is found in individuals who have severe corpus atrophy and intestinal metaplasia. It is the pattern of the infection and its severity which is important (*Fig. 1*). Antral predominant infection is not positively associated with the development of cancer. Few studies have addressed the issue as to whether the pattern and severity of gastritis varies between populations. A recent paper prospectively studied age matched cohorts of patients in England and Japan [25]. The incidence of cancer in Japan is substantially higher than that in England. The study showed the prevalence of infection to be only slightly higher in the Japanese group, but the major difference between the populations was the pattern of the gastritis. English patients were more likely to have an antral predominant gastritis as compared to the Japanese where there was a higher prevalence of pan and corpus gastritis. Furthermore corpus atrophy and intestinal metaplasia was enormously higher in the Japanese than in the English patients especially in the older cohorts. It is possible that some international differences in the incidence of cancer may relate not only to the number of individuals infected, but to the phenotype of the gastritis.

The severity of gastritis varies between patients and is influenced by the virulence of the infecting *H. pylori* strain. The presence, for example, of the Cag pathogenicity island increases the pathogenic potential of the organism [26]. There are other variations between the strains including sub-types of the vacuolating cytotoxin that increase the virulence of the organism. *Helicobacter* strains vary considerably and recent work shows that there are geographical differences, for example, a higher percentage of strains in the Far East are CagA positive as opposed to those in Europe [27]. It is likely that there are other strain differences as yet unknown that have a geographical selection, causing a greater severity of inflammation in certain parts of the world than others.

A low acid secretion is associated with a pan gastritis or corpus predominant gastritis and a high acid with antral gastritis. If an acid suppressant is prescribed, the pattern of antral gastritis

changes to a pan gastritis [28]. The amount of acid secreted by individuals varies enormously. Not all of the factors responsible for this are known, however, malnutrition is a recognised cause of hypochlorhydria [29] and gastric acid secretion is proportional to lean body mass [30]. Populations vary in their nutrition and in the size of their citizens so these are possible factors that might influence the pattern of gastritis.

Individuals respond to infection with *Helicobacter pylori* by the production of a series of cytokines. These are subject to variation. In particular, interleukin-1 beta has a number of polymorphisms, that is to say the molecular composition of the protein varies slightly according to the genetic make up of the individual. Tiny changes in these molecules may radically alter their activity. Certain polymorphisms are positively associated with gastric cancer [31] so it is possible that racial differences may influence the inflammatory response that certain populations have to an infecting organism both in terms of the pattern of gastritis and its severity.

Summary

The discovery of *Helicobacter pylori* has revolutionized our understanding of the pathology of the stomach and duodenum. There is strong evidence to suggest that this organism is responsible for nearly all non-cardia gastric cancer. The disease, however arises primarily in individuals who have had a severe corpus predominant inflammation whilst those with an antral predominant gastritis are relatively protected from the disease. This suggests that *Helicobacter pylori* is not directly responsible for the development of cancer, but that by destroying the acid secreting part of the stomach and inducing a generalized inflammation it sets the scene for other mechanisms to act directly on the mucosa and it is these that cause the transition from a diseased, albeit stable mucosa, to neoplasia. It is unclear which factors are ultimately responsible for the malignant change and more work is required in this area to elucidate the pathogenesis of the disease. Nevertheless, *Helicobacter pylori* is responsible for inducing the premalignant lesion that develops into cancer, in its absence gastric cancer would be extremely rare.

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Clinical management of autoimmune pancreatitis

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Abstract

Autoimmune pancreatitis (AIP) is a newly described entity with characteristic clinical, radiological, serological, and histological features, in which autoimmune mechanisms seem to be involved in pathogenesis. Many new clinical aspects of AIP have been clarified during 10 years, and AIP has become a distinct entity recognized worldwide. However, precise pathogenesis or pathophysiology remains unclear. As AIP responds dramatically to steroid therapy, accurate diagnosis of AIP is necessary to avoid unnecessary laparotomy or pancreatic resection. It is importance to misdiagnose pancreatic cancer as AIP as well as to misdiagnose AIP as pancreatic cancer. In the absence of a diagnostic serological marker for AIP, its diagnosis rests on identifying unique patterns of abnormalities. Japanese criteria are based on the minimum consensus features of AIP and aim to avoid misdiagnosis of malignancy. It contain 3 items: (1) enlargement of the pancreas and narrowing of the main pancreatic duct; (2) high serum gammaglobulin, IgG, or IgG4, or the presence of autoantibodies; (3) histological findings of lymphoplasmacytic infiltration and fibrosis in the pancreas. For diagnosing AIP, the presence of the imaging criterion is essential. Other clinical characteristics of AIP are elderly male preponderance, fluctuating obstructive jaundice without pain, occasional association with diabetes mellitus and extrapancreatic lesions, and favorite responsiveness to oral steroid therapy. Elevation of serum IgG4 levels and infiltration of abundant IgG4-positive plasma cells in various organs are rather specific in AIP patients. In an elderly male presenting obstructive jaundice and pancreatic mass, AIP should be considered as one of differential diagnoses.

Key words: autoimmune pancreatitis, IgG4, chronic pancreatitis, steroid.

Introduction

Sarles et al. [1] first reported a condition called "primary inflammatory sclerosis of the pancreas" and suggested an autoimmune cause for this condition. Some researchers also have proposed the possible role of autoimmunity in causing chronic pancreatitis. Since Yoshida et al. [2] proposed the concept of autoimmune pancreatitis (AIP) in 1995, many cases of AIP have been reported in the Western countries as well as in Japan, and AIP has become a distinct entity recognized worldwide. However, precise pathogenesis or pathophysiology remains unclear.

AIP has many clinical, radiological, serological and histopathological characteristics. However, in the absence of a diagnostic serological marker for AIP, AIP should be diagnosed currently on the basis of combination with these unique patterns of abnormalities. It is uppermost importance to misdiagnose AIP as pancreatic cancer. In North America, about 2.5% of pancreaticoduodenectomies are performed for AIP because of a mistaken diagnosis of pancreatic cancer [3], and AIP cases represent between 21% [4] and 23% [3] of pancreaticoduodenectomies performed for benign conditions. Although AIP dramatically responds to oral prednisolone therapy, there is no consensus on regimen of steroid therapy for AIP. This review focuses on clinical management of AIP, based on our experience of 35 cases of AIP.

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Figure 1. **Ultrasonography of a patient with autoimmune pancreatitis showing a diffusely enlarged hypoechoic pancreas**

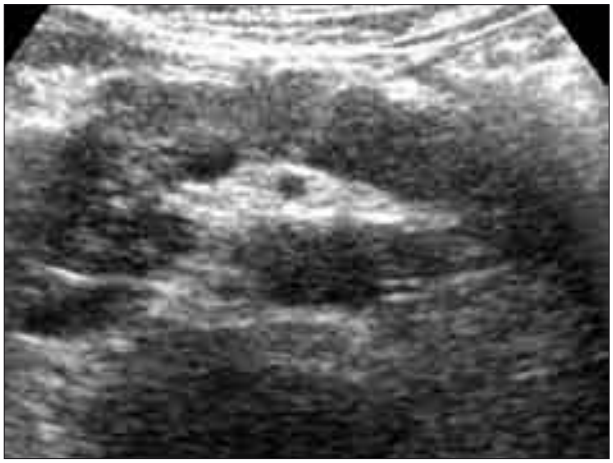
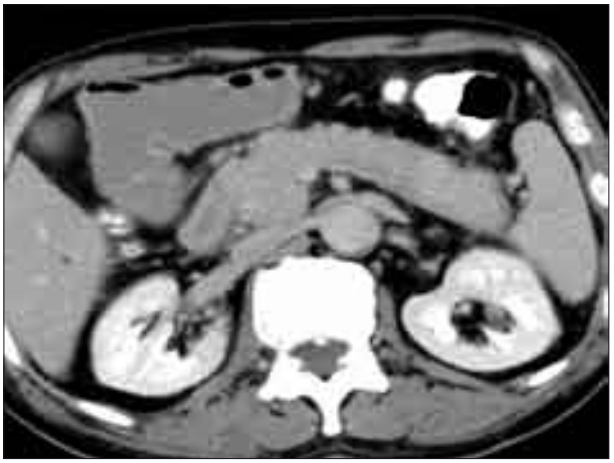


Figure 2. **Dynamic computed tomography showing delayed enhancement of the diffusely enlarged pancreatic parenchyma**



Diagnosis of AIP

Clinical features

AIP is diagnosed more commonly in elderly males [5]. The mean age of the patients is 68.5 years (range 29-83 years) and the male-to-female ratio is 4:1 in our series. In Korea, the mean age was 59.1 years (45-75 years), and the male-to-female ratio was 15:2 [6]. Typical presentation with severe abdominal pain and clinically acute pancreatitis is rarely seen. Obstructive jaundice due to associated sclerosing cholangitis occurs frequently (65% [6]-86% [7]). The jaundice sometimes fluctuates. Failure of pancreatic exocrine or endocrine function is frequently seen. Up to 50% of AIP patients present with glucose intolerance [8]. The diagnoses of diabetes mellitus and AIP are made simultaneously in many cases, but some cases show exacerbation of preexisting diabetes mellitus with the onset of AIP [8].

AIP patients frequently have various extrapancreatic lesions such as sclerosing cholangitis, sclerosing sialadenitis and retroperitoneal fibrosis [9]. Sclerosing cholangitis is most frequently associated with AIP. Stenosis of the lower bile duct is usually detected. When stenosis is found in the intrahepatic or the hilar hepatic bile duct, the cholangiographic appearance is very similar to that of primary sclerosing cholangitis [10,11]. Swelling of the bilateral salivary glands was detected in 8 (23%) of 35 patients with AIP in our series. Hydronephrosis due to retroperitoneal fibrosis was detected in 4 AIP patients. All these extrapancreatic lesions show similar histopathological findings to those in the pancreas and also respond well to steroid therapy [10-14].

Laboratory findings

Marked elevation of serum pancreatic enzymes is rarely seen. Hypergammaglobulinemia (>2.0 g/dl) and elevated serum IgG levels (>1 800 mg/dl) are detected in 59%-76% [12-14] and 53% [6]-71% [12] of AIP patients, respectively. A diagnostic autoantibody for AIP has not been detected. Autoantibodies including antinuclear antibody and rheumatoid factor are present in 43%-75% and 13%-30% of them, respectively [13-15]. Serum IgG4 levels are rather significantly and specifically high (>135 mg/dl) in AIP patients. The sensitivity of elevated serum IgG4 levels is reported to be 63%-95% [6,16-18]. Elevation of serum IgG4 levels was reported in a patient with pancreatic cancer [19].

Radiological findings

On ultrasonography (US), an enlarged hypoechoic pancreas is characteristically detected in AIP (*Fig. 1*) [14,20]. On dynamic computed tomography (CT), there is delayed enhancement of the enlarged pancreatic parenchyma (*Fig. 2*) [14,20]. Typical AIP cases show diffuse enlargement of the pancreas, the so-called sausage-like appearance. Since inflammatory and fibrous changes involve the peripancreatic adipose tissue, a capsule-like rim surrounding the pancreas, which appears as a low density on CT, is detected in some cases [14,20]. Pancreatic calcification or pseudocyst is rarely seen. Some cases show a focal enlargement of the pancreas, similar to that seen with pancreatic cancer (*Fig. 3*). Endoscopic retrograde cholangiopancreatography (ERCP) discloses an irregular, narrow (<3 mm in diameter) main pancreatic duct (*Fig. 4*). In patients with segmental narrowing, absence of upstream dilatation of the main pancreatic duct is characteristic [14]. Stenosis of the extrahepatic or intrahepatic bile duct is frequently observed. Marked wall thickening of the extrahepatic bile duct or gallbladder is sometimes detected on US or endoscopic ultrasonography (EUS) [20,21]. Magnetic resonance cholangiopancreatography does not adequately show the narrow portion of the main pancreatic duct, but it can adequately demonstrate stenosis of the bile duct with dilatation of the upper biliary tract [22].

Histopathological findings

The histological finding of AIP is characteristic, that is, dense lymphoplasmacytic infiltration with fibrosis of the pancreas (*Fig. 5*). Lymphoid follicles are occasionally formed. The acinar cells are replaced by inflammatory cells and fibrosis, and the lobular architecture of the pancreas is almost lost. Pancreatic duct is narrowed by periductal fibrosis and lymphoplasmacytic infiltration. Another characteristic histological finding is obliterative phlebitis involving minor and major veins, includ-

***Figure 3*. Ultrasonography showing a focal enlargement of the pancreas similar to pancreatic cancer**

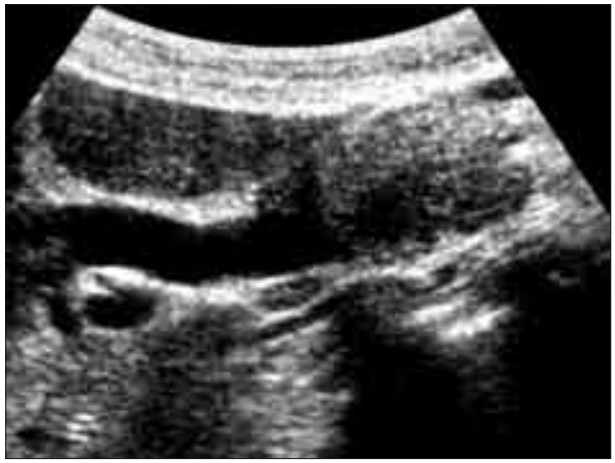


***Figure 4*. Endoscopic retrograde pancreatography showing diffuse irregular narrowing of the main pancreatic duct**

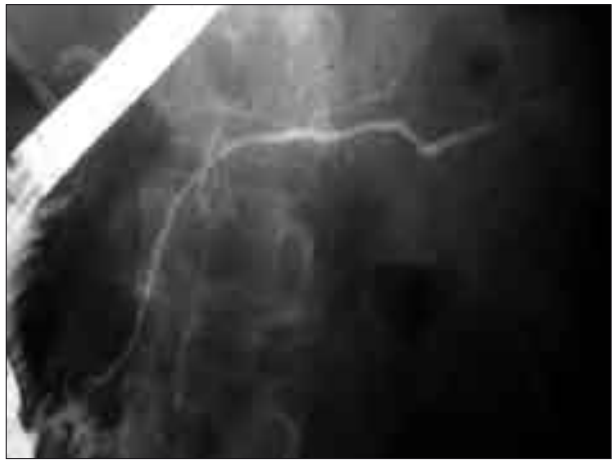


***Figure 5*. Histological findings of the pancreas of a patient with autoimmune pancreatitis showing dense lymphoplasmacytic infiltration and fibrosis with acinar destruction (Hematoxylin-eosin)**

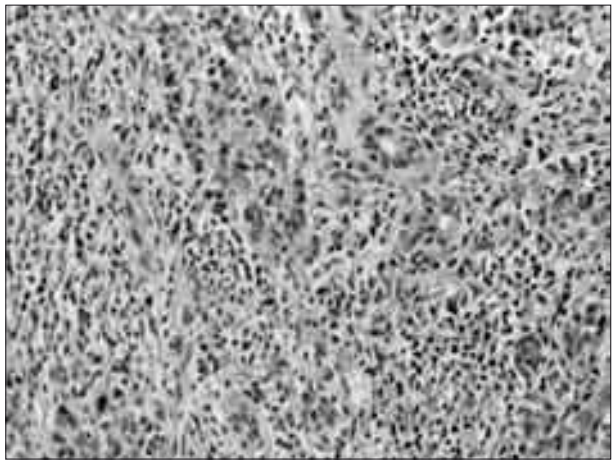
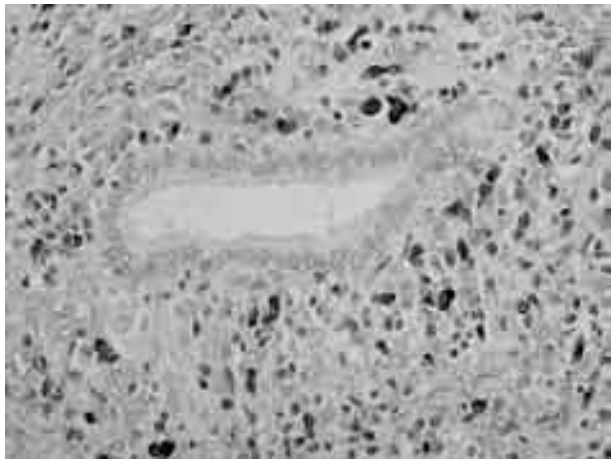


***Figure 6*. IgG4-immunostaining of the pancreas of a patient with autoimmune pancreatitis showing dense infiltration of IgG4-positive plasma cells**



ing the portal vein. Such an inflammatory process widely and intensely involves the contiguous soft tissue, peripancreatic retroperitoneal tissue, and the thickened wall of the bile duct and gallbladder [14,23,24].

These characteristic histological findings of AIP are detected during its active phase, and can be gold standard for diagnosing AIP [25]. However, diagnosing AIP on biopsy or endoscopic ultrasound-guided fine needle aspiration biopsy (FNAB) is sometimes difficult, because of small sample size.

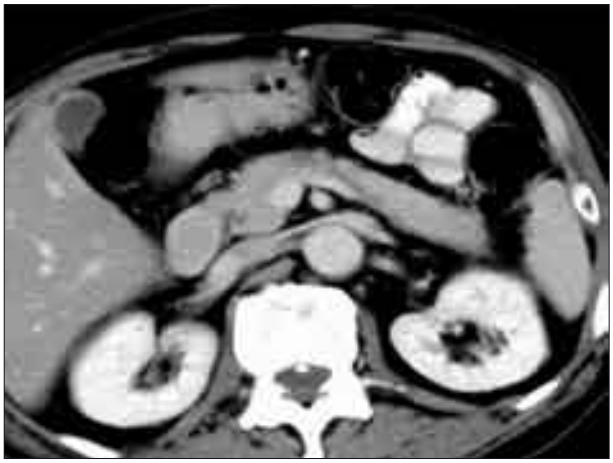
Immunohistochemically, infiltrating inflammatory cells in the pancreas consist of CD4- or CD8-positive T lymphocytes and IgG4-positive plasma cells (*Fig. 6*). Dense infiltration of IgG4-positive plasma cells in the pancreas is not observed in chronic alcoholic pancreatitis or pancreatic cancer. Infiltration of abundant IgG4-positive plasma cells is also detected in various organs such as peripancreatic retroperitoneal tissue, major duodenal papilla, biliary tract, intrahepatic periportal area, salivary glands, gastric mucosa, colonic mucosa, lymph nodes and bone marrow of AIP patients [14,23,24]. We suggested that AIP might be a pancreatic lesion of IgG4-related systemic disease [23,26].

Diagnostic criteria and differential diagnosis

The Japan Pancreas Society has proposed "Diagnostic Criteria for Autoimmune Pancreatitis" in 2002 [27,28], and it was revised in 2006 [29]. It contains three items: (1) radiological imaging showing diffuse or localized enlargement of the pancreas and diffuse or segmental irregular narrowing of the main pancreatic duct; (2) laboratory data demonstrating high serum gammaglobulin, IgG or IgG4, or the presence of autoantibodies, such as antinuclear antibodies and rheumatoid factor; and (3) histological examination of the pancreas showing marked interlobular fibrosis and prominent infiltration of lymphocytes and plasma cells. Diagnosis of AIP is established when criterion 1, together with either criterion 2 and/or criterion 3, are fulfilled. The presence of the imaging criterion is essential for diagnosing AIP. These criteria are based on the minimum consensus features of AIP to avoid a misdiagnosis pancreatic cancer as far as possible.

The most important disease that should be differentiated from AIP is pancreatic cancer. Clinically, patients with pancreatic cancer and AIP share many features, such as being elderly,

***Figure 7*. Computed tomography of the patient of *Fig. 2*, which was taken 3 weeks after commencement of steroid therapy. Enlarged pancreas was normalized**



having painless jaundice, developing new-onset diabetes mellitus, and having elevated tumor markers [14]. Radiologically, focal swelling of the pancreas, the "double-duct sign" representing strictures in both the biliary and pancreatic ducts, as well as angiographic abnormalities, can sometimes be seen in both pancreatic cancer and AIP [14]. As AIP responds dramatically to steroid therapy, accurate diagnosis of AIP can avoid unnecessary laparotomy or pancreatic resection. Imaging findings, such as a mass showing delayed enhancement and a capsule-like rim on dynamic CT, and segmental narrowing of the main pancreatic duct associated with less dilated upstream pancreatic duct, are all useful in differentiating pancreatic cancer from AIP. Measurement of serum IgG4 levels is a useful tool to differentiate between the two diseases. IgG4-immunostaining of biopsy specimens taken from the major duodenal papilla of AIP patients may be useful to support the diagnosis of AIP [30]. Although improvement in clinical findings with steroid therapy may be useful in the differential diagnosis of AIP from pancreatic cancer, diagnostic steroid trial should be avoided not to misdiagnose pancreatic cancer as AIP. It is of uppermost important to consider the presence of AIP in elderly patients presenting obstructive jaundice and pancreatic mass.

Treatment and prognosis

The dramatic response to corticosteroid is a well-known phenomenon in AIP, but a regimen of steroid therapy has not been established [6,31,32]. Before steroid therapy is started, endoscopic or percutaneous transhepatic biliary drainage must be done in cases with obstructive jaundice, and glucose levels must be controlled in cases with diabetes mellitus. Oral prednisolone is usually initiated at 30-40 mg/day and it is tapered by 5 mg every 1-2 weeks. Serological and imaging tests are followed periodically after commencement of steroid therapy. Usually, pancreatic size is normalized within a few weeks (*Fig. 7*), and biliary drainage becomes unnecessary during 1-2 months. Patients in whom complete radiological improvement is documented can stop their medication. To prevent relapses without complete discontinuation of steroid, continued maintenance therapy with prednisolone 5 mg/day is sometimes required. In half of steroid-treated patients, impaired exocrine or endocrine function improved [8]. Some AIP patients relapse during maintenance therapy or after stop of steroid medication, and should be retreated with high-dose steroid therapy. The indications for steroid therapy in AIP include obstructive jaundice due to stenosis of the bile duct or the presence of other associated systemic diseases, such as retroperitoneal fibrosis. Steroid therapy is also effective for sclerosing cholangitis which relapses after surgery [33].

The long-term prognosis of AIP is not well known. It is reported that recurrent attacks of AIP resulted in pancreatic stone formation in some cases [7].

Conclusion

AIP is a distinctive type of pancreatitis that shows reversible improvement with oral steroid therapy. AIP has many clinical, serological, morphological, and histopathological characteristic features. AIP should be diagnosed carefully based on combination of these findings. In an elderly male presenting obstructive jaundice and pancreatic mass, AIP should be considered as one of differential diagnoses to avoid unnecessary surgery.

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Transcatheter arterial chemoembolization for superficial hepatocellular carcinoma induces adhesion

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Introduction

The therapies for hepatocellular carcinomas (HCCs) have remarkably developed during the recent one or two decades and the prognosis of HCC patients has subsequently much improved. At present we have been able to perform several therapeutic modalities for HCC, such as surgical resection, transplantation, loco-regional therapies including percutaneous ethanol injection (PEI), radiofrequency ablation (RFA), and microwave coagulation therapy (MCT), chemotherapy, and transcatheter arterial embolization (TAE) and/or transcatheter arterial chemoembolization (TACE) [1]. According to the latest report of Liver Cancer Study Group of Japan during January 1, 2002 to December 31, 2003, 33.6% of 15,681 patients with HCC underwent surgical resection, 31.2% underwent loco-regional therapy, 29.6% underwent TAE/TACE, 4.9% underwent chemotherapy, and 0.8% underwent the other therapy [2]. As the report, TA(C)E is one of the most frequently performed modalities for HCC in Japan and has been validated effective in the treatment for advanced stages of HCCs. However, the efficacy of ordinary TAE/TACE has not been able to expect complete response of the targeted HCC nodules. The rate of complete response of TAE/TACE was 27.8%, and the 5-year survival rate was 22.6%. These results of TAE/TACE seemed to be lower in comparison to those of loco-regional therapies including PEI, MCT, and RFA, whose complete response was 82.2% and 5-year survival 42%. In these situations, we have experienced membranous adhesions between superficial position of HCC nodules and peritoneum and/or omentum in some patients with these HCCs nodules treated with TAE/TACE prior to the laparoscopic loco-regional treatments for these nodules performed at our hospitals in order to achieve complete response [3]. With respect to the adhesion, we would like to consider the positive or negative efficacy of TAE/TACE for these HCC nodules and the usefulness of laparoscopic thermal ablation for these HCC nodules.

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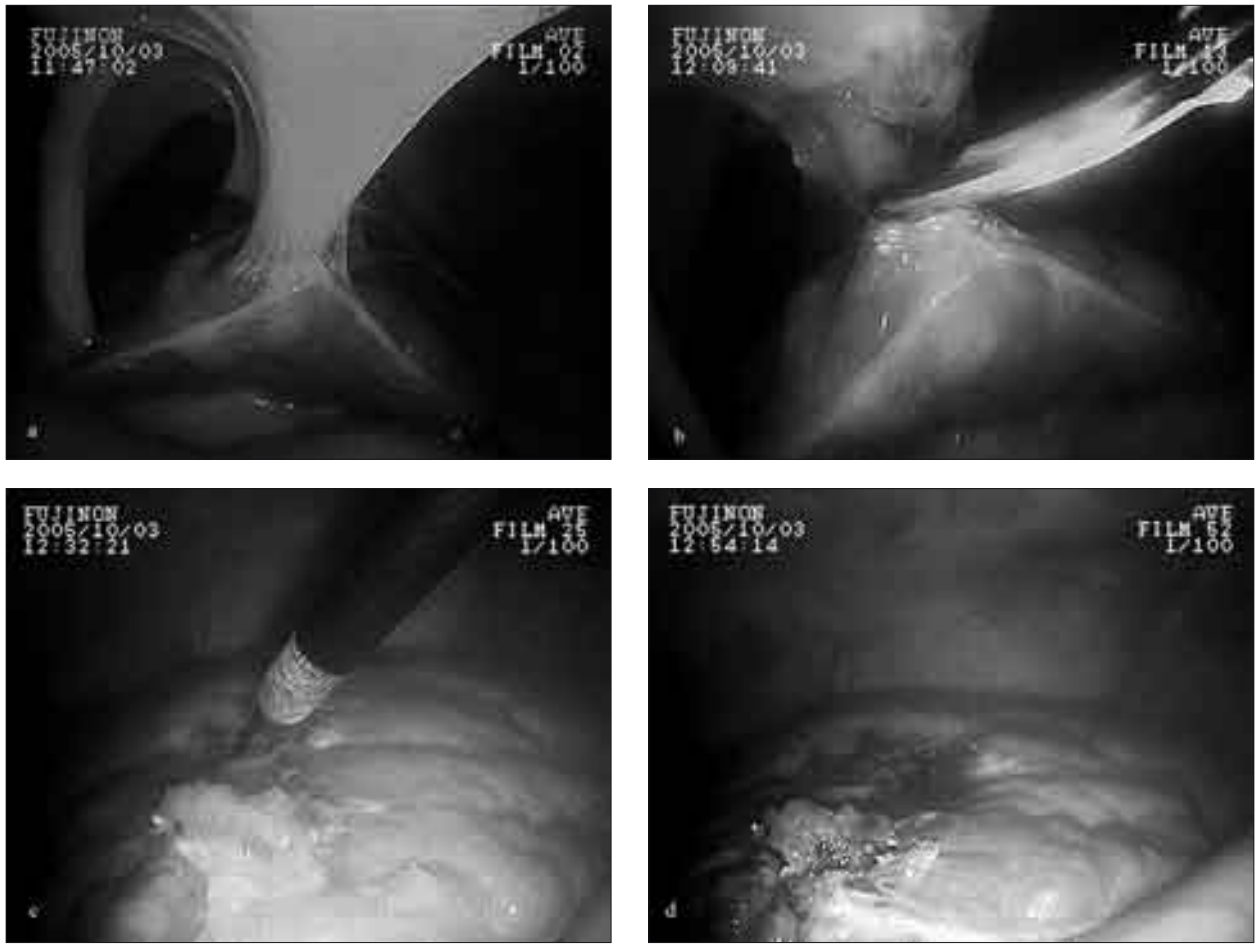
Patients and methods

During April 1, 1995 to October 31, 2005, we performed laparoscopic thermal ablation (LTA), MCT or RFA, for 119 consecutive patients with superficial position of HCC nodules at our hospital. Eighty-eight of the 119 patients (73.9%) underwent the LTA for their primary HCC nodules, and the remaining 31 (26.1%) underwent LTA for their secondary or tertiary HCC nodules. At laparoscopy, we found the adhesion between superficial HCC nodule and peritoneum and/or omentum in seven patients of the 119 (5.9%) patients. We analyzed the correlation between adhesion and history of the treatments performed prior to LTA in the 7 patients. Demographic and clinical data on the 119 patients who underwent LTA were summarized in *Tab. 1*. The 119 patients consisted of 72 males and 47 females. Mean age of the 119 patients was 66 yr (range 46-87). With regard to Child-Pugh classification, 79 were classified into class A, 39 in class B, and 1 in class C, respectively. The causative agent of chronic liver disease in the 119 patients was hepatitis C virus in 110, hepatitis B virus in 3, alcohol in 4, Wilson disease in one, and primary biliary cirrhosis in one. The mean diameter of HCC was 18.8 (minor axis) x 20.6 (major axis) mm (SD 6.0 x 6.6 mm). Histological examinations by fine needle biopsy were performed in 37 patients. Well differentiated HCC was diagnosed in 21 patients, moderately differentiated HCC in 14, and poorly differentiated HCC in 2. Alpha-fetoprotein was measured in 91 patients.

Figure 1. **Laparoscopic microwave coagulation. a. Adhesion between superficial HCC nodules and peritoneum. b. Adhesions are cut with harmonic scalpel. c. Superficial HCC nodules are treated with MCT. d. Coagulation necrosis forms decompression with LMCT**



Statistical analysis

Categorical variables were compared statistically using the chi-squared or Fisher's exact test where appropriate. Continuous variables were compared using the Wilcoxon rank sum test. The correlation between continuous variables was tested using Spearman correlation coefficients. Multivariate analysis was performed using logistic regression analysis. All statistic analyses were performed using the Statistic Analysis System (SAS Institute Inc.). P values of less than 0.05 were considered significant.

Results

At laparoscopy, we found adhesion between superficial HCC nodules treated previously and peritoneum and/or omentum in 7 patients. Of the 7 patients, one underwent treatment with LTA for the primary HCC after TAE performed at other hospital about one month prior to admission to our institution. The other 6 patients were treated for local progression tumor of the secondary or tertiary HCC on the liver surface after the initial therapy at our institution (*Fig. 1*). We cut the adhesion with harmonic scalpel and treated HCC with MCT or RFA. Some adhesion tissues contained vessels. Demographic and clinical data on the 7 patients were summarized in *Tab. 2*. They consisted of three men and four women. Superficial HCC nodule

Table 1. **Baseline data on 119 patients treated for superficial hepatocellular carcinoma nodules**

Men/women	**72/47**
Initial/second laparoscopic therapy	88/31
Mean age, years	66 (range 46-87)
Child-Pugh classification	
A /B/C	79/39/1
Cause of liver disease	
HCV/HBV/Alcohol/Wilson/PBC	110/3/4/1/1
Diameter of HCC (mm)	18.8 x 20.6 (SD 6.0 x 6.6)
Histological findings for HCC (n=37)	
Well/Moderately/Poorly	21/14/2
Alph-fetoprotein (ng/ml) (n=91)	
0-20/21-200/201<	44/36/11

HCV – hepatitis C virus; HBV – hepatitis B virus; PBC – primary biliary cirrhosis; HCC – hepatocellular carcinoma; SD – standard deviation

***Table 2*. Baseline data on the 7 patients with adhesion between HCC and peritonium and/or omentum**

	Age/Sex	Location/size of HCC	Previous therapy	Primary or local progression
1	72/M	S3/14 x 17 mm	TAE	primary
2	57/M	S3/23 x 25	TAE + PEI	local progression
3	75/F	S3/13 x 21	TAE + PEI	local progression
4	77/F	S3/28 x 28	TAI	local progression
5	73/M	S6/24 x 26	TAE	local progression
6	61/F	S6/30 x 30	TAE + PEI	local progression
7	81/F	S3/16 x 17	TAE	local progression
		S3/17x 18		

HCC – hepatocellular carcinoma; M – male; S – subsegment; TAE – transcatheter arterial embolization; PEI – percutaneous ethanol injection; F – female; TAI – transcatheter arterial infusion

***Table 3*. Data on 25 patients without adhesion who underwent LLRT for secondary or tertiary HCC**

Men/women	14/11
Mean age years (range)	67 (51-82)
Child-Pugh, number A/B/C	14/10/1
Cause of liver disease	
HCV/HBV/Alcohol	23/2/0
Diameter of HCC, minor x major (mm)	20.5 x 20.6 (SD 7.0 x 7.4)
Histology of HCC (differentiation)	
Well/moderately/poorly (n=5)	1/3/1
Alpha-fetoprotein (ng/ml)	
0-20/21-200/201< (n=23)	8/11/3
Previous therapy (consecutive)	
PEI/TAE/TAE +PEI/MCT/RFA/TAE;RFA	12/10/7/5/2/1

LLRT – laparoscopic loco-regional therapy; HCC – hepatocellular carcinoma; HCV – hepatitis C virus; HBV – hepatitis B virus; PEI – percutaneous ethanol injection; TAE – transcatheter arterial embolization; MCT – microwave coagulation; RFA – radiofrequency ablation

with adhesion was located in subsegment 3 in 5 patients and in subsegment 6 in two. The diameter of HCC ranged from 13 to 30 mm. All seven patients previously underwent TAE, TAI, or combination of TAE and PEI for the superficial HCC as the therapy (*Fig. 2*). After the therapy with TAE, TAI, or TAE + PEI for the primary, secondary, or tertiary HCC, local progression tumor developed in the 7 patients. We completely treated these local progression tumors with LTA.

Next, we examined 25 patients without adhesion, who were treated for secondary or tertiary HCC with LTA (*Tab. 3*). The 25 patients consisted of 14 men and 11 women. The mean age was 67 years (range 51-82 years). With respect to Child-Pugh classification, 14 patients were classified into class A, 10 in class B, and one in class C. Most of patients contracted cirrhosis with HCV infection. The mean diameter of HCV was 20.5 mm (minor axis) x 20.6 mm (major axis) (SD 7.0 x 7.4 mm). Diverse therapies were previously performed for HCCs which were located in comparatively deep positions of the liver or dorsal subsegments. Twelve patients were treated with PEI, 10 with TAE, 7 with TAE + PEI, 5 with MCT, 2 with RFA, and one with TAE + RFA. In this study, PEI and TAE dominated in several forms of therapies, but we could not find any adhesion in these patients by laparoscopy. Therefore, TAE and PEI for HCCs in deep positions or dorsal subsegments might not induce adhesion.

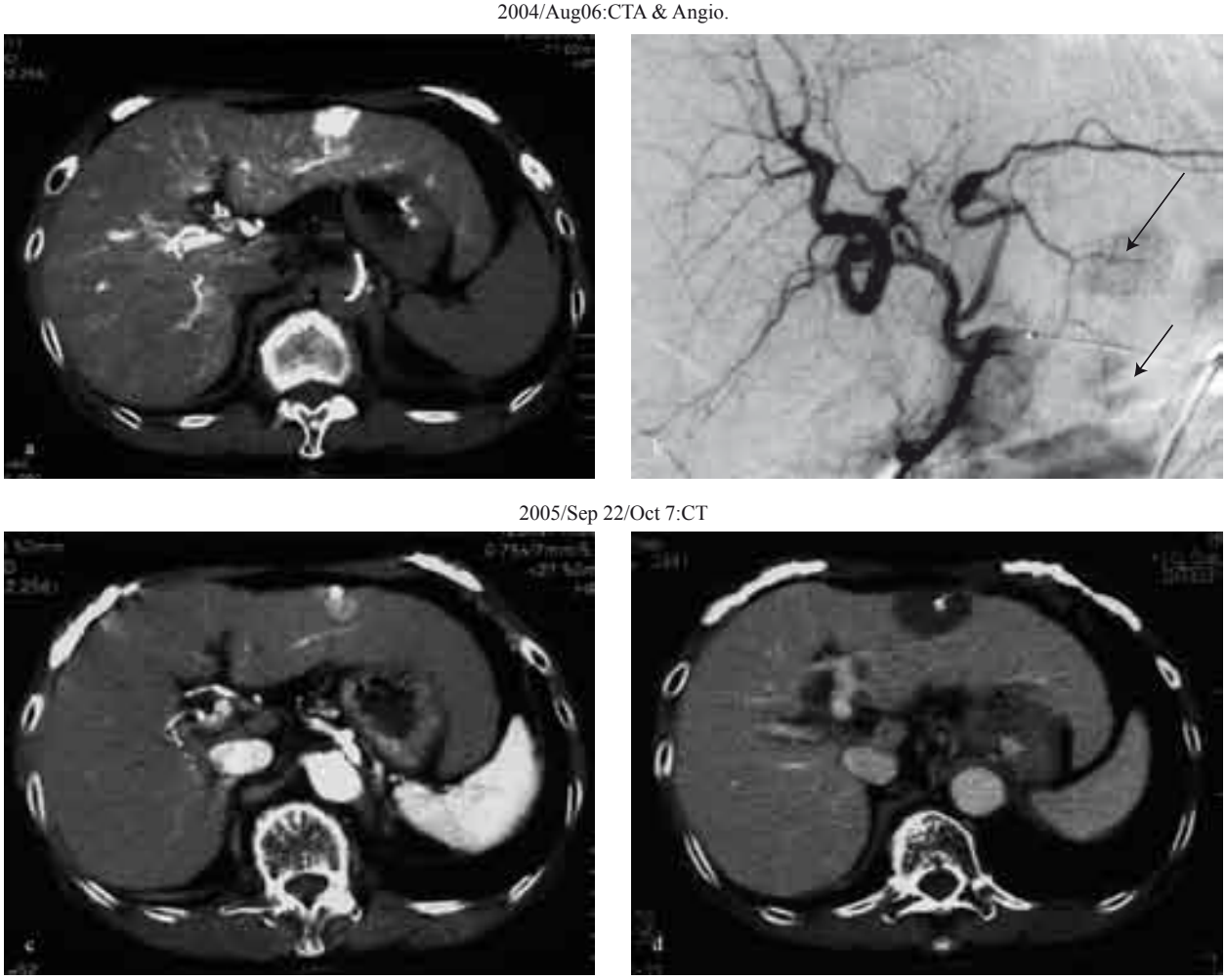
In statistical analysis, any variables, such as sex, age, causes of chronic liver disease, primary or secondary treatment, and Child-Pugh classification, were not significant (data not shown). There is the history of TACE for the superficial HCC nodule as a different factor between the superficial HCC patients with and without adhesion.

Discussion

In current years, selection range of therapeutic modalities for hepatocellular carcinoma has much expanded. Generally speaking, in many countries, in particular Japan, physicians perform loco-regional therapies including PEI, MCT, and RFA, radiologists perform transcatheter arterial chemo-embolization (TACE) including transcatheter arterial infusion (TAI) and radiation, and surgeons perform hepatic resection and transplantation. With the improvement in skills and implements of therapeutic modalities, some suggested algorithms of therapies for HCC have become available [1,4]. As curative treatments, hepatic resection, liver transplantation, PEI, and RFA have been selected [1,5-8], but TAE has been selected for HCCs at relatively progressed stages [9-12]. However, among these therapeutic modalities for the advanced HCCs, TAE including TACE and TAI dominated in Japan [2].

We have performed laparoscopic loco-regional therapy for superficial position of HCC nodule and the therapeutic efficacy and complications of LTA have been reported in recent years [3]. In these situations, we found adhesions between the treated HCC and peritoneum and/or omentum containing vessels. As

Figure 2. **Radiological study and intervention. a. Computed tomographic hepatic arteriography reveals positive staining of HCC nodule in subsegment 3. b. Positive staining of HCC nodules (arrows) is visible. TACE was performed. c. Local progression is found about one year after TACE. d. Complete response is confirmed after LMCT for local progression of HCC**



shown *Tab. 2*, all 7 patients with adhesion had histories of treatments with TAE for the superficial HCC. On the other hand, all 25 patients had the histories of treatments with diverse modalities including TAE and PEI for the HCCs in deep positions of the liver or dorsal subsegments had no adhesions. Taken together, therefore, TAE for superficial HCC might induce adhesions between the treated HCCs and other tissues including vessels. The rates of complete response of TAE for HCC have been reported, 50%, 63.3%, and 23.5%, respectively [5-7]. It is very difficult for TAE alone to achieve complete response for superficial HCC, so we should add the other therapeutic modalities, PEI, MCT, or RFA when we hope complete response. Rather than the additional therapy, avoidance of TAE for the superficial HCC may be preferential in the treatments when we expect curative treatments. In any case, we could completely treat the adhered HCC nodules with laparoscopic MCT or RFA, in addition to laparoscopic resection [13]. Therefore, we would like to emphasize that LMCT or LRFA is very useful for the superficial HCCs, if they had adhesion.

In conclusions, we have experienced 7 patients with adhesions between the treated superficial HCCs and omentum and/or peritoneum including vessels, who had histories of TAE for these HCCs. Therefore, we should avoid the treatment with TAE alone for superficial HCC when we hope to achieve complete response as a primary curative treatment. However, LMCT and LRFA are useful therapeutic procedures for the superficial HCC nodules, even if HCC nodules have adhesions.

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Autoimmune pancreatitis: the classification puzzle

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Introduction

In 1961, Sarles et al. [1] reported the case of a non drinker patient suffering from pancreatitis associated with hypergammaglobulinemia. The authors hypothesized that the disease in this patient was an autonomous pancreatic disease of autoimmune origin. After this report, other authors around the world reported similar cases and they named the disease in several manners: chronic pancreatitis with diffuse narrowing of the pancreatic duct, primary inflammatory pancreatitis, non-alcoholic duct destructive chronic pancreatitis, lymphoplasmacytic sclerosing pancreatitis, granulomatous pancreatitis, idiopathic tumefactive chronic pancreatitis, and sclerosing pancreatocholangitis [2,3]. In 1995, Yoshida et al. [4] suggested the term "autoimmune pancreatitis" for this disease and, therefore, this term has become largely accepted for pancreatic disease of an autoimmune origin. In the last 10 years, there has been an increasing number of cases reported in Japan and Europe [5]. In this review article, we will briefly describe the main characteristics of autoimmune pancreatitis and then we will concentrate on our aim, namely, evaluating the clinical characteristics of patients having recurrence of pain from the disease.

Key words: autoimmune pancreatitis, classification, pathogenesis, diagnosis, therapy.

Incidence

At present, the exact incidence of the disease is not known. The only available data are those reported in Japan and in Italy. In these two countries, the estimated incidence of autoimmune pancreatitis is quite similar, 4.6% and 6.0% in Japan and in Italy, respectively [5] and we are awaiting data from the United States as well as from other countries in order to define the real incidence of the disease around the world. Autoimmune pancreatitis seems to have a preference for the male gender, in fact, about 80% of the cases described are males [5]. However, a geographic variation may be observed because, in Italy, the male:female ratio is 1:1. At diagnosis, the patients were more than 55 years of age [5]. Diabetes mellitus is present in about half of the patients [5].

Pathogenesis

From a pathological point of view, the disease is characterized by diffuse or focal pancreatic swelling with a narrowing of the pancreatic duct and/or common bile duct and the histological hallmark of this type of pancreatitis is lymphoplasmacytic infiltration, especially concentrated on the pancreatic ducts [6-8]. Some authors have defined autoimmune pancreatitis [9] as the simultaneous involvement of the pancreas, the salivary glands and the liver (primary biliary cirrhosis) by means of an immune-mediated inflammatory process. Thus, the still open question is the differentiation of autoimmune pancreatitis as a primary or a secondary disease based on the absence or presence of other autoimmune diseases.

Clinical aspects

From a clinical point of view, patients with autoimmune pancreatitis rarely complain about the typical severe abdominal pain of pancreatitis and are usually hospitalized for painless jaundice [10]; other symptoms of autoimmune pancreatitis include non-specific mild abdominal pain and weight loss. The diagnosis is sometimes quite intriguing because the disease may be mistaken for pancreatic cancer [11].

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***Table 1*. Diagnostic criteria for autoimmune pancreatitis released by the Japan Pancreas Society [18]**

Criteria	Definition
I. Imaging criterion	Diffuse narrowing of the main pancreatic duct with an irregular wall (more than 1/3 length of the entire pancreas) and enlargement of the pancreas
II. Laboratory criterion	Abnormally elevated levels of serum gammaglobulin and/or IgG, or the presence of autoantibodies
III. Histopathologic criterion	Marked lymphoplasmacytic infiltration and dense fibrosis

For diagnosis, criterion I must be present, together with criterion II and/or III

***Table 2*. Italian diagnostic criteria for autoimmune pancreatitis [5]**

Criteria	Definition
Criterion I.	Histology and cytology
Criterion II.	Association with other postulated autoimmune disease
Criterion III.	Response to steroid therapy

One or more criteria must be present in order to diagnose autoimmune pancreatitis

***Table 3*. Korean diagnostic criteria for autoimmune pancreatitis released by the Asian Medical Center [20]**

Criteria	Definition
Criterion I.	Pancreatic imaging (essential): (1) CT – Diffuse enlargement (swelling) of pancreas and (2) ERCP – Diffuse or segmental irregular narrowing of main pancreatic duct
Criterion II.	Laboratory findings: (1) elevated levels of IgG and/or IgG4 or (2) detected autoantibodies
Criterion III.	Histopathologic findings: Fibrosis and lymphoplasmacytic infiltration
Criterion IV.	Response to steroids

Definite diagnosis: Criterion I and any of criteria II-IV

Laboratory data

Laboratory analysis is undergoing continuous evolution. Serum amylase and lipase may often be normal or a mild elevation of the serum pancreatic enzymes may be observed, and in only a few cases is there a marked elevation of these pancreatic damage markers [12]. Hypergammaglobulinemia and IgG serum increase have been reported in percentages ranging from 37 to 76% [13,14]. Japanese authors have claimed that elevated serum levels of IgG4, a subtype of IgG, are a biochemical hallmark of autoimmune pancreatitis [15]; however, other authors have recently questioned the specificity of the IgG4 because elevated IgG4 levels are present in patients suffering from pancreatic carcinoma and other types of chronic pancreatitis [5].

Non-specific autoantibodies, such as antinuclear antibodies, antimitochrondial antibodies and so on have a low sensitivity in diagnosing autoimmune pancreatitis; the detection rate of specific antibodies such as antilactoferrin and anticarbonic anhydrase II antibodies have not been widely assessed in clinical setting because they require a special laboratory for their measurement which is available to only a low number of clinicians. A number of groups have tried to find other laboratory indicators of autoimmune pancreatitis and evaluation of the alleles of major histocompatibility complex genes seems to be a promising tool for identifying patients susceptible to autoimmune pancreatitis. One report mentioned that DRB1*0405 and DQB1*0401 are significantly more frequent in patients with autoimmune pancreatitis when compared to chronic calcifying pancreatitis [16]. At the present time, however, further studies are required to evaluate the value of each laboratory indicator and to find a more reliable one.

Imaging evaluation

Imaging evaluation is essential in the diagnosis of autoimmune pancreatitis [17]. Ultrasound is often the first imaging technique to be utilized in a patient with obstructive jaundice or with upper abdominal pain and a hypoechoic diffuse swelling in the pancreas (sausage-like appearance), or a focal swelling of the pancreas simulating a neoplastic lesion can be observed as well as a dilation of the extrapancreatic bile duct, secondary to an involvement of its intrapancreatic portion. Contrast-enhanced ultrasonography can successfully visualize fine vessels in pancreatic lesions and may play a pivotal role in the depiction and differential diagnosis of pancreatic tumors. In particular, some Authors have analyzed the enhancement of focal pancreatic lesions and it has been shown that, while most of the inflammatory pancreatic masses

***Table 4*. A proposal of revised Korean diagnostic criteria for autoimmune pancreatitis [20]**

Criteria	Definition
Criterion I.	Pancreatic imaging (essential): (1) CT – Diffuse enlargement (swelling) of pancreas and (2) ERCP – Diffuse or segmental irregular narrowing of main pancreatic duct
Criterion II.	Laboratory findings: (1) elevated levels of IgG and/or IgG4 or (2) detected autoantibodies
Criterion III.	Histopathologic findings: fibrosis and lymphoplasmocytic infiltration
Criterion IV.	Association with other postulated autoimmune disease

Definite diagnosis: I+II+III+IV or I+II+III or I+II or I+III; Probable diagnosis: I+IV (Rediagnosed as “definite” if “response to steroids” is present); Possible diagnosis: I (Rediagnosed as “definite” if “response to steroids” is present)

Table 5. **A proposal of revised Japanese diagnostic criteria for autoimmune pancreatitis (modified) [21]**

Criteria	Definition
I. Clinical criteria	
Criterion 1	Diffuse or segmental narrowing of the main pancreatic duct with irregular wall and diffuse or localized enlargement of the pancreas by imaging studies, such as abdominal ultrasonography (US), computed tomography (CT), and magnetic resonance imaging (MRI).
Criterion 2	High serum γ-globulin, IgG, or IgG4, or the presence of autoantibodies, such as antinuclear antibodies and rheumatoid factor.
Criterion 3	Marked interlobular fibrosis and prominent infiltration of lymphocytes and plasma cells in the periductal area, occasionally with lymphoid follicles in the pancreas.
II. Relationship to extrapancreatic lesions and other associated disorders	AIP may be associated with sclerosing cholangitis, sclerosing sialadenitis, or retroperitoneal fibrosis. Most AIP patients with sclerosing sialadenitis show negativity for both anti-SSA and anti-SSB antibodies, which may suggest that AIP differs from Sjögren's syndrome. Sclerosing cholangitis-like lesions accompanying AIP and primary sclerosing cholangitis respond differently to steroid therapy and have different prognoses, suggesting that they are not the same disorder.
Diagnosis of autoimmune pancreatitis is established when criterion 1, together with criterion 2 and/or 3, are fulfilled. However, it is necessary to exclude malignant diseases such as pancreatic or biliary cancers	

Description notes	
A. Imaging studies	
1. Diffuse or localized swelling of the pancreas	
a.	On US, pancreatic swelling is usually hypoechoic, sometimes with scattered echogenic spots
b.	Contrast-enhanced CT generally shows delayed enhancement similar to a normal pancreas with sausage-like enlargement, and/or a capsular-like low-density rim.
c.	MRI shows diffuse or localized enlargement of the pancreas with lower density in the T1-weighed image and higher density in the T2-weighed image compared with the corresponding liver image.
2. Diffuse or localized narrowing of the pancreatic duct	
a.	Unlike obstruction or stricture, narrowing of the pancreatic duct extends over a larger range, where the duct is narrowed with irregular walls. In typical cases, more than one-third of the entire length of the pancreatic duct is narrowed. Even in cases where the narrowing is segmental and extends to less than one-third of the total length, the upper part of the main pancreatic duct rarely shows notable dilatation.
b.	When the pancreatic images show typical findings but laboratory data do not, AIP is possible. However, without histopathological examination, it is difficult to distinguish AIP from pancreatic cancer.
c.	To obtain images of the pancreatic duct, it is necessary to use endoscopic retrograde cholangiopancreatography in addition to direct images taken during an operation or of specimens. Currently, it is difficult to depend for the diagnosis on magnetic resonance cholangiopancreatography.
3. The pancreatic image findings described above may be observed retrospectively from the time of diagnosis	
B. Laboratory data	
1.	In many cases, patients with AIP show increased levels of serum γ-globulin, IgG, or IgG4. High serum IgG4, however, is not specific to AIP, since it is also observed in other disorders such as atopic dermatitis, pemphigus, or asthma. Currently, the significance of high serum IgG4 in the pathogenesis and the pathophysiology of AIP is unclear.
2.	Although increased levels of serum γ-globulin (≥2.0 g/dl), IgG (≥1800 mg/dl), and IgG4 (≥135mg/dl) may be used as a criterion for the diagnosis of AIP, further studies are necessary.
3.	Autoantibodies such as antinuclear, anti-lactoferrin, anti-carbonic anhydrase antibody and rheumatoid factor are often detected in patients with AIP.
C. Histopathological findings of the pancreas	
1.	Fibrotic changes associated with prominent infiltration of lymphocytes and plasma cells, occasionally with lymphoid follicles, are observed. In many cases, infiltration of IgG4-positive plasma cells is observed.
2.	Lymphocytic infiltration is prominent in the periductal area, together with interlobular fibrosis, occasionally including intralobular fibrosis.
3.	Inflammatory cell infiltration involves the ducts and results in diffuse narrowing of the pancreatic duct with atrophy of acini.
4.	Obliterative phlebitis is often observed.
5.	Although fine-needle biopsy under ultrasonic endoscopy is useful for differentiating AIP from malignant tumors, diagnosis may be difficult if the specimen is too small.
D. Endocrine and exocrine function of the pancreas	
	Some patients with AIP show a decline of exocrine pancreatic function and develop diabetes mellitus. In some cases, steroid therapy improves endocrine and exocrine pancreatic dysfunction.

show a pattern of enhancement similar to the normal pancreatic gland ("isovascular"), a focal pancreatic tumor is hypovascular to the surrounding normal parenchyma. A focal or diffuse swelling of the pancreatic gland can be observed at both computed tomography and magnetic resonance imaging. Dynamic imaging at computed tomography or magnetic resonance imaging shows

a delayed enhancement of the segments of the gland which are involved. In some cases, minimal peripancreatic stranding suggesting inflammation can be seen. Moreover, a capsule-like smooth rim can be observed which is hypodense on computed tomography and hypointense on T2 weighted images, showing delayed enhancement on dynamic imaging. This is thought to correspond to an inflammatory process involving peripancreatic tissues and appears to be a characteristic finding of autoimmune pancreatitis. Pancreatic calcifications are rarely seen in autoimmune pancreatitis. Involvement of the main pancreatic duct and the biliary duct is well-described in the literature. Endoscopic retrograde cholangiopancreatographic criteria for the diagnosis of autoimmune pancreatitis include diffuse irregular narrowing of the main pancreatic duct and abnormalities which normalized after steroid therapy. The same alterations can be observed at MR cholangiopancreatography. The invasion of vessels, vascular encasement, mass effect and fluid collections are absent in autoimmune pancreatitis.

Diagnostic criteria

There are no internationally accepted diagnostic criteria for the diagnosis of autoimmune pancreatitis. The diagnostic criteria widely used for autoimmune pancreatitis are those proposed by the Japan Pancreas Society [18] and are reported in *Tab. 1*; interestingly, the criteria do not include symptoms or common laboratory findings as they are not specific to autoimmune pancreatitis [12,19]. Italian criteria include some differences with respect to the Japanese diagnostic criteria (*Tab. 2*) [5] such as the association with other autoimmune diseases and the response of the disease to steroid treatment. Korean researchers utilize a third classification which takes into account the Japanese and the Italian diagnostic criteria (*Tab. 3*) [20]. Furthermore, new classification systems have been proposed from Korean researchers (*Tab. 4*) [20], and, very recently, by Japanese Research Committee of Intractable Diseases of the Pancreas (*Tab. 5*) [21].

Therapeutic options

Autoimmune pancreatitis usually responds to steroid therapy. There are numerous reports of dramatic response of this disease to above mentioned therapy. However, spontaneous resolution without treatment has also been noted. Autoimmune pancreatitis is a fibro-inflammatory disease and intense inflammation is often accompanied by intense fibrosis; thus, even if the inflammatory component responds to steroid therapy; the fibrosis often permanently disfigures, damages and sometimes destroys the organ [22].

Open questions

There is a need for a classification system for such a rare disease; therefore, an international consensus statement releasing widely accepted guidelines for autoimmune pancreatitis would be welcome in order to help in evaluating the possible presence of autoimmune pancreatitis in patients with an undefined etiology; in fact, a recent study has reported that clinical or biochemical autoimmune stigmata are present in 40% of patients with idiopathic chronic pancreatitis and, therefore, autoimmune mechanisms may be frequent in idiopathic pancreatitis [23].

We also need to know the duration of steroid treatment and the possible cause of failure of steroid therapy in some patients; finally, we need to evaluate the reason why some patients experience more attacks of pain in a disease characterized by a painless course.

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Application of summary receiver operating characteristics (sROC) analysis to diagnostic clinical testing

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Abstract

Summary receiver operating characteristics (sROC) analysis is a recently developed statistical technique that can be applied to meta-analysis of diagnostic tests. This technique can overcome some of the limitations associated with pooling the sensitivities and specificities of published studies. The sROC curve is initially constructed by plotting the sensitivity (true positivity) and false positivity (1 – specificity) of each study. After mathematical manipulation of the true and false positivities, linear regression is performed to calculate the slope and y-intercept. These coefficients are then entered into the sROC equation to generate the sROC curve. There are three commonly used methods to assess the accuracy of the test: the exact area under the curve (AUC) for the sROC function, the homogeneous AUC, and the index Q*. Statistical formulas can compare these values from different diagnostic tests. With the introduction of sROC software and better understanding of this method, the application of sROC analysis should continue to increase.

Key words: summary receiver operating characteristic (sROC) curve, meta-analysis, diagnostic tests.

Introduction

New diagnostic tests are continuously being introduced in medicine. Clinical trials usually attempt to demonstrate the superiority of a new test by comparing its sensitivity and specificity to a conventional test. In many cases, published studies comparing diagnostic tests may have inconclusive or conflicting results. A meta-analysis of the published studies can be useful in evaluating these comparisons. Most meta-analysis studies of diagnostic tests generally provide a pooled estimate of the sensitivity and specificity. Recently, meta-analysis studies have begun to use summary receiver operating characteristics (sROC) analysis. This method consists of constructing a receiver operating curve from published studies that have determined the sensitivity and specificity of a test. The sROC curve can then be evaluated by a variety of statistical techniques. As many clinicians are unfamiliar with sROC analysis, the purpose of this review is to summarize the principles involved in this technique. Although the authors are gastroenterologists and the hypothetical example relates to colonic polyps, the concepts apply to most tests in clinical medicine.

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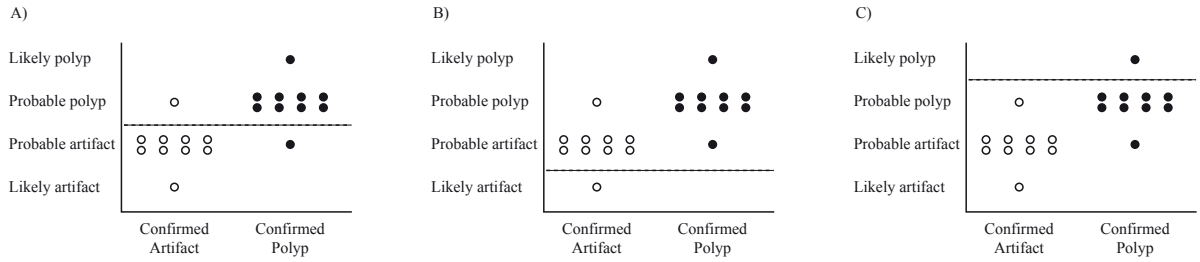
Limitations of sensitivity and specificity

The statistical technique for pooling sensitivities and specificities generally consists of weighing these rates by the inverse of their variance, summing the weighted rates, and dividing this sum by the sum of the inverses of their variance [1-3]. While pooled estimates of sensitivity and specificity are useful, they have a variety of limitations. First, sensitivity and specificity represent a trade-off as the threshold changes. By loosening the criteria (i.e., generally lowering the threshold), a test will become more sensitive but less specific. Raising the threshold will make a test more specific but less sensitive. Furthermore, sensitivity and specificity by themselves do not provide an overall evaluation of the accuracy of a test. For example, one may wish to compare two tests, one with a sensitivity and specificity both equal to 90% and the other with a sensitivity of 98% and a specificity of 80%. It is difficult to determine which one is more accurate from these characteristics.

Figure 1. **Three radiologists are evaluating the ability of CT colonography to discriminate polyps vs artifacts. In panel A, the first radiologists selects a threshold to optimize discrimination, resulting in a sensitivity and specificity both equal to 90%. In Panel B, the second radiologist selects a threshold to avoid missing polyps, resulting in a sensitivity of 100% and a specificity of 10%. In panel C, the third radiologist selects a threshold to minimize false positives, resulting in a sensitivity of 10% and a specificity of 100%**



In addition, pooling the sensitivities and specificities from multiple studies can occasionally result in a distorted estimate [4,5]. In a hypothetical example shown in *Fig. 1*, three radiologists are asked to determine whether filling defects seen on computed tomographic (CT) colonography represent polyps or artifacts. The radiologists classify the findings using the following scale: likely polyp, probable polyp, probable artifact, and likely artifact. All three of these radiologists have identical skills in visual perception and their samples of polyps are similar. When evaluating the ten endoscopically confirmed polyps, each one independently reports identical results as follows: likely polyp (n=1), probable polyp (n=8), and probable artifact (n=1). When evaluating the ten endoscopically confirmed artifacts, each one independently reports identical results as follows: likely artifact (n=1), probable artifact (n=8), and probable polyp (n=1). The radiologists are then asked to select a threshold and then report their results using a dichotomous scale: polyp or artifact. The first radiologist uses a threshold that maximizes the correct classification, resulting in a sensitivity of 90% and a specificity of 90% (*Fig. 1A*). The second radiologist, concerned about the legal consequences of a missed polyp, will diagnose a lesion as a polyp even it appears to be a probable artifact. The overly anxious radiologist will then report a sensitivity of 100% and a specificity of 10% (*Fig. 1B*). A third radiologist is more cavalier, believing that most polyps rarely progress to cancer. In order to minimize the number of colonoscopies being performed at his hospital, he will only diagnose a polyp on CT colonography if it has the appearance of a definite polyp. He then reports a sensitivity of 10% and a specificity of 100% (*Fig. 1C*). Using the statistical methods for pooling rates [1-3], the pooled sensitivity and specificity will both be equal to 67% (95% confidence intervals: 47-83%). These pooled values represent a distortion from the optimal sensitivity and specificity (both equal to 90%) that one would have obtained using an appropriate threshold. Analogous to Gresham's law of currency ("bad money drives good money out of circulation"), bad studies are able to distort good studies in a statistical pooling of sensitivity and specificity.

Advantages of sROC

In contrast, meta-analysis using sROC analysis will generate a composite statistic that reflects the discriminating ability of a diagnostic test. A traditional ROC curve plots the true positivity (sensitivity) as a function of false positivity (equal to 1-specificity) of a test at different thresholds [6]. In sROC analysis, one first plots the sensitivity and false positivity for each study [7]. A sROC curve is then constructed to fit these points. The area under the sROC curve is then determined to assess the discriminating ability. An area under the curve (AUC) close to 1.0 signifies that the test has almost perfect discrimination while an AUC close to 0.5 suggest poor discrimination [6]. An AUC significantly less than 0.5 would indicate that the criteria for "normal" and "abnormal" should be reversed. This scoring system is analogous to that of a true-false test. A student who knows all of the answers would score 100% while a student who knows none of the material should be able to score 50% by random guessing. A score significantly less than 50% would suggest an aberrant testing technique (e.g., confusing the symbols for true and false).

Many clinicians have not been well acquainted to sROC analysis for several reasons. This statistical technique is relatively new, having first been described in 1993 [7]. In addition, there have been relatively few published review articles which attempt to explain the mathematical techniques to most clinicians. Finally, many commonly used statistical software packages do not currently include sROC analysis. On the other hand, the techniques for performing sROC analysis are not complicated. The techniques include transformation using logarithms, linear regression, curve fitting, and understanding the relationship between integration and the area of the curve. These mathematical techniques are taught in most pre-medical school curriculums. Thus, sROC analysis should be accessible for most clinicians. In addition, sROC software is available to simplify the process.

Method of sROC

The sROC curve is a plot of the true positive rate (sensitivity) as a function of the false positive rate (1-specificity). The sROC equation is as follows:

$$TPR = \frac{e^{a/(1-b)} \times (FPR/(1-FPR))^{(1+b)/(1-b)}}{1+e^{a/(1-b)} \times (FPR/(1-FPR))^{(1+b)/(1-b)}} \quad (1)$$

where TPR is the true positive rate, FPR is the false positive rate, and a and b are coefficients which need to be deter-

Table 1. **Diagnostic characteristics of a test**

	Patients with disease	Patients without disease
Abnormal test	True positives	False positives
Normal test	False negatives	True negatives
Total	Number of patients with disease	Number of patients without disease

Table 2. **Transformation of the sensitivities and specificities in order to construct the sROC curve using linear regression**[1]

Study	Sensitivity (true positive rate)	Specificity	False positive rate (1-specificity)	Corrected sensitivity (true positive rate)[2]	Corrected false positive rate[2]	Logit of the true positive rate (TPR)[3]	Logit of the false positive rate (FPR)[4]	D (logit TPR - logit FPR)	S (logit TPR + logit FPR)
Optimal radiologist	9/10 (0.9)	9/10 (0.9)	1/10 (0.1)	9.5/11 (0.86)	1.5/11 (0.14)	1.85	-1.85	3.69	.00
Anxious radiologist	10/10 (1.0)	1/10 (0.1)	9/10 (0.1)	10.5/11 (0.95)	9.5/11 (.86)	3.04	1.85	1.20	4.89
Cavalier radiologist	1/10 (0.1)	10/10 (0.0)	10/10 (1.0)	1.5/11 (0.14)	0.5/11 (0.05)	-1.85	-3.04	1.20	-4.89

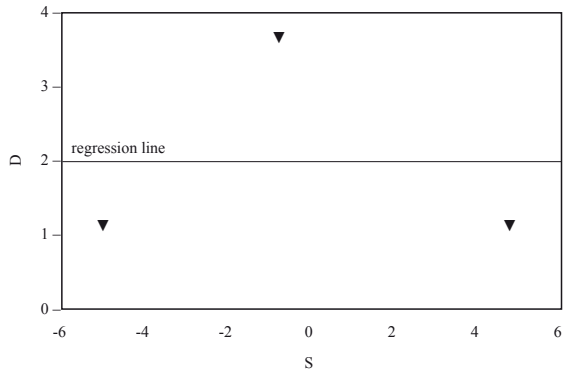
[1] – Number in parenthesis indicate rate; [2] – The rates were corrected by adding 0.5 to the numerator and 1.0 to the denominator; [3] – The logit of the true positive rate is the natural log of [true positive rate/(1-true positive rate)]; [4] – The logit of the false positive rate is the natural log of [false positive rate/(1-false positive rate)]

mined in order to fit the sROC curve [8]. At a first glance, the equation appears to be too daunting for a simple curve fitting technique. Nevertheless, Moses et al. [7] proposed a method that is relatively straightforward. The diagnostic characteristics of each study (i.e., true positivity and false positivity) can be transformed to other variables and then fitted using linear regression. As shown in *Tab. 1*, one first has to determine the number of true positives, false negatives, true negatives, and false positives for each study. One then has to calculate their sensitivity (true positives/number of patients with disease), specificity (true negatives/number of patients without disease), and false positive rate (1-specificity). If any study has a sensitivity or specificity that is either equal to 0 or 1.0, a continuity correction is required. Moses et al. [7] suggests adding 0.5 to all of the four cells of *Tab. 1* for all included studies.

One has to then transform the true positive rate (TPR) and false positive rates (FPR) into their corresponding logits. The logit of the true positive rate is the natural log of [TPR/(1-TPR)] while the logit of the false positive rate is the natural log of [FPR/(1-FPR)]. If the values for the TPR or FPR are either 0 or 1, the continuity correction will prevent obtaining an undefined value which would either occur by dividing by zero or taking the natural log of zero. One then calculates two parameters, D and S. D is defined as the difference of the logits (logit TPR - logit FPR) while S is defined as the sum of the logits (logit TPR + logit FPR). These transformations can be readily calculated using spreadsheet software by specifying the equations. Alternatively, many sophisticated statistical packages permit users to develop short programs for custom transformation of variables. *Tab. 2* illustrates the transformations of true and false positive rates using the example of the three radiologists.

These transformations will facilitate constructing the sROC curve using linear regression. A linear model using these transformed variables (D and S) can be constructed as follows:

Figure 2. **Linear regression of the transformed D and S variables from the hypothetical study of the three radiologists. D is defined as the difference of the logit of the true positive rate and the logit of the false positive rate. S is defined as the sum of the logit of the true positive rate and the logit of the false positive rate. The y-intercept, also known as coefficient *a*, is 2.03±1.18 while the slope, also known as coefficient *b* is 0±0.29. The sROC curve can be generated by plugging in the values for coefficients *a* and *b* in the sROC equation**



$$D = b \times S + a \qquad (2)$$

in which coefficients *a* and *b* are the y-intercept and slope, respectively. It can be mathematically shown that the coefficients a and b are identical to those in the sROC equation. These coefficients can be determined by performing linear regression using D as the dependent variable and S as the independent variable. Using the example of the three radiologists, coefficient *a* (the y intercept) is 2.03 while coefficient *b* (the slope) is 0 (shown in *Fig. 2*). The standard errors of coefficients *a* and *b* are 1.18 and 0.29, respectively.

One can construct the sROC curve by substituting the values for coefficients *a* and *b* into the sROC function (equation 1) and then plotting the true positive rate over the range

***Figure 3*. Comparison of two sROC curves. Panel A shows an sROC curve constructed assuming that coefficient *a* is 100 and coefficient *b* is 0. The curve approximates a right angle with an AUC near 1, signifying high discriminating ability. Panel B shows an sROC curve constructed assuming that coefficient *a* and *b* are zero. The curve is a 45 degree straight line with an AUC equal to 0.5, signifying poor discriminating ability**

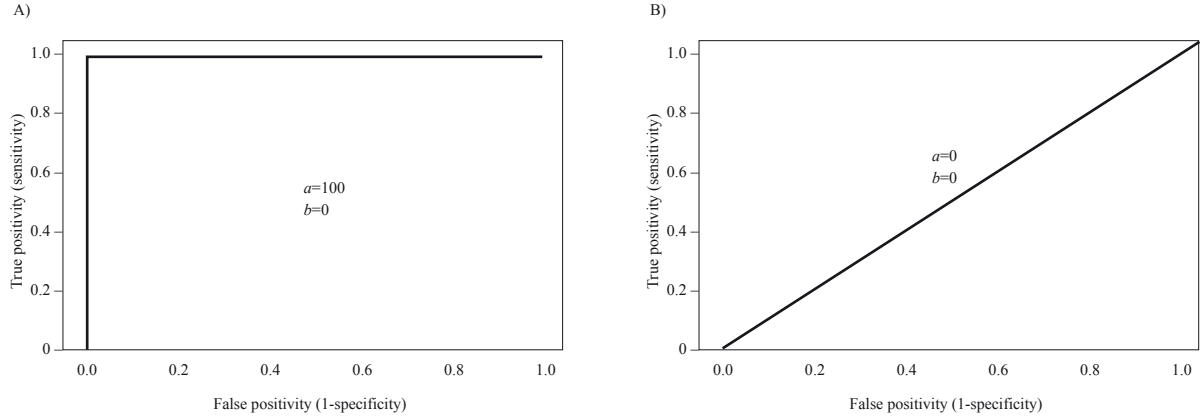
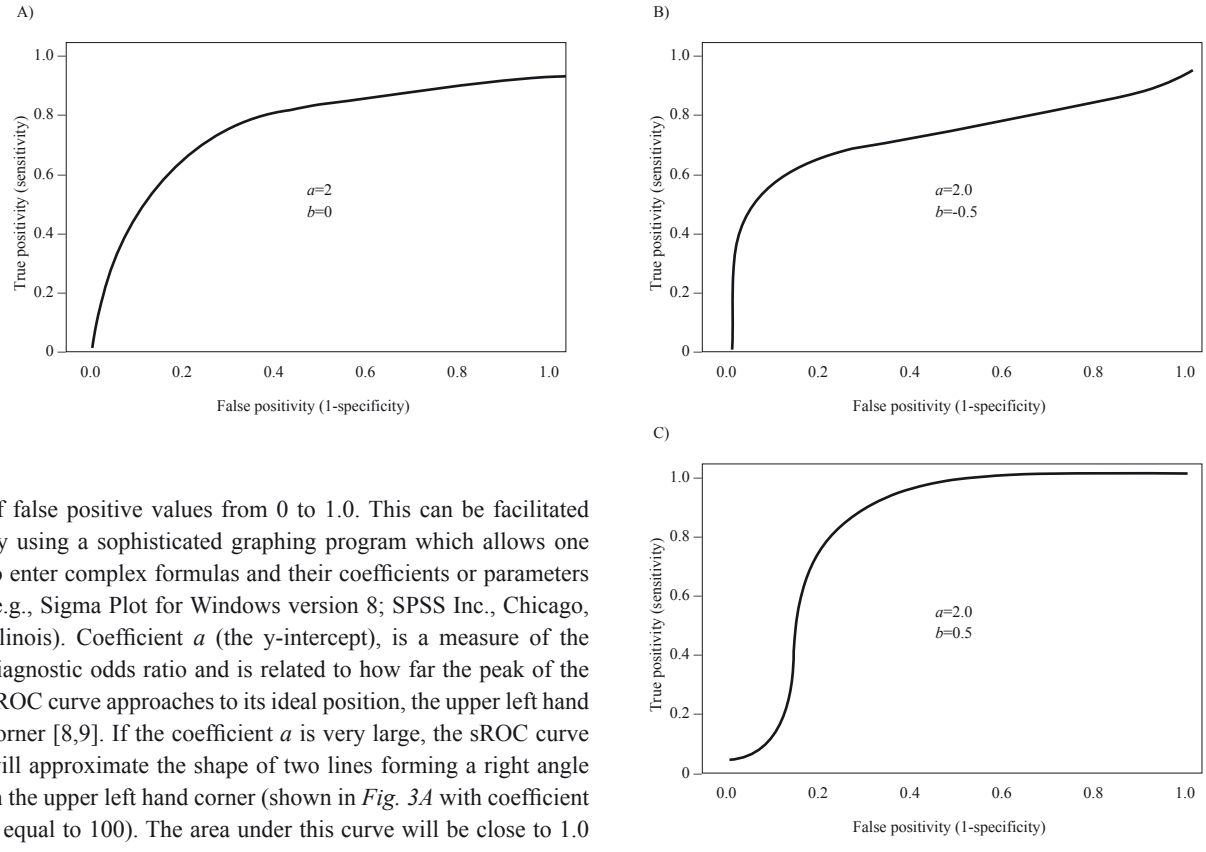


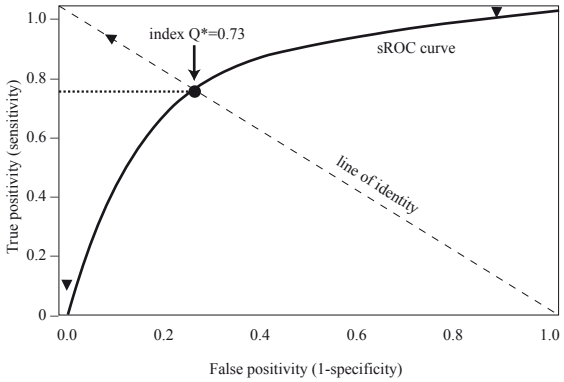
***Figure 4*. Comparison of three sROC curves. All three curves assume that coefficient *a* equals 2. Panel A shows an sROC curve constructed assuming that coefficients *b* equals 0, resulting in a symmetric shape. An sROC curve with coefficient *b* equals 0 is called the homogeneous case and has properties that make the AUC easier to calculate. Panels B and C assume the coefficient *b* is -0.5 and 0.5, respectively. These curves have an asymmetric shape and their exact AUCs are more complicated to calculate**



of false positive values from 0 to 1.0. This can be facilitated by using a sophisticated graphing program which allows one to enter complex formulas and their coefficients or parameters (e.g., Sigma Plot for Windows version 8; SPSS Inc., Chicago, Illinois). Coefficient *a* (the y-intercept), is a measure of the diagnostic odds ratio and is related to how far the peak of the sROC curve approaches to its ideal position, the upper left hand corner [8,9]. If the coefficient *a* is very large, the sROC curve will approximate the shape of two lines forming a right angle in the upper left hand corner (shown in *Fig. 3A* with coefficient *a* equal to 100). The area under this curve will be close to 1.0 and the test will have a high degree of discrimination. In contrast, if coefficient *a* is close to 0, the sROC curve will assume the shape of 45 degree line (shown in *Fig. 3B*) [4,8]. The area under this curve is 0.5 and the test will have poor discriminating ability. Coefficient *b* (i.e., the slope of the regression line) will affect the shape of the sROC curve. If coefficient *b*=0, the sROC curve will be symmetrical (shown in *Fig. 4A*). If coefficient *b* is significantly less than or greater than zero, the sROC curve will be markedly asymmetric (shown in *Fig. 4B* and *4C*) [8]. The sROC curve of the example of the three radiologists is shown in *Fig. 5*.

Once the sROC curve has been constructed, there are three methods to assess the discriminating ability of the test: the exact AUC, the homogeneous AUC, and the index Q* [8]. However, an exact calculation of the AUC for an sROC curve can be somewhat complicated. In many situations, the area under a curve can be calculated by integrating the function. However, the general sROC function (equation 1) cannot be integrated using calculus. Thus, the exact AUC needs to be determined by numerical integration [8] in which the sROC curve is bro-

Figure 5. **The sROC curve summarizing the diagnostic characteristics of the three radiologists. The true and false positivities of each radiologist are plotted using a triangle, ▼. The exact and homogenous AUC are both equal to 0.80±0.01. The "identity line" corresponds to the point in which sensitivity (true positivity) equals specificity (1 - false positivity). Index Q* can be visualized as the intersection of the sROC curve and the "line of identity". The point of intersection is shown as a closed circle, ●. Index Q* corresponds to the value of the true positivity at the point of intersection (shown as a horizontal line projecting from the point of intersection to the y-axis)**



ken up into very small rectangles or trapezoids (depending on the algorithm being used). Mathematical software programs such as Mathcad (MathSoft, Inc., Cambridge, MA), Mathematica (Wolfram Research, Inc., Champaign, IL), and Maple (MapleSoft, Waterloo, Ontario) can readily perform numerical integration of most functions. Walter [8] also provided the formulas for calculating the standard error of the exact AUC. This calculation also involves numerical integration and using the standard errors of the coefficients *a* and *b* and their covariance. These three terms are generally provided by by many statistical programs that perform linear regression. In the hypothetical example of the three radiologists, the exact AUC is 0.80 with a standard error of 0.01.

A simpler method for estimating the AUC was also provided by Walter [8]. Most sROC curves of clinical tests have a coefficient *b* close to zero. In mathematical models in which one assumes that one of the coefficients is zero, the example is then called the homogenous case. In the homogenous case of the sROC function, one assumes that coefficient *b*=0. The sROC equation can then be simplified to:

$$TPR = \frac{e^{a} \times (FPR/(1\text{-}FPR))}{1 + e^{a} \times (FPR/(1\text{-}FPR))} \qquad (3)$$

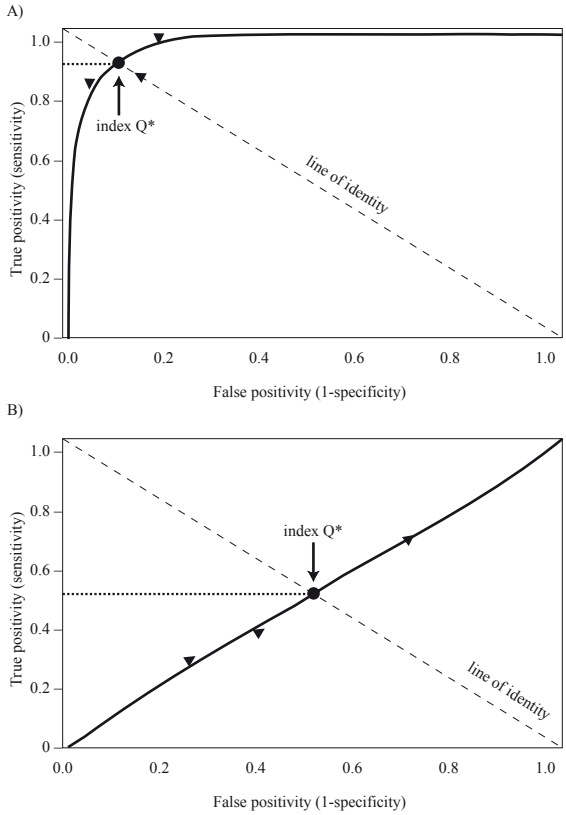
This function can be integrated with respect to FPR using standard calculus techniques, resulting in the following equation for the area under the homogeneous sROC curve:

$$\text{Homogeneus AUC} = \frac{e^{a} \times (e^{a}\text{-}1\text{-}a)}{(e^{a}\text{-}1)^{2}} \qquad (4)$$

The standard error of the homogeneous AUC [SE (homogenous AUC)] was shown by Walter [8] as follows:

$$SE(\text{Homogeneous}) = \frac{e^{a} \times [(e^{a}+1) \times a\text{-}2(e^{a}\text{-}1)] \times SE(a)}{(e^{a}\text{-}1)^{3}} \qquad (5)$$

Figure 6. **Comparison of a test with good discrimination (Panel A) with a test with poor discrimination (Panel B). Three hypothetical studies of the "good" test (shown in Panel A) have reported the following pairs of sensitivities and specificities: 90% and 88%, 98% and 85%, and 88% and 97%. The test's sROC curve and the points for the true and false positivities (using a triangle, ▼) are plotted in Panel A. The exact and homogeneous AUCs are both equal to 0.97±0.01. Index Q* corresponds to the point of intersection of the sROC curve and the "line of identity". The value for index Q*, 0.93±0.02, corresponds to the true positivity of the intersection (shown as a horizontal line projecting from the point of intersection to the y-axis). The exact AUC, homogeneous AUC, and index Q* are all close to 1, the value for a test with perfect discrimination. Three hypothetical studies of the "poor" test (shown in Panel B) have reported the following pairs of sensitivities and specificities: 40% and 60%, 30%, and 75%, and 70% and 30%. The test's sROC curve and the points for the true and false positivities (using a triangle, ▼) are plotted in Panel B. The exact and homogeneous AUCs are both equal to 0.51±0.01. The index Q*, the point of intersection of the sROC curve and the "line of identity" is also equal to 0.51±0.01. These values are close to 0.5, signifying that the test has poor discrimination**



where SE (a) is the standard error of coefficient *a* (i.e., the y-intercept). This value is generally provided by most statistical programs that perform linear regression. While these formulas are tedious to calculate by hand, they are much easier to compute than numerical integration. The homogenous AUC will be very close to the exact AUC in cases in which coefficient *b* is close to zero. When coefficient *a*=0, equation 3 will degenerate into an undefined value. Using an alternative formula, Walter [8] showed that the AUC of this case is equal 0.5.

Comparing two sROC curves

The AUC of two sROC curves can be statistically compared using a formula provided by Hanley and McNeil [10] for evaluating tradition ROC curves:

$$Z = \frac{AUC_1 - AUC_2}{\sqrt{SE(AUC_1)^2 + SE(AUC_2)^2}} \qquad (6)$$

where z is the z statistic, AUC_1 and AUC_2 are the area under the sROC curves of the two tests, and $SE(AUC_1)$ and $SE(AUC_2)$ are their standard errors. The p value for the z statistic can be determined by using a z table or an internet-based calculator.

Another method for assessing the accuracy of an sROC curve is the index Q*. This method is less intuitive to clinicians than AUC but is easier to calculate. The index Q* corresponds to the upper most point on the sROC curve in which true positivity (or sensitivity) equals specificity [7,8]. This can be shown graphically by drawing a "line of identity" in which true positivity = specificity on the sROC graph. As the x-axis on the sROC curve is the false positivity, one has to transform the variable specificity to 1-false positivity. Thus, the appropriate equation for this "line of identity" would be true positivity =1 – false positivity. This line would have a y-intercept of 1 and a slope of -1. Graphically, the index Q* corresponds to the value of the true positive rate at the point of the intersection of the sROC curve and the "line of identity" (shown in *Fig. 5*). A test close to ideal has an sROC curve that approximates a right angle; therefore, it would intersect the "line of identity" close to the upper left hand corner, resulting in an index Q* close to 1 (shown in *Fig. 6A*). In contrast, a test of poor discriminatory ability has an sROC curve that approximates a diagonal line. It would intersect the "line of identity" near their mid-points, resulting in an index Q* close to 0.5 (shown in *Fig. 6B*). The index Q* can also be calculated using the formula described by Moses et al. [7] and Walter [8]:

$$\text{index } Q^* = \frac{e^{a/2}}{1+e^{a/2}} \qquad (7)$$

The formula for the standard error of the index Q* is as follows:

$$SE(\text{index } Q^*) = \frac{e^{a/2}}{2(1+e^{a/2})^2} \times SE(a) \qquad (8)$$

where is the standard error of coefficient *a* (y-intercept).

The index Q* values of two tests can be statistically compared using a formula analogous to that of AUC as described by Moses et al. [7]:

$$z = \frac{\text{index } Q_1^* - \text{index } Q_2^*}{\sqrt{SE(\text{idex } Q_1^*)^2 + SE(\text{idex } Q_1^*)^2}} \qquad (9)$$

where z is the z statistic, index Q_1* and index Q_2* are the index Q* for test 1 and test 2, respectively, and $SE(\text{index } Q^*_1)$ and $SE(\text{index } Q^*_2)$ are their corresponding standard errors.

The following example will illustrate how sROC can be used to compare two hypothetical tests. The first test has good discrimination as three studies have reported the following pairs of sensitivities and specificities: 90% and 88%, 98% and 85%, and 88% and 97%. After applying linear regression, coefficients *a* and *b* are computed to be 5.08±0.65 and 0.06±0.43, respectively. *Fig. 6A* shows the sROC curve constructed using these coefficients. The exact and homogeneous AUCs are both equal to 0.97±0.01. These values are close to 1, suggesting that the test has nearly perfect discrimination. The point of intersection of the sROC curve and the "line of identity" has a true positivity (or sensitivity) equal to 0.93±0.02 which corresponds to the index Q*. The second test has poor discrimination as three studies have reported the following pairs of sensitivities and specificities: 40% and 60%, 30%, and 75%, and 70% and 30%. The coefficients *a* and *b* are 0.06 ±0.08 and -0.06±0.05, respectively. *Fig. 6B* shows the sROC curve constructed using these coefficients. The exact and homogenous AUCs are both equal to 0.51±0.01. Index Q* for this test (shown in *Fig. 6B*) is equal to 0.51±0.01. All of these values are close to 0.5, the value for a worthless test. Statistical comparisons of the exact and homogeneous AUCs result in a z-test of 25 ($p<0.0001$). Statistical comparisons of their index Q* result in a z-test of 17 ($p<0.001$).

Software has been developed in order to facilitate sROC analysis. Meta-test is a DOS based program that was developed by Dr. Joseph Lau at New-England Medical Center in Boston [11,12]. Meta-DiSc is a Windows based program that uses a graphic interface that was developed at the Clinical Biostatistics Unit at the Ramón y Cajal Hospital in Madrid [13].

Use of sROC

From 1995 to June 2007, approximately 180 published articles have discussed sROC analysis (SilverPlatter's MEDLINE, Ovid Technologies, New York). Furthermore, the number of papers has increased in the last 5 years. Published studies which used sROC analysis have evaluated CT colonography for polyp detection [14,15], diagnostic tests for hepatocellular carcinoma [16], diagnosing intravascular device-related bloodstream infection [17], ultrasonography for temporal arteritis [18], helical CT scans for diagnosing pulmonary embolism [19,20], and stress tests for risk stratification of coronary artery disease [21]. It is the hope of the authors that this paper will further familiarize clinicians with the principles of sROC analysis and further increase its application.

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Molecular basis of sodium butyrate-dependent proapoptotic activity in cancer cells

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Abstract

This review outlines the molecular events that accompany the antitumor action of sodium butyrate (NaBt). Butyrate, a low-molecular weight four-carbon chain volatile fatty acid (VFA) has been previously shown to withdraw cells from cell cycle or to promote cell differentiation, and finally to induce programmed cell death. Recent advances in molecular biology indicate, that this product of large bowel microbial fermentation of dietary fiber, might evoke the above-mentioned effects by indirect action on genes. NaBt was shown to inhibit histone deacetylase activity, allowing DNA binding of several transcription factors. Higher genomic activity leads to the higher expression of proapoptotic genes, higher level of their protein products and elevated sensitivity to death ligand-induced apoptosis. Cancer cells might be arrested in G1 phase of cell cycle in a p21-dependent manner. Proapoptotic activity of NaBt includes higher expression of membrane death receptors (DR4/5), higher level and activation of Smad3 protein in TGF-β-dependent apoptotic pathway, lower level of antiapoptotic proteins (cFLIP, XIAP) and activation of proapoptotic tBid protein. Thus, both intrinsic and extrinsic apoptotic pathways are stimulated to ampify the apoptotic signals. These effects are specific for tumor but not for regular cells. Unique properties of NaBt make this agent a promising metabolic inhibitor to retard tumorigenesis to suppress tumor growth.

Key words: sodium butyrate, apoptosis, cancer cells.

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Introduction

The "immune escape", also known as immunoediting, is evolutionary developed ability of cancer cells to avoid elimination by the immune system [1-3]. The main strategies used, such as ignorance, impaired antigen presentation, expression of immunosuppressive factors and molecules, tolerance induction and apoptosis resistance allow tumor cells to grow and develop [3]. The current efforts are focused on identification of the molecular mechanisms responsible for the inhibition of apoptotic signals and sensitization of cancer cells to natural cell death induction by metabolic inhibitors. Among various tested compounds also naturally derived substance i.e. sodium butyrate is a promising agent for future anticancer immunotherapy.

Sodium butyrate – multifunctional short-chain volatile fatty acid

Butyrate in a non-toxic short-chain fatty acid that is produced naturally during the microbial fermentation of dietary fiber in the colon [4]. Butyrate plays an important role in homeostasis of the colonic mucosa by inducing pathways of cell maturation, including cell cycle arrest, differentiation and apoptosis [5,6]. More interestingly, butyrate-mediated regulation of apoptotic pathways occurs also in colon cancer cells [7-9]. Noteworthy, the proapoptotic action of sodium butyrate (NaBt) is not limited to gastrointestinal tract but was also reported in chronic myelogenous [10] and myeloid leukemia [11], breast [12], prostate [13,14] and many other cancer types [15,16]. Despite of numerous studies demonstrating the antiproliferative effect of NaBt treatments, there is no universal explanation for this phenomenon. The review presents some of the postulated molecular mechanisms of sodium butyrate-mediated regulation of apoptosis process.

Sodium butyrate regulates gene expression by inhibiting histone deacetylase activity

Sodium butyrate and other short-chain fatty acids (SCFAs) are histone deacetylase (HDAC) inhibitors. The major biochemical change that occurs in cells treated with HDAC inhibitors is the hyperacetylation of histones [17]. Histone proteins package DNA into nucleosomes, and core histones can be acetylated on lysine residues of NH2-terminal tails. Acetylation and deacetylation are catalyzed by specific enzymes, histone acetyltransferese and HDAC, respectively [18]. Sodium butyrate causes histone hyperacetylation through a noncompetitive and reversible inhibition of HDAC [19]. Histone hyperacetylation neutralize the charge between histone tails and DNA, freeing this region of DNA for access to transcription factors and is generally associated with activation of specific genes [20,21]. NaBt is intensively tested in cancer research, keeping in mind, that chromatin modification is a key factor in the development of neoplasia (for example certain oncogenic transcription factors, such as leukemogenic transcription factor promote oncogenesis by deregulation of chromatin structure) [22].

Sodium butyrate regulates Sp1 transcription factor

The according to Kim et al. [7] one of the NaBt cellular targets is Sp1 transcription factor. NaBt treatment [2, 3 mM, 6 h] disrupted association of histone deacetylase with Sp1 in colon cancer HCT-116 and HT-29 cell lines. At the same time, the supershift analysis confirmed increased binding of Sp1 to DNA in sodium butyrate-treated nuclear cells extracts, which in turn led to chromatin decondensation and activation of DR5 gene transcription. As a consequence, the HCT-116 and HT-29 cells became refractory to TRAIL (tumor necrosis factor-related apoptosis-inducing ligand) by up-regulated expression of DR5 protein, the TRAIL specific transmembrane receptor. Interestingly, NaBt was not able to activate the DR4 gene expression, which codes the second transmembrane TRAIL receptor. The selective expression of TRAIL receptors was also demonstrated in bladder [23] and breast cancer cells [12]. In colon cancer cells the NaBt-stimulated [0.5, 2 mM, 24 h] up-regulation of DR5 receptor allowed TRAIL to induce apoptosis process, which was visualized by procaspase-3 activation and PARP degradation. Simultaneously, the Western blot analysis showed the reduction of XIAP but not cIAP-1 and cIAP-2 antiapoptotic proteins level [7]. However, the authors did not prove that the expression of cIAPs is also Sp1-dependent.

Sodium butyrate induces G1 cell cycle arrest and sensitizes cancer cells to death ligands-induced apoptosis by p21-dependent pathway

The other target for NaBt action is p21. The NaBt-stimulated [5 mM, 24 h] histone hyperacetylation resulted in accumulation of acetylated histones H3 and H4 in colon cancer COLO-320 and SW1116 cell lines [24]. It is noteworthy, that the level of acetylated histone H3 and H4 at the domain containing the transcriptional start site in *p21/WAF-1* promoter and the binding sites of E2A transcription factor was significantly higher than that at another domain in promoter. Thus the authors observed the extended *p21* mRNA and p21 protein level [24]. p21/WAF-1 is the major suppressor of cyclins (A-H) and their associated cyclin-dependent kinases (cdk) [25]. The balance between the activation and inhibition of cyclin/cdk activities determines whether or not a given cell will proceed through the cell cycle and, as such, may contribute to the development of neoplasia. Archer et al. [26] demonstrated that NaBt administration [5 mM, 1-48 h] retarded HT-29 colon cancer cells growth and concomitantly decreased cyclin B1 (cB1) protein level. Further studies revealed that the delayed reduction in cB1, beginning at 6 h with maximal changes at 24 h (90%), contrasted with the early induction of p21 mRNA at 2 h. To verify the relationship between cB1 and p21 proteins, the HCT-116 p21 wild-type (+/+), heterozygote (+/-) and mutant (-/-) cells were used. The Northern-blot analysis showed that NaBt treatment caused dramatic decrease of cB1 expression in +/+ and +/-, but not in -/- cells. These results proved that p21 plays a critical role in butyrate-mediated repression of cB1 in colon cancer cells. This repression occurs through cis-element within 90-bp of the *cB1* gene transcriptional start site. The authors hypothesized, that histone hyperacetylation allows direct binding of p21 to DNA through protein-protein interactions. Although p21 is known to contain a zinc finger motif, generally seen in transcription factors, it has not been shown to play a role of transcription factor. It is possible that the p21-DNA interaction is mediated by cdk proteins, which could be bound to the amino-terminal portion of p21 [27]. Interestingly, Archer et al. [26] reported that in 90-bp region upstream of the *cB1* transcriptional start site, several consensus sequences for various transcription factors are localized, such as: heat shock factor (HSF), NF-Y and Sp1. Thus the transcription regulation of gene expression could be more complex and hard to predict.

On the other hand, Wang et al. [16] claimed that in primary effusion lymphoma (PEL), a peculiar type of B cell non-Hodgkin lymphoma cells, co-infected with Epstein-Barr virus (BCBL-1 cells) NaBt regulates cell cycle-related proteins and cause the growth inhibition but in a p21/WAF-1-independent manner. The Western blot analysis revealed the decreased cyclin-dependent kinase (cdk) 2, cdk4 and cyclin A proteins level in NaBt-treated cells, but at the same time there were no changes observed in p21/WAF-1 expression. The authors hypothesized that distinct results could be explained by the presence of virus, which could modulate cell response [16].

The p21/WAF-1 action was also examined by VanOosten et al. [13] in three prostate cancer cell lines: ALVA-31, DU-145 and LNCaP and by Earel et al. [23] in bladder tumor cells. Similarly to Archer et al. [26], NaBt [5 mM, 24 h] stimulated p21/WAF-1 activity, that in turn increased the percentage of cells in G1 cell cycle phase [13,23]. Additionally, in prostate cancer cells NaBt increased the responsiveness to TRAIL-induced cell death, further confirmed by the flow cytometry analysis [13]. Moreover, the quantitative real-time PCR revealed a modest up-regulation

in DR5 (TRAIL-R2) mRNA level after NaBt treatment, but no changes in DR5 death receptor protein level on cell surface was detected. These observations are contradictory to the previously described results published by Kim et al. [7], Earel et al. [23] and Chopin et al. [12] who found significantly higher level of DR5 surface protein after NaBt administration. VanOosten et al. [13] concluded that in prostate cancer cells additional molecular mechanism exists, which supports TRAIL-induced apoptosis. Based on Izeradjene et al. [28,29] and Ravi et al. [30] studies, the role of casein kinase II (PKCK2), which is also engaged in TRAIL resistance, was evaluated. The mechanism of the change in phenotype was found to lie in the connection between PKCK2 and caspase-2 [31]. When PKCK2 is down-regulated, procaspase-2 is dephosphorylated, allowing it to dimerize and become activated. The activated caspase-2 then processes procaspase-8 monomers between the large and small subunit, so that procaspse-8 can be fully activated by cleavage whenever TRAIL is recruited to DR4/5 death initiating signaling complex (DISC) after TRAIL-DR4/5 ligation. Interestingly, for the first time VanOosten et al. [13] demonstrated that PKCK2 activity is regulated by HDAC inhibitors, such as NaBt. The NaBt administration resulted in highly significant inhibition of PKCK2 activity, accompanied by the increased caspase-2 activity. Moreover, the immunoprecipitation analysis confirmed the elevated level of cleaved p43/41 procaspase-8 fragment in DR5-DISC complex. According to previously described scenario, the addition of the specific procaspase-2 inhibitor, Z-VDVAD-fmk totally abrogated NaBt-induced increase in TRAIL sensitivity. Finally, if cells were treated with 4,5,6,7--tetrabromobenzotriazole (TBB) prior to TRAIL treatment, the PKCK2 inhibitor caused a number of cells to undergo apoptosis, when compared to either agent used individually. Summing up, these results demonstrated that NaBt can sensitize tumor cells to TRAIL-mediated apoptosis by inhibiting PKCK2 activity, which in turn leads to caspase-2 activation and the processing of procasapse-8 into active form when tha latter is recruited to the DR-DISC complex. Within the context of apoptotic signal transduction pathways, the location of caspase-2 in this pathway has been historically lacking. Nevertheless, the activation of caspase-2 in primary effusion lymphoma after NaBt administration [3 mM, 18 h] was previously reported by Wang et al. [16]. the authors did not explain the mechanism of NaBt-induced p48 procaspase-2 to p33 cleavage. They supposed that the observed increase in active caspase-2 protein level resulted from oxidative stress [16]. However, the use of various antioxidants, such as vitamin C or catalase, did not protect tested cells from NaBt-induced apoptosis. Therefore, the above-mentioned observations reported by VanOosten et al. [13] shade more light on the molecular mechanism of NaBt-mediated sensitization of cancer cells to TRAIL-induced cell death.

Sodium butyrate sensitization of cancer cells to death ligands-induced apoptosis is mediated by down-regulation of antiapoptotic proteins

In accordance to former chapter, the NaBt is able to sensitize various cancer cells to TRAIL-induced cell death [7,13,23]. The identification of cellular targets for NaBt led these authors to draw similar conclusions pointing to the NaBt-dependent molecular mechanism of restored susceptibility of tumor cells TRAIL. Hitherto, no detailed study was done concerning the co-operation of NaBt with other death ligands (TNF-α, FasL). However, some reports suggest the presence of other cellular targets for sodium butyrate. NaBt up-regulates signals of death ligand-induced apoptosis. According to Chopin et al. [12] NaBt [1 mM, 6-48 h] modulates the TNF-R1, TNF-R2, Fas-R/CD95 death receptors in MCF-7 breast cancer cell line. At the same time, in NaBt-treated cells the Western blot analysis clearly showed the elevated level of FADD protein, one of the DISC complex components. The stimulated expression of transmembrane receptors and DISC components resulted in extensive cell death after TNF-α or FasL exposure [0.1 ng/mL, 18 h], what was visualized by Hoechst staining and confirmed by caspase-8 activation. Similar observations in colon cancer cells were previously demonstrated by Giardina et al. [32] and Hara et al. [33]. Additionally, Chopin et al. [12] indicated that mitochondria are involved in NaBt-induced apoptosis. TNF-α or FasL and NaBt co-treatments increased the level of tBid (truncated Bid), which is able to translocate to mitochondrial membrane and to induce release of cytochrome c from mitochondria to cytosol. Cytosolic cytochrome c favors the activation of caspase-9, which in turn activates downstream caspases [34]. In MCF-7 breast cancer cells the release of cytochrome c and caspase-9 activation were inhibited in the presence of Z-LETD-fmk, caspase-8 inhibitor. It was concluded that mitochondria-dependent apoptotic pathway is activated as a consequence of ligand-receptor complexes formation. It is not clear, whether NaBt modulates the mitochondrial apoptotic pathway, or just initiates execution of programmed cell death (PCD).

The second possible scenario of sensitization of various cancer cells to death ligands-induced cell death is the elimination of antiapoptotic proteins, which are able to inhibit trunsduction of death signal in the cell interior. One of such proteins is cFLIP (FLICE-inhibitory protein) protein bound to DISC complex of TNF-R1, DR4 and -5, and Fas-R [35]. By direct interaction with FADD protein, cFLIP diminishes or totally blocks death signals by competitive inhibition of caspase-8 activation [35]. A critical role of cFLIP in the resistance of certain cancers to death ligand-induced cell death was demonstrated. Upon treatment with certain cytokines increased sensitivity to cell death of cancers cells was associated with apparent reduction in cellular levels of cFLIP [36,37]. According to Hernandez et al. [38] NaBt could be considered as another potent agent that could serve as a useful adjunct for the treatment of metastatic colorectal cancer. They found that NaBt treatment [5 mM, 24 or 48 h] inhibits cFLIP expression in three human colon cancer cell lines: KM12C, KML4A and KM 20. Moreover, when cells were co-treated with NaBt [5 mM] and TRAIL [100 ng/mL], both the caspase-3 assay and Annexin-V immunofluorescenct assay showed apoptosis induction. The similar results were obtained by Natoni et al. [39], who observed the significant reduction of cFLIP level after NaBt administration in pancreatic cancer cells. As a result, cells became responsive to FasL-induced programmed cell death. Noteworthy, at the same

time the intrinsic apoptotic pathway was activated, which is in accordance to data previously reported by Kim et al. [7] in MCF-7 breast cancer cell line. The same authors also noticed NaBt-mediated elimination of the antiapoptotic proteins, such as XIAP. It was supposed, that NaBt could efficiently regulate the presence of various antiapoptotic proteins in cancer cells, supporting the objectives for immunotherapy.

Sodium butyrate up-regulates TGF-β signaling pathway in cancer cells

Transforming growth factor beta (TGF-β) is expressed, for instance in gut epithelium and serves an important role in negative regulation of the proliferation of enterocytes and colonocytes. TGF-β is also a potent tumor suppressor by inhibiting cellular proliferation and inducing apoptosis [40, 41]. However, most cancer cells are resistant to the TGF-β-induced apoptosis by acquiring defects of various components of TGF-β signaling pathway. For example, TGF-β I receptor [42], type II receptor [43], Smad2 [44] and Smad4 [45] have been shown to be either mutated or down-regulated in human colorectal cancers. TGF-β mediate signals through its binding to a cell surface receptor complex, which subsequently phosphorylates Smad2 and Smad3. The phosphorylated Smad2 or Smad3 form a heteromeric complex with Smad4, which translocates into nucleus and regulates transcription of target genes [46]. Nguyen et al. [8] described for the first time, the sodium butyrate-mediated regulation of TGF-β pathway. In HT-29, KM12C, KM12L4A, and KM20 colon cancer cell lines NaBt significantly induced the Smad3 protein expression, what was visualized by Western blot analysis. Moreover, NaBt up-regulated Smad3 activation by its extensive phosphorylation, which allowed Smad3 to translocate to the nucleus. The quantitive RT-PCR analysis revealed that the consequences of increased Smad3 activation were the higher plasminogen activator inhibitor-1 (PAI-1) and cyclooxygenease-2 (COX-2) mRNA levels, gene products engaged in proces of carcinogenesis. Since NaBt enhances TGF-β signaling and TGF-β is an important tumor suppressor the authors next examined whether NaBt enhances the tumor suppressor function of TGF-β. The tumor suppressor function was attributed to its ability to inhibit cell cycle progression and induce apoptosis. Although, the authors did not identify the molecular mechanism, it was found that NaBt and TGF-β synergistically inhibit anchorage-independent growth of colon cancer cells. The described data revealed a novel mechanism that may explain in part the beneficial effects of sodium butyrate in decreasing risk of colon cancer.

Sodium butyrate specifically affects malignant cells

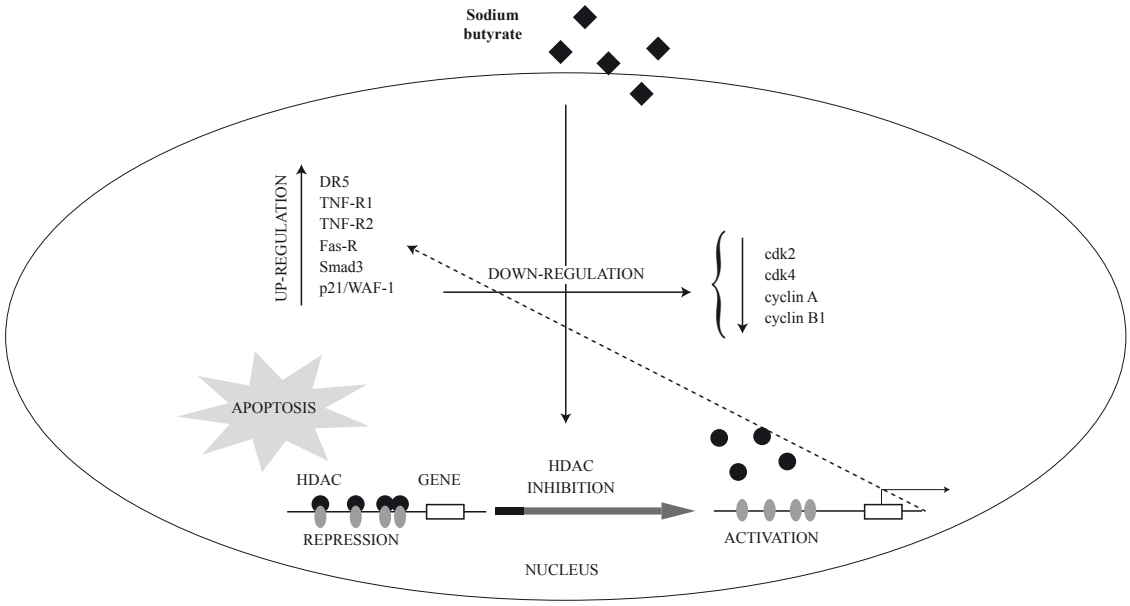
Studies of intensive immunotherapy revealed several metabolic inhibitors, such as cycloheximide [37], actinomycin D [47], anisomycin, harringtonine [48] and other metabolic inhibitors, which are able to modulate the resistance of various cancer cells to cytokine-induced cell death. However, the clinical use of several tumor cell death promoting agents is limited, because they act non-specifically and are often cytotoxic. Thus, non-toxic NaBt seems to be the ideal agent in future anticancer immunotherapy. Earel et al. [23] showed that the NaBt-mediated sensitization to TRAIL-induced PCD is specific ultimately for cancer cells, whereas do not affect normal cells in bladder. In normal bladder epithelial cells (SVHUC-1) co-treatment with NaBt [5 mM] and TRAIL [10-1000 ng/mL] caused only 20% of cells to die, and barely if TRAIL was used in the highest concentration. To understand why the SVHUC-1 cells were not sensitized by NaBt to TRAIL, the TRAIL-R1 (DR4) and TRAIL-R2 (DR5) expression levels were assessed after NaBt administration. In both cases the expression of transmembrane TRAIL receptors was unchanged. These observations are in contrast to those described previously for bladder cancer represented by T24 cell line, They responded with increased DR5 expression after NaBt treatment. Thus, the authors concluded that NaBt may be a viable alternative treatment.

Other interesting studies were described by Liu et al. [49], who evaluated the affect of sodium butyrate on 1,2-dimethylhydrazine (DMH)-induced colon tumorigenesis in mice. The mice of Kunming species were divided into five groups and received relevant treatments: control group (saline), DMH alone group (with subcutaneous injection of 30 mg/kg of DMH weekly for eleven weeks), DMH plus low dose of NaBt group (1.25 mol/kg, 24-week coloclysis), DMH plus high dose of NaBt group (2.5 mol/kg, 24-week coloclysis) and high dose of NaBt group. The mice were killed in batches at the 12th, 18th and 24th weeks of carcinoma induction separately. The incidence of colorectal tumor in each group was evaluated. Meanwhile, the general condition, body weight, liver and renal functions and pathological changes of liver, kidney, lungs and pancreas of the mice were also measured. The obtained results revealed that at the 24th week of study the tumor incidence was 95% in DMH mice group, 45% in DMH plus low dose of NaBt group, and 15% in DMH plus high dose of NaBt group. More importantly, no tumors were observed in control group and high dose of NaBt group. No differences in general condition, body weight and liver and renal functions of mice were observed between control and high dose of NaBt group ($P>0.05$). Furthermore, no pathological changes in lungs, livers and kidneys were observed in the mice with high dose of NaBt group. The described results confirmed that NaBt is nontotoxic for normal cells, moreover, they suggest that NaBt could protect against experimentally-induced colon carcinogenesis.

Summary

Herein, the various molecular mechanisms of proapoptotic sodium butyrate action in cancer cells are described. On transcription level, NaBt affects histone deacetylase activity. The up- or down-regulation of specific genes results in the antiapoptotic proteins elimination, such as cFLIP, XIAP and/or extensive synthesis of transmembrane receptors or components of various apoptotic signaling pathways (TGF-β,TRAIL) (*Fig. 1*). All these cellular changes help to restore the natural processes of cancer cell deletion. Additionally, the presented *in vitro* and

Figure 1. **Tentative model for the mechanism of sodium butyrate-mediated apoptosis induction. Butyrate-dependent inhibition of histone deacetylase (HDAC) activity results in the up-regulation of DR5, TNF-R1, TNF-R2, Fas-R, Smad3 and p21/WAF-1 gene transcription. As a consequence of p21/WAF-1 activation the cdk2, cdk4, cyclin A and cyclin B1 are down-regulated. Presented changes in gene transcription facilitate apoptosis induction in cancer cells [based on Kim et al., Carcinogenesis 2004; 25(10): 1813-20]**



in vivo studies showed that sodium butyrate specifically affects malignant cells but not the normal ones. Thus, sodium butyrate should be seriously considered as an anticancer treatment or an adjuvant in novel immunotherapy.

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Effect of oxidative phosphorylation uncoupler FCCP and F1F0-ATPase inhibitor oligomycin on the electromechanical activity of human myocardium

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Abstract

Purpose: The purpose of the study was to determine the influence of oxidative phosphorylation uncoupler FCCP (carbonyl cyanide p-trifluoromethoxy-phenylhydrazone) and F_1F_0-ATPase inhibitor oligomycin on the parameters of electromechanical activity in human myocardium.

Material and methods: The experiments were performed on isolated human ventricle strips from patients undergoing cardiac corrective open heart surgery. Effect of investigative agents was registered using conventional method of registration of cardiac electromechanical activity.

Results: FCCP (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} mol/L) caused the gradual reduction of contraction force (P). The maximal decrement of P (to 8.3±3.1% (n=5) vs control), was achieved at 10^{-6} mol/L FCCP concentration. The duration of action potential at 50% of repolarization (AP_{50}) was decreased only at 10^{-7} and 10^{-6} mol/L FCCP concentrations, i.e. to 94.3±1.9% and 55.5±3.1% (n=4), respectively, vs control. Oligomycin ($2x10^{-5}$ mol/L) alone decreased P only to 77.8±5.1 % (n=5) and slightly reduced AP50 to 94.2±6.2% (n=4) vs control. Application of FCCP on top of oligomycin decreased P at the smaller extent than under the action of FCCP alone: the highest concentration of FCCP (10^{-6} mol/L) reduced P to 21.1±4.5% (n=5) vs effect of oligomycin. The duration of AP_{50} was also less shortened after application of FCCP in the presence of oligomycin. The highest concentration of FCCP (10^{-6} mol/L) reduced AP_{50} to 73.5±10.1% (n=4) vs effect of oligomycin.

Conclusions: In conclusion, our data show that the inhibition of F_1F_0-ATPase reduces the impairment of electromechanical activity caused by oxidative phosphorylation uncoupler FCCP in human myocardium.

Key words: human myocardium, contraction force, action potential duration, FCCP, oligomycin.

Introduction

Cardiac contractility strongly depends on mitochondria, which supply ATP for ionic channels, ATPases and participate in calcium homeostasis. The most part of cellular ATP (80--90%) is generated by mitochondrial oxidative phosphorylation, which comprises the electron transport chain (complexes I to IV) and F_1F_0-ATPase (ATP synthase). Under normal physiological conditions F_1F_0-ATPase generates ATP from ADP using the proton gradient, established by the electron transport chain of the inner mitochondrial membrane. Under these conditions, the mitochondrial ATP synthase provides the cell with ATP, which is then used in diverse cell functions. ATP level in the myocyte is a critical key for normal cardiac function as ATP is used by actomyosin ATPase and various sarcolemmal, as well as sarcoplasmic reticulum, ion channels (L-type Ca^{2+} channel, Ca^{2+} ATPases, ATP sensitive K^+ channel, Na^+-K^+ ATPase) during contraction and relaxation. Under oxygen deficiency conditions, i.e. during ischemia, mitochondrial electrochemical gradient collapses, and F_1F_0-ATPase starts hydrolyzing ATP for the proton gradient recovery. Then F_1F_0-ATPase instead of producer becomes the main consumer of ATP in failing cardiomyocytes and contributes to the heart failure progression [1-4]. Experimental observations demonstrated that this ATPase consumed 35-50% of the overall high-energy phosphates during heart ischemia [1,5]. Studies showed that inhibition of F_1F_0-ATPase could reduce this undesirable effect and protect ischemic myocardium from ATP depletion [1,6,7]. For that purpose could be used an inhibitor oligomycin, a macrocyclic compound produced by actinomycetes, which binds to the F0

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***Table 1*. Clinical characteristics of the patients**

Patient No	Sex	Age	Tissue	NYHA	Medication	Comorbidities	Operation
1	M	74	endo	III	Hep, AC, ACE, N, D	Hypertonic disease	AoVpr, TVa
2	F	70	endo	IV	AC, N, D	Strume diffuse-nodose	AoVpr, MVpr
3	F	74	endo	III	AC, ß, ACE, N	Diabetes mellitus I	CABG, AoVpr, MVpr.
4	M	42	endo	III	Hep, ß, ACE, D	Hypertonic disease	CABG, MVpr.
5	F	60	endo	III	Hep, AC, ACE, D	Hypertonic disease	AoVpr, TVa, MVa
6	F	72	endo	III	ß, ACE, N, D	Hypertonic disease	AoVpr
7	M	73	endo	IV	AC, ACE, D	Hypertonic disease, diabetes mellitus I	CABG, TVa, MVa
8	F	66	endo	III	ß, N, D	-	CABG, AoVpr, MVa, TVa
9	F	81	endo	III	Hep, ß,	-	AoVpr
10	M	43	endo	III	ACE	Hypertonic disease	CABG, Bentall procedure

Abbrevations: M, male; F, female; endocardium; NYHA, New Your Heart Association Clases I to IV; AoVpr, aortic valve prosthesis; MVpr, mitral valve prosthesis; MVa, mitral valve annuloplastic, TVa, Tricuspid valve annuloplastic; CABG, coronary artery bypass grafting. Medication: Hep, heparin; AC, anticoagulant; ß, beta AR blocker; ACE, angiotensin converting enzyme inhibitor; N, nitrates; D, diuretics

domain and blocks proton flow through the F_1F_0-ATPase [8]. In the experimental models the decrease of mitochondrial potential can be evoked by mitochondria uncoupling of oxidative phosphorylation with the agents such as FCCP (carbonyl cyanide p-trifluoromethoxy-phenylhydrazone) which inner mitochondrial membrane makes permeable to protons [3], or with an inhibitors of the respiratory chain complexes. Our previous studies with rat hearts showed that oligomycin significantly reduced myocardial injury evoked by inhibitors of complexes III or IV of the mitochondrial respiratory chain [9], or by mitochondrial uncoupling [10]. The aim of the present study was to investigate the effect of oxidative phosphorylation uncoupler FCCP and F_1F_0-ATPase inhibitor oligomycin on the electromechanical activity of human myocardium.

Materials and methods

The experiments were performed on human ventricular muscle strips (0.25-1 cm^2) that were resected from patients undergoing open-heart surgery (general anesthesia) – mid sternal longitudinal sternotomy – just before cannulating heart and instituting cardiopulmonary bypass. The investigations were approved by the institutional Ethics Committees and conform to the European Community guiding principles. Clinical characteristics of the patients are shown in *Tab. 1*. A pharmacological pretreatment was stopped 24 h before surgery. In addition, all patients received sedatives, anesthesia. The pieces of human tissue were transported in cold (10°C) St. Thomas cardioplegic solution composed of (in mmol/L): NaCl 110, KCl 16, $CaCl_2$ 1.2, $MgCl_2$ 16, glucose 5, Hepes 10, pH 7.4 (adjusted with NaOH). After transportation, muscles were placed in an experimental chamber and perfused (for 30 min) with oxygenated Tyrode solution (pO_2 580-600 mmHg) composed of (in mmol/L): NaCl 137, KCl 5.4, $CaCl_2$ 1.8, $MgCl_2$ 0.9, glucose 5, Hepes 10, pH 7.4 (adjusted with NaOH). Perfusion was kept at a rate of 6 ml/min and temperature was continuously monitored at 36±0.5°C. All preparations were continuously paced at frequencies of 1 Hz with pulses of 2-5 ms duration and twice the diastolic threshold. Isometric contraction was recorded using a linear force-displacement transducer (Harvard Apparatus, USA). Transmembrane action potentials were recorded with glass microelectrodes filled with 3 mol/L KCl (resistance 7-10 MΩ). The microelectrodes were connected to the input stage of a high-impedance amplifier (MEZ-7101; Nihon Kohden US, Inc., Foothill Ranch, CA). The amplified signals were displayed on a dual-beam oscilloscope (C1-69) and sampled at 10 kHz using a 16-bit analog-to-digital converter (PCL816; Advantech) [11]. The data was recorded and analyzed with specialized computer program.

The effect of oxidative phosphorylation uncoupler FCCP and F1F0-ATPase inhibitor oligomycin was registered after an equilibration period of 40-50 min. In the first group of experiments (n=5) we determined the effect of FCCP (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} mol/L) on contraction force (P) and action potential duration at 50% of repolarization (AP50) of human myocardial strips. In the second group (n=5) – the effect of FCCP on these parameters was investigated in the oligomycin treated ventricular strips. The experiments were performed as follows: after 30 min perfusion of ventricular strips with a Tyrode solution containing oligomycin, the increasing concentrations of FCCP were added to this solution and the perfusion with each concentration was continued for a 20 min. We used $2x10^{-5}$ mol/L of oligomycin, i.e. concentration, which induced inhibition of mitochondrial F_1F_0-ATPase activity [12].

Changes of the parameters were expressed in percentage: under the influence of FCCP or oligomycin alone – in respect to control (Tyrode solution), and under the influence of FCCP in the presence of oligomycin – in respect to the effect of oligomycin. All values were presented as means ±S.E.M. The significance of data was assessed using Student's t-test and the results were considered significant at $p<0.05$.

All agents used in experiments were from "Sigma" (USA).

Results

It was established that under control conditions, i.e. at perfusion of human ventricle strips with Tyrode solution, an average of contraction force was 1.21±0.3 mN (n=10) and action potential duration (AP_{50}) – 214.56±29.87 ms (n=9).

***Figure 1*. Time-course of the effect of FCCP on the contraction force of human ventricle strip, slow speed recordings. Arrows indicate the moment administration of FCCP (concentrations in mol/L are shown above arrows)**

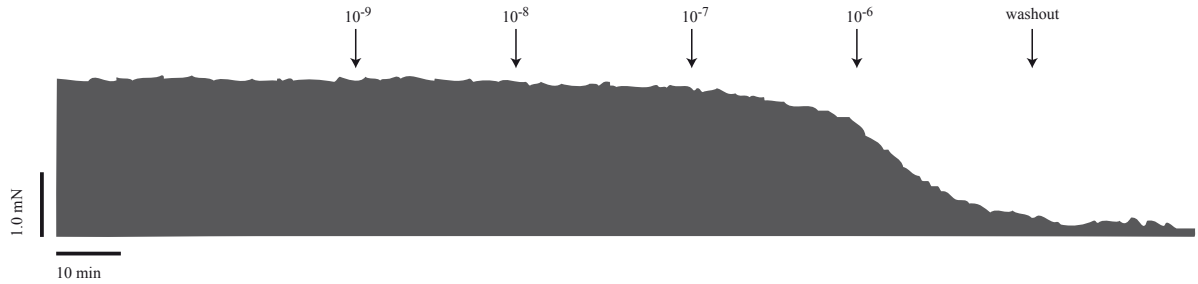


***Figure 2*. Effect of FCCP, an uncoupler oxidative phosphorylation, and oligomycin, an inhibitor of F1F0-ATPase, on the contraction and action potential of human ventricle strips. A – the action of FCCP (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} mol/L) alone on contraction (a) and action potential (a'); B – the action of oligomycin ($2x10^{-5}$ mol/L) alone and with FCCP (10^{-6} mol/L) on contraction (b) and action potential (b')**

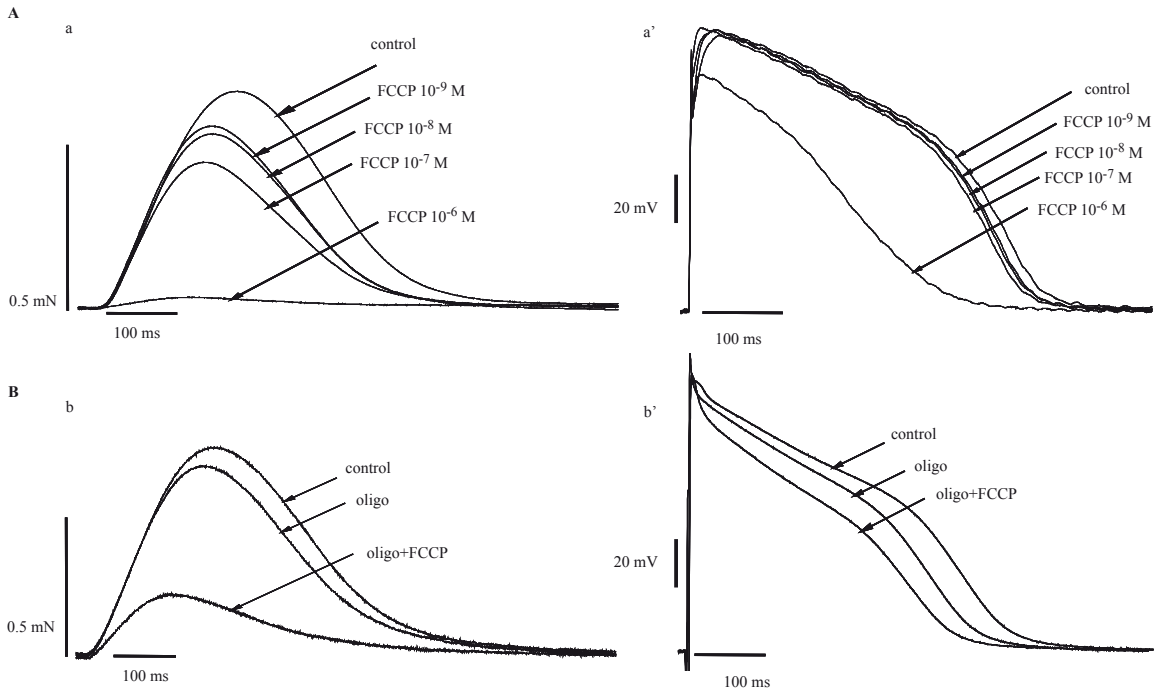
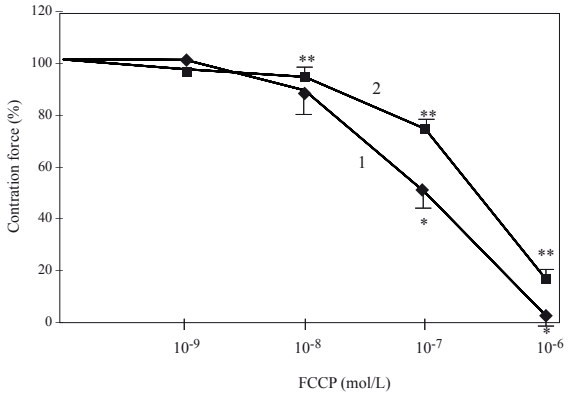


Fig. 1 depicts a representative example of the time-course of the effect of FCCP. In this example up to 10^{-8} mol/L of FCCP (of about 40 min in the control and 20 min in the presence of 10^{-9} mol/L FCCP) the contraction force was stationary. The negligible action of FCCP started at the 10^{-8} mol/L whereas 10^{-7} and 10^{-6} mol/L of FCCP caused substantial decrease in contraction force.

Fig. 2 (A) demonstrates the original traces from a typical experiment, showing contractions (A, a) and action potentials (A, a') in control conditions, and after 20 min of the action of FCCP (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} mol/L). The averaged data of the changes in contraction force under the influence of different concentrations of this uncoupler are presented in *Fig. 3* curve 1). FCCP caused the reduction of P in a dose-dependent manner and the maximal decrement of P to 8.3±3.1% (n=5) vs control, was obtained at 10^{-6} mol/L FCCP. AP_{50} decreased with drug concentration only at 10^{-7} and 10^{-6} mol/L to 94.3±1.9% and 55.5±3.1% (n=4), respectively, vs control. At the end of experiments the perfusion of myocardial strips with the Tyrode solution did not restored P and AP_{50}, i.e. the inhibitory effect of FCCP was irreversible.

In order to test the influence of F_1F_0-ATPase inhibition on alterations of human myocardium contraction force and action potential duration caused by FCCP, myocardial strips were pretreated with oligomycin for 30 minutes before the increasing concentrations of FCCP were added. Oligomycin ($2x10^{-5}$ mol/L) alone decreased the contraction force to 77.8±5.1% (n=5), and slightly reduced the action potential duration to 94.2±6.2% (n=4) vs control. *Fig. 2* (B) demonstrates the original traces of myocardial contractions (B, b) and actions potentials (B, b') recorded during experiments under the influence of oligomycin alone and together with FCCP. *Fig. 3* shows the averaged data of action of FCCP (10^{-9}, 10^{-8}, 10^{-7}, 10^{-6} mol/L) together with oligomycin ($2x10^{-5}$ mol/L) (curve 2) on the contraction force of human ventricular strips. It was established that in the presence of oligomycin FCCP decreased P at the smaller extent than

***Figure 3*. Effect of FCCP alone (curve 1) and with oligomycin ($2x10^{-5}$ mol/L) (curve 2) on the contraction force of human ventricle strips. Data are given as mean ±S.E.M., * p<0.05, vs control , ** p<0.05, vs oligomycin effect**



under the action of FCCP alone: the highest concentration of FCCP (10^{-6} mol/L) P reduced to 21.1±4.5% (n=5) vs effect of oligomycin. The duration of AP_{50} under the same experimental conditions was also less shortened, as compare with the FCCP action without oligomycin, i.e. AP_{50} at 10^{-6} mol/L FCCP in the presence of oligomycin decreased to 73.5±10.1% (n=4) vs effect of oligomycin.

Discussion

The present study describes the effect of mitochondrial oxidative phosphorylation uncoupler FCCP and F_1F_0-ATPase inhibitor oligomycin on the electromechanical activity parameters of human ventricular myocardium. Our experiments show that under the influence of FCCP the contraction force and duration of action potential decreased in a dose response manner. One of the major consequences of uncoupling of oxidative phosphorylation by FCCP is a decrease of intracellular ATP and creatine phosphate pools in myocardial cells [13-15]. The depletion of high-energy phosphates leads to impaired functioning of all energy-dependent systems, which regulate ion movement and the contraction of myocardial cells, i.e. sarcolemmal L-type Ca^{2+} channels, Ca^{2+} pump, Na^+-K^+ pump dependent Na^+-Ca^{2+} exchange, ATP-sensitive potassium channels and sarcoplasmic reticulum Ca^{2+}-ATPase (SERCA2), phospholamban, ryanodine receptors. This results in an elevation of the intracellular concentration of Ca^{2+} and Na^+ ions, development of diastolic contracture, diminishing of myocardial contraction force and heart failure [13]. Our data and a numerous other experimental investigations corroborate this explanation [9,13,16]. It has been shown that L-type Ca^{2+} current is decreased under conditions of metabolic inhibition in guinea pig and rabbit ventricular myocytes [14,16]. The decrease of ATP evokes shortening of action potential duration not only for the reduction of L-type Ca^{2+} current but also for an increase of K^+ current through the ATP dependent K^+ channels [17,18].

Our experimental data show that F_1F_0-ATPase inhibition with oligomycin significantly slowed the decrease of contraction force and reduced shortening of action potential duration evoked by FCCP in human myocardium. It is necessary to point out that oligomycin is nonselective inhibitor of F_1F_0-ATPase and inhibits both synthase and hydrolase activities of that enzyme [1,8]. This might explain our observation that oligomycin alone caused a moderate decrease of contraction force and action potential duration of human ventricular muscle. However, it has been shown that during ischemia oligomycin, as well as aurovertin, an inhibitor of F_1F_0-ATPase, attenuated the rate of ATP depletion in myocardium of rats and dogs [1,6]. Grover et al. demonstrated that oligomycin, or specific inhibitor of F_1F_0-ATPase BMS-199264, which selectively blocks hydrolase activity and has no or weak effect on synthase activity, significantly increased ATP concentration during ischemia in rat cardiomyocytes [8]. Recent studies demonstrated similar effect of oligomycin on electromechanical activity in rat myocardium at metabolic inhibition with anoxia, antimycin A, or FCCP, i.e. the inhibition of F_1F_0-ATPase by oligomycin not only slowed the diminishing of myocardial contraction, but also delayed the development of contracture [9,10].

It is known that human as well as larger animals express relatively high amounts of IF1 protein, a selective inhibitor of ATP hydrolase activity of F_1F_0-ATPase [1,19]. Fall of mitochondrial electrochemical gradient and intracellular pH to 6.7 as might be seen during ischemia causes reversibly binding of IF1 protein to F_1F_0-ATPase and blocking ATP hydrolase activity [20]. However, it was demonstrated that IF1 does not completely inhibit the hydrolase activity; therefore, further inhibition will confer the added benefit [8,21]. This suggests that during metabolic inhibition human cardiac cells are not capable to stop ATP hydrolysis in full by protein IF1, and the addition F_1F_0-ATPase inhibitor, such as oligomycin, can contribute to maintenance of electromechanical activity. Our experiments with FCCP and oligomycin in human myocardium support this explanation.

In conclusion, our data show that the inhibition of F_1F_0-ATPase reduces the impairment of electromechanical activity caused by oxidative phosphorylation uncoupler FCCP in human myocardium.

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Pregnancy-associated osteoporosis: an underestimated and underdiagnosed severe disease. A review of two cases in short- and long-term follow-up

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Abstract

Pregnancy-associated osteoporosis is an uncommon condition characterized by the occurrence of painful fractures during late pregnancy or lactation. To date the pathophysiology of this entity of bone disorder is still uncertain, and its therapeutical management is poorly defined.

We report two clinical cases: a 10-years follow-up with pain medication and intermittent antiresorptive therapy courses, subsequent traumatic vertebral fracture and actually fracture of scaphoid after inadequate trauma. Beside this long-term course a young female patient with pregnancy-associated osteoporosis and painful lumbar and also thoracic vertebral fractures is described. She was treated with an osteoanabolic therapy, at the timepoint of first follow-up at 6 months of treatment a solid increase of bone mineral density and sustained pain reduction was observed.

Key words: osteoporosis, pregnancy, vertebral fractures, osteoanabolic therapy.

Introduction

During pregnancy and lactation different significant alternations in the maternal environment proceed, particularly estrogen and prolactin, which may alter bone density. Therefore pregnancy and lactation might have significant impact on bone density and depending on the extent of loss in bone density this might result in pregnancy-associated osteoporosis and vertebral fractures. At the moment exist no guidelines for the therapy of pregnancy-associated osteoporosis. The existing literature reveals several case reports [1,2] but evaluates the pregnancy associated osteoporosis alltogether as rare disorder with unknown pathophysiology. With consideration of severity of the clinical findings an individual therapy concept should be intiated by specialised therapycentres. Furthermore a referencecenter for pregnancy-associated osteoporosis was initiated in Germany to collect and analyze cases to be able to develop a risk profile. Although rare, diagnosis of pregnancy-associated osteoporosis should be suspected when lumbar or thoracic spine pain occur during pregnancy or in the post-partum period as it can lead to multiple vertebral fractures.

Case report of complex course

We report a case of a 32-years old female patient, sectio half a year ago, who was treated in a hospital nearby because of severe and therapy-resistant back pain. With the suspicion of a pregnancy-associated osteoporosis the patient was transfered for further diagnosis and therapy.

We started with a bone mineral density (by DXA: dual x-ray-absorption) measurement. We diagnosed an osteoporosis with a T-score of the lumbar spine: L1-L4: -2.12, L4: -2.71, the T-score of the left femur was: -1.81. X-rays of thoracic and lumbar spine (*Fig. 1*) as the MRI of the whole spine (*Fig. 2*) showed changes of the vertebrae (wedge gibs) of the thoracic vertebra 5 and 7; fracture of thoracic vertebra 12 and changes of the cover plates of thoracic vertebrae 8, 10, and lumbar spine 2 and 3.

Due to a history of pulmonary embolism six months ago, the patient was treated with low-molecular-weight heparine (Clexane 40 sc twice daily), actually a therapy with an oral anticoagulans (Phenprocoumon) was initiated.

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***Figure 1*. Initial x-rays of thoracic and lumbar spine**

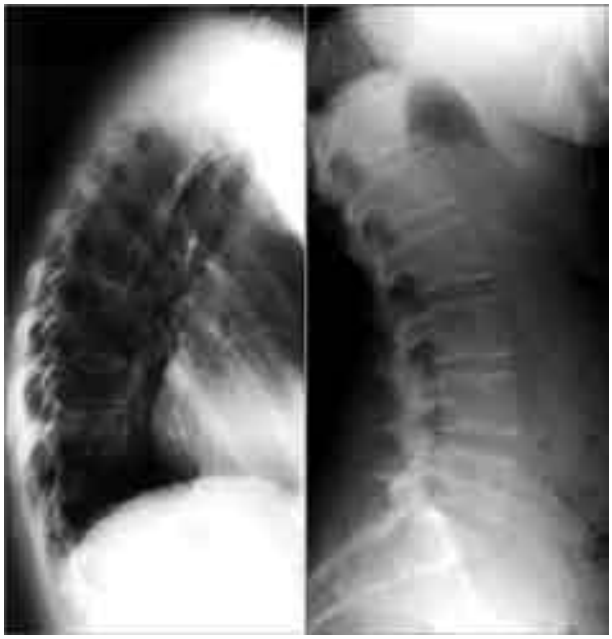
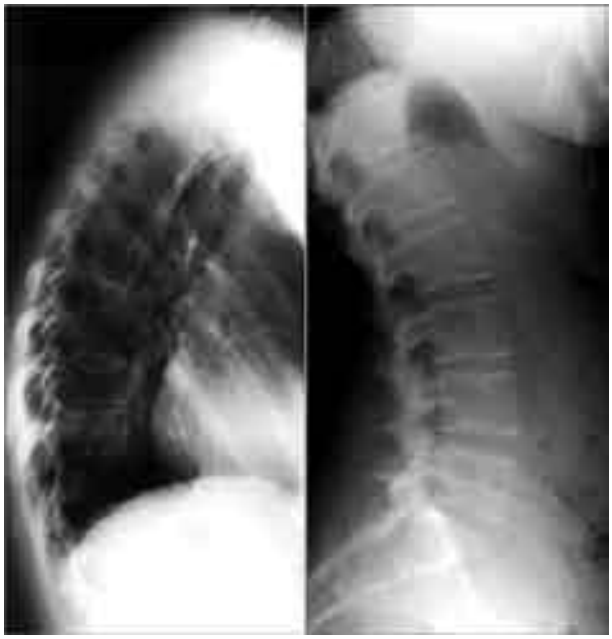


***Figure 3*. Control x-ray (6 weeks after fracture) of thoracic and lumbar spine**

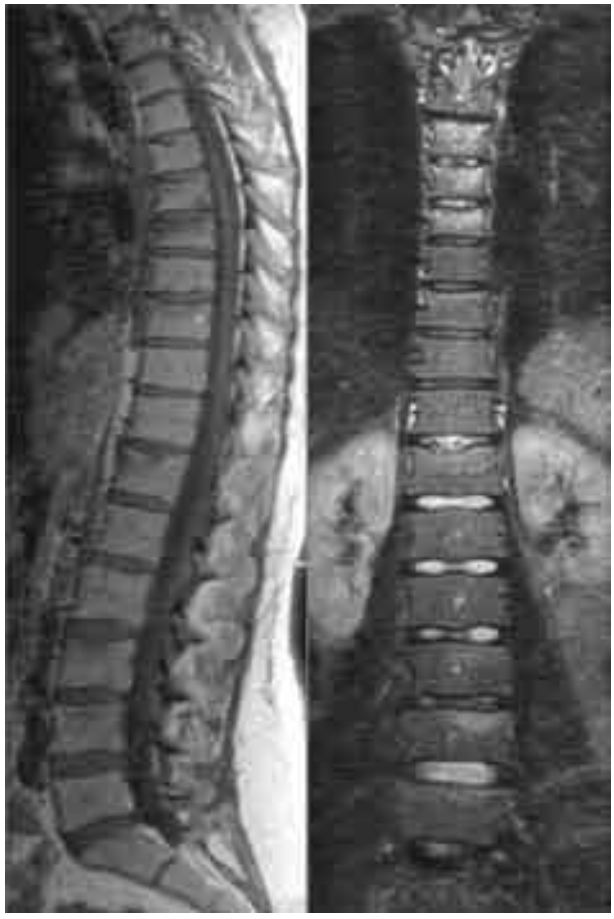


In the clinical chemistry as in 24 hours-urine assessment no pathologic values were observed.

Therapy

In accordance with the reference center for pregnancy-associated osteoporosis of the university of Marburg we initiated an osteoanabolic therapy with teriparatide (1,34 rhPTH 1-34). In the recent literature of reporting cases of pregnancy-associated osteoporosis an antiresorptive therapy with bisphosphonates was chosen as first-line therapy [1,3]. Altogether the authors

***Figure 2*. MRI of thoracic and lumbar spine before therapy: changes of the vertebrae (wedge gibs) in thoracic vertebra 5 and 7; fracture of thoracic vertebra 12 and changes of the cover plates of thoracic vertebrae 8, 10, and lumbar spine 2 and 3**



reported bisphosphonates as well tolerated and effective in the long follow-up, despite to date unknown potentially long-term side-effects of bisphosphonate treatment in premenopausal women. In our patient several risk factors were condensed, so a potent and fast treatment was necessary. Because of the already progressive course of disease, the existence of multiple vertebral fractures, the high risk for secondary osteoporosis due to ongoing anticoagulant therapy and the fertile premenopausal status of the patient we decided to use a substance with specific action on bone formation not being incorporated into the bone matrix [4,5]. After full and adequate explanation to the patient and her family written informed consent was obtained from the patient in view of off-label therapy. We started therapy with teriparatide (rhPTH 1-34), accompanied with supplementation of 1000 mg calcium and 800 iE 25OH-Vit D3 per day [6]. Due to persisting back pain performed x-ray-controls after 6 weeks of treatment showed no further progress of vertebral fractures (*Fig. 3*) compared with the x-rays at the beginning of our therapy. And we could not measure progressive changes in the already existing vertebral fractures.

Altogether a consilidation of the disease could be diagnosed. Collateral therapy with immobilisation of the spine by a corset (Spinomed©) [7] was run for 3 months. Further accom-

***Figure 4*. CT of instable fracture L1**

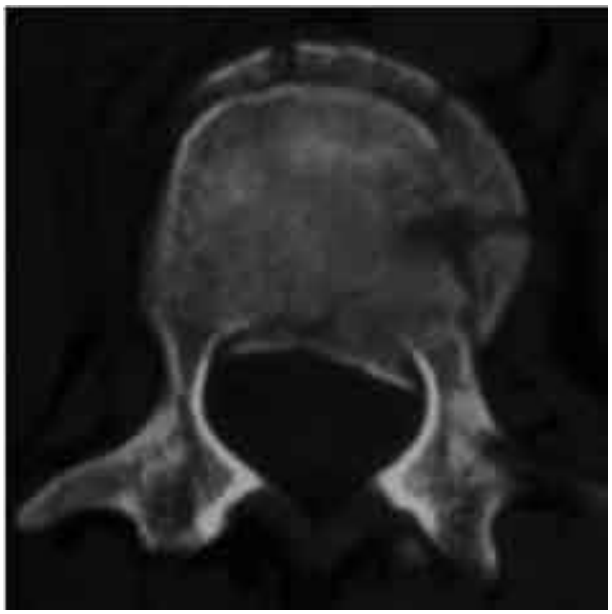
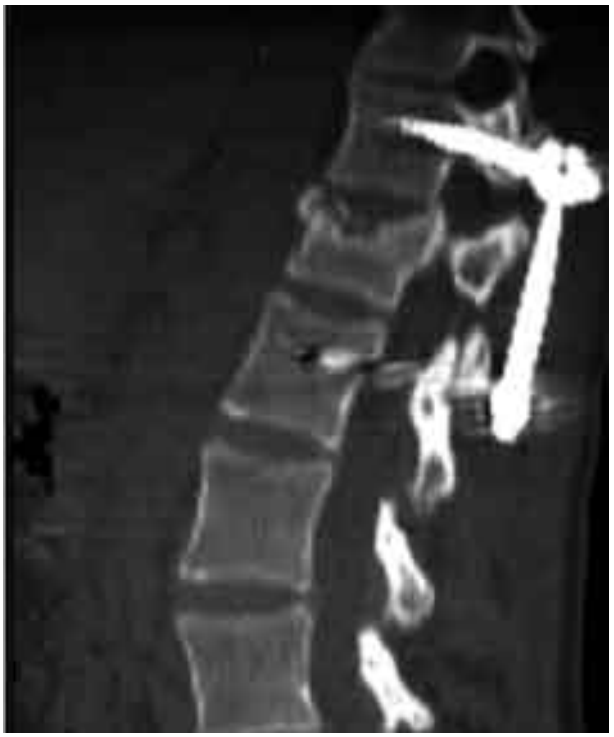


***Figure 5*. Internal fixator from Th 12 to L2**



panying therapy was pain therapy and physiotherapy. Control measurements of bone mineral density were done after 6 month of treatment.

The mean value of T-score L1-L4 was -0.9, corresponding to a gain of 42.45%. No further non-traumatic vertebral fractures occurred. In conclusion the applied therapy showed an immediate effect on biomechnical properties of bone and reduced future fracture risk.

Case report with 10-years-follow-up

A 41-years-old, premenopausal, slim (BMI=19) woman is accompanied by pain of the whole spine and the musculoskeletal system in general since 10 years. Within her adolescence she suffered from traumatic fractures of the jaw, metatarsus and toes. She had two pregnancies 10 and 8 years ago. Since the first pregnancy a PAO was known, which impaired after the second pregnancy. A possible cause for secondary osteoporosis is the treatment for asthma bronchiale over 12 years with inhalative, intermittent oral, corticosteroids (CS) in the range between 5-16 mg/die [8]. Seven years ago, the first DXA-scan was performed and showed a T-score (L2-L4) of -2.1 (z-score: -2.0), specific antiosteoporotic therapy was initiated with etidronate, calcitonin and ongoing supplementation of calcium and vitamin D3. In the DXA-scan after 1 year of therapy, a densitometric consolidation of the PAO could be observed (T-score L2-L4: -1.7; z-score-1.6). With this medication also back pain was improved and well tolerated by the patient. 5 years ago, the patient had a horse-riding accident with an instable burst-fracture of L 1 (*Fig. 4*), which was stabilised by internal fixator from Th12 to L2 (*Fig. 5*).

In the actual DXA-scan an increase of bone mineral density up to a T-score L2-L4 of –1.3 (z-score:-1.3) was observed. In the first laboratory assessment of biochemical markers of bone metabolism an overall low turnover of bone was observed, due to corticosteroid therapy without accelerated bone resorption, PTH and vitamin D3 in normal range. Futhermore, a low estradiol (24 pg/ml range: 0.0-270.00 female) despite regular menstrual cycle was noticed.

Therapy

We continued with a combined antiosteoporotic (ibandronate 3 mg iv./12 weeks) and pain treatment (level I WHO) with supplementation of 1000 mg calcium and 800 IU vitamin 25OH-Vit.D3 per day.

Discussion of differential therapy

Effective treatment of pregnancy-associated osteoporosis is discussed controversial due to few reported cases in the literature and widespread underestimation of this distinct entity of bone metabolism disturbance. The lack of controlled studies and the severity of disease courses deserve effective therapy regimen. Recent case histories report usefulness and rapid improvement under antiresorptive treatment with bisphosphonates even in an younger patient [9].

Balancing existing risk factors and the progressive course of the disease against possible risk factors and the off-label-use of teriparatide [6,10] we decided interdisciplinary for this new therapeutic option: to use teriparatide in the treatment of severe pregnancy-associated osteoporosis. The decision to use teriparatide was supported by the fact of incompleted family planning. Accepting

the possibility of further pregnancies we did not use bisphosphonates in this case. There is no prediction of long-term follow-up adverse events concerning prenatal impairments. To enable further healthy pregnancies we decided to use teriparatide, which is not stored in bone matrix for years like the bisphosphonates.

We observed the beneficial effects of anabolic treatment with rhPTH 1-34 (teriparatide), especially in gain of bone mineral density and the prevention of further vertebral fractures. Up to now the treatment is well tolerated and showed the expected positive gain in bone mineral density and fracture-protective effect: no further detoriation or new fractures occurred. Also she reported substantial pain relief as an additional benefit from the osteoanabolic treatment.

In the long-term follow-up of the other patient sequentially bisphosphonates and calcitonin were used as antiresorptive treatment options. The premenopausal patient underwent bisphosphonate therapy as an off-label-therapy as well. Due to the age of 41 of this female patient and completed personal family planning we decided to use ibandronate. The iv.-medication was very well tolerated by the patient and after 4 weeks of therapy a substantial reduction of back pain was achieved.

Conclusion

As we can see in this long-term follow-up the PAO accompanies the patient their whole life and should not be underestimated. We conclude that it is necessary to avoid a long course of pain and fractures in these young women in order to treat the PAO initially adequate with antiresoptive or even osteoanbolic medication to obtain a fast consolidation of BMD and to substantially reduce the risk of further fractures and chronification of pain.

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Food allergies, cross-reactions and agroalimentary biotechnologies

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Abstract

The discrepancy between what the general public and specialist in allergic diseases regard as a true food allergy can in part depend on the frequent evidence of subjects in whom clinical symptoms elicited by a given food allergen are frequently not reproducible: this suggests the existence of allergens variably present in certain foods. In adults and older children common is a form of food allergy associated with inhaled allergens, especially pollens. In this allergic form pollens and various vegetal food often cross react but the underlying scientific rationale is largely unclear. From the study of the "latex-fruits allergic syndrome" and the "oral allergic syndrome" emerged that the cross reactivity depends on epitopes of pollens and vegetables belonging to one of the 14 classes of the "pathogenesis related proteins" (PRPs). Vegetables produce PRPs in response to infection or after plant injury or application of chemicals: long-term conservation and methods used for rapid artificial ripening of vegetables can cause plant to produce PRPs or other allergens. A genetic selection of vegetables "protecting themselves against infection and infestation" by mean of PRPs production is practiced in agroalimentary biotechnology. We deem it urgent that the two realms, Medical Science (Allergology) and Agricultural Biotechnology begin to communicate openly in order to produce food as efficiently as possible but without harming the large part of the population which is predisposed to allergy and react to PRPs.

Key words: food allergy, cross-reactions, biotechnology.
Background: the prevalence of food allergies.

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Introduction

The discrepancy between what the general public, medical practitioners and specialists in allergic diseases regard as a true food allergy is beyond doubt. Whereas a large percentage (from 12 to 20%) of the general population complain of symptoms that they blame on food, double-blind controlled studies using oral challenge tests disclose food allergies or food-related disorders in no more than 1% of the population [1-2].

One reason for this situation is that there are no straightforward diagnostic tests for food allergy-intolerance with sufficient sensitivity [3]. The double-blind placebo-controlled oral challenge is a laborious undertaking (especially in children) [4-6]. It hinges upon the concept that a certain limited quantity of a given food administered in the course of 2 hours is sufficient to trigger the reported symptoms. Yet even in the most clear-cut cases, that is, patients with positive histories and positive prick tests, it actually does so in no more than half the cases [7,8]. In the other 50% of subjects with a clear-cut history and in all those whose history is less clear or who have negative prick tests we doubt whether clinical double-blind testing is sensitive enough to exclude a food allergy. If it is not, then we presume that the prevalence of allergy-intolerance in the general population considerably exceeds 1%.

Clinical types of food allergy and cross-reactions between vegetal allergens

Food allergies can be subdivided into two types. The first type is provoked by cow's milk, hen's egg, legumes and other foods containing allergens resistant to digestion that induce gastrointestinal tract sensitisation. It tends to disappear after the first years of life and is later replaced by other clinical forms of atopy [9]. The second type of food allergy, more commonly seen in older children or adults, is associated with sensitisation

to inhaled allergens (especially pollens) that often cross react with other pollens or various vegetal foods [10,11].

The scientific rationale underlying cross-reactions between vegetables, although extensively discussed in the literature [12--17] remains largely unclear. The chief reason is that natural extracts of plant proteins are extremely labile. Hence diagnostic procedures *in vivo* and *in vitro* often yield irreproducible results.

The history of unexpected allergic cross reactions began in 1970 with a report describing coincident allergies to *Parietaria* and banana [18] followed by reports describing a frequent coupling of allergies to birch and apple [19], mugwort and celery [20], and latex-banana-avocado [21]. Over recent years the list of syndromes involving cross-reactivity between pollens, fruits and vegetables has steadily lengthened.

The most favoured hypothesis to explain cross-reactivity is that an individual produces IgE that can recognise structurally similar epitopes on the proteins of the vegetables in question. Until today these epitopes were regarded as phylogenetically related, probably being conserved throughout the various evolutionary processes.

To illustrate the problem of cross-reactivity as seen in clinical practice we describe here two of the better known, though complex and poorly understood syndromes.

"Latex-Fruits Allergy" and Chitinase type 1

Allergy mediated by IgE specifically directed against latex from natural rubber creates major problems for certain health professionals (2-10%) and for children with congenital anomalies (bifid spine, 28-37%) that are daily exposed to latex products. The prevalence of the syndrome (currently less than 1% in the general population and 3% in children with severe allergic diseases) is high in urban areas where particles from the wear-and-tear of car tyres are continually released into the air [17]. A high frequency (50-70%) of cross-reactions has been shown between latex and food allergens such as banana, avocado, chestnut, as well as kiwi, papaya, peach, apricot, grapes, pineapples, passion fruit, potatoes and tomatoes [22-24].

Another potential allergen is the whitish secretion that exudes from injured parts of *Ficus benjamina* (the commonest houseplant in Europe). When the secretion dries, organic substances it contains are released into the environmental air, and if inhaled can provoke allergic symptoms in people with the "latex-fruits syndrome" [25]. The latex-fruits syndrome [22,26] aroused great interest among allergists because the lack of an apparent taxonomic relationship between the vegetal species involved made it difficult to imagine that they each possessed a structurally similar epitope. Finally attention turned up to an allergen from latex (*Hevea brasiliensis*) denominated "Hevein", which derives from a precursor. Its amino acid sequence with an N-terminal permits to adhere to chitin, one of the structural components in the walls of numerous fungi and the skeleton of many insects. Because Hevein adheres to and hydrolytically degrades chitin, it belongs to the plant defence system: by digesting the outer covering of fungi and insects it increases the plant's defences against numerous vegetal pathogens [24,25,27-29]. As well as the principal chitinase of latex (chitinase 1) other chitinase classes abound in nature (some in latex itself), for example: potatoes, turnips and tomatoes produce chitinase type 2.

Latex also contains many other allergens: some (gluconases and esterases) are recognized by specific IgE from patients allergic to vegetal food [27].

A high percentage of subjects who are allergic to the vegetables in the latex-fruits syndrome are allergic also to pollens [30,31].

It is important to underline that chitinases are produced when (and mostly only if) plants are damaged, infected or chemically treated [14,32,33]. Therefore the correspondence between the allergen content of a vegetable, positive skin tests and the presence of clinical symptoms is a highly complex matter that depends on the environmental conditions under which the vegetal was grown, stored, and processed as a foodstuff.

Many explanations can be put forward to explain why an individual who ingests a certain food in the group, contrary to expectations derived from previous experiences, has no symptoms: aside the possible allergen degradation (qualitative and quantitative) caused by digestion or cooking it is possibly because the vegetal ingested contains no chitinases or other allergens that plants produce in excess only in response to a noxious stimulus.

The oral allergy syndrome (OAS), Bet v 1 and lipid transfer proteins (LTPs)

The oral allergy syndrome [34-36] is a disorder involving the sensitisation to birch pollen allergens and apple, mugwort, hazelnut, walnuts, green beans and various fruits belonging to the *Rosaceae* family (pears, cherries, plums, and apricots) and vegetables belonging to the *Apiaceae* species (parsley, potatoes, carrots, courgettes, lettuce and celery). The number of foods involved in this syndrome is continuously growing.

Patients with OAS manifest a wide range of symptoms, caused by direct contact of plant food with the oral mucosa ranging from swelling and angioedema of the lip, itching and sudden desquamation of the oral mucosa, oedema of the glottis, gastroenteritis and diarrhoea to occasional systemic reactions such as urticaria, asthma and shocking [34]. More than 70% of patients with OAS react to two foods or more, they typically tolerate cooked foods [35,37]. The association between pollen and plant-derived food allergy can be explained by the presence of specific IgE against allergens (panallergens) that share a homologous structure and are thus cross-reactive. Allergy to apple (Mal d 1) and to major birch pollen allergen (Bet v 1) is frequently associated with OAS. Some of patients are also sensitized to minor birch pollen allergen, profilin Bet v 2, but recent studies suggested that profilin sensitization has little or no clinical relevance [35].

Studies conducted in the early 1990s recognised that this association between birch and apple allergens depended on the homology of its antigenic determinants, in particular the Bet v 1 antigen from apple and birch [29]. This antigen, like

chitinase in the latex-fruit syndrome, belongs to one (class 10) of the 14 classes of the pathogenesis-related proteins (PRPs), namely proteins that vegetals produce for defence or functional purposes (for a complete scientific classification of PRPs see references 14 and 38).

Later studies in Southern Europe noted that many subjects sensitised to apple were not sensitised to birch, a relatively rare allergen in the various Mediterranean areas [39-42]. In these subjects, many of whom are sensitive to cherries, peaches, plums and apricots and occasionally to soybean and barley, the common allergen is a protein that transfers phospholipids across the vegetal cell, hence the name lipid transfer protein (LTP). LTPs are located in the skin or hull of vegetables [39]. Their function is to defend the plant from fungi and bacteria [40]. These substances provoke allergic sensitisation through the oral route because they are extremely resistant and readily survive oral and gastrointestinal digestive processes [42-43]. The recent studies indicate that the IgE cross-reactivity patterns and the clinical relevance is still not clear and that only some of patients with confirmed IgE cross allergy to Bet v 1 and Mal d 1 demonstrated clinical symptoms after ingestion of apple [8,39].

Pathogenesis-related proteins (PRPs) and other plant food allergens

Faced with the growing problem of cross-reactivity among pollens-vegetables and fruits that has now extended to encompass taxonomically distant plant derivatives, immunologists and specialists in allergy have devoted their most recent efforts to discovering "panallergens" as ubiquitous substances in the vegetal world, panallergens could underlie the ability of the various vegetables to elicit identical IgE in predisposed subjects [43-48].

The "latex-fruits syndrome" and the "oral allergy syndrome" have probably provided the clearest evidence so far that rather than being constitutively present in a given list of vegetables, many panallergens have precise defence functions in the vegetal world (like chitinase 1 in latex and BET v1 in birch) – innumerable vegetables may produce them when necessary, e.g. in response to infection by pathogens (fungi, bacteria, and virus) or after plant injury or application of chemicals [45,49].

Despite their enormous and emerging complexity, "plant defense-related proteins" or "stress-inducible plant proteins" or "pathogenesis-related proteins" (PRPs) engender new concepts that help to put the problems of cross-reactivity in vegetables, and some of those related to food allergy into perspective [14,38]. PRPs have been classified into 14 classes some of which (classes 2, 3, 4, 5, 9, 10, 14) are richer in substances with allergenic properties – together with other classes of proteins (alpha amylases and proteases inhibitors) they form the "plant defence system". Several basilar papers have described these substances and their clinical meaning [14,50,51].

Vegetables may also contain other substances that are potent allergens and have different biological activities. Foods that are especially rich in these allergens are seeds and tubers [14]. Many of these substances are proteolytic and glycolytic enzyme inhibitors, seeds use them to resist invasion and digestion by microorganisms and insects. These allergens are frequently found in seeds from cereals (including Kunitz-trypsin inhibitor from soybean; alfa-amylases inhibitors in barley rice, grain and rye that cause baker's asthma) and make up a family (alfa-amylase-trypsin inhibitors) with functional and structural homologies [14].

Proteolytic plant enzymes, especially those belonging to the family of thiol-proteases have been frequently found to exert the function of cross-reactive allergens, for example ficin and papain from fig and papaya. Moreover, antibodies reacting papain and ficin cross-reacted with allergens of house dust mites (Der p 1 and Der p 2) which also belong to the family of thiol-proteases. However, no clinical association between house dust mite allergy and allergy to tropical fruits has been reported [14,36].

The profilins (proteins that help to regulate the cyto-skeletal components of vegetables) were for long considered the allergens responsible for the "mugwort-celery-spice" syndrome. Later studies recognised them as relatively common allergens in tree pollens (birch), grass (*Graminaceae*) and mugwort. Antigens cross-reacting with profilins are found in various vegetables including carrots, hazelnuts, peanuts, tomatoes, pumpkins, soybeans and pears [14,52,53].

It must be said that plant food allergens belong to a limited number of protein families: they are in general characterized by a number of biochemical and physiochemical properties like resistance to proteolysis and enhanced ability to bind ligands such as lipids (membranes or other lipid structures) or enhanced stability, for example thermal stability, which is a frequent characteristic of allergens [51].

Tropomyosins are a family of proteins which are heat-stable cross-reactive food allergens (e.g. boiled shrimps contain Pen a 1 and water soluble allergens that are released into boiling water). In this family heat-stability probably derives from the presence in their structure of numerous repeat series (40 or more) of heptads of amino acids – these proteins adopt an helical structure with two molecules wound around each other [54,55].

Also the globulin seed storage proteins share the propensity to become heat resistant (forming large structures, from trimers to dodecamers) depending on salt concentration in the environment and on wet or dry thermal processing; often proteins become more thermostable during thermal processing at low water levels, like roasting (e.g. peanuts and other nuts) but also baking, grilling, frying, etc. This involves sugars reacting with free amino groups of proteins with the consequent production of advanced glycation-glycosylation end products (AGEs). In addition to forming during dry heating procedures these products are also slowly formed over days and months as a consequence of the aging process of foods. However, AGEs ingestion in humans largely depends by the consumption of heat-processed foods (in general degree and time of heat exposure determine AGEs content of different foods) [51]. It must be said that the problem represented by the allergenic properties of AGEs containing foods are far outweighed by their detrimental metabolic effects ranging from multiple gene activation to pro-atherosclerotic and glomerulosclerotic effects involving cytokine and growth factor modulation, lipid oxidation and albuminuria [56].

Other groups of allergenic substances are proteins contained in seeds (seed-storage proteins). These usually exist as dimers, tetramers or hexamers but their subunits, released during the processes of ripening and conservation, have strong allergenic potential and can provoke symptoms when inhaled or ingested. Albumins are water soluble at low salt concentration and, in appropriate environmental conditions, they are cleaved into large and small subunits held together by a disulfide bond (2S albumins). 2S albumins are major allergens in Brazil nut, peanut, yellow mustard. Seed storage globulin is soluble at high salt concentration – to this class belong most of the allergens of soybeans and of peas [14,51].

The seed storage proteins therefore represent example of proteins which can become allergenic when conservation conditions modifies them.

In conclusion, current evidence shows that most of the cross-reacting allergens contained in vegetables are functional substances that vegetables may contain depending on the conditions the vegetal encountered during growth and maturation, conservation and food processing.

Food allergies and biotechnology in the production of fruit and vegetable foodstuffs

Over the past few years, immunologists have laboriously become aware that cross allergies to fruits, vegetables, and pollens depend on substances found widespread in the vegetal world. Plants use them as tools for functioning or to prevent or combat the action of pathogens or environmental stress. No wonder these substances have long been known to those botanists and scientists who strive to seek more efficient ways of producing vegetal foodstuffs. Research conducted some years ago showed that if a transgenic plant is induced to express high concentrations of chitinases it will become far more resistant to chitin-containing pathogens [57-61].

Intense research efforts are of course underway to exploit this field commercially: chitinase-producing microorganisms may in future be disseminated in the soil to create a space into which nematodes and fungi cannot penetrate [57]. Species of cereal, fruits and vegetables acclimatized to be cultivated in cold or glacial temperatures survive thanks to chitinases or LTPs [59-60]. A further promising field of research is that of using transgenic plant technology aimed to induce plant production of inhibitors of the various digestive enzymes present in the intestine of predator insects [61].

Another cause of concern is the widespread use of ethylene gas in controlling the ripening of fruit and vegetable foodstuffs before they go on sale. Ethylene is the final product of a major metabolic amino acid pathway present not only in plants but also in bacteria and fungi. Ethylene is a hormone that has complex actions: it stimulates cell respiratory activity thus enabling cells to mature, and by interacting with other substances (auxines), seems to have a central directive role in plant life. Ethylene applied to batches of fruit and vegetable products (especially apples, bananas, tomatoes and avocado pears) to accelerate ripening induces the production of high chitinase concentrations in the treated vegetables ultimately destined for sale to consumers [45-62].

In this way repeated trauma (imagine for example the periodic bark cutting that rubber trees producing latex suffer), the application of phytohormones or other chemical substances, long-term conservation, methods used for ripening, or even genetic selection of vegetables to make production cheaper can cause a plant to produce allergens. A recent article entitled "Will genetically modified foods be allergenic?" states that only "few" genetically modified vegetables have already been introduced commercially: these few include staples such as potatoes, soybeans and maize [63]. But within years dozens of new vegetables capable of "protecting themselves against infection and infestation" will come onto the market.

Delving more deeply into a highly technical subject is outside the scope of this article. These few data should nonetheless suffice to delineate a possible "conflict of interest" between the Food and Agriculture Industry and the Public Health Service. Their duty is on a planetary level to use scientific knowledge to feed the largest number of people as efficiently as possible and to ensure that commercially available foods are in general healthy and, in particular, non allergenic for the many people who are predisposed.

We deem it urgent that the two realms, Medical Science and Agricultural Biotechnology begin to communicate openly. As so often happens, they may well discover that an ethical and rational approach will identify the problems we need to worry about so that much damage can be avoided with modest expense.

By example, if chitinase is useful in agriculture then we need to be told which foods contain it. Being largely degradable by heat it should pose no danger for those who consume cooked foods. People who have an allergy to chitinases could therefore avoid eating raw vegetables and fruits labelled as containing this enzyme in large amounts. Technology could then certainly use less economically attractive methods aimed at producing "anallergic foods" for persons who are sensitised. Another practical point is that the public needs to know that many of the quoted "panallergens" are contained in the external parts of fruits and vegetables.

In practice, we consider that generic assurances by the food and agricultural industry that their products are harmless are not enough. Industry and experts in allergy must collaborate so as to guarantee food for all yet avoid damaging the vulnerable part of the population.

Concluding remarks

Concern over the steady rise in food allergy-intolerance and vegetal cross reactions over the past ten years prompted us to fill in the gaps in our knowledge of this emerging health problem and hypothesize changes in future practice that might help to solve it.

Evidence that vegetables contain cross-reacting substances in variable amounts provides the scientific rationale for certain clinical observations related to allergy heretofore poorly understood. It might for example explain the ever increasing

prevalence or incidence of intolerance or allergy to foods of plant origin, or the wide variability of symptoms in an individual, even if that person avoids the food that previously caused symptoms and follows a strict, unchanging diet [36].

An important concept to understand is that a vegetable may not be tolerated because it contains one or more of the vegetal "pan allergens" that elicited in that individual the production of specific IgE. Yet because the presence of panallergens depends strongly on the environmental conditions under which the vegetal was grown, manured, treated, harvested and conserved, the specific clinical reactions to an ingested vegetal depend on its origin. Various cross-reacting foods, each containing panallergens in highly variable amounts, can elicit allergic reactions even when the recognized offending food has been removed from the diet. This could also, at least partially, explain the low sensitivity of challenge tests.

To apply these concepts in clinical practice we need ask several questions. Instead of the array of vegetable extracts obtained from each food shall we soon use an allergen panel including the most important PR proteins, the "seed storage proteins", alpha-amylase, or proteases inhibitors? [63] Could this allergen panel also be used to desensitise subjects with food allergies? Will industries learn to produce non-allergic foods eventually diversifying their production so as to offer people who are sensitised (up to 20% of the population) safer products? Could we make foods less allergenic by conserving them better (length of storage, temperature, and humidity)? We certainly would like to stop recommending empiric diets for patients who complain of food-associated disorders and instead, prescribe proper diets based on new diagnostic procedures able to cope with multifaceted reality that is emerging.

Our children along with their families and we ourselves have the right to receive information. To be properly informed means collaborative efforts to improve communication between the general public, scientific institutions, and industrial authorities. Being dogmatic about the matter would do more harm than good. In facing one of the possibly less pleasant aspects of progress we need to keep an open mind. But we need to know more.

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Soluble CD40 and its ligand CD154 in patients with Graves' ophthalmopathy during combined therapy with corticosteroids and teleradiotherapy

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Abstract

Aim: To assess the role of CD40/CD154 interaction in GO pathogenesis and to estimate usefulness of soluble CD40 (sCD40) and CD154 (sCD154) measurements as markers of GO activity.

Material and methods: 61 individuals in 4 groups: 1) 15 euthyroid patients with clinical symptoms of ophthalmopathy (GO) who underwent corticosteroid therapy consisting of intravenous infusions of methylprednisolone (MP) and subsequent treatment with oral prednisone (P) and teleradiotherapy (TR); 2) 14 patients with hyperthyroid GD (GDtox); 3) 22 patients with GD in euthyreosis treated with methimazol (GDeu); 4) 10 healthy volunteers age and sex-matched to group 1-3. The serum samples were collected 24 hours before MP, 24 hours after MP, after TR and at the end of therapy. Serum CD40, CD154 and TPOab were determined by ELISA and TSHRab by RIA.

Results: Serum concentrations of CD40 (in pg/ml) and CD154 (in ng/ml) were increased in GO patients: 84.9 (74.7-93.9) and 4.0 (2.5-7.3) respectively in comparison to controls ($p<0.001$ and $p<0.05$ respectively). Serum CD154 in GO group was elevated as compared to both hyperthyroid and euthyroid GD without clinical ophthalmopathy ($p<0.001$ both). The sCD40/sCD154 quotient was significantly elevated during GO therapy with CS and TR in nonresponders after MP ($p<0.05$) and at the end of the study ($p<0.01$).

Summary: Our data suggest an important role of CD40/CD154 interaction in the pathogenesis of autoimmune process leading to inflammatory infiltration in Graves' ophthalmopathy, however usefulness of sCD40 and sCD154 measurements in prediction of effects of GO treatment and its monitoring needs further investigations.

Key words: sCD40, sCD154, TSHRab, TPOab, Graves' ophthalmopathy.

Introduction

Severe, progressive ophthalmopathy (GO) associated with Graves' disease (GD) is still the most difficult clinical problem in the treatment of this chronic autoimmune disorder [1]. Despite recent progress in understanding of GO pathogenesis it remains a pathogenetic enigma [2]. The characteristic manifestations of progressive GO such as proptosis, extraocular muscle disfunction, periorbital edema or loss of vision are a consequence of an increase in volume of extraocular muscles as well as connective and fatty tissues within a space confined by the orbit bones. The enlargement of retroorbital tissues is predominantly a result of an infiltration of mononuclear cells and accumulation of fibroblast-derived hydrophilic glycosaminoglicans (GAGs). Severe GO need to be intensively treated with immunosuppressive medication, most often by corticosteroids (CS) and teleradiotherapy (TR) [3]. However, the efficacy of both CS and TR is limited and their use entails considerable risk [1]. There have been many attempts to find reliable predictors of response to immunosuppressive treatment, but very few of appeared to be useful in the clinical practice.

CD40 – a member of the TNFα receptor superfamily is expressed on antigen-presenting cells and B and T cells. CD40 ligand (CD40L, CD154) was found on T cells. Ligation of CD40L on T cells by CD40 on B cells or other antigen-presenting cells was shown to be necessary for efficient activation of T-cell effector functions [4,5]. On the other hand binding of CD40 on B cells by its ligand promotes B cell survival and differentiation [6,7]. It has been shown recently that orbital

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Table 1. **Clinical features of the studied patients with Graves' ophthalmopathy**

N	age	sex	initials	GO duration (months)[a]	strumectomy (months)[a]	radioiodine history (months)[a]	smoking history	CAS	response to therapy of GO
1	57	*f*	MR	2	-	3	current	5→1	+
2	68	*f*	GM	4	-	-	current	5→0	+
3	52	*f*	ZJ	8	-	-	quit 12 m ago	5→0	+
4	56	*f*	GE	2	-	4	current	4→0	+
5	49	*f*	KE	2	84	48	current	4→0	+
6	48	*m*	KK	4	-	36	current	5→0	+
7	52	*f*	KH	3	-	8	no	4→1	+
8	53	*f*	FJ	6	48	24, 10	current	4→0	+
9	67	*f*	NT	2	-	172	no	4→1	+
10	66	*f*	SA	6	-	-	current	4→1	+
11	38	*m*	PP	6	-	-	current	5→2	-/+
12	52	*f*	GZ	6	-	-	no	4→2	-/+
13	54	*f*	PA	6	-	-	current	4→4	-
14	52	*f*	ST	6	-	7	current	4→4	-
15	41	*m*	CS	6	-	2	current	5→4	-

[a] – period before GO treatment

fibroblasts express intensively CD40 and its ligation by CD40L stimulates proinflammatory cytokines such as IL-6 and IL-8, GAGs synthesis, cyclooxygenase-2 activity and in consequence PGE2 production [8-10]. CD40/CD154 interaction in GO pathogenesis is suggested an important pathway of T cells induced fibroblast activation and proliferation.

Thus the aim of the study was to assess the role of CD40/CD154 interaction in GO pathogenesis and to estimate usefulness of soluble CD40 (sCD40) and CD154 (sCD154) measurements as markers of GO activity.

Material and methods

The study was carried out in 61 individuals divided into 4 groups:

1) 15 patients with clinical symptoms of ophthalmopathy (GO) (12 females and 3 males, mean age 54.4±8.8 years). Clinical features of subjects included to the study are shown in *Tab. 1*. Patients were euthyroid with thiamazol or levothyroxine (patients 5-9). In all patients Clinical Activity Score of eye changes (CAS) was ≥4 and anamnesis of GO ≤1 year. All of them underwent corticosteroid therapy consisting of intravenous infusions of methylprednisolone (MP) (6 series, 3 grams each time) and subsequent treatment with oral prednisone (P) (30 mg per day for 2 months and then a gradual tapering schedule with reduction of 5 mg per week). During prednisone administration teleradiotherapy (TR) was used in 10 fractions of 2 Gy per day. Magnetic Resonance Imaging (MRI) was used to assess retroorbital muscle volume, a presence of inflammation inside muscle tissue and features of proptosis;

2) 14 patients with hyperthyroid GD (GDtox): 10 females and 4 males aged 41±21 years with duration of the disease from 3-12 months. 3) 22 patients with GD in euthyreosis treated with methimazol (GDeu): 19 females and 3 males in mean age 40±13 years with a duration of the disease from 7-18 months;

4) 10 healthy volunteers age and sex-matched to group 1-3 (ctrl): 8 females and 2 males aged 41±16 years who had either no family history of Graves disease nor other autoimmune diseases. Clinical euthyreosis in groups of 1 and 3 was confirmed by thyrotropin and free thyroxine estimation. No acute infections were observed in patients 3 weeks prior to the study.

The serum samples were collected 24 hours before MP, 24 hours after MP, after TR and at the end of therapy. All the sera were kept frozen at -70°C until used. ELISA commercial kits were used to determine serum levels of CD40 and CD154 (Bender Medsystems, Vienna, Austria; sensitivity respectively 12 pg/ml and 0.062 ng/ml; intra-assay coefficience of variation (CV) 5.5% and 6.8%). To estimate antiperoxidase antibodies (TPOab) AxSYM Anti-TPO kit was used (Abbot Laboratories, USA; normal values <12 IU/ml; CV =9.2%). The serum levels of thyrotropin receptor antibodies (TSHRab) were determined by the RIA method (TRAK kit, BRAHMS, Berlin, Germany; sensitivity 0.9 IU/L; CV 7.0%).

The statistical significance was estimated by Mann-Whitney test. To evaluate relationships between variables Spearman's test was performed using Statistica 6.0 for Windows XP (StatSoft, Tulsa, USA).

Results

Fig. 1 and *2* show medians and interqurtile ranges of sCD40 (in pg/ml) and sCD154 (in ng/ml) in GO patients: 84.9 (74.7--93.9) and 4.0 (2.5-7.3) respectively, GDtox: 50.5 (30.3-62.9) and 2.9 (1.4-4.8) respectively and GDeu patients: 41.6 (23.4--63.5) and 3.6 (1.5-6.2) respectively in comparison to the control group: 33.0 (18.3-40.6) and 2.2 (1.4-2.9) respectively.

The results of serum CD40 (in pg/ml), CD154 (in ng/ml), TSHRab (in IU/l) and TPOab (in IU/ml) in GO patients who responded to the therapy (satisfactory clinical effect, decrease of CAS ≥1) (n=10) and nonresponders (no clinical effect, no difference in CAS after treatment) (n=5) are shown in *Tab. 2* as median and interquartile range. Also statistical significance

***Table 2*. The medians and interquartile ranges of sCD40 (in pg/ml), sCD154 (in ng/ml), TSHRab (in IU/l) and TPOab (in IU/ml) in patients with Graves' ophthalmopathy in consecutive periods of treatment are shown in table 2 as median and interquartile range**

	before MP	after MP	after TR	after therapy
		sCD40		
responders	82.5 (74.7-91.8)	81.5 (74.3-90.7)	79.6 (74.4-90.5)	98.0 (86.3-102.5)
nonresponders	87.8 (80.1-92.1)	80.4 (69.3-89.3)	98.9 (91.2-110.9)	87.0 (76.5-89.5)
		sCD154		
responders	2.9 (2.3-6.0)	4.9 (3.1-7.2)	5.5 (4.2-6.4)	7.9 *# (3.8-11.1)
nonresponders	5.6 (5.4-7.7)	0.9 ** (0.8-1.9)	6.4 ## (6.1-7.6)	1.0 **•• (0.6-1.1)
		TSHRab		
responders	4.7 (3.4-15.5)	2.2 * (1.2-6.4)	3.1 (1.6-6.4)	2.0 *** (1.2-3.7)
nonresponders	16.8 (15.7-39.5)	3.9 (2.5-13.9)	3.2 (2.3-12.5)	4.6 (2.9-13.4)
		TPOab		
responders	182.5 (44.6-621.0)	60.1 (25.1-64.7)	60.1 (19.6-191.7)	59.5 * (16.9-132.8)
nonresponders	531.6 (349.4-657.0)	210.9 (178.6-235.0)	162.5 (120.3-166.2)	76.9 (64.3-82.5)

*p<0.05 vs I; **p<0.02 vs I; ***p<0.01 vs I; #p<0.05 vs II; ##p<0.02 vs II; •• p<0.02 vs III

***Figure 1*. Serum CD40 in GO (n=15), GDtox (n=14) and GDeu patients (n=22) in comparison to the control group (n=10)**

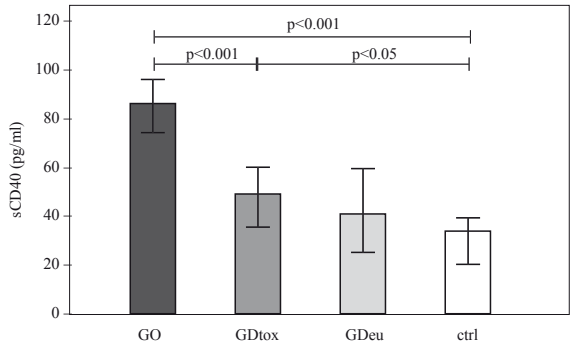


***Figure 2*. Serum CD154 in GO (n=15), GDtox (n=14) and GDeu patients (n=22) in comparison to the control group (n=10)**

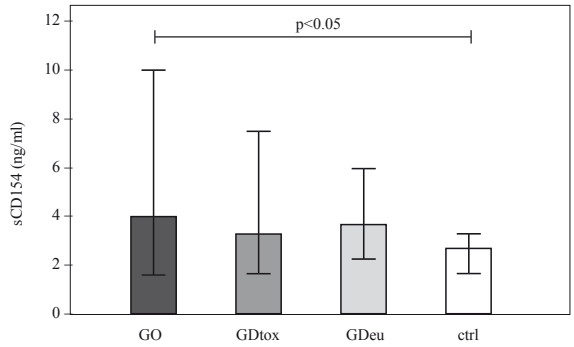


***Figure 3*. The quotient of sCD40/sCD154 in patients with Graves' ophthalmopathy during therapy**

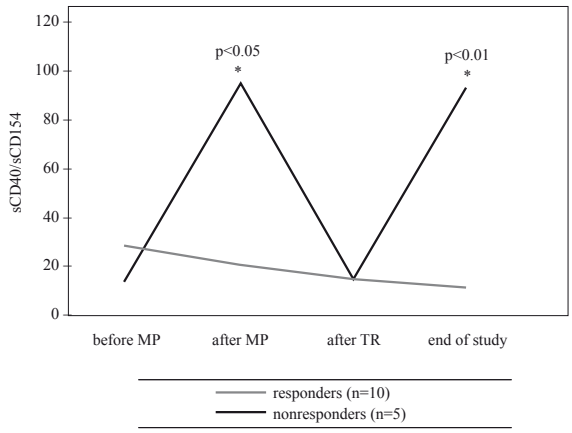
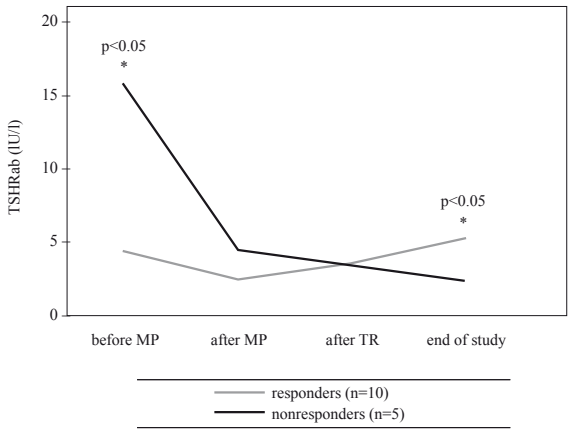


***Figure 4*. The median TSHRab values in patients with Graves' ophthalmopathy during therapy**



of differences in values of studied parameters in consecutive periods of treatment was shown in *Tab. 2*.

Fig. 3 and *4* show differences between the quotient of sCD40/sCD154 and values of TSHRab in GO groups of responders and nonresponders during therapy.

We have found no correlations between sCD40, sCD154, TSHRab and TPOab.

Discussion

TSHR has been hypothesized in GO pathogenesis to be a crucial autoantigen shared by thyroid and orbital tissues [11,12]. TSHRab measurement was found to be useful as a marker of GO activity and as a predictor of relapse in the treatment of Graves' hyperthyroidism [13-15]. Combined determination of TSHRab and TPOab has been recently shown to improve prediction of relapse of Graves' thyrotoxicosis [16]. Lai et al. have confirmed lately the presence of TPO in orbital tissues of GO patiens and suggested modulatory role of the immune responses directed against orbital TPO in clinical expression of GO [17]. Our present data of higher TSHRab in GO nonresponders both at the beginning and at the end of the study confirm previous results of TSRab usefulness in prediction and monitoring of GO therapy [13,14]. Significantly reduced TPOab concentration in responders suggest that TPOab measurement may be helpful as additional marker of immune process activity in GO.

Serum immunoglobulins from Graves' patients were shown to stimulate orbital fibroblasts to produce GAGs and T cell chemoattractants [18,19]. Fibroblasts, functioning as facultative antigen presenting cells, activate T cells via an antigen-dependent mechanism requiring an interaction of class II Major Histocompatibility Complex (MHC) and T cell receptor (TCR) (signal 1) and CD40–CD40L transduction (signal 2) [20]. In the antibody blocking experiments, blockade of class II MHC resulted in signal 2 without signal 1, whereas blockade of CD40 or CD40L costimulation resulted in signal 1 with no signal 2, both of which conditions result in T cell anergy [21,22]. These findings underline the importance of CD40-CD154 signaling pathway in the maintenance of autoimmunity. The candidate gene approach has led to findings of CD40 polymorphism associations with Graves' disease prevalence in some populations [23].

We found increased serum concentrations of CD154 and CD40 in GO patients in comparison to controls. Serum CD154 level in GO group was elevated as compared to both hyperthyroid and euthyroid GD without clinical ophthalmopathy. Moreover the sCD40/sCD154 quotient was significantly elevated during GO therapy with CS and TR in nonresponders suggesting CD40/CD154 pathway activity in ongoing autoimmune inflammatory process and intense infiltration of retrobulbar tissues by immunocompetent cells. An enhanced expression of CD154 on T cells and increased serum concentration of soluble CD154 was detected in patients with active systemic lupus erythematosus and rheumatoid arthritis in correlation with the relevant auto-antibodies and with the clinical disease activity [24,25]. Circulating CD154 may be cleaved from the cell surface of activated T cells and shares properties of the membrane form to stimulate CD40 receptor [26]. Elevated CD154 in the serum was suggested to be derived from the membrane-bound form of CD154 on activated T cells and to reflect autoimmune process intensity in lupus erythematosus [27]. Soluble form of CD40, produced by B cells cocultured with activated T cells, has been shown in vitro to hamper the binding of CD154 to CD40 through competition [28]. Our previous results suggest that soluble CD40 plays an important regulatory role in pathogenesis of Hashimoto's thyroiditis (in press). Contin et al. have demonstrated that soluble CD40 inhibits CD154 ligation onto membrane CD40 and strongly diminishes the production of immunoglobulins by CD154-activated B lymphocytes [29]. On the other hand, increased level of soluble CD40 may reflect more massive T cells infiltration of thyroid gland and/or retrobulbar tissues as degree of surface CD40 expression was shown to closely correlate with intensity of lymphocyte infiltration [30].

In summary, data of the present study suggest an important role of CD40/CD154 interaction in the pathogenesis of autoimmune process leading to inflammatory infiltration in Graves' ophthalmopathy. However, an assessment of usefulness of sCD40 and sCD154 measurements in prediction of effects of GO treatment and its monitoring needs further investigations.

Acknowledgements

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Concentrations of ssDNA in liver tissue and its correlation with sFas and sFasL in serum of patients infected with HBV, HCV, HCV and HIV

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Abstract

Purpose: The concentration of nucleic acids that undergo apoptosis (ssDNA) determines the actual activity of programmed cell death. ssDNA concentrations in liver tissue of patients with chronic HBV, HCV and HCV and HIV infections were assessed. The concentration of this nucleic acid was analyzed in relation to the concentrations of serous apoptosis indicators, sFas and sFasL receptor proteins, the activity of inflammatory processes and fibrosis in liver tissue as well as HBV, HCV and HIV viraemia.

Patients: The study included 153 patients: 48 chronic HBV infected, 86 chronic HCV infected and 19 HCV and HIV infected.

Patients and methods: The concentrations of HBV-DNA, HCV-RNA and HIV-RNA were determined by use of RT-PCR method. CD3+, CD4+ and CD8+ lymphocytes count were detected in HIV infected patients' blood by use of a flow cytometer. The concentration of ssDNA was determined by use of monoclonal antibodies and ELISA tests. The concentrations of sFas and sFasL in serum were determined by use of an immunoenzymatic method (ELISA).

Results: The concentration of ssDNA in liver tissue of both HCV and HBV infected patients was higher in comparison to those co-infected with HCV and HIV ($1\,332\times10^{-6}$ µg/mg, ±664×10-6; vs $1\,508\times10^{-6}$ µg/mg, $\pm810\times10^{-6}$; vs 886×10^{-6} µg/mg, $\pm388\times10^{-6}$; $p<0.004$). No correlation between ssDNA concentration and HBV and HCV viraemia was observed. In patients infected with HCV genotype 3, the concentration of ssDNA was $1\,343\times10^{-6}$ µg/mg, $\pm700\times10^{-6}$, comparable from patients infected with genotype 1, 296×10^{-6} µg/mg, $\pm615\times10^{-6}$. The highest concentration of ssDNA in liver tissue was detected in HBV infected patients with low inflammatory activity ($1\,645\times10^{-6}$ µg/mg, ±987) and low fibrosis ($1\,606\times10^{-6}$ µg/mg, $\pm876\times10^{-6}$). Mild inflammatory changes and low fibrosis were observed in all HCV and HIV infected patients. No correlation between ssDNA concentration in liver tissue and HIV viraemia ($r=0.03$; $p=0.90$), HCV, CD8+ and CD4+ count ($r=-11$; $p=0.66$) was observed. The concentration of ssDNA among HCV and HIV infected patients correlated with the concentration of sFas in serum ($r=0.52$; $p<0.02$).

Conclusions: HCV, HBV and HIV viraemias do not correlate with ssDNA concentration in liver tissue. In patients with HCV and HIV infections, CD4+ and CD3+ counts do not correlate with the concentration of ssDNA in liver tissue. HIV infection seems to inhibit apoptosis processes in liver tissue of HCV and HIV co-infected patients. In the case of HCV and HIV infections, the concentration of sFas in serum correlates with the concentration of ssDNA in liver tissue.

Key words: HCV, HBV, HIV infection, hepatocytes apoptosis.

Introduction

Hypo- or hyperactivity of apoptosis processes can influence the persistence of a chronic inflammatory state in the liver. Most of examinations assessing apoptosis activity in HBV and HCV infected patients are based on the determination of the concentrations of programmed death cell indicators in blood. The concentration and activity of these indicators can be modified by modulators, such as Bcl-2, or indicate simultaneous inflammatory necrotic processes. The determination of the concentration of cellular nucleic acids that undergo apoptosis (ssDNA) unambiguously indicates a programmed cell death process in progress. The aim of the study was to assess the concentration

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of ssDNA in liver tissue of patients with chronic HBV, HCV, HCV and HIV infections. The concentration of the nucleic acid was analyzed in relation to the concentration of serous apoptosis indicators, sFas and sFasL receptor proteins, the activity of inflammatory processes and fibrosis in liver tissue as well as HBV, HCV and HIV viraemia in serum.

Patients and methods

The study included 153 patients: 48 chronic HBV infected (17 women and 31 men aged 19 to 63), 86 chronic HCV infected (32 women and 54 men aged 18 to 66) and 19 HCV and HIV infected (4 women and 15 men aged 19 to 48). All the patients under study had been qualified and waiting for antivirus treatment due to chronic viral hepatitis.

HBV-DNA quantitative assay

HBV-DNA was detected by use of PCR method using a conservative pre-S/S primer. The concentration of HBV-DNA in serum was determined by means of RT-PCR method, using TaqMan chemistry (Applied Biosystems, USA) reagents. Amplifications were performed by use of a 25-µl TaqMan Universal Master Mix. For detection, Sequence Detector V1.6.3 (PEB bisosystems) was used. A standard line for HBV-DNA copies was drawn by means of standard sera. Copies count could range from 10 to 108 in a milliliter.

HCV-RNA quantitative assay

The RNA of HCV virus was amplified in a RT-nested – PCR reaction system with two pairs of nested primers, complementary to the conservative part of viral genome (external primers: sense 5'-TCT AGC CAT GGC GTT AGT ATG AGT GT-3', antysense 5'-CAC TCG CAA GCA CCC TAT CAG GCA GT-3'; internal primers: sense 5'- GGC GAC ACT CCA CCA TAG AT-3', antysense 5'-GTG CAC GGT CTA CGA GAC CT-3', (Sigma, USA). Amplification products were detected by means of electrophoresis in 2.0% agarose gel stained with ethidium bromide. Electropherograms were visualized in a Syngen Biotech UVI-KS400i/Image PC documentation and computer analysis system and quantitative concentration was determined.

HIV-RNA quantitative assay

The infection was diagnosed on the basis of double detection of HIV antibodies in blood by means of an immunoenzymatic method (ELISA, ABBOTT, USA) and Western-blot[1] confirming test (Cambridge Biotech Corporation, USA). HIV virus copies count was determined by means of RT-PCR method using Cobas Amplicor HIS 1.5 (Ultra Sensitive)[2].

Studied lymphocytes count

CD3+, CD4+ and CD8+ lymphocytes counts were determined in HIV infected patients' blood by means of a Becton Dickinson flow cytometer[2].

The concentration of ssDNA in liver tissue

The formamide is an agent that denatures DNA in apoptotic cells, but not in necrotic cells or in the cells with DNA breaks in the absence of apoptosis. In apoptotic cells, formamide denatures DNA to single-stranded DNA (ssDNA) [1].

The concentration of ssDNA was determined by means of Apoptosis ELISA Kit ssDNA test, (CHEMICON, Germany). The tissue obtained during biopsy was examined morphologically and its fragment was placed in 0.9% NaCl buffer solution. The tissue was emulgated and then the concentration of proteins in it was determined. The suspension of emulgated cells was transported to chambers for 24 hours in order to fix hepatocytes to their walls. Formamide, which denatured DNA in cells undergoing apoptosis facilitated the detection of ssDNA. The complexes of ssDNA and monoclonal antibody were stained and absorbance was determined by means of a 405 nm beam spectrometer. The concentrations of ssDNA were assessed by means of a plotted absorbance curve. The obtained results were re-calculated to one gram of liver tissue.

The concentration of sFas and sFasL

The concentrations of sFas and sFasL in serum were performed twice, using an immunoenzymatic method (ELISA, Bender MedSystems, Austria). The sFas and sFasL proteins were bound with monoclonal antibodies and then stained and optical density of sera at the wavelength of 450 nm was measured. The concentrations of sFas and sFasL were determined by comparing optical density to the plotted standard curve of the concentrations of sFas or sFasL.

Morphological assessment of liver

Liver tissue obtained from transcutaneous bioptates of this organ was analyzed morphologically in accordance with Scheuer's classification.

All patients and control group individuals gave their consent to take part in the study. The approval for the study was obtained from the Bioethical Committee of the Medical University of Białystok.

Statistical analyses

Statistical analyses were performed using Mann-Whitney and Spearman tests. Significance level $p<0.05$.

Results

The concentration of ssDNA in liver tissue does not depend on sex and age of patients [TW Lapiński et al. World J Gastroenterol, 2005; 11: 6130-3].

The concentration of ssDNA was higher in liver tissue of HCV infected patients ($1\,332\times10^{-6}$ µg/mg, $\pm664\times10^{-6}$), in comparison to those co-infected with HCV and HIV (886×10^{-6} µg/mg, $\pm388\times10^{-6}$; $r=2.84$; $p<0.004$) and in HBV

[1] The Western-blot test was performed in Laboratory and Experimental Institute of Department of Venerology Medical University of Warsaw, Head: Z. Solibórska, M.D., Ph.D.

[2] The examination was performed in Department of Immunology and Molecular Diagnosis of Regional Infectious Hospital in Warsaw, Head: J. Stańczak, M.D., Ph.D.

Table 1. **Concentration of ssDNA in liver tissue of HBV, HCV and HCV and HIV infected patients in relation to inflammation and fibrosis (Scheuer's classification)**

patients		ssDNA, µg/mg			
		inflammation 0-2	inflammation 3-4	fibrosis 0-1	fibrosis >1
HBV infected	x	1 645*	1 331	1 606**	1 135
	±SD	987	466	876	277
HCV infected	x	1 361*	1 301	1 344**	1 300
	±SD	613	722	662	682
HCV and HIV infected	x	886*	-	886**	-
	±SD	388	-	388	-

*, ** – statistical differences

Table 2. **Differences of studied parameters in HIV and HCV co-infected patients in relation to the number of CD4**

Group of patients	n		ssDNA x 10^{-6} µg/mg	CD8+ µl	HCV copy/mL	HIV copy/mL
CD4+ <410 µl, x=322 µl; ±SD=59	9	x	829	625	3.8 x 2 Log10	3.0 x 3 Log10
		±SD	327	170	1.7 x 1 Log10	6.1 x 3 Log10
CD4+ >410 µl, x=495 µl; ±SD=54	10	x	937	960	3.5 x 3 Log10	6.9 x 3 Log10
		±SD	447	494	2.4 x 1 Log10	8.9 x 3 Log10
p<0.0002			p=0.62	p=0.08	p=0.19	p=0.46

Table 3. **The concentration of ssDNA in liver tissue in relation to viraemia and HCV genotype**

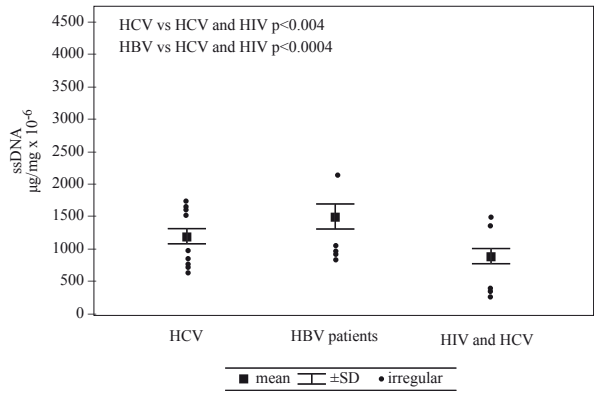
Group of patients	viraemia or genotype	n	ssDNA, x 10^{-6} µg/mg	
			x	±SD
HCV	HCV > 3 log10	54	1356	704
	HCV < 3 log10	32	1209	470
	genotype 1	68	1461	530
	genotype 3	18	1104	448
HCV/HIV	HCV > 2 log10	11	892	339
	HCV < 2 log10	8	878	471
	HIV > 3 log10	8	948	520
	HIV < 3 log10	11	841	275

infected patients (1 508×10^{-6} µg/mg, ±810×10^{-6}) in comparison to those co-infected with HCV and HIV (886×10^{-6} µg/mg; ±388×10^{-6}; r=3.55; p<0.0004). No difference as to the concentration of ssDNA in liver tissue between HBV and HCV infected patients was observed (r=-1.02; p=0.3) – *Fig. 1*.

Among HBV and HCV infected patients, the concentration of ssDNA in liver tissue was higher in persons with higher viraemia but it did not correlate with HBV (r=0.21, p=0.14) and HCV (r=-0.01; p=0.92) viraemias. Among patients infected with HCV genotype 3 the concentration of ssDNA was comparable from infected with genotype 1 (1 343 µg/mg × 10^{-6}, ±700 × 10^{-6}; vs 1 296 10^{-6} µg/mg, ±615 × 10^{-6}). No correlation between the concentration of ssDNA and ALT activity was detected – *Tab. 3*.

The highest concentration of ssDNA in liver tissue was detected in HBV infected patients, with low inflammatory activity (1 645 × 10^{-6}µg/mg, ±987 × 10^{-6}) and low fibrosis (1 606 × 10^{-6} µg/mg, ±876 × 10^{-6}). Mild inflammatory changes and low fibrosis were observed in all HCV and HIV infected patients. HBV and HIV infected patients, in comparison to HBV infected patients with similar histopathological changes, had lower concentration of ssDNA (886 x 10^{-6} µg/mg, ±388 × 10^{-6}; vs 1 645 × 10^{-6} µg/mg; ±987 × 10^{-6}; r=3.13, p<0.002). As regards HCV infection, there were similar correlations of the

Figure 1. **The concentration of ssDNA in liver tissue of HBV, HCV and HCV and HIV infected patients**



concentration of ssDNA in relation to HCV and HIV co-infection (1 361 × 10^{-6} µg/mg, ±613 × 10^{-6}; vs 886 × 10^{-6} µg/mg, ±388 × 10^{-6}; r=3.08, p<0.002). When comparing patients with similar fibrosis progression, differences in the concentration of ssDNA were observed between HBV infected patients and those with HCV and HIV (1 606 × 10^{-6} µg/mg vs 886 x 10^{-6} µg/mg; r=3.61; p<0.0003) as well as between HCV infected patients and those

***Figure 2*. The concentration of sFas in serum of HBV, HCV and HCV and HIV infected patients**

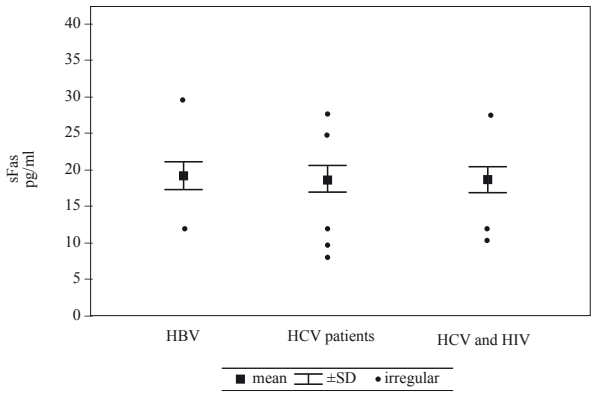
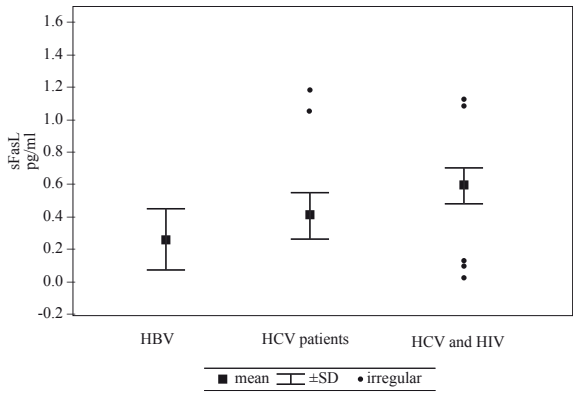


***Figure 3*. The concentration of sFasL in serum of HBV, HCV and HCV and HIV infected patients**



with HCV and HIV (1 344 × 10^{-6} µg/mg vs 886 × 10^{-6} µg/mg; z=2.88; p<0.004) – *Tab. 1*.

No correlation between ssDNA concentration in liver tissue and HIV viraemia (r=0.03; p=0.90), HCV, CD8+ and CD4+ counts (r=-11; p=0.66) was observed. Among HIV and HCV infected patients, the concentration of ssDNA in liver tissue was higher in patients with higher HIV (841 × 10^{-6} µg/mg, ±275 x 10^{-6}; vs 948 × 10^{-6} µg/mg, ±520 x 10^{-6}) and HCV (878 × 10^{-6} µg/mg, ±471 × 10^{-6}; vs 891 x 10-6 µg/mg, ±339 × 10^{-6}) viraemias. These differences, however, were not statistically significant – *Tab. 2*.

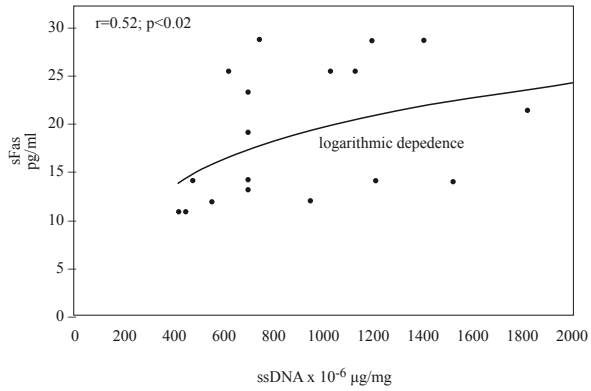
The mean concentrations of sFas in sera of patients infected with HBV, HCV, HCV and HIV were comparable – *Fig. 2*.

The concentration of sFasL was the highest among HCV and HIV infected patients. The differences were not statistically significant – *Fig. 3*.

Only among HCV and HIV infected patients was a correlation between the concentration of ssDNA in liver tissue and sFas in serum (r=0.52; p<0.02) observed.

No correlation between CD4+, CD8+ counts, HCV, HIV viraemias, and ssDNA in liver tissue of HCV and HIV infected patients was observed – *Tab. 2*.

***Figure 4*. The correlation between ssDNA in liver tissue and the concentration of sFas among HCV and HIV infected patients**



Discussion

It is argued that HBV stimulates apoptosis in hepatocytes stronger than HCV [2]. Strong stimulation of such process by HBx antigene of HBV viruses can be one of the reasons. In liver tissue of HCV infected patients, Mutluat et al. [3] detected higher concentration of Bcl-2 in comparison to those infected with HBV. Bcl-2 inhibits apoptosis, which can be another reason for the stronger course of programmed cell death processes among patients infected with HBV. In own studies, the highest concentration of ssDNA in liver tissue was observed among HBV infected patients. However, no significant difference between HBV and HCV infected patients was observed. This result suggests that programmed cell death activations by HBV as well as HCV are comparable. In the studies by Bondini and Younossi [4], it was shown that HCV genotype 3 stimulates apoptosis stronger than genotype 1. Also in own studies, the concentration of ssDNA among patients infected with HCV genotype 3 was higher in comparison to those infected with genotype 1. These observations can explain biological differences as well as immunity to antivirus treatment due to HCV genotype 1 or 3 infection.

HIV infection activates apoptosis of CD4+ lymphocytes, but not of hepatocytes [5]. HCV and HIV co-infection, in comparison to HCV-infection alone, predisposes to a more frequent occurrence of primary liver neoplasms. Esposito et al. [6] argue that the reason for this can lie in reduced apoptosis of hepatocytes among HCV and HIV co-infected patients. In own studies, the concentration of ssDNA in liver tissue of HCV and HIV co-infected patients was lower in comparison to those infected with HCV or HBV. It seems that HIV inhibits the processes of apoptosis in liver tissue, either in a direct or indirect way. HCV and HIV co-infected patients with higher CD4+ count show a slightly stronger activity of programmed hepatocyte death processes (with no significant statistical difference). No HCV or HBV viraemia influence on programmed cell death processes was detected. In own studies it was shown that inflammatory activity and fibrosis intensification in liver tissue do not influence the concentration of ssDNA in HCV infected patients. In the group of HBV infected patients, lower inflammation and less intense fibrosis correlated with the concentration of ssDNA. These observations suggest that there is a relation between fibrosis in the liver and programmed cell death processes.

HBV and HCV viruses stimulate programmed cell death indicators such as TNF, Fas, TRAIL, IL-1 [7]. In own studies, the concentration of sFas among HBV infected patients was slightly higher in comparison to those infected with HCV or HCV and HIV. Macias et al. [8] showed that HCV and HIV infections cause a higher expression of Fas in hepatocytes. However, the correlation between the concentration of sFas in serum and the concentration of ssDNA in liver tissue was detected only among HCV and HIV infected patients. This undermines the importance of the assessment of sFas and sFasL concentrations as a programmed hepatocyte death indicatior among patients infected with HBV and HCV with no HIV co-infection.

Balasubramanian et al. [9] argue that the activity of apoptosis in hepatocytes among patients infected with HCV and HIV is regulated by the stimulation of Fas/FasL receptors; moreover, HCV E2 and HIV gp 120 stimulate the expression of caspase 3. In own studies, a higher concentration of ssDNA was present in patients with higher HCV and HIV viraemia. It seems that, in infections with these viruses, HIV viruses inhibit programmed cell death activity, as assessed by the concentration of ssDNA, either in a direct or indirect way.

Conclusions

HCV, HBV and HIV viraemias do not correlate with ssDNA concentration in liver tissue. In patients with HCV and HIV co-infection, CD4+ and CD3+ counts do not correlate with ssDNA concentration in liver tissue. HIV infection seems to inhibit apoptosis processes in liver tissue of HCV and HIV co-infected patients. In the case of HCV and HIV co-infection, the concentration of sFas in serum correlates with the concentration of ssDNA in liver tissue. This phenomenon is not observed among HBV or HCV infected patients.

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Matrix metalloproteinases and their tissue inhibitors in children with chronic hepatitis B treated with lamivudine

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Abstract

Purpose: To evaluate if measurements of MMP-2, MMP-9, TIMP-1 and TIMP-2 have clinical applicability as markers of liver fibrosis and to assess the effect of long-term lamivudine treatment on liver fibrosis in children with chronic hepatitis B (chB).

Material and methods: The observation was carried out on 41 children with biopsy proven chB (HBe/+/, HBVDNA/+/) who were nonresponders to previous IFNα therapy. Lamivudine was administered in the group of 29 children (3 mg/kg/day, maximum 100 mg/daily). The serum concentration of examined markers was measured with ELISA before and after 24 months of therapy. ROC analysis was used to calculate the power of the assays to detect advanced liver fibrosis (score >2 according to Batts&Ludwig).

Results: Serum TIMP-1 and TIMP-2 levels were significantly higher and MMP-9 lower in children with chB compared to controls. There was a significant positive correlation between serum MMP-2 and negative correlation between MMP-9 level and the stage of liver fibrosis. The ability of serum MMP-9 to differentiate children with mild fibrosis from those with advanced fibrosis was significant (AUC=0.75; p=0.03). Other serum markers did not allow a useful prediction. 2-year lamivudine treatment did not improve histological fibrosis but it caused significant decrease of serum TIMP-2 (p=0.01) and increase of MMP-9 level (p=0.00005).

Conclusions: MMP-9 is a better serum fibrosis marker than MMP-2, TIMP-1 and TIMP-2 to diagnose children with advanced liver fibrosis. The significant decrease of TIMP-2 and increase of MMP-9 level during therapy suggest antifibrotic effect of lamivudine in children with chB.

Key words: liver fibrosis, HBV, lamivudine, MMP, TIMP.

Introduction

HBV is still considered one of the most important epidemiological problems because nearly 400 million people all over the world have been chronically infected with this hepatotropic virus [1]. This issue is also important for about 40 millions of Polish citizens, although there was a significant drop from 13 296 HBV incidents in 1993 to 1 473 in 2004 [2]. Due to possible development of liver cirrhosis or primary hepatic carcinoma, chronic hepatitis B is also regarded a significant clinical problem.

During progression of chronic liver disease an imbalance occurs between synthesis and breakdown of extracellular matrix (ECM) components. Matrix metalloproteinases (MMPs) are involved in degrading ECM while tissue inhibitors of matrix metalloproteinases (TIMPs) prevent their fibrolytic action. Hepatic stellate cells (HSC) play a central role in the pathogenesis of liver fibrosis and their activation leads to release of both MMPs and TIMPs [3]. However, MMP-9 are mainly derived from Kupffer cells [4]. These components, which can be measured in serum, are thought to play an essential role in liver injury associated with tissue remodeling but it is still unclear whether their circulating levels are liver-specific [5].

So far the treatment of chronic hepatitis B in children has been based on interferon alpha. The use of this drug allows to expect the inhibition of HBV replication in approximately 30--50% patients [6]. This is certainly not sufficient and therefore, new attempts are made to introduce new antiviral agents. Presently, lamivudine, a nucleoside anologue is considered the most promising drug in the treatment of chronic hepatitis B in

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children. One of the main goals of lamivudine therapy, even in the absence of virus supression is improvement of liver histology, especially of liver fibrosis, which is a predictor of progression to cirrhosis [7].

To our knowledge, serum fibrosis markers predicting liver fibrosis have not been assessed before in children with chronic hepatits B during long-term lamivudine treatment. Therefore, the aim of the study was to evaluate if measurements of MMP-2, MMP-9, TIMP-1 and TIMP-2 have clinical applicability as markers of liver fibrosis and to examine the effect of lamivudine treatment on liver fibrosis by direct assessment of histological scores and by indirect assessment of serum levels of selected fibrosis biomarkers (MMP-2 and 9 and TIMP-1 and 2) in children with chronic hepatitis B.

Material and methods

Patients

The study was carried out with 41 Caucasian children (mean age 10.5 years, range 6-17, 29 boys and 12 girls) with serologically and biopsy-verified chronic hepatitis B, who were nonresponders to previous intrerferon alpha therapy (3 MU tiw for 20 weeks). The children were still positive for HBs, HBe antigens and serum HBVDNA. Patients with HCV coinfection and with liver cirrhosis were excluded from the study. None of the children was treated with antiviral and immunomodulating drugs during the 12-month period before inclusion into the study. Informed consent was obtained from all patients' parents and the protocol was approved by the local ethical committee of the Medical University of Białystok, Poland. Serum samples were evaluated twice: at the beginning and after 24 months of lamivudine treatment. Serum samples were stored at -70ºC until use. Standard liver tests were measured by validated automated methods and included total bilirubin, albumin, ALT, AST, GGT and ALP. HBsAg and HBeAg were determined by MEIA (Microparticle Enzyme Immunoassay) and HBV DNA were determined by PCR (qualitative) method.

Lamivudine treatment and definition of response

Lamivudine was applied in the group of 29 children at the dose of 3 mg/kg/day up to 100 mg daily for 24 months. HBeAg/antiHBe seroconversion with concomitant clearance of serum HBVDNA after 2 years of treatment was considered the criterion of treatment response.

Measurement of serum fibrosis markers

The concentration of MMP-2, MMP-9, TIMP-1 and TIMP-2 were measured with EIA technique in serum using R&D Systems commercial kits. Nine children (mean age 10 years) were included as control group without anamnestical, clinical or laboratory signs of liver diseases or other chronic diseases.

Histological analysis

Percutaneous liver biopsies were obtained in all patients before treatment and in 13 children after 24-months of lamivudine treatment. The liver specimens were fixed in buffered formalin and embedded in paraffin. Histological sections were stained using hematoxylin-eosin, Masson-Goldner, Masson's trichrome and reticulin stains. Fibrosis stage and inflammation grade were assessed in a blinded fashion by a single pathologist without knowledge of the patients' laboratory or clinical data. In order to determine specificity and sensitivity of the assay we arbitrarily defined advanced liver fibrosis as a score >2 and advanced inflammation as a score >1 according to Batts and Ludwig [8].

Statistics

Results are expressed as means ± SD. Statistical analysis was performed with the Mann-Whitney U test for independent samples and Wilcoxon signed rank test for paired samples. The relationship between non-invasive markers and liver histology scores were analysed by the Spearman rank-correlation test for non-parametric data and by the Pearson method for parametric data. The tests were considered statistically significant at $p<0.05$.

Receiver operating characteristics (ROC) analysis (AccuROC, Montreal, Canada) was used to calculate the power of the assays to detect advanced liver fibrosis [9]. Sensitivity of the assays was plotted against the false positivity (1-specificity). Comparison of the area under the curve (AUC) was performed using a two-tailed p test, which compares the AUC to the diagonal line of no information (AUC 0.5).

Results

Serum concentration of biomarkers in children with chronic hepatitis B

Serum concentration of TIMP-1 and TIMP-2 levels were significantly higher and MMP-9 was lower in children with chronic hepatitis B /n=41/ compared to controls /n=9/ (142.0±32.0 vs 123.2±17.5 ng/ml, p=0.043; 86.7±11.0 vs 72.6±13.8 ng/ml, p=0.008; 185.2±135.7 vs 261.6±107.0 ng/ml; p=0.016, respectively).

There were no significant correlations of the examined biomarkers with age, ALT, AST, GGT, ALP, bilirubin and albumin or histological inflammation according to Batts and Ludwig. However, there was a significant positive correlation between serum MMP-2 and negative correlation between MMP-9 and the stage of fibrosis (r=0.31, p=0.046; r=-0.32, p=0.041, respectively).

Diagnostic value of serum fibrosis markers for identification of patients with advanced liver fibrosis and inflammation

All children had liver fibrosis. Thirty-three children (80.5%) had mild liver fibrosis: 25 of them – score 2 and 8 – score 1, and 8 children (19.5%) had advanced fibrosis (score = 3 according to Batts and Ludwig).

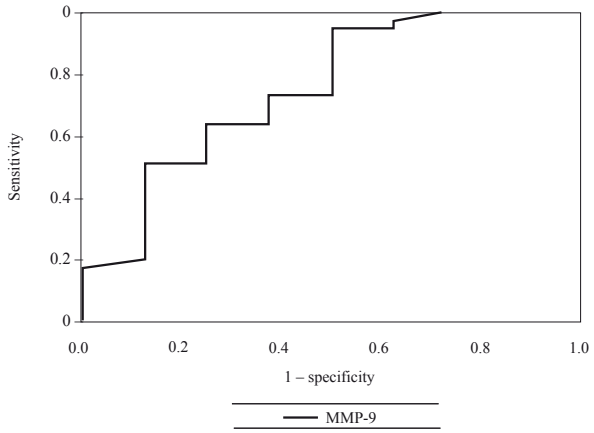
The patients with advanced liver fibrosis did not have significantly higher serum level of liver enzymes, bilirubin, albumin and histological inflammation than patients with mild liver fibrosis. The level of examined biomarkers was not different in children with mild liver fibrosis and with advanced fibrosis except MMP-9 (p=0.03) (*Tab. 1*).

Table 1. **Comparative characteristics of children with mild and advanced liver fibrosis**

Data of the patients	Mild fibrosis (n=33) mean ± SD	Advanced fibrosis (n=8) mean ± SD	p-value
Age (years)	10.4±3.2	11.5±3.5	0.43
ALT (IU/l)	64±35	97±57	0.11
AST (IU/l)	56±23	65±30	0.47
GGT (IU/l)	12±5	12±3	0.99
ALP (IU/l)	277±80	261±116	0.57
Bilirubin (μmol/l)	9.06±4.1	9.75±2.05	0.60
Albumin (%)	63.2±2.8	63.1±3.5	0.79
MMP-2 (ng/ml)	261.7±48.3	283.8±42.6	0.29
MMP-9 (ng/ml)	200.8±145.2	120.6±54.3	0.03*
TIMP-1 (ng/ml)	144.5±27.9	131.9±46.3	0.47
TIMP-2 (ng/ml)	85.4±10.2	92.1±12.2	0.28
Grading	1.36±0.49	1.62±0.74	0.45

* p < 0.05; Normal ranges: AST – 10-40 IU/l, ALT – 10-40 IU/l, GGT – 9-35 IU/l, ALP – 110-350 IU/l, bilirubin – 1.71-18.81 μmol/l, albumin – 58.8-69.6%

Figure 1. **Receiver Operating Characteristic Analysis**



ROC curve of ability of serum MMP-9 to detect mild liver fibrosis according to Batts and Ludwig in children with chronic hepatitis B

Table 2. **The examined serum biomarkers AUC value to detect advanced liver fibrosis (for MMP-9 – mild liver fibrosis) and advanced inflammation according to Batts & Ludwig in children with chronic hepatitis B**

Biomarker	Fibrosis	Inflammation
MMP-2	AUC=0.625±0.1147 p=0.2776	AUC=0.5538±0.097 p=0.5655
MMP-9	AUC=0.75±0.1089 p=0.03*	AUC=0.4013±0.098 p=0.291
TIMP-1	AUC=0.4148±0.1234 p=0.4591	AUC=0.6088±0.0907 p=0.245
TIMP-2	AUC=0.6269±0.1070 p=0.2703	AUC=0.6525±0.0985 p=0.1029

* p< 0.05

The ability of these markers to differentiate the children with mild liver fibrosis from those with advanced fibrosis was not significant except MMP-9 (AUC=0.75±0.1089, p=0.03) (*Fig. 1*, *Tab. 2*). A serum MMP-9 level above 94.6 ng/ml had a sensitivity of 93.9% and specificity of 50%; PPV= 88% and NPV= 66.6%.

None of these markers was a good predictor of histological inflammation (*Tab. 2*)

Effect of lamivudine on serum MMP-2, MMP-9, TIMP-1 and TIMP-2 level in children with chronic hepatitis B

Children with chronic hepatitis B who were treated with lamivudine were analysed according to the type of response. There were 4 responders (14%) and 25 nonresponders (86%).

There were no significant differences between the groups regarding age, the levels of bilirubin and albumin, the activity of AST, ALP, GGT and grade of inflammation as well as the stage of fibrosis. However, the responders displayed a significantly higher activity of ALT (p=0.012). The level of examined serum fibrosis markers before lamivudine therapy was not significantly different in responders and nonresponders either (*Tab. 3*).

During 24 months of lamivudine treatment there were significant changes of examined serum fibrosis markers: TIMP-2 significantly decreased (p=0.01) and MMP-9 level significantly increased (p=0.00005). No significant changes of TIMP-1 and MMP-2 were found at the end of 24 months of lamivudine treatment (*Tab. 4*).

Effect of lamivudine on liver histology in children with chronic hepatitis B

There were no significant changes in liver fibrosis as well as in liver inflammation after 24 months of lamivudine therapy (1.77±0.6 vs 1.77±0.44 and 1.38±0.51 vs 1.69±0.48, respectively).

Discussion

The advanced liver fibrosis and even cirrhosis had been regarded irreversible until quite lately. However, experimental and clinical studies confirmed possibility of stopping or even decreasing the stage of liver fibrosis through causal factor

***Table 3*. The baseline characteristics of the groups of examined children with chronic hepatitis B treated with lamivudine**

Data of the patients	Responders (n=4) mean± SD	Nonresponders (n=25) mean± SD	p-value
Age (years)	12± 3.5	10.2±2.9	0.37
ALT (IU/l)	122±35	63±37	0.008*
AST (IU/l)	69±21	56±26	0.14
GGT (IU/l)	12±3	13±6	0.77
ALP (IU/l)	272±99	279±91	0.83
Bilirubin (μmol/l)	8.38±3.08	8.72±3.08	0.76
Albumin (%)	62.0±3.6	63.6±2.4	0.35
Staging	2.5±0.6	1.96±0.61	0.11
Grading	1.75±0.5	1.48±0.59	0.41
MMP-2 (ng/ml)	278.3±40.8	267.5±45.9	0.47
MMP-9 (ng/ml)	126.6±56.8	164.9±99.8	0.49
TIMP-1 (ng/ml)	160.5±23.8	145.8±34.8	0.23
TIMP-2 (ng/ml)	93.8±5.5	87.0±12.6	0.11

* $p < 0.05$

***Table 4*. Effect of 24-month lamivudine treatment on serum fibrosis markers in children with chronic hepatitis B (n=29)**

Marker	Before treatment	After treatment	p-value
MMP-2 (ng/ml)	269.0±44.7	261.7±59.0	0.24
MMP-9 (ng/ml)	159.6±95.2	359.1±229.0	0.000046*
TIMP-1 (ng/ml)	147.8±33.6	142.8±39.3	0.28
TIMP-2 (ng/ml)	87.9±12.0	82.4±12.2	0.014*

* $p < 0.05$

elimination or application of pharmacological preparation of potential antifibrotic activity, e.g., interferon alfa with ribavirin or lamivudine [10,11]. Therefore, reliable diagnostics is necessary for monitoring hepatic fibrogenesis in patients with chronic hepatitis. Although, the morphological examination of liver biopsy is still regarded a standard method in assessment of the stage of liver fibrosis, it is an invasive procedure and has several limitation such as sampling error and it only provides static information about the amount of fibrotic tissue [12,13]. Recently, several biochemical blood tests for liver fibrosis have been evaluated in adults which give possibility of long-term monitoring of the course of disease and possible changes caused by treatment. The noninvasive markers of fibrosis include extracellular matrix (ECM) components (e.g. hyaluronan, laminin, collagens, MMPs, TIMPs) as well as non-ECM biochemical panels (FibroTest, Forns index, APRI) [14,15].

To our best knowledge non-invasive biomarkers that predict liver fibrosis due to chronic hepatitis B in children are lacking except our previous studies. We found that the combination of serum hyaluronan and laminin-2 as well as APRI can accurately predict significant liver fibrosis [16,17]. The results of our next studies showed that the ability of serum TGF beta 1 or cystatin C to differentiate children with advanced liver fibrosis from those with mild fibrosis was not significant [18,19].

In this study we found significantly higher level of TIMP-1 and TIMP-2 and lower level of MMP-9 in the group of children with chronic hepatitis B than in controls. Our results are in agreement with data presented by Flisiak et al. [20], Mitsuda et al. [21] and Murawaki et al. [22] who also confirmed higher TIMP-1 level and with Leroy et al. [23] and Walsh et al. [24] who showed higher level of TIMP-1 and -2 in patients with chronic hepatitis than in controls. Reif et al. [25] and Walsh et al. [24] found that patients and control group had similar serum level of MMP-2. This finding is not with agreement with data presented by Chen et al. [26] who showed higher level of MMP-2 in patients with hepatitis but they also confirmed, like in our study, that MMP-9 level in patients with chronic liver disease were lower than in controls. The study of Reif et al. [25] is not consistent with ours and Chen et al. [26] findings because they demonstrated that serum activity of MMP-9 was increased in patients with hepatitis C compared to controls. Such discrepancies could be explained by analytic methods used (ELISA or zymography), subject sampling or severity of the disease in the examined group of patients with chronic viral hepatitis.

Although we confirmed significant correlation of MMP-2 and -9, which are the major MMPs in circulation, with the stage of liver fibrosis, using ROC analysis we found that only the ability of MMP-9 to differentiate the children with mild liver fibrosis from those with advanced fibrosis was significant.

In adults with chronic viral hepatitis, mainly caused by HCV, the role of MMPs and TIMPs as markers of liver fibrosis was discrepant. Our findings are in agreement with Leroy et al. [23] who also demostrated a correlation of serum level of MMP-2 and -9 with fibrosis stage. However, in contrast, Reif et al. [25] found that MMP-2 and -9 are markers of inflammation but not of the degree of fibrosis. Morever, Murawaki et al. [27] showed that TIMP-1 correlated both with histological inflammation and fibrosis. Walsh et al. [24] demonstrated TIMP-1 correlation with liver inflammation and TIMP-2 with histological fibrosis, however, MMP-2 was related neither to fibrosis nor histological activity index. Using ROC analysis both TIMP-1 and TIMP-2 (but not MMP-2) had significant diagnostic ability

in detecting advanced liver disease. The usefulness of TIMP-1, which was better than MMP-2, in detecting severe liver fibrosis was also confirmed by Leroy et al. [23] and Boeker et al. [28].

These discrepencies in the estimation of the role of MMPs and TIMPs in liver fibrosis might be related to the use of a different fibrosis scoring systems as well as multiple versus single pathologists scoring the biopsies. Moreover, non-invasive markers will not have complete concordance with histological staging because histological scoring systems are not sensitive enough to detect small changes in fibrosis stage and biomarkers may even be more accurate than biopsy in staging disease [15].

The main aim of this study was to examine the effect of lamivudine treatment on liver fibrosis by direct assessment of histological scores and by indirect assessment of serum levels of fibrosis markers in children with chronic hepatitis B who were nonresponders to previous interferon alpha therapy. Data regarded the effect of lamivudine treatment on liver histology in children are scarce. Only Ozgenc et al. [29] evaluted retrospectively histological response in 29 children who received first combination therapy (interferon alpha and lamivudine) and then continued with prolonged treatment with lamivudine and they found significant decrease of inflammation and fibrosis scores. In our prospective study, neither significant changes in histological evolution of fibrosis nor in inflammation after 2-year lamivudine therapy were found. All 13 children with repeated liver biopsy did not seroconvert to antiHBe. Therefore, probably for that reason we did not observe statistically significant improvement in liver histology, especially in fibrosis.

Only few studies analysed the effect of lamivudine treatment on the level of serum fibrosis markers in adults with chronic hepatitis B. Flisiak et al. [20] showed that lamivudine treatment resulted in a significant decrease of TIMP-1 and TGF beta 1 and increase of MMP-1 level during treatment. Although in our study we examined different kind of MMPs and TIMPs, we found similar changes induced by lamivudine treatment; we demonstrated the significant decrease of TIMP-2 and increase of MMP-9. Other authors analysed other serum fibrosis markers. Poynard et al. [30] found that in patients with chronic hepatitis B a 24-month course of lamivudine treatment lead to significant decrease in fibrosis assessed by FibroTest. Grzeszczuk et al. [31] demonstrated that hyaluronan level decreased during lamivudine treatment both in patients with HBeAg seroconversion and without it. Maxwell et al. [32] showed that amino-terminal propeptide of type I procollagen (PINP)/carboxy-terminal telopeptide of type I collagen (ITCP) ratio was sensitive and specific in detecting responders to 48-week lamivudine treatment.

We conclude that MMP-9 is a better serum fibrosis marker than MMP-2, TIMP-1 and TIMP-2 to diagnose children with advanced liver fibrosis. The significant decrease of TIMP-2 and increase of MMP-9 level during therapy suggest antifibrotic effect of lamivudine in children with chronic hepatitis B but this finding needs to be confirmed in larger studies.

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Serum level of YKL-40 does not predict advanced liver fibrosis in children with chronic hepatitis B

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Abstract

Purpose: The aim of the study was to evaluate the serum concentration of YKL-40 (human cartilage glycoprotein-39) in the assessment of fibrosis stage in children compared to biopsy and prior to antiviral treatment for chronic hepatitis B.

Material and methods: We determined serum level of YKL-40 (METRA, EIA kit, Quidel Corporation, San Diego, USA) after an overnight fast in 63 children (age range 4-17 years, mean 10 years) with biopsy-verified chronic HBeAg-positive hepatitis B. Fibrosis stage and inflammation grade were assessed in a blinded fashion according to Ishak et al. We defined advanced liver fibrosis as a score >2. Receiver operating characteristics (ROC) analysis was used to calculate the power of the assay to detect advanced liver fibrosis (AccuROC, Canada).

Results: Serum concentration of YKL-40 was significantly higher in patients with chronic hepatitis B compared to controls (n=16) (38.5±19.2 vs 27.9±8.75 ng/mL; p=0.032). The ability of serum YKL-40 to differentiate children with advanced liver fibrosis (n=31; 49.2%) from those with mild fibrosis was not significant (AUC=0.387±0.072, p=0.12). This marker was not a good predictor of histologic inflammation either.

Conclusion: Serum level of YKL-40 does not predict advanced liver fibrosis in children with chronic hepatitis B.

Key words: biomarker, children, chronic hepatitis, HBV, HC gp-39, fibrosis, YKL-40.

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Introduction

YKL-40 also known as human cartilage glycoprotein-39 (HC gp-39) or chondrex is a 40 kilodalton glycoprotein first described in whey secretions of nonlactating cows [1]. It is a mammalian member of a chitinase family (18-glycosylhydrolases) [2]. The exact biological functions of YKL-40 are still unknown but its growth factor activities for chondrocytes, synovial cells [3] and fibroblasts [4] have been reported. It also plays a role in angiogenesis by stimulating the migration and reorganization of vascular endothelial cells [5]. Haemodynamic studies have shown that this biomarker is released from the hepatosplanchnic area [6]. The expression of YKL-40 in normal and disease conditions suggest that it is involved in inflammatory processes and in remodelling the extracellular matrix (ECM) [1,2,7]. Subsequent studies have shown that YKL-40 may be used as a potent marker of arthritic disorders [8-10], inflammatory bowel disease [11], purulent meningitis [12], Streptococcus pneumoniae bacteremia [13], breast cancer [14] and colorectal cancer [15].

According to Johansen et al. [16] YKL-40 can be secreted by hepatic stellate cell (HSC) which is principal effector cell in liver fibrogenesis. Therefore this glycoprotein can be regarded as biomarker of liver fibrosis.

To our knowledge, serum level of YKL-40 predicting liver fibrosis has not been assessed before in children. Normal serum concentration of this marker is not significantly affected by growth and therefore appear to be more useful in assessing ECM metabolism in paediatric liver diseases [7]. Therefore the serum concentration of this marker was measured in children with chronic hepatitis B and compared to liver histology to determine if measurement of this biochemical test has any clinical usefulness as marker of liver fibrosis.

***Table 1*. Initial characteristics of children with chronic hepatitis B**

Data of the patients	Mean	SD	Minimum	Maximum
Age (years)	10	3.41	4	17
ALT (IU/L)	84	57	12	312
AST (IU/L)	68	37	27	264
GGT (IU/L)	15	9	3	69
Bilirubin (μmol/L)	10.1	4.45	2.74	32.8
YKL-40 (ng/mL)	38.5	19.2	16.2	119.2
Staging	2.55	0.88	1	5
Grading	3.76	1.43	1	8

Normal ranges: ALT – 10-40 IU/L, AST – 10-40 IU/L, GGT – 9-35 IU/L, bilirubin – 1.7-18.8 μmol/L

Material and methods

Patients

The study was carried out on 63 consecutive children (mean age 10 years, range 4-17, 41 boys and 22 girls) with biopsy-verified chronic HBeAg positive and HBV DNA positive hepatitis B prior to antiviral therapy. Other causes of chronic liver disease, such as HCV coinfection, autoimmune hepatitis and metabolic liver disorders were excluded. Children with diagnosed liver cirrhosis and evidence of other acute or chronic infections were excluded from this study. Informed consent was obtained from all patients' parents and the protocol was approved by the ethics committee of the Medical University of Bialystok, Poland. As a control group, 16 children (mean age – 10 years) were included without anamnestical, or laboratory signs of organic liver diseases or other systemic diseases. Standard liver tests were measured directly by validated automated methods and included total bilirubin, alanine aminotransferase (ALT), aspartate aminotransferase (AST) and gamma glutamyltranspeptidase (GGT). HBsAg and HBeAg were determined by ELISA and HBV DNA – by PCR method.

Measurement of serum YKL-40

YKL-40 was measured in serum samples (obtained after an overnight fast) using commercial kit (METRA, EIA kit, Quidel Corporation, San Diego, USA).

Histological analysis

All children underwent liver biopsy on the day after serum sampling. Liver specimens were fixed in buffered formalin and embedded in paraffin. Histological sections were stained using hematoxylin-eosin, Masson's Goldner, Masson's trichrome and reticulin stains. Fibrosis stage and inflammation grade were assessed in a blinded fashion by a single pathologist without knowledge of the patients' laboratory or clinical data. In order to determine specificity and sensitivity of the assay we arbitrarily defined advanced liver fibrosis as a score >2 and advanced inflammation as a score >3 according to Ishak et al. [17].

Statistics

Serum concentration of biochemical tests were expressed as mean values ± standard deviation (SD). Statistical analysis was performed with the Mann-Whitney two-sample test for nonparametric data. The relationship between biochemical tests and liver histology scores was analyzed by the Spearman rank-correlation test for nonparametric data and by the Pearson method for parametric data. Tests were considered statistically significant at $p<0.05$. Receiver operating characteristics (ROC) analysis (AccuROC, Montreal, Canada) was used to calculate the power of the assay to detect advanced liver fibrosis. Comparison of the area under curve (AUC) was performed using a two-tailed p-test, which compares the AUC to the diagonal line of no information (AUC 0.5) [18].

Results

Patients characteristics

Selected biochemical and histological data are presented in *Tab. 1*.

Serum concentration of YKL-40

Serum concentration of YKL-40 was significantly higher in patients with chronic hepatitis B compared to controls (38.5±19.2 vs 27.9±8.75 ng/mL; p=0.032). There were no significant correlations of YKL-40 with age (r=0.059, p=0.65), ALT (r=0.075, p=0.563), AST (r=-0.065, p=0.617), GGT (r=-0.0062, p=0.96), bilirubin (r=0.045, p=0.728) or liver fibrosis and inflammation according to Ishak et al. (r=-0.168, p=0.18; r=0.005, p=0.97, respectively).

Diagnostic value of YKL-40 for identification of patients with advanced liver fibrosis and inflammation

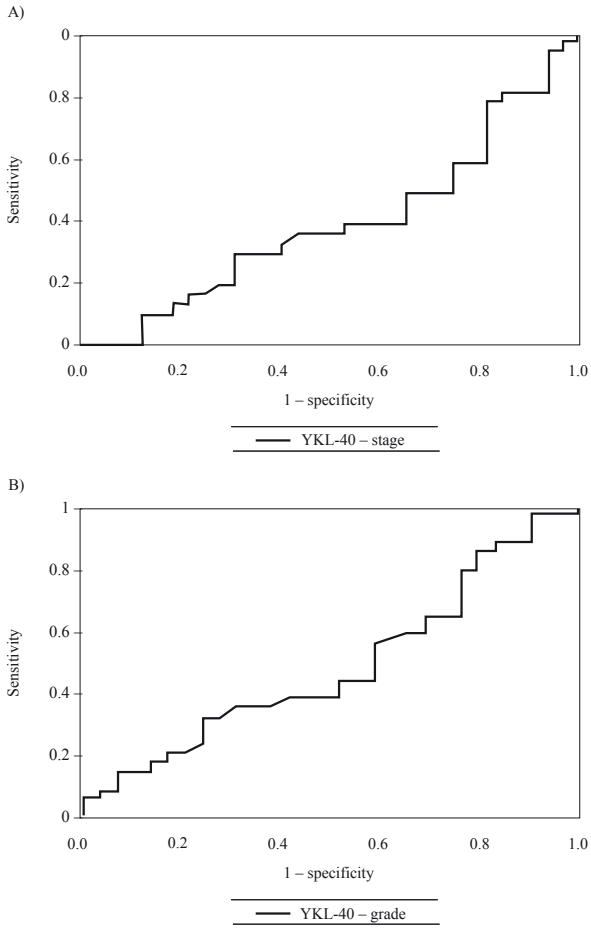
All the examined children had liver fibrosis – 32 children (50.8%) had mild liver fibrosis: 27 of them – score 2 and 5 – score 1 and 31 children (49.2%) had advanced fibrosis (score >2 according to Ishak et al.): 24 of them – score 3, 4 – score 4 and 2 score 5. Children with advanced liver fibrosis had significantly higher activity of ALT and AST and higher grade of histological inflammation than children with mild fibrosis (*Tab. 2*).

The ability of YKL-40 to differentiate the children with advanced liver fibrosis from those with mild fibrosis was not significant (AUC=0.387±0.072, p=0.12) (*Fig. 1A*). The ability of YKL-40 to differentiate children with advanced inflammation (n=17) from those with mild inflammation was not significant either (AUC=0.488±0.075, p=0.87) (*Fig. 1B*).

***Table 2*. Characteristics of 32 children with mild fibrosis and 31 children with advanced liver fibrosis**

Data of the patients	Mild fibrosis (n=32) mean±SD	Advanced fibrosis (n=31) mean±SD	p-value
Age (years)	10±3.2	10.4±372	0.69
ALT (IU/L)	64±37	106±66	0.003
AST (IU/L)	54±19	82±45	0.02
GGT (IU/L)	14±11	15±8	0.51
Bilirubin (μmol/L)	10.1±5.1	10.1±3.42	0.91
YKL-40 (ng/mL)	42.5±22.6	34.3±14.0	0.12
Grading	3.06±1.07	4.48±1.41	0.0001

***Figure 1*. Receiver Operating Characteristic Analysis**



ROC analysis of YKL-40 in predicting advanced liver fibrosis (A) and inflammation (B) according to Ishak et al. in children with chronic hepatitis B

Discussion

Liver biopsy is still regarded as the standard method to assess fibrosis stage but it is an invasive procedure with possible complications [19]. Furthermore biopsy sampling error can reach 30% for the difference in one stage, when using systems which range from 0 to 4 (cirrhosis). Finally, biopsy only provides static information about the fibrotic process [20,21]. For that reasons there is a clinical need for noninvasive measurement of liver fibrosis both to diagnose significant fibrosis and to monitor the effects of antiviral or antifibrotic treatment. An ideal serum fibrosis marker should be liver-specific, independent of metabolic alteration, easy to perform, minimally influenced by impaired urinary and biliary excretion, and should reflect fibrosis in all types of chronic liver diseases, correlate with matrix deposition or removal. It should also be sensitive enough to discriminate between different stages of fibrosis and reflect the response to antifibrotic therapy but no single marker fulfills all the criteria sufficiently to merit routine clinical use yet [22,23].

Serum level of extracellular matrix components have been studied in children recently, but almost exclusively in patients with biliary atresia and cystic fibrosis [24-26]. To our knowledge noninvasive markers that predict advanced fibrosis due to chronic hepatitis B in children are lacking, except our previous studies. From the broad panel of matrix-derived serum markers (collagen IV, collagen VI, PIIINP, laminin-2, hyaluronan, MMP-2, TIMP-1, MMP-9/TIMP-1 complex, tenascin – C) the combination of serum hyaluronan and laminin-2 can accurately predict significant liver fibrosis [27]. We have also previously shown that serum TGF beta 1 and cystatin C does not predict advanced liver fibrosis in children with chronic hepatitis B [28,29]. We also found that APRI (aspartate aminotransferase to platelet ratio index) may be an accurate and simple index in predicting advanced liver fibrosis in children [30].

In our present study for the first time we assessed the potent fibrosis marker – YKL-40 in children with chronic hepatitis B and we found its significantly higher level in this group of children than in controls. However, there was no significant correlation of this biomarker with stage of liver fibrosis.

Data regarding assessment of YKL-40 in adults with chronic viral hepatitis are scarce. Our results are in agreement with data presented by Johansen et al. [31] and Nojgaard et al. [32] who also confirmed that YKL-40 level in patients with chronic liver disease were higher than in controls. However, they were not consistent with their other findings, because they showed significant positive correlation of this marker with histological stages. The similar results presented Saitou et al. [33] in patients with chronic hepatitis C, Zheng et al. [34] in patients with hepatic fibrosis due to schistosomiasis japonica and Nojgaard et al. [35] in patients with alcoholic liver disease.

Recently ROC analysis has been recommended to calculate the power of the assays to detect advanced liver fibrosis [18,36]. According to Kelleher et al. [37] in this study we arbitrarily

defined advanced liver fibrosis as a score >2 ("substantial" fibrosis) and mild fibrosis as a score ≤2 ("minimal" fibrosis). The ability of YKL-40 to differentiate children with advanced liver fibrosis from those with mild fibrosis was not significant. The studies in adults with chronic hepatitis C were not consistent with our findings. Saitou et al. [33] based on ROC analysis demonstrated that YKL-40 was superior to other serum fibrosis markers (hyaluronan, collagen type IV and PIIINP) in predicting severe fibrosis (stage 2-4) from mild fibrosis (stage 0-1). However, the ability of serum hyaluronan exceeded the ability of YKL-40 to determine fibrosis score 4 from scores 0-3. These data are in keeping with previous results in HIV/HCV co-infected patients with hepatic fibrosis [37].

The lack of correlation between fibrosis marker and liver histology in our study could be explained by findings that liver biopsy is not necessarily a gold standard for assessing liver histology and for that reason noninvasive markers will not have complete concordance with histological staging. It was established that the best correlation was found at the extreme spectra of fibrosis, i.e. low stage of fibrosis and cirrhosis [37]. In the examined group most of the children (51 out of the 63) had moderate disease (Ishak stage 2 and 3) and probable this was a reason for lacking the correlation between YKL-40 and histological staging in our group of children. According to Poynard et al. [38] inadequate liver biopsy rather than inaccuracy of serum markers was more commonly the cause for divergent results between biochemical panel of biomarkers (FibroTest) and biopsy. Other authors suggests that histological scoring systems are not sensitive enough to detect small changes in fibrosis stage and biomarkers may even be more accurate than biopsy in staging disease [39,40].

We conclude that YKL-40 does not predict advanced liver fibrosis in children with chronic hepatitis B but usefulness of this biomarker in this group of children needs to be evaluated in larger studies.

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Primary sternoclavicular septic arthritis in patients without predisposing risk factors

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Abstract

Background: Septic arthritis (SA) of the sternoclavicular joint (SCJ) is an uncommon form of arthritis, generally described in patients with predisposing risk factors such as primary or secondary immunosuppressive disorders, systemic or localized infections and central venous catheters. More rarely the infection occurs in patients without these risk factors, thus rendering difficult an early diagnosis.

Material and methods: We report two cases of SA of the SCJ occurred in two patient, without known predisposing risk factors, hospitalized in our Internal Medicine Unit.

Results: The clinical characteristics didn't significantly differ from clinical course of the disease occurring in patients with predisposing risk factors. Imaging techniques were useful to suspect diagnosis, but only fine-needle aspiration biopsy with culture of specimens leaded to identify the pathogen and its antibiotic sensitivity (in both patients *Staphylococcus aureus*). One patient was treated with surgical adequate curettage, drainage and intravenous methicillin, while the other one received only medical treatment with intravenous teicoplanin and ceftazidime. The outcome was uneventful with a complete recovery in both cases.

Conclusions: Even if SA of SCJ is uncommon in subjects without predisposing risk factors, the clinician must have a high index of suspicion to consider this disease in differential diagnosis of arthritis also in previously healthy subjects with negative or unsettling instrumental investigations. In fact, prompt diagnosis is essential to obtain a successful outcome, avoiding the prolongation of the hospitalization and the sequelae of a chronic infection.

Key words: sternoclavicular joint, septic arthritis, *Staphylococcus aureus*, infection.

Introduction

Septic arthritis (SA) of the sternoclavicular joint (SCJ) is an uncommon condition, usually affecting immunocompromised patients with contiguous or distant foci of infection. As a rule, SCJ infection occurs in patients with predisposing risk factors, as intravenous drug use, hemodialysis, infected central venous line, diabetes mellitus and rheumatoid or other inflammatory arthritis [1,2]. Other reported risk factors are alcohol abuse, corticosteroid treatment, cancer, trauma, radiation therapy, chronic liver disease, surgery with median sternotomy [3]. In addition, most common noncontiguous foci of infection are pneumonia, cellulitis, endocarditis, urosepsis, septic pulmonary emboli, spontaneous bacterial peritonitis, epidural abscess, intra-abdominal abscess, gingivitis and disseminated tuberculosis [4,5].

SCJ infection is a potentially life-threatening condition because of tight anatomic connection with the most important chest vascular structures. Very rarely SCJ infection occurs in previously healthy adults. On the clinical ground a high index of suspicion is required in these subjects to estabilish the diagnosis early in the course of the disease [6,7].

In this article, we report two cases of primary septic arthritis of SCJ due to *Staphylococcus aureus* infection occurred in patients without predisposing risk factors.

Case Report 1

A 58-years-old man was admitted to our Unit because of five days-lasting fever (about 38°C) with severe pain and

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serious limitation of movements right shoulder's movements. A similar symptomatology occurred a month before and the symptoms disappeared after paracetamol treatment. The patient had an history of chronic cerebrovasculopathy with two brain transient ischemic attacks, with focal blindness and arterial hypertension. During a previous admission to hospital, laboratory investigations revealed neither metabolic nor haemocoagulative alterations and the patient did not underwent central vein catheter placement.

At the admission in our Unit, clinical examination showed an aching swelling at the SCJ and at the first and at the second sternochondral joints (*Fig. 1*). Significant laboratory data showed erytrocyte sedimentation rate (ESR) 45 mm/Ist h; white blood cell count (WBC) 13000/mm^3 (neutrophils); C-reactive serum protein 28 mg/dl (n.v.: <5), serum fibrinogen 817 mg/dl. Other rheumatic investigations were in the normal range. Chest and right shoulder x-rays were negative. SCJ echography and computerized tomography (CT) did not show pathologic features. Empiric pharmacologic treatment with ceftazidime and non-steroidal anti-inflammatory drugs was started, but only poor symptomatic improvement was observed. Chest magnetic resonance (MR) imaging with focal examination of SCJ and sternochondral joints showed structural alteration of SCJ with the presence of a tissue of unhomogeneous low signal in T1-weighted images, involving the first two sternochondral joints and surrounding retrosternal areas (*Fig. 2*). The absence of predisposing factors for local infection, physical examination, laboratory and MR investigation suggested a neoplasm, so an open biopsy was performed. Surgical exploration of anterior chest surface at SCJ level revealed copious purulent fluid with an inflammatory process defined histologically as osteomyelitis involving the joint and the clavicular bone. Strains of *Stapylococcus aureus* grew within fluid cultures. *In vitro* antibiotic sensitivity was also obtained. The patient was treated with surgical adequate curettage and drainage; intravenous methicillin was administered. A progressive improvement with resolution of symptoms was observed; as a consequence, the patient was discharged two weeks after surgical treatment. Follow-up for 8 months showed complete recovery without sequelae or relapses.

***Figure 1*. The aching swelling at the sternoclavicular joint and at the first and the second sternochondral joints**

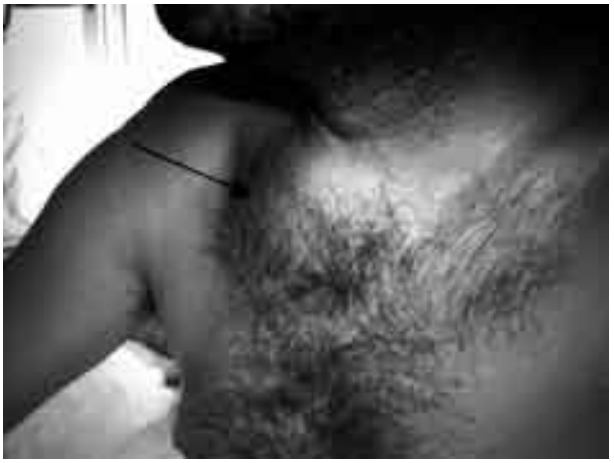
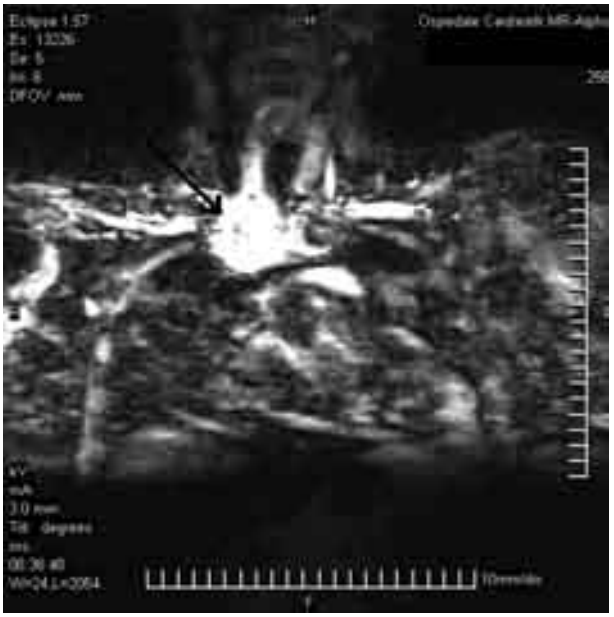


***Figure 2*. Chest magnetic resonance: structural alteration of sternoclavicular joint with the presence of a tissue of unhomogeneous low signal in T1-weighted images, involving the first two sternochondral joints and surrounding retrosternal areas**

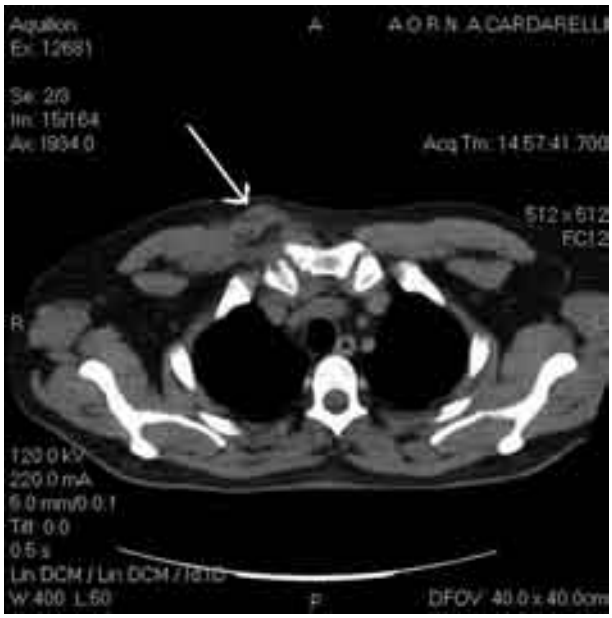


Case Report 2

A previously healty 40 years-old male without predisposing risk factors for SA of SCJ was admitted to the hospital because of a SCJ severe pain that appeared about 20 days before. Few days before admission pain became associated with fever and aching swelling. Administration of ciprofloxacin and non-steroidal anti-inflammatory drugs did not reach significant improvement of symptoms.

At the admission, the patient presented fever (38.2°C) and pain of SCJ and right sternochondral joints. Examination was unremarkable except for erythema, swelling and tenderness over the right SCJ, without limitation of right shoulder movement. Laboratory data showed an increase of ESR (67 mm/Ist h) and WBC (13400/mm^3). C-reactive serum protein was 177.3 mg/L Routine blood chemistries and urinalysis were normal and numerous blood cultures were negative. Only urinalysis yielded growth of *Escherichia coli*. Plain radiographs of the chest with focal examination of right SCJ and clavicle were normal. 99 mTc radionuclide scintigraphy disclosed increased uptake in the SC and costochondral joints; CT scan showed right clavicular cortical and subcortical irregularity near the SCJ with soft tissue swelling, documenting the presence of osteoarthritis (*Fig. 3*). The patient was submitted to MR of sternum and SCJ that showed the presence of solid tissue with structural abnormalities of SCJ. This tissue (7 x 4 x 6 cm) wrapped the sternoclavicular joint and surrounded retrosternal areas. Fine-needle aspiration biopsy (FNAB) was performed and exploration of the right SCJ revealed purulent fluid and an inflammatory process. Cultures from fluid aspi-

***Figure 3*. Chest computerized tomography: right clavicular cortical and subcortical irregularity near the sternoclavicular joint with soft tissue swelling, documenting the presence of osteoarthritis**



rated from the joint yielded growth of strains of *Staphylococcus aureus*. In order to exclude predisposing factors or underlying conditions, determination of serum immunoglobulins, cancer serum markers and sonographic evaluation of the heart and the abdomen were performed and all resulted negative or within the normal limits. Intravenous therapy with teicoplanin and ceftazidime was initiated and continued for a month; the patient progressively improved becoming afebrile in a few days. After discharge (3 months later), normal functioning of the affected joint was observed with CT-scan feature, compatible with a resolving flogistic lesion. Six months follow-up did not show additional sequalae or relapses.

Discussion

In a recent review of 180 cases of SCJ infection (4), pathogens more frequently isolated were *Staphylococcus aureus*, *Pseudomonas aeruginosa*, *Brucella Melitensis*, *Escherichia coli*, Group B *streptococcus*, *Mycobacterium tuberculosis*, *Streptococcus pneumoniae*, *Brucella* spp, *Hemophilus influenzae*, *Salmonella* spp, *Serratia marcescens* and *Candida albicans*. Sthaphylococcus aureus represents the most common cause of bacterial SC septic arthritis in adults (49% in the above-cited series [4]), particulary when the disease originates from infected central venous line. The pathogenesis of SCJ infection is not well understood but it appears to result from either haematogenous or contiguous spread from an infected central venous line [8]. In almost all cases, the disease is unilateral. In selected cases it may result difficult to identify the primary site of infection and also in the two cases we described there is the high probability of primitive joint involvment. The differential diagnosis of a swollen sternoclavicular joint includes primary or metastatic tumor of the SCJ area or non-infectious inflammatory processes, as rheumatoid arthritis, osteoarthritis, rheumatic fever, gout and Tietze's syndrome (TS) [9]. This last one is a rare condition, occurring with recurrent episodes of chondral inflammation causing pain and swelling of rib cartilages. For a correct identification of TS, some clinical features can help the clinician: in fact, TS rarely affects SCJ and presents a favorable outcome; in addition, fever is uncommon and there aren't laboratory and radiological alterations typical of septic arthritis. The SCJ infection usually presents with an insidious onset and the diagnosis may be missed until a complication occurs, as osteomyelitis with joint destruction, sepsis, fistula formation, mediastinitis and superior vena cava syndrome. Also cutaneous, mediastinal or chest wall abscess may develop [10,11]. These complications are very serious: particularly, mediastinitis presents a high percentage of mortality both in immunocompromised patients both in ones with normal immunoresponse [12].

The diagnosis is especially difficult in patients without predisposing risk factors or previous/recent central venous access [13,14]. It is indispensable a high index of suspicion in investigators to estabilish the correct diagnosis early in the course of the disease, because there are frequent false negative observations and non-conclusive results from standard radiology and CT [15].

MR may be more useful even if the exact kind of the lump involving the SCJ is difficult to identify when the course has already been going on for many days or weeks, causing a diffuse inflammation and a fibrotic reaction [16]. In fact, in the majority of cases, SCJ is diagnosed only after exploratory surgery with aspiration and biopsy. Percutaneous blind fine-needle aspiration of the SCJ may prove difficult and often unsuccessful due to the small size of the joint and the presence of intra-articular disc. Echography and CT scan-guided aspiration procedure can realize an easier approach to SCJ and it is recommended in presence of a well identifyable area (hypoechogenic area at ultrasonography, hypodense area at CT). The culture of speciments obtained from fine-needle aspiration biopsy permits to identify the pathogen and its antibiotic sensitivity [17].

SCJ infection is treated both medically and surgically. Antibiotic treatment should be started as soon as possible and continued for at least 4 weeks. Conservative treatment represents the first therapeutic option and medical therapy alone may be successful in a lot of patients. Percutaneous drainage may be carried out with excellent results in selected cases. Surgical exploration is usually performed when the diagnosis is late or uncertain. Open exploration of the joint with drainage and debridement with adequate curettage is the most frequent surgical procedure, while joint resection is indicated only in some selected cases such as extensive bone destruction, chest wall phlegmon or abscess, retro-sternal abscess, mediastinitis or pleural extension [18].

Conclusions

The two cases reported suggest that SA of SCJ should be considered also in patients without known predisposing risk factors and with negative or unsettling instrumental investiga-

tions. Prompt diagnosis is essential to obtain a successful outcome, avoiding the prolongation of the hospitalization and the sequelae of a chronic infection.

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Correlation of peripheral blood monocyte and neutrophil direct counts with plasma inflammatory cytokines and TNF-α soluble receptors in the initial phase of acute pancreatitis

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Abstract

Material: The relationship between direct count of peripheral blood leucocyte populations and plasma concentrations of IL-6, IL-8, sTNFR-55 and sTNFR-75 during five initial days of acute pancreatitis was studied.

Results: Most significant relationship was found for monocytes, which correlated with sTNFR-55 ($R=0.38$, $p<0.05$) and sTNFR-75 ($R=0.41$, $p< 0.05$ and $R=0.55$, $p<0.01$ during 1st and 2nd day, respectively). Later, in days 2, 3 and 4 an interrelation between monocytes and IL-6 ($R= 0.49$ to $R=0.41$, $p <0.01$) was observed. Monocytes also correlated with IL-8 in days 2 and 3 ($R=0.41$, $p<0.05$ and $R=0.43$, $p<0.01$, respectively). Neutrophil count correlated with IL-6 in days 3 and 4 ($R=0.34$, $p<0.05$ and $R=0.56$, $p<0.01$, respectively) and with IL-8 in the 4th day only ($R=0.39$, $p<0.05$). No significant correlations of lymphocyte, eosinophil and basophil direct counts with cytokines and receptors during the initial 5 days of AP were found.

Conclusions: Observed relationships between monocyte direct counts and plasma cytokine levels reflect monocytes involvement in the development of acute pancreatitis.

Key words: acute pancreatitis, inflammatory mediators, white blood cell counts.

Introduction

Acute pancreatitis (AP) involves interstitial activation of pancreatic secretory proteases and damage of the pancreatic tissue [1,2] which process is a source of chemotactic peptides stimulating blood phagocytes [3]. Subsequently, stimulated phagocytes release a set of cytokines, including IL-1, TNFα, IL-8 and IL-6 [4], and mobilize blood neutrophils to accumulate in the inflammed pancreas. In effect, the increase of white blood cell (WBC) count is one of the most pronounced signs, reflecting functional stimulation of neutrophils and mononuclear phagocytes in developing AP [4-6]. Therefore, beside the organ-visualization methods presently used, the WBC count is still considered as a useful sign of inflammatory diseases in patients with abdominal pain [7,8]. WBC count may also indicate a severe disease course with generalized response to inflammation [9,10].

White blood cells include several populations of various cells which play different roles in the immune reaction to infection, tissue injury and tissue necrosis. Mutual interaction between inflammatory cytokines and phagocytes play a critical role in the development of AP. Several recent papers focused on the effects of AP on functional stimulation of blood lymphocytes [11-13] and monocytes [4,11,14]. These studies, employing flow cytometry, focused on specific antigens on the surface of monocytes and lymphocytes [4,11,12,14]. On the other hand, rather few studies refer to changes in direct counts of individual blood inflammatory cells and inflammatory cytokines in the course of AP. The aim of this study was to follow the assumed relationships between direct counts of the WBC populations and plasma concentration of interleukines -6 and -8 (IL-6 and IL-8) and soluble receptors of tumor necrosis factor (sTNFR55 and sTNFR75) in patients with the initial phase of AP.

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Material and methods

The studied group consisted of 56 patients (21 females and 35 males, age 22 to 92 years, mean: 52.8±16.3) admitted to the 2nd Department of General Surgery, Jagiellonian University School of Medicine in Kraków that were diagnosed as AP. All patients in the studied were admitted to the hospital no later than 48 hours after an attack of acute symptoms. Reference group for comparison was composed of 30 healthy subjects (15 females and 15 males, age 36.6±10.3 years) who were subjected to routine health check-up. These persons agreed for use of their test results for research purposes. The study was approved by the Bioethical Committee of the Jagiellonian University and all patients provided an informed consent for their participation.

Diagnosis of AP was based on typical clinical symptoms, such as abdominal pain, fever, nausea and vomiting associated with increased serum amylase activity three times above the upper reference limit (normal range: 0-220 U/L). All patients were subjected to standard physical examination followed by ultrasound examination of the abdomen. In patients diagnosed in respect of developing pancreatic necrosis, the contrast-enhanced abdominal computed tomography (CT) scans were performed at admission and after 48 hours. The severity of AP and concomitant complications were assessed according to the Atlanta Classification. 36 patients were finally diagnosed as mild AP and 20 patients as severe AP. Among 20 patients with severe AP, 7 developed organ failure and 13 local complications such as: necrosis (n=9), pseudocyst (n=3) and abscess (n=1). Seven patients underwent surgical intervention (necrosectomy and lavage) due to infected pancreatic necrosis and a deteriorating clinical condition despite ICU treatment. Five patients died. The etiology of AP included: gallstones in 35, alcohol in 16 and idiopathic origin in 5 patients.

Blood samples for standard laboratory tests and cytokine assays were collected by puncturing cubital vein in to Becton Dickinson Vacutainer tubes (Beckton Dickinson – USA) from each patients on admission and then daily for five consecutive days. Laboratory blood tests assessing patients clinical status were routinely performed at admission and then at 24, 48, 72 and 96 hours respectively. Cytokine concentrations were measured in serum. IL-8 concentration was determined with a solid phase Enzyme Amplified Sensitivity Immunoassay (IL-8 EASIA™ Biosource Europe S.A., Belgium). Concentration of IL-8 was expressed as pg/ml and minimum detectable concentration (MDC) for this test was 0.7 pg/ml. IL-6 concentration expressed as pg/ml was measured using the IL-6 EASIA™ kit (Medgenics Diagnostics S.A., Belgium) and the MDC of this assay was 2.0 pg/ml. Serum sTNFR55 (sTNFRI) and sTNFR75 (sTNFRII) were measured by an ELISA assay with monoclonal and polyclonal anti-sTNFR55 and anti-sTNFR75 antibodies (MEDGENIX COMBO sTNFRI/sTNFRII kit, Biosource Europe S.A., Belgium). Purified sTNFR55 and sTNFR75 were used to construct standard curves. The lower limits of detection for assays were 0.05 ng/ml and 0.02 ng/ml for sTNFR55 and sTNFR75, respectively.

Blood for hematological examination was collected into Vacutainer tubes with $EDTAK_2$ solution. The hematology test profile included: total erythrocyte count and erythrocyte indice values, platelet count, and direct and differential counts of neutrophils, lymphocytes, eosinophils, basophils and monocytes. Blood cell counting was performed with ABX Vega Retic hematological analyzer using 5-diff leucocyte differentiation system. Results are expressed as direct WBC count $\times 10^3/\mu l$. Individual populations of the white blood cells were counted in three separate measuring channels designed for:

1. Total white blood cells counting (WBC – channel);
2. Basophils counting (BASO – channel);
3. Lymphocyte, monocyte, neutrophil and eosinophil counting (LMNE – channel).

The ABX differential WBC counting is automatically performed by the analyzer. Differentiation of the individual cells was based on assessment of cell volume by high frequency alternative current impedance and laser light scattering mode depending on size of the nucleus and number of cytoplasmic granules (MAPS technique). At the first step of leukocyte differential counting the procedure of erythrocyte removal from the sample by addition of 500 µl of VEGALYSE™ solution was performed. The ABX Vega hematological analyzer also differentiates "large lymphocytes", registered as "Atypical Lymphocytes" (ALY) and calculates abnormal immature forms of polymorphonuclear neutrophilic granulocytes marked as "Large Immature Cells" (LIC). However, fractions of ALY and LIC cells were not followed in the present study. Results of differential percentage counts and direct counts are expressed in number of cells $\times 10^3/\mu l$ of the studied blood. The results of the automated blood counting were validated by systematic daily quality control using EightCheck-3WP; ICN blood control samples covering "normal", "high" and "low" ranges obtained from RIQAS USA. Occasional tests of random blood samples from healthy blood donors were also verified by microscopic analysis (to follow performance of the analyzer on fresh blood samples).

All variables had non-normal distribution and were presented as median and range. Mann-Whitney U-test was used to assess differences between groups and correlations between variables were calculated with Spearman coefficient. P level of <0.05 was considered statistically significant. Statistical analysis was performed with Statistica 6.0 software (StatSoft Inc., Tulsa, USA).

Results

As expected, in patients with acute pancreatitis the total WBC count values were significantly elevated in the entire observation period. The highest mean WBC count value was observed in the first day of hospitalization, and then steadily decreased in the following days (*Tab. 1*). A markedly wide scatter range of the WBC counts was observed in all days following admission. This was an apparent effect of increasing diversification of disease severity in individual patients, along with time of initial appearance of the disease symptoms.

The white blood cell populations which counts most significantly correlated with plasma inflammatory cytokine levels were peripheral blood monocytes. Monocyte direct counts in the initial days of AP were significantly higher than that

Table 1. **White blood cell counts and cytokines concentrations in patients with AP in five consecutive days since admission. Data are expressed as median and range. Day 1 is the day of patient admission to hospital**

Parameter studied	Patients group (N=56)					Reference group (N=30)
	Day 1	Day 2	Day 3	Day 4	Day 5	
Total WBC count (10^3/μl)	11.40* 3.20-21.20	9.70* 2.40-22.40	8.70* 3.70-17.60	7.40 3.10-19.50	7.65 3.80-18.60	5.6 3.5-11.7
Neutrophils (10^3/μl)	8.15* 2.75-16.20	6.10 1.12-16.30	5.86 2.13-15.69	4.46 1.85-13.10	5.27 2.21-14.00	5.11 4.23-6.47
Lymphocytes (10^3/μl)	1.10 0.39-3.27	1.15 0.22-2.61	1.25 0.32-3.24	1.11 0.49-2.63	1.12 0.64-2.28	1.7 1.5-3.0
Monocytes (10^3/μl)	0.75* 0.25-1.56	0.54 0.20-1.40	0.57 0.18-2.26	0.52 0.17-2.54	0.58 0.13-2.05	0.40 0.3-0.5
Eosinophils (10^3/μl)	0.11 0.00-0.70	0.13 0.00-0.55	0.18 0.05-0.56	0.18 0.03-0.56	0.18 0.04-0.49	0.20 0.05-0.25
Basophils (10^3/μl)	0.02 0.00-0.12	0.02 0.00-0.12	0.02 0.00-0.15	0.02 0.00-0.13	0.02 0.00-0.19	0.19 0.1-0.27
IL-8 (pg/ml)	47.11 0.50-1043.8	73.16*** 1.25-1204.0	21.07*** 1.14-101.5	18.06*** 1.18-118.9	18.10*** 0.28-86.07	2.25*** 0.07-4.11
IL-6 (pg/ml)	474.79*** 2.0-10000	330.19*** 2.0-4300	171.29*** 2.0-2100	83.50*** 2.0-510.0	78.71*** 2.0-332.0	8.55*** 0-10.0
sTNF55 (ng/ml)	8.93*** 0.68-73.6	7.45** 0.45-40.32	6.96** 0.98-27.28	6.26** 0.88-23.28	6.53** 0.45-21.04	2.19** 0.58-3.26
sTNFR75 (ng/ml)	14.96*** 3.56-64.51	12.26*** 2.66-46.56	11.66* 2.32-32.24	11.49*** 2.19-31.98	11.24*** 2.29-33.40	3.54** 1.67-6.40

P<0.05*; P<0.01**; P<0.001***

Figure 1. **Monocytes direct count values (mean ±SD; 10^3/μl) in patients with AP during initial five days after admission. The means did not differ significantly (p<0.05)**

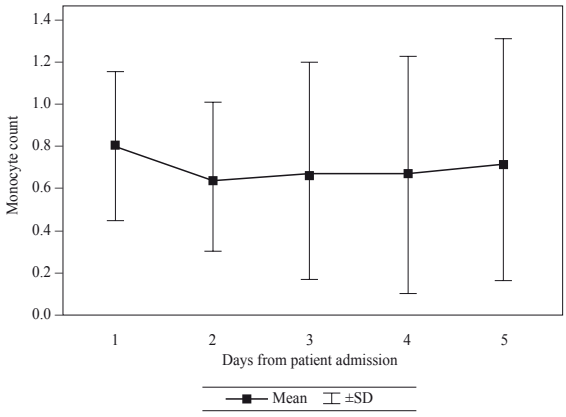
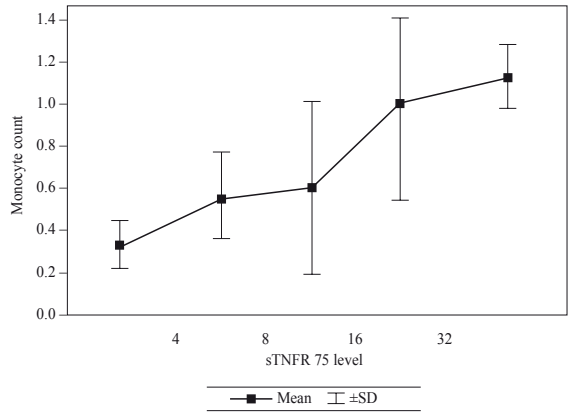


Figure 2.* **Interrelation between monocyte mean values (10^3/μl) and the respective means of sTNFR75 (ng/ml) on the 2nd day after admission. The bars represent SD for the calculated mean values. The same relationship is expressed by regression equation: monocytes (10^3/μl)=0.066+0.184 x $\log_2$ sTNFR75 (ng/ml). The correlation coefficient between monocytes and sTNFR75 (R=0.55; p<0.01)**



in healthy patients (*Tab. 1*). The scatter range of monocyte counts increased in days following admission (*Fig. 1*). There was an evident interrelation between the sTNFR75 and direct monocyte count (*Fig. 2*). On the 2nd day after admission, this relationship in terms of regression manifested as the following equation: monocytes (10^3/μl) =0.066+0.184 x $\log_2$ sTNFR75 (ng/ml). Monocyte direct count significantly correlated with both sTNFR55 and sTNFR75 in the initial two days of hospitalization (*Tab. 2*). Then, in days 2, 3 and 4 there was a significant interrelation between monocytes and IL-6 (*Tab. 2*). This interrelation manifested as a highly significant correlation in days 2 and 3 (R=0.49, p<0.01) and at day 4 (R=0.41, p<0.05) (*Fig. 3*). There was also a moderate correlation with IL-8 at days 2 and 3, respectively (*Tab. 2*, *Fig. 4*).

The total WBC count significantly correlated with IL-6 plasma level in days 2, 3, 4 and 5 after admission; whereas IL-8 correlated with WBC count in days 2 and 3 only (*Tab. 3*). Also sTNFR55 correlated with WBC count in days 3 and 4 only. On the other hand no significant correlation in the entire 5-days observation period was found for WBC count and sTNFR75. Also, none of the cytokines and receptors studied correlated with the WBC count in the first day of observation (*Tab. 3*). Relationship between IL-6 levels and WBC counts in day 3 was expressed as regression equation: WBC (10^3/μl) =6.131+2.111 x $\log_{10}$ IL-6 (pg/ml).

***Table 2*. Correlations of direct monocyte counts with proinflammatory cytokine plasma levels in patients with AP during five consecutive days since admission**

Observation day	Spearman rank correlation coefficient R			
	sTNFR-55	sTNFR-75	IL-6	IL-8
1	0.38 (p=0.007)	0.41 (p=0.005)	0.26 (p=0.055)	0.22 (p=0.121)
2	0.38 (p=0.007)	0.55 (p<0.001)	0.49 (p<0.001)	0.41 (p=0.005)
3	0.25 (p=0.077)	0.20 (p=0.199)	0.49 (p<0.001)	0.43 (p=0.002)
4	0.24 (p=0.095)	0.25 (p=0.078)	0.41 (p=0.003)	0.26 (p=0.057)
5	0.23 (p=0.103)	0.26 (p=0.057)	0.25 (p=0.077)	0.26 (p=0.058)

Observation day 1 is the day of admission

***Table 3*. Correlations of total WBC counts with cytokine plasma and soluble TNF-α receptors levels in patients with AP during five consecutive days since admission**

Observation day	Spearman rank correlation coefficient R			
	sTNFR-55	sTNFR-75	IL-6	IL-8
1	0.18 (p=0.201)	0.16 (p=0.264)	0.13 (p=0.376)	0.12 (p=0.406)
2	0.12 (p=0.410)	0.23 (p=0.100)	0.30 (p=0.033)	0.27 (p=0.049)
3	0.31 (p=0.026)	0.21 (p=0.144)	0.44 (p=0.001)	0.29 (p=0.041)
4	0.34 (p=0.016)	0.20 (p=0.174)	0.39 (p=0.005)	0.16 (p=0.262)
5	0.26 (p=0.058)	0.19 (p=0.189)	0.39 (p=0.005)	0.22 (p=0.120)

Observation day 1 is the day of admission

***Figure 3*. A linear regression for interdependence between monocyte direct count (10^3/μl) and IL-6 levels (pg/ml) in plasma on the 3rd day after admission. The calculated regression is expressed by equation: monocytes (10^3/μl) =0.153+0.401 x $\log_{10}$ IL-6 (pg/ml)**

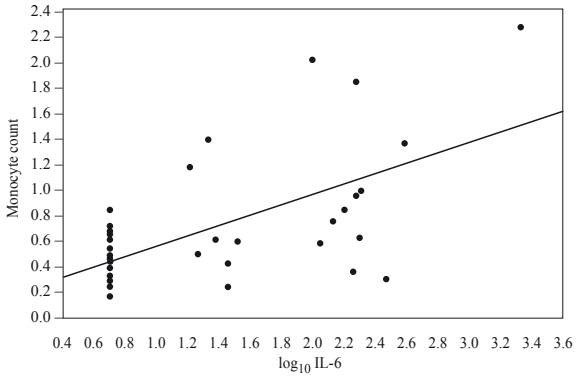
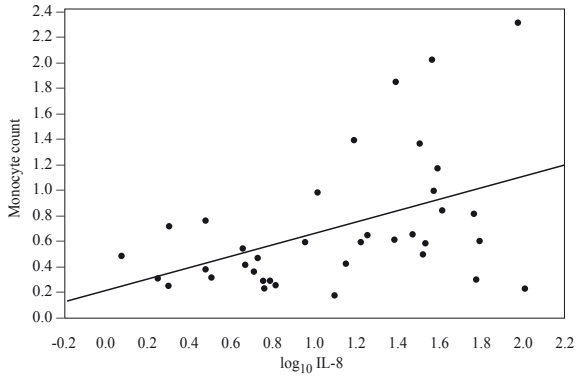


***Figure 4*. Regression for interdependence between monocytes direct count (10^3/μl) and IL-8 (pg/ml) on the 3rd day after admission. The calculated regression is expressed by the equation: monocytes (10^3/μl)=0.219+0.439 x $\log_{10}$ IL-8 (pg/ml)**



Neutrophils which compose a major fraction of the WBC count tended to follow the total WBC count changes. A markedly wide scatter range of the neutrophil direct counts in days following admission were observed. The direct neutrophil counts showed an increasing correlation with IL-6 in days 3 and 4 (R=0.34, p<0.05 and R=0.56, p<0.01, respectively); while IL-8 correlated with direct neutrophil count in the 4th day only (R=0.39, p<0.05).

The lymphocyte counts in AP are present in *Tab. 1*. Though patients with AP had generally lower direct lymphocyte count values than healthy persons, no significant trend of changes in the studied days was observed. Also, no significant changes in eosinophil and basophil direct counts in patients with AP during the initial 5 days after admission were found. Moreover, no significant correlations between lymphocyte, eosinophil and basophil direct counts and cytokines or soluble receptors studied were found.

Discussion

The initial phase of AP development is difficult to study due to inevitable delay between the onset of disease symptoms and presentation of patients in the admission room. Mean delay time of the studied patients was about 24 hours. To study interrelation between inflammatory cytokine levels and individual WBC subsets collection of a group of patients both with mild and severe course of AP was necessary. Therefore, our AP group represents the whole range of symptoms related to developing inflammation, broad enough to disclose existing correlations between the parameters studied. Such correlations may remain unnoticeable in studies concerning mechanisms of inflammation in acute pancreatitis, especially in experimental animals representing uniform severity and course of disease. As expected, results of carried out studies have shown some regularity in relationship between subsets of white blood cell populations, plasma inflammatory cytokines and soluble TNFα receptors.

The most pronounced correlation was found for monocytes which significantly correlated with sTNFR55 and sTNFR75 in the first and second observation days; and then with IL-6 in days 2, 3 and 4. Monocyte count also correlated with IL-8 in days 2 and 3, respectively (*Tab. 2*). The initial correlations with sTNFR55 and sTNFR75 are replaced by correlations with IL-8 and IL-6 that dominated in days 3 and 4. These results are in accordance with results of other studies indicating that blood monocytes play a main role in the enhancement of the inflammatory process and in the development of life threatening systemic inflammatory reaction syndrome (SIRS) [15]. The assay of peripheral blood monocyte direct count provides immediate information on the cytokine output in the developing inflammation.

There is a common notion that an increase in the number of neutrophils, which are directly involved in the pancreas injury process [16-18], reflects severity of developing acute pancreatitis [5,9,19]. In our studies, neutrophils correlated with plasma IL-6 and IL-8 levels in days 3 and 4. This is in accord with data indicating that IL-8 is the cytokine responsible for neutrophil stimulation in the inflammatory site [20,21]. However, in our studies the neutrophils – IL-8 interrelation was less pronounced than that of monocytes and sTNFR55 and IL-6, as well as the neutrophils – IL-6 relationship .The total WBC counts correlate similarly to that of neutrophils with IL-8, IL-6 in days 2 and 3 and with IL-6 in day 4. This is in accord with the phenomenon that in acute inflammation the WBC count increase is mostly due to the increase in neutrophils direct count. Neutrophil count (as well as total WBC count) increase in the later disease phase when IL-6 and IL-8 stimulation dominates. This may reflect the evolution of pancreas injury, which (in humans) usually leads to necrotic lesions at 48 hours after onset of pancreatitis acute symptoms [25]. Neutrophils react secondarily to monocytes and macrophages – releasing the TNFα, IL-1β, IL-6 and IL-8 [3,18] as primary mediators of developing inflammation [4]. On the other hand, neutrophils are the main source of IL-8 in the later phase of the inflammatory process [3].

De la Mano et al. [22] studied the role of circulating inflammatory blood cells in pathogenesis of AP in rats subjected to pancreatic duct obstruction. The authors noticed a significant increase in direct neutrophil and monocyte counts, which peaked at the 6th hour after pancreatic duct obstruction and returned to normal thereafter. The activation of circulating monocytes was reflected by CD11b antigen expression and the TNF output. Lymphocytes, as well as CD4+ and CD8+ cell subsets increased at the earlier stages after pancreatic duct obstruction and progressively decreased thereafter. The TNFα level increased in the 12th h after inducing AP and was paralleled by a spontaneous production of TNFα by monocytes; while no TNFα neither IL-10 were produced by circulating T cells. This study indicates a central role of peripheral blood monocytes in the systemic inflammatory response induced by severe AP caused by pancreatic duct obstruction. Blood monocytes in AP increase interleukin 1 receptor expression and decrease the HLA-DR surface antigen expression both in the initial phase of disease and in the subsequent days of the disease. Onset of AP is accompanied by low levels of anti-inflammatory cytokines IL-4, IL-10 and IL-13. Moreover, the severity of the disease is related to the concentration of IL-6 and IL-10 at the admission time [13,14]. Development of acute pancreatitis depends on contradictory action of stimuli enhancing the inflammatory process, derived from activated neutrophils infiltrating the injured pancreas [23] and on action of anti-inflammatory cytokines attenuating release of IL-8 and TNFα [24]. Usually the activity of the inflammatory process is regulated by a feed-back type relationship between the pro-inflammatory cytokine IL-1β and anti-inflammatory IL-10 [25]. However, a high concentration of neutrophil deriving mediators originating from the inflammatory core in the pancreas may eventually abolish this regulatory mechanism, leading to an uncontrolled general inflammatory reaction. Therefore, monocytes are both the source of pro-inflammatory cytokines and the effectors cells reacting to various inflammatory stimuli [4,11,26].

In acute pancreatitis, a decrease in direct lymphocyte count indicates the development of severe course of disease [11], while increase in neutrophile/lymphocyte ratio above 5.3 predicts pancreatic necrosis. In patients with severe acute pancreatitis with pancreatic necrosis, activated lymphocyte subset CD19+ was significantly higher (46±16.6% versus 26.4±14.6%) then in mild acute pancreatitis [11]. This effect, however, was observed when severe complications of disease were already fully developed.

Results obtained in this study indicating increase in concentration of inflammatory IL-6 and soluble plasma TNF-α receptors in early phase of AP are in accord with recent findings that AP development involves counteraction between primary proinflammatory stimuli with systemic release of anti-inflammatory cytokines [14-16]. This process influences mobilization of blood inflammatory cells and the described relationships may add to symptoms disclosing development of “severe acute pancreatitis”.

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Typeability of AmpFlSTR SGM Plus loci in kidney, liver, spleen and pancreas tissue samples incubated in different environments

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Abstract

Purpose: The aim of the study was assessment of environmental effect on typeability of AmpFlSTR SGM Plus loci: D3S1358, VWA, D16S539, D2S1338, D81179, D21S11, D18S51, D19S433, TH01, FGA and gender marker amelogenin.

Material and methods: Kidney, liver, spleen and pancreas tissue specimens collected during autopsies of five persons aged 20-30 years were incubated at 21°C and 4°C in different environmental conditions. DNA was extracted by organic method from tissue samples collected in 7-day intervals and subsequently typed using AmpFlSTR SGM Plus PCR Amplification Kit and ABI 310.

Results: A fast decrease in typeability rate was seen in all tissue specimens incubated in peat soil and in sand. The specimens immersed in pond water and in salt water were partially typeable in all SGM Plus loci within 126 days. Increased air access and higher temperature during our experiments favoured desiccation and preservation of the material resulting in longer typeability of full SGM Plus.

Conclusion: Decomposed soft tissues are potential material for DNA typing.

Key words: forensic science, tissue decomposition, environmental conditions, DNA typing, AmpFlSTR SGM Plus.

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Introduction

Automated fluorescence analysis of PCR amplified short tandem repeat systems (STRs) by capillary electrophoresis has gained a widespread usage in forensic medical practice [1-4]. An implementation of multiplex PCR kits and fluorescence based DNA detection allow increased sensitivity, unmatched accuracy and high throughput of samples for forensic casework analysis and paternity tests. STR alleles can be rapidly determined using commercially available kits. Typing of STR from highly degraded body is usually based on DNA extracted the from most resistant tissues, e.g., teeth, bones, hairs [5-9]. On the other hand, DNA extraction from soft tissue is easier and less time consuming. Organic extraction of DNA is reported by Takahashi et al. as a useful method in case of decomposed human tissue [10]. AmpFlSTR SGM Plus kit was validated as highly specific and sensitive for human DNA and suitable in typing of degraded samples [11].

The aim of the study was assessment of typeability of AmpFlSTR SGM loci in kidney, liver, spleen and pancreas specimens depending on different environmental conditions.

Material and methods

Kidney, liver, spleen and pancreas specimens were collected according to recommended anatomical body sections (abdomen) during autopsies of five persons aged 20-30 years with post mortem interval (PMI) limited to 14 hours. All the persons died due to hypothermia and early signs of body decomposition were prevented by storage in morgue refrigerator. Tissue specimens of dimensions 2×2×2 cm were incubated at 4°C and 21°C in closed 40 ml containers and at 21°C in closed 40 ml containers filled with sand, garden peat soil, pond water or salt water and at 21°C in open 40 ml containers. Five samples of each tissue were collected in 7-day intervals. DNA was extracted from 5 mg tissue by modified organic pro-

Table 1. **Typeability of AmpFlSTR SGM Plus loci in kidney specimens**

Conditions	D3S1358	VWA	D16S539	D2S1338	XY	D8S1179	D21S11	D18S51	D19S433	TH01	FGA
4ºC, closed container	91/119	84/105	70/98	63/84	105/126	56/84	70/98	56/84	98/119	91/119	63/91
21ºC, closed container	70/98	63/84	49/77	42/63	84/105	35/63	49/77	35/63	77/98	70/98	42/70
21ºC, open container	77/105	70/91	56/84	49/70	91/112	42/70	56/84	42/70	84/105	77/105	49/77
21ºC, salt water in closed container	42/84	35/77	28/70	21/49	56/105	42/77	28/70	14/49	49/98	42/77	21/56
21ºC, pond water in closed container	63/98	49/84	42/84	28/63	77/126	56/91	42/84	21/49	70/98	56/91	35/77
21ºC, sand in closed container	7/21	7/21	1/21	1/21	14/21	7/21	1/21	1/21	14/21	7/21	7/21
21ºC, peat soil in closed container	1/21	1/14	1/14	1/14	1/21	1/21	1/14	1/14	1/21	1/21	1/14

Table 2. **Typeability of AmpFlSTR SGM Plus loci in liver specimens**

Conditions	D3S1358	VWA	D16S539	D2S1338	XY	D8S1179	D21S11	D18S51	D19S433	TH01	FGA
4ºC, closed container	35/70	21/63	21/56	21/42	56/84	28/63	21/56	14/42	35/77	28/63	21/49
21ºC, closed container	21/54	7/35	7/35	1/35	21/56	14/35	7/35	7/35	21/56	14/42	7/35
21ºC, open container	28/49	14/56	14/56	14/49	28/77	21/56	14/56	14/42	28/63	21/49	14/49
21ºC, salt water in closed container	63/126	63/126	56/112	56/98	70/140	56/119	56/119	56/98	70/126	56/126	56/105
21ºC, pond water in closed container	70/126	63/126	56/112	49/98	70/133	56/126	56/119	49/98	70/126	56/126	56/105
21ºC, sand in closed container	14/21	14/21	7/21	7/21	7/21	14/21	7/21	7/21	14/21	7/21	7/21
21ºC, peat soil in closed container	14/21	14/21	14/21	7/14	14/21	14/21	7/21	7/14	14/21	7/21	7/21

cedure. The specimens were placed in 1.5 ml eppendorf tubes and incubated overnight at 56°C for 12 hrs in 0.5 ml digest buffer pH 7.5 (10 mM Tris-HCl, 10 mM EDTA, 50 mM NaCl, 2% SDS) with 0.3 mg/ml proteinase K (Sigma). Centrifuged pellets (Eppendorf, 16500 rpm, 1 min) were discarded and aspirated supernatants were transferred to fresh tubes containing 0.5 ml phenol-chloroform-isoamyl alcohol mix (Sigma). After centrifugation at 16500 rpm for 5 min (Eppendorf), resulting supernatants were transferred to fresh tubes. The latter step was repeated 2-3 times until the phenol phase became transparent. DNA preparations were concentrated and purified using QIAquick PCR Purification Kit (Qiagen). Reference DNA profiles were typed in fresh blood samples collected from respective corpses on autopsy. Recovered DNA was quantitated fluorometrically [12,13]. DNA quality was assessed by ethidium bromide 2% agarose gel electrophoresis. Ten polymorphic systems: D3S1358, VWA, D16S539, D2S1338, D81179, D21S11, D18S51, D19S433, TH01, FGA and gender marker amelogenin included in AmpFlSTR SGM Plus PCR Amplification Kit were amplified following the manufacturer's instructions (Applera, USA) with the exception, that the all reaction reagents were reduced proportionally so that volume of the reaction mix was 10 µl. Electrophoresis and genotyping were performed in ABI 310 Genetic Analyzer (Applera, USA) using Genescan v. 3.11 and Genotyper v2.5 software. As a threshold value a signal of 150 RFU was assumed.

Results

Extracted DNA yield ranged 0-5 ng. AmpFlSTR SGM Plus typeability limits for the tissues under study are presented in *Tab. 1*, *2*, *3*, *4*. First values denote time limits in days, when full AmpFlSTR SGM Plus profiles were typeable in all samples. Second values denote time limits in days, after which no AmpFlSTR SGM Plus profiles were seen for the set of 5 × 5 samples as a whole. In time spans between the two values partial profiles were observed.

***Table 3*. Typeability of AmpFlSTR SGM Plus loci in spleen specimens**

Conditions	D3S1358	vWA	D16S539	D2S1338	XY	D8S1179	D21S11	D18S51	D19S433	TH01	FGA
4ºC, closed container	49/91	42/84	35/70	21/56	63/126	42/91	35/70	21/49	56/98	42/91	28/56
21ºC, closed container	28/70	21/63	14/49	14/42	42/105	21/70	14/49	7/14	35/77	21/70	7/35
21ºC, open container	35/77	28/70	21/56	21/49	49/112	28/77	21/56	14/21	42/84	28/74	14/42
21ºC, salt water in closed container	35/77	28/70	14/49	7/42	70/119	28/70	14/49	7/35	42/84	28/70	14/49
21ºC, pond water in closed container	49/84	42/77	35/70	21/42	56/105	42/84	35/70	21/42	49/98	42/84	21/42
21ºC, sand in closed container	14/21	14/21	7/21	7/21	7/21	14/21	7/21	7/21	14/21	7/21	7/21
21ºC, peat soil in closed container	14/21	14/21	14/21	7/14	14/21	14/21	7/21	7/14	14/21	7/21	7/21

***Table 4*. Typeability of AmpFlSTR SGM Plus loci in pancreas specimens**

Conditions	D3S1358	vWA	D16S539	D2S1338	XY	D8S1179	D21S11	D18S51	D19S433	TH01	FGA
4ºC, closed container	63/105	49/98	42/84	42/77	77/126	56/98	42/84	42/70	63/105	56/98	42/84
21ºC, closed container	42/84	28/77	21/63	21/56	56/105	35/77	21/63	21/49	42/84	35/77	21/63
21ºC, open container	49/91	35/84	28/70	28/63	63/112	42/84	28/70	28/56	49/91	42/84	28/70
21ºC, salt water in closed container	28/70	21/56	14/49	7/35	42/91	28/63	14/49	7/35	28/84	28/63	14/35
21ºC, pond water in closed container	49/84	42/77	35/63	21/56	56/105	42/84	35/70	21/42	49/98	42/84	21/56
21ºC, sand in closed container	7/21	7/21	1/21	1/21	14/21	7/21	1/21	1/21	14/21	7/21	7/21
21ºC, peat soil in closed container	7/21	7/21	1/14	7/14	7/21	7/21	1/14	7/14	7/21	7/21	7/14

Discussion

The authors evaluated typeability of AmpFlSTR SGM Plus kit loci D3S1358, VWA, D16S539, D2S1338, D81179, D21S11, D18S51, D19S433, TH01, FGA and gender marker amelogenin in kidney, liver, spleen and pancreas specimens incubated at 21°C and 4°C in different environmental conditions in the interval of 7 to 140 days. In our experiment fast DNA degradation was observed in tissue material stored in peat soil and in sand, which may result from humus acid content, microbial action or acid pH [14-16]. On the other hand, increased air access and higher temperature during our experiments favoured desiccation and preservation of the material [17,18] resulting in the prolonged typeability of full SGM Plus profiles in specimens stored at 21ºC in open containers when compared to that in closed containers. Incubation of kidney specimens in closed containers at 21ºC resulted in partial DNA degradation after 35 days. Liver specimens immersed in pond water and in salt water were typeable in all SGM Plus loci within 49 days. Hoff-Olsen et al. reported typing of seven STRs in the liver of decomposed corpse recovered from a lake after 90 days and from a river after 17 days [19]. In our experiment storage of liver specimens in closed containers at 21ºC resulted in partial SGM Plus profiles after 7 days except D2S1338 which was typed in all samples only on first day of the experiment. Hoff-Olsen et al. typed three STRs in liver samples collected from a decomposed body recovered from a house after 27 days [19]. Piasecka-Pazik et al. [20] reported lack of longer alleles of AmpFlSTR Identifiler loci typed in liver samples after 7-day incubation. Spleen specimens incubated in different conditions of our experiment were partially typeable within 119 days. Pancreas specimens incubated in different conditions were partially typed within 126 days. The experimental model assumed in our study does not reflect a typical process of decomposition, but can be used for identification of fragmentary tissue samples from victims of airplane, train and car accidents recovered from water, and sand or soil [21,22].

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Results of small intestinal bacterial overgrowth testing in irritable bowel syndrome patients: clinical profiles and effects of antibiotic trial

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Abstract

Purpose: Small intestinal bacterial overgrowth (SIBO) may coexist with irritable bowel syndrome (IBS) and eradication therapy has been reported as effective in reducing IBS symptoms. Aims of this study were to: 1. Assess the clinical profiles of IBS patients, who underwent breath testing with a glucose substrate – glucose breath test (GBT); 2. Evaluate hydrogen and methane parameters in various IBS groups; 3. Assess the role of inhibition of gastric acid in contributing to SIBO; 4. Investigate efficacy and safety of non-absorbable antibiotic rifamixin for eradication and symptom relief.

Methods: 204 IBS patients met the ROME II criteria for IBS (170F & 34M; mean age 46.4; range 18-88) and underwent GBT. 8 of these patients with positive GBT were treated with rifaximin 200 mg, 4 times a day for 1 month and symptom assessments and GBT were repeated.

Results: 93 (46%) had a positive GBT. 68 (73%) of these 93 IBS-diarrhea dominant (IBS-D), 12 (13%) were constipation dominant (IBS-C) and 13 (14%) IBS with alternating bowel pattern. 48% of SIBO positive patients were receiving PPI therapy compared to 40% of IBS patients with negative GBT. 61 (66%) produced only hydrogen, 27 (29%) methane only, and 5 (5%) both-hydrogen and methane. There were more methane producers in IBS-C then IBS-D group (58% vs 28%) while IBS-D had more hydrogen formers (71% vs 42%).

8 patients with SIBO (7F & 1M; mean age 55, range 31-85) received rifamixin 800 mg/day. Repeat GBT was normal in 6 (75%), 1 patient (12.5%) normalized according to hydrogen criteria but methane remained positive. Symptoms score improved in 7 (87.5%) patients and no adverse events were noted.

Conclusions: 1. SIBO was present in nearly half of this large cohort of IBS patients based on the results of GBT; 2. Chronic PPI use was not associated with SIBO; 3. Methane formers on the GBT are more likely to be constipated; 4. Rifaximin is effective in treatment of SIBO in IBS and controlled trials are warranted.

Key words: irritable bowel syndrome, glucose breath testing, rifaximin, proton pump inhibitors.

Introduction

Small intestinal bacterial overgrowth (SIBO) is a condition where there are excessive numbers of bacteria (>10^5 bacteria/ml in jejunal fluid), mainly colonic type species present in the lumen of the small bowel. It may be caused by variety of reasons, such as: I) Weak gastrointestinal motility with ineffective clearance permitting colonic flora to migrate proximally, II) Surgery, such as resection of the ileocecal valve or creation of a blind loop during gastric by-pass predisposes to migration, retention and colonization of the bacteria in the proximal small bowel, and III) Conditions which are less hostile for the growth of bacteria in the small intestine, e.g., less acidic from the use of proton pump inhibitors (PPI). This overgrowth of microbiota in the small intestine and accompanying malabsorption can cause symptoms such as, diarrhea or constipation, excessive gas production, bloating, and abdominal pain [1,2].

Noninvasive breath tests are widely used to diagnose SIBO. These tests analyze production of gases, which are generated by enteric bacteria and are exhaled after ingestion of a fermentable carbohydrate like glucose or lactulose. Most common detectable gases are hydrogen and methane, which are indicators of bacterial metabolism in gut. Cultures of the jejunal fluid demonstrating >10^5 organisms/ml is the current gold standard test for

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diagnosis of SIBO, however, it is usually not practical to carry out in clinical practice. Usually the first step in the treatment of SIBO is to suppress bacterial colonization in the small bowel with a course of broad-spectrum antibiotics. The management may also include therapy for promoting small intestinal motility using prokinetics and/or maintaining proper intestinal flora with probiotics [3].

Rifamixin is a rifamycin derivative with a broad antibacterial spectrum. Less than 0.5% of the oral dose is absorbed and therefore has very low risk of systemic toxicity, adverse events, and drug interactions [4].

Aims of this study were to: 1. Assess the clinical profiles of IBS patients, who underwent breath testing with a glucose substrate – the glucose breath test (GBT); 2. Correlate hydrogen and methane production with various sub-groups of IBS; 3. Assess the role of inhibition of gastric acid in contributing to SIBO; and 4. Investigate efficacy and safety of rifamixin in eradicating SIBO and providing symptom relief from IBS.

Materials and methods

The study was completed in two phases:

Phase I

Subjects: 204 subjects (170 females, 34 males) who met the ROME II criteria for IBS were included in the study. All the subjects were being followed in the functional bowel diseases clinic supervised by one of the authors (RWM). The following procedures were performed in all patients

Clinical data: Demographic data and information regarding the dominant bowel movement pattern with IBS was recorded. Subjects were subsequently sub grouped based on the predominant bowel movement pattern into, IBS-constipation dominant (IBS-C), IBS-diarrhea dominant (IBS-D), and IBS with alternating bowel habits and predominance of gas and bloating (IBS-other). In addition subjects were also asked to comment on their PPI medication usage.

Breath testing: Hydrogen and methane excretion were measured using a glucose breath test (GBT). Patients were asked to have a low carbohydrate dinner on the day before and then fast for at least 12 hours before testing. Use of antimicrobial agents within the previous 4 weeks, pregnancy, and breast-feeding were exclusion criteria. Smoking and heavy physical exertion were not allowed 1 hour prior to the test. Just before sampling, patients used a mouthwash with 40 mL of 1% chlorhexidine. Two samples were obtained at baseline and then at 30, 45, 60, 75 and 90 minutes following ingestion of 50 g of glucose in 150 cc water (iso-osmotic solution). The results of the glucose breath test were analyzed and expressed as mean (+/- SD). For every patient the baseline and peak values for hydrogen and methane were recorded and their total excretion of either hydrogen or methane was calculated as an area under the time-concentration curve.

End expiratory breath samples were taken to ensure alveolar gas sampling using a commercial device (Gasampler Quintron, Milwaukee, WI), which allows the first 500 mL of dead space air to be separated from remaining alveolar air collected in a gas-tight bag. Samples were analyzed immediately after collection. To detect hydrogen and methane in air samples, a gas chromatograph was used (Model DP, Quintron Instruments, Milwaukee, WI). The concentration of hydrogen and methane in the breath was expressed in parts per million (p.p.m.).

The GBT was considered as positive for SIBO if: 1) There was a hydrogen and/or methane peak >20 ppm when the baseline was <10 ppm; or 2) In cases where the patient started with baseline of >10 ppm a further increase of >12 ppm indicated a positive result.

Table 1. **Summary of glucose breath test results**

	IBS - total	IBS-D	IBS-C	IBS other	Receiving PPI
GBT positive	93 (45.6%)	68 (73.1%)	12 (12.9%)	13 (14.0%)	45 (48.4%)
GBT negative	111 (54.4%)	81 (73.0%)	18 (16.2%)	12 (10.8%)	44 (39.6%)

Table 2. **Specific gas production and IBS subgroups in SIBO patients**

	H_2	CH_4	Both Gases	Total
IBS-D	48 (70.6%)	19 (27.9%)	1 (1.5%)	68 (100%)
IBS-C	5 (41.7%)	7 (58.3%)	0 (0.0%)	12 (100%)
IBS-other	8 (61.5%)	1 (7.7%)	4 (30.8%)	13 (100%)
Total	61 (65.6%)	27 (29.0%)	5 (5.4%)	93 (100%)

Phase II

The following procedures were done in phase II of the trial:

Subjects: 8 subjects (7 females, 1 male) from the initial cohort of subjects who had a positive GBT were selected for this phase of the study after they agreed to take rifaximin to treat SIBO.

Antibiotic Treatment Protocol: Subjects were asked to take 200 mg of rifaximin four times a day for 4 weeks and were instructed to report any adverse events during the treatment.

Symptom Score: Symptom assessment, and an overall score were obtained by analyzing frequency of stools, abdominal pain, bloating and gas pre and post therapy with rifaximin.

Breath Test: GBT was performed using the above protocol after the completion of the antibiotic treatment. A negative GBT signified the eradication of SIBO.

Results

Mean age of the subjects was 46 years, (range 18-88). 93 (46%) had a positive GBT. 68 (73%) were IBS-D, 12 (13%) had IBS-C and 13 (14%) IBS-other. 48% of IBS patients who had SIBO were receiving PPI therapy compared to 40% without SIBO negative GBT (*Tab. 1*).

With regards to the specific gas production: 61 (66%) produced hydrogen, 27 (29%) methane, and 5 (5%) both-hydrogen and methane (*Tab. 2*). There were more methane producers in

Figure 1. **Hydrogen concentration in ppm (X-axis) detected in the breath test before (dashed line) and after (solid line) treatment with rifaximin. Y-axis represents time in minutes**

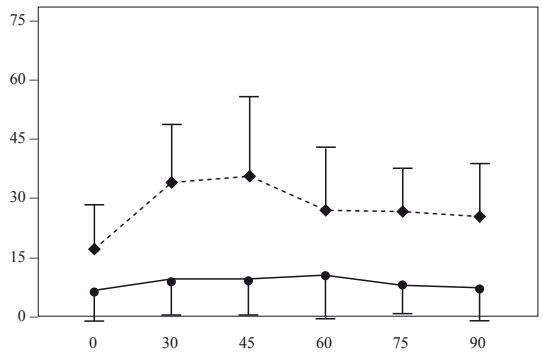
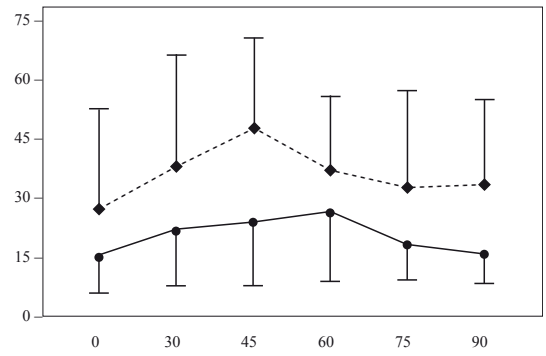


Figure 2. **Methane concentration in ppm (X-axis) detected in the breath test before (dashed line) and after (solid line) treatment with rifaximin. Y-axis represents time in minutes**



IBS-C then IBS-D group (58% vs 28%) and more hydrogen formers in IBS-D (71% vs 42%).

In the 8 subjects who were treated with rifaximin for SIBO, the mean age was 55 (range 31-85). Repeat GBT was normal in 6 (75%), 1 patient (12.5%) had a normal GBT according to hydrogen criteria but methane remained positive (*Fig. 1* and *2*). Improvement in overall symptom score was observed in 7 (87.5%) patients: 4 subjects had >75% improvement, 2 – 50-75%; 1 – 25-50% and 1 had no symptom improvement (<25% improvement). No adverse events were noted in any of the subjects.

Discussion

IBS is the most common functional bowel disease that affects up to 20% of the population. The criteria for the diagnosis is a symptom-based set of observations that are required because no consistent biological marker or unifying framework is available to explain the different symptoms and findings of IBS [5]. In addition to the degrees of constipation, diarrhea, or pain approximately 90% of the IBS patients also have a significant bloating component to their presentation [5]. This is usually associated with the perception of abdominal distention and recent data suggest that abnormalities in gas production and its transit through the small bowel can be present and could explain these symptoms. Whether SIBO contributes to some of the distressing symptoms such as gas and bloating in IBS remains an area of active investigation. In a study of 202 patients meeting Rome II criteria for IBS by Pimentel et al. abnormal breath test results suggesting SIBO was found in 78% [6]. In another study by the same group the incidence of an abnormal lactulose breath test was 84% vs 20% in the control of subjects who did not meet the Rome II criteria [7]. In a randomized placebo controlled trial using neomycin, there was a significant improvement in IBS symptoms in patients receiving neomycin compared to placebo [8]. Symptom improvement after antibiotics was more consistent in the group where SIBO was eradicated.

Up to 75% of their IBS patients had an abnormal lactulose test consistent with the presence of the SIBO [9]. The interpretation of the lactulose breath test is affected by the individual oral-cecal transit times. The lactulose breath test has the underling principle that lactulose is a non-absorbed sugar that would arrive in the cecum and right colon and be metabolize into hydrogen and in some cases methane depending on the specific bacteria species present. Separating SIBO in the distal ileum from cecal arrival time is usually not possible by the lactulose breath testing unless there is an identification of a subsequent second peak or second rise in hydrogen. When lactulose goes into the cecum and right colon there would be a second and substantially higher arise in hydrogen production, so-called second peak phenomenon. Unless the double-peak criteria is included, it is very difficult to interpret a lactulose breath test when the rise in breath hydrogen or methane begins 60 or later minutes after lactulose ingestion as it could also represent rapid small bowel transit [10].

Glucose on the other hand will identify bacterial overgrowth beginning in the proximal small bowel and extending to the proximal ileum. A positive result is a rise in the breath hydrogen above 20 ppm in the first 90 minutes. There are no false positive tests as could occur with the lactulose breath test. We therefore chose the glucose breath test technique to assess the incidence of SIBO in this large group of IBS patients who met the ROME II criteria. 46% of the patients in our cohort had a positive breath test for SIBO as opposed to 80 or 90% being reported by investigations relying on the lactulose breath test methodology. Nevertheless, the figure of 46% still indicates that a substantial number of patients previously regarded as pedestrian IBS patients have SIBO which could contribute to some of the symptoms of their condition [11].

We hypothesized that a contributing factor predisposing to SIBO is the loss of gastric acid in the stomach that usually provides a hostile environment in the small bowel for the growth of bacteria [12]. The use of PPI's could be one reason for low gastric acidity and in turn causing SIBO [12]. We therefore evaluated the risk of SIBO in IBS patients using PPI. Though, the rate of PPI use was slightly more in the IBS with SIBO, no definite correlation could be found. Other hypotheses that have been proposed include the previous history of gastrointestinal infection which would have initiated an imbalance of bacteria flora, or the migration of colonic flora into the small bowel because of impaired defense mechanisms in the small bowel, related to damage to the small bowel migrating motor complex apparatus (the housekeeper of the gut) induced by the gastrointestinal infection or hitherto present in IBS patients.

The focus of the second phase of our trial was to assess the efficacy of rifaximin therapy in reducing symptoms of

IBS and normalizing the GBT. Rifamixin was chosen for the study as it had a low probability for side effects with long-term use, given that it is a non-absorbable antibiotic. A dose finding study evaluating different doses of rifamixin showed that a higher dose (1 200 mg/day) was more efficacious in eradicating bacteria than the dose of 600 mg/day for 7 days. In our study we chose a dose of 800 mg/day for four weeks [13]. We hypothesized that the longer treatment period would result in greater SIBO eradication and a more sustained symptom free interval following treatment. The majority of the patients we summarized had been colonized for many months or even years based on the history of the presentation. We therefore felt that a higher eradication rate would be achieved by more sustained dosing. Indeed we achieved an eradication rate of 85% after four weeks, which is comparable to the response using a higher dose of rifaximin (1 200 mg/day) for 7 days. Whether another dosing cycle or a higher dose of rifaximin should be given to those not eradicated, the duration of the symptom free interval after antibiotic treatment or whether probiotic therapy should be added for maintenance of the SIBO free state are questions that remain to be answered.

Another interesting aspect of our study was the quantitation of hydrogen and methane proportions in the subsets of IBS patients. In normal subjects the elimination of hydrogen produced by bacteria fermentation in the colon depends on methanogenic and sulfate reducing bacteria that convert hydrogen to methane and hydrogen sulfide. These organisms are highly competitive so the stool of an individual contains high concentrations of only one of these two organisms. A study by Pimentel et al. [14] documented the excretion of methane alone in constipated IBS patients. Our data confirms that there is a predominance of methane production in constipated individuals. In IBS-D patient's hydrogen production is greater than methane. Methane production is also higher in patients in whom gas and bloating symptoms are dominant. Since some patients had a positive GBT only by the methane criteria, it is very important to measure both hydrogen and methane when a breath test for SIBO is performed.

One of the limitations of this study is diagnosing SIBO on the basis of a positive GBT and not performing the gold standard bacterial culture of the jejunal fluid as the latter technique is not performed at most institutions.

We conclude that a substantial number of patients with IBS have SIBO by using the GBT, which we believe is a more specific test than the lactulose breath test [15]. Hydrogen and methane are the predominant gases associated with IBS-D and IBS-C respectively. Rifaximin is effective in treating SIBO and improvement of IBS symptoms correlated with SIBO eradication. Optimal dosing regimens for rifaximin to eradicated SIBO and the-long term follow up of these IBS patients are subjects of further studies.

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The effect of granulocyte colony stimulating factor on neutrophil functions in children with neutropenia after chemotherapy in the course of neoplasma

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Abstract

Purpose: Granulocyte-colony stimulating factor stimulates proliferation and maturation of granulocyte precursor cells. The influence of this hematopoietic factor on phagocytic function of granulocytes was performed in *in vitro* experiments. The aim was to find, whether G-CSF applicated to children with neutropenia after chemotherapy influences phagocytic functions of neutrophils and whether evaluated parameters depend on a time of G-CSF injection?

Material and methods: The investigation was conducted on a group of 26 children with cancer, treated with granulocyte-colony stimulating factor in the cause of neutropenia after chemotherapy. The control group included 29 healthy children. The blood was taken before the stimulator injection and after 2 and 5 granulocyte-colony stimulating factor injections. The percentage of phagocyting cells and the phagocytic index of granulocytes were determined in heparinized whole blood samples. Oxygen metabolism was evaluated in the absence and presence endotoxin by nitroblue tetrazolium reduction.

Results: It was found that granulocyte-colony stimulating factor activates phagocytic functions of neutrophils by normalizing low values of phagocytic index and number of granulocytes, reducing dye nitroblue tetrazolium reduction and increasing the number of phagocytic cells.

Conclusion: Based on obtained results we can conclude that granulocyte-colony stimulating factor apart from granulopoiesis stimulation can also increase phagocytic and oxidative capacity of granulocytes after chemotherapy.

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Key words: granulocyte-colony stimulating factor, neutropenia, cancer.

Introduction

Granulocyte-colony stimulating factor (G-CSF) is a cytokine belonging to a group of haematopoietic growth factors. It takes part in haematopoiesis regulation by stimulation proliferation and maturation of granulocyte precursor cells [1]. Scientific coverage from last years reports that this cytokine apart from haematopoiesis regulation can also influence the activity of mature granulocytes. Majority of published investigations concern the evaluation of neutrophil functions *in vitro* conducted on isolated and stimulated cells by G-CSF where it was found that it activates phagocytosis and increases production of superoxide anions in mature neutrophilic granulocytes [2-7]. Experiments conducted *in vivo* on animal models indicate the significant G-CSF effect on bactericidal abilities, oxygen metabolism and phagocytosis of mature neutrophils [6]. There are several data [7,8] on neutrophil functions after G-CSF stimulation in various diseases in adults. Therefore the aim of our study was to find out whether G-CSF applicated to children with neutropenia after chemotherapy in the course of cancer influences phagocytic functions of neutrophils, as evaluated by:

– percentage of phagocyting cells;
– phagocytic index;
– oxygen metabolism measured with nitroblue tetrazolium (NBT) reduction in stimulated and non-stimulated cells?

Materials and methods

The investigation was conducted on a group of 26 children (13 boys and 13 girls) in the age of 1.5-17 years (average age

was 8.5 years) with cancer disease (acute lymphoblastic leukaemia – 9, lymphomas – 7, solid tumors – 10). One week after chemotherapy, children displayed neutropenia (absolute number of granulocytes – 328/µl±). In order to stimulate granulopoiesis, G-CSF (Neupogen) was given to children subcutaneously at a dose of 4.4 to 12.9 µg/kg/day (average 6.66 µg/kg/day) for the whole period of experiments, lasting from 5 to 7 days.

The control group consisted of 29 healthy, not treated children (15 boys and 14 girls) in the age of 5-17 years (average age – 12.5 year). An interview was a criteria qualifying to the group. They included lack of immunological disorders and absence of clinical infection for the period of 2 months and during the tests.

The blood was collected with heparin from elbow vein after overnight fasting in the morning before the G-CSF injection (time 0) and after 2 and 5th, stimulator injection (3rd and 6th day). In the control group the blood was also taken with heparin from elbow vein on an empty stomach in the morning. The tests were repeated 2-5 times in every healthy child.

Percentage of phagocyting cells

The investigation was done on whole blood collected with heparin. After the blood was centrifuged, volume 100 µl of leukocyte layer was taken and transfered to a test-tube with 10 µl of latex beads (by Sigma-Aldrich). The mixture was incubated for 30 minutes in 37°C and 30 minutes in a room temperature. From the mixture, smears were made on a microscopic slides and stained with Giemsa. Under the light microscope phagocyted latex beads granule cells were counted in 100 evaluated granulocytes.

Phagocytic index

Phagocytic index was calculated as the number of latex beads absorbed by total phagocyting cells divided by the number of phagocyting cells.

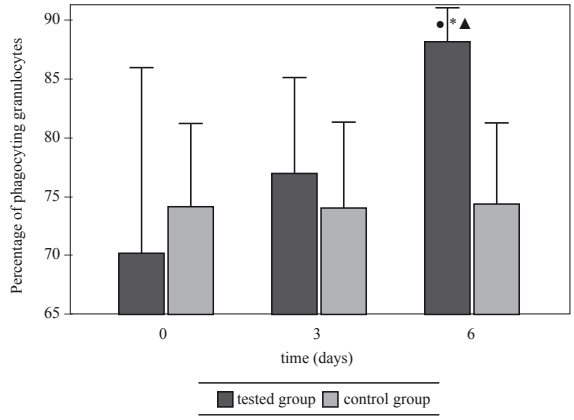
Test of NBT reduction [9]

Test of NBT reduction was made with the whole heparinised blood. For resting NBT reduction test, 0.1 ml blood sample was mixed with 0.05 ml phosphate buffer 0.15 M (pH=7.2) and 0.05 ml of NBT mixture (2 mg Nitro Blue Tetrazole Chlorine in 1 ml 0.9% NaCl). For stimulation test 0.1 ml lipopolisaccharide endotoxin B of E. Coli 055:B5 (0.05 mg endotoxin in 0.01 ml 0.9% NaCl) was added. The mixture was incubated for 15 minutes at 37°C in a water bath. Subsequently microscopic-slide smears were made and stained with Pappenheim. Fraction of granulocytes that contained blue formazan crystals was assessed.

Statistics calculations

Kolmogorov-Smirnov test was used to check fulfil of the data to gaussian distribution pattern ($p \geq 0.1$ was considered to be indicative for gaussian distribution). Differences between 2 experimental groups were tested by unpaired Student t-test. Results obtained after treatment of the same person were compared by the paired Student t-test. $P \leq 0.05$ was considered as indicator of significant difference. Standard deviations (SD) are indicated on the figures.

Figure 1. **The percentage of phagocyting granulocytes after G-CSF injection**



* – the difference statistical significant to control group; ● – the difference statistical significant to 0 day; ▲ – the difference statistical significant to 3 day; statistical significance when $p \leq 0.05$

The investigation was approved by the Bioethical Committee of Medical University of Białystok and written informed consent was obtained from parents of each tested subject's (R-I-003/84/2002).

Results

Percentage of phagocyting cells

Changes in the percentage of phagocyting cells in patients after receiving G-CSF are presented on *Fig. 1*. Before the G-CSF injection the percentage of phagocyting cells in the whole patients group (70.4%) was similar to the healthy group (73.7%). After 2 injections no significant rise of phagocytic capacity was found. However, after 5 injections of G-CSF (day 6) the percentage of phagocyting cells increased to 87.5%. The results obtained on day 6 were also significantly higher than those obtained in the day 3.

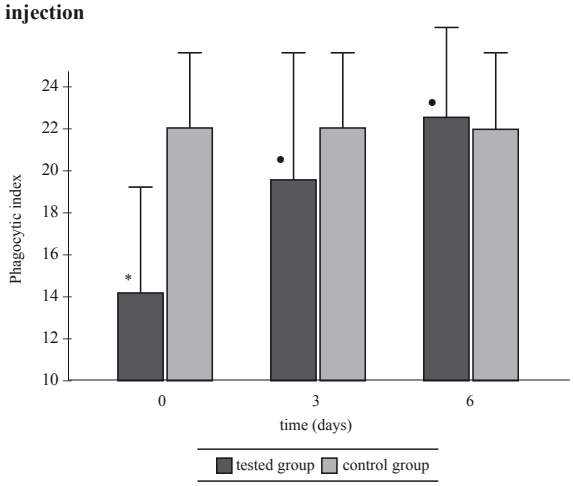
Phagocytic index

Fig. 2 represents the index of phagocytosis. On the day 0 the phagocytic index was lower in the patients (14.2%) than in the control group (22.1%) After G-CSF treatment of patients group the values of index significantly increased reaching values 19% and 22% on the 3rd and 6th treatment day. In those days the differences between tested and control group were statistically insignificant.

NBT non-stimulated test

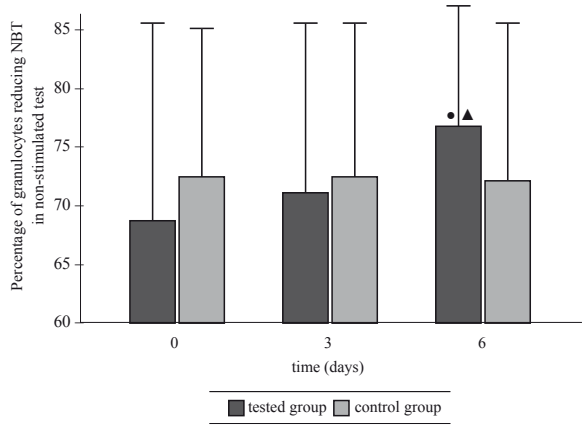
Changes in the percentage of cells reducing NBT are presented in *Fig. 3*. In the patients group percentages of cells reducing NBT before the G-CSF injection (day 0) (68.7%) and after 2, G-CSF injections (71.0%) were similar as in control group (73.0%). On day 6th significant increase in of this parameter to 76.7% took place. This increase was also significant in comparison to day 3.

***Figure 2*. The phagocytic index of granulocytes after G-CSF injection**



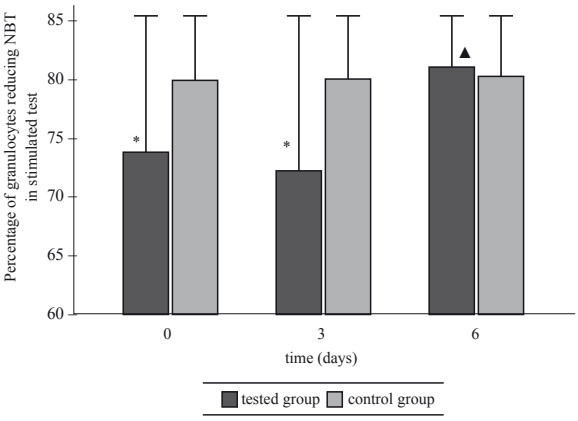
* – The difference statistical significant to control group; ● – the difference statistical significant to 0 day; statistical significance when p≤0.05

***Figure 3*. The percentage of granulocytes reducing NBT in non-stimulated test after G-CSF injection**



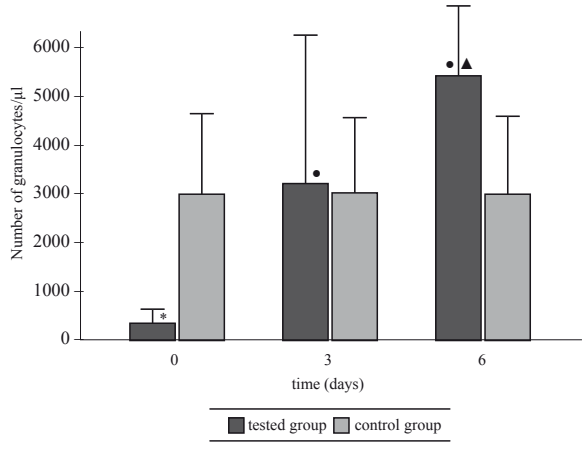
● – the difference statistical significant to 0 day; ▲ – the difference statistical significant to 3 day; statistical significance when p≤0.05

***Figure 4*. The percentage of granulocytes reducing NBT in stimulated test after G-CSF injection**



* – the difference statistical significant to control group; ▲ – the difference statistical significant to 3 day; statistical significance when p≤0.05

***Figure 5*. The number of granulocytes after G-CSF injection**



* – the difference statistical significant to control group; ● – the difference statistical significant to 0 day; ▲ – the difference statistical significant to 3 day; statistical significance when p≤0.05

NBT stimulated test

The results received in NBT lipopolisaccharide stimulated test are presented on *Fig. 4*. In day 0 and 3 percentage of cells reducing NBT in patients group (73.8% and 72.7%) was significantly lower than in the control group (80.2%). On day 6 percentage of NBT reducing cells in patients group was similar to the values of the control group (80.8%) and was statistically higher in comparison with values from day 3 (*Fig. 4*).

Number of granulocytes

Changes in the number of granulocytes are presented in *Fig. 5*. Before the G-CSF injection number of granulocytes (328/µl) was significantly lower than in the control group (1394/µl). After the G-CSF treatment of patients group number of granulocytes significantly increased (3284/µl day 3 and 5430/µl day 6) in comparison to the day 0. The results obtained on day 6 were also significantly higher than those obtained in the day 3.

Discussion

There are only a few works testing influence of G-CSF on the function of mature neutrophils *in vivo* in humans conducted on healthy adult volunteers. Turzański et al. [10] found no significant increase of phagocytic index. The authors claimed that negative finding was due to too small number of participants of the experiment (12 – people). Hoglund et al. [7] conducted a similar investigations on 4 groups of healthy adult volunteers (6 people in each group). Every one received G-CSF at a dose of 3-10 µg/kg/d for 6 days. The authors evaluated phagocytic function of neutrophils before administration of G-CSF and on

the day 2, 5 after injection. They report increase in phagocytic activity of granulocytes upon this treatment. Thus our findings remain in accord with those date.

Ishikawa et al. [11] tested the G-CSF influence on functions of mature neutrophils in adult patients with neutropenia in the course of septicaemia. G-CSF at a dose of 2 µg/kg/d for 5 consecutive days, caused significant rise of phagocytic activity of patients neutrophils.

Gerber et al. [12] using flow cytometry evaluated the influence of Neupogen on neutrophils functions in adult patients without neutropenia, requiring intensive supervision and surgical treatment. They found significant increase of phagocytic activity during G-CSF injection but without the increase percentage of phagocyting cells. On the other hand our data (*Fig. 1*) demonstrate rise of both parameters after G-CSF treatment. This discrepancy may result from different experimental groups and different assay procedures employed in these studies [12].

Obtained results of our investigation and the remarks of above-mentioned authors prove that G-CSF *in vivo* influences the phagocytic activity of mature granulocytes. It corresponds with the results of the investigations conducted *in vitro* on humans [2,9,11,12] and *in vivo* on animals [6] .

The NBT tests helped to assess decreased oxygenic metabolism in patient-children granulocytes in tested group before the treatment (day 0) in comparison to the control group (*Fig. 3*,*4*). Our date prove that G-CSF injection caused improvement of chemotherapy impaired oxygen metabolism in granulocytes. Increased percentage of granulocytes reducing NBT in lipopolisaccharyde stimulation test means that G-CSF increases reduction potential of the cell which became capable to react to additional stimuli.

Ahmad et al. [13] proved the effect of recombinant human G-CSF *in vivo* on phagocytic function and oxidative burst activity in neonates with septic neutropenia. These parameters increased after G-CSF injection but did not achieve matching control values, despite of that absolute neutrophil count increased of a 2 to 12-fold [13]. These results suggest that septic neonates may remain susceptible to infection due to deficient neutrophil-killing capacity, even thought their absolute neutrophil count returns to normal ranges. On the contrary our data indicate, that in chemotherapy depressed granulocytes. G-CSF is efficient factor restoring proper granulocyte functions (*Fig. 2*,*3*,*4*).

Cancer patients with post chemotherapy leukopenia had decreased levels of cytokines and their receptors in neutrophils [16]. Patients after G-CSF therapy increased density cytokine receptors to values seen in healthy patients [16]. It may explain the effect of that cytokine on neutrophil functions and reduction of evaluated parameters before G-CSF injection in our investigation.

Conclusions

1. G-CSF administrated to children with neutropenia after chemotherapy of the cancer activates the phagocytic functions of neutrophils:

– normalizing the low values of phagocytic index and the percentage of granulocytes reducing NBT in the absence and presence endotoxin;

– increasing normal values of phagocytosis granulocyte percentage.

2. The improvement of granulocyte function is time dependent.

3. G-CSF except of granulopoiesis stimulation has also the ability to activate the non-specific immunity *in vivo* in humans.

Our study complement finding, with data concerning cancer children patients. We demonstrated that children granulocytes loose their functional capacites during chemotherapy. Our data also indicate that like in Terashi et al. study the restoration of phagocytic and oxidative capacity of granulocytes by G-CSF may be due to increased density of cytokine receptors.

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Are elevated serum levels of IGFBP-2 after intensive chemotherapy of childhood acute lymphoblastic leukemia a risk factor of relapse?

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Abstract

Introduction: In the study we investigated the association between IGFs, their binding proteins and pathogenesis as well as prognostic factors of relapse of childhood ALL.

Material and methods: In 43 children suffering from ALL, we observed 7 cases of relapse. We studied the serum levels IGF-I, IGF-II, IGFBP-3 and IGFBP-2 (expressed in SDS) in a subgroup with relapse (A) and in a subgroup without relapse (B) at diagnosis (1), after induction of remission (2) and after intensive chemotherapy (3). All comparisons were made with age-and sex-matched controls.

Results: It was found that in subgroup A, the values of IGFBP-2 remained high at each stage of the investigation: 3.92±2.50 (1) 3.68±0.99 (2) 3.52±1.26 (3), whereas in the subgroup B they underwent a significant reduction from 3.87±1,86 (1) 3.45±1.25 (2) 2.15±1.84 (3), p=0.02. In comparison to a control group, the correlations between IGF-I and IGFBP-3, and IGF-I and IGFBP-2 were disturbed for the whole group of children at each stage of the investigation. However, at diagnosis we obserwed a negative correlation between IGFBP-2 and hemoglobin (r=-0.57 p=0.0001).

Conclusion: Increased values of IGFBP-2 after intensive chemotherapy in children who subsequently underwent a relapse of the disease, suggest that IGFBP-2 levels might constitute a prognosis factor. However, this requires verification with a larger group of children. The negative correlation between values of hemoglobin and IGFBP-2 observed at diagnosis might further suggest the involvement of this protein in the process of leukemogenesis in children.

Key words: IGFBP-2, acute lymphoblastic leukemia, children, relapse.

Abbreviations: ALL acute lymphoblastic leukemia; AML – acute myeloblastic leukemia; IGF – insulin-like growth factor; IGFBP – IGF binding protein; IGFBP-rP – IGFBP related protein; ALS – acid labile subunit; BFM – Berlin-Frankfurt-Münster; NHL – Non-Hodgkin lymphoma; BMI – body mass index; GH – growth hormone.

Introduction

Insulin-like growth factors – IGFs, belong to a family of peptides involved in the proliferation and differentiation of cells. They also have an insulin-like metabolic effects. Together with their binding proteins (IGF binding proteins – IGFBPs), proteases of IGFBPs, activators and inhibitors of these proteases, as well as two cell-surface receptors mediating the biological activity of IGFs, they constitute a system of great significance within physiology and pathology [1-5].

The primary function of IGFBPs is the modulation of the biological activity of IGFs, through prolongation their half-life and the influence on their bioavailability (1,9-11). The physiological activity of IGFBPs is based on two mechanisms: IGF-dependent and IGF-independent. The function of IGFBPs in the mechanism IGF-dependent, is the transport of IGFs across the capillary barrier, enhance or inhibit the presentation of IGFs to their receptor. The regulation of the growth, migration and metabolism of cells, through IGFBPs, also occurs in the mechanism independent on IGFs [4,6,10-12].

Recent studies have confirmed the involvement of the IGF system in the pathogenesis of cancers (breast, prostate, lung, ovarian, bladder cancer, childhood acute lymphoblastic leuke-

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mia, Wilms tumour, neuroblastoma), and have verified their significance in prognosis [13-21].

The aim of the study was to investigate the involvement of IGF-I and IGF-II, and their binding proteins IGFBP-3 and IGFBP-2 into the pathogenesis of acute lymphoblastic leukemia (ALL) in children, as well as learn if the investigated factors play a role in relapse prognosis.

Material and methods

Patients and control

Forty-three children with newly diagnosed ALL (30 males and 13 females), from Department of Pediatric Oncology in Bialystok were studied prospectively from February 2000 to January 2004. The patients were investigated at three different points: at diagnosis (n=43), after induction of remission (n=32) and after the end of intensive chemotherapy (n=38). Thirty-seven of the patients (86%) suffered from B-precursor cell ALL, whereas four (9.3%) suffered from T-cell ALL. The immunophenotype ALL-common with coexpression of the myeloid markers was determined in two patients (4.65%). Initial leukocytes count ranged from 1.200 to 540.000 $x10^9$/l. According to the protocol ALL IC-BFM 2002 children with initial leukocytosis over 20 000 /mm^3 face higher risk of relapse. In analysed group 14/43 (30,23%) of the patients had initial leukocytosis >20 000/mm^3 and only 3/14 (21,42%) of them presented the relapse of ALL – *Tab. 1*. The median age of the patients was 4.66 (range 1.5-16) at the start of chemotherapy. Three of the patients died at the time induction remission (as the consequence of severe infection during bone marrow aplasia), one continued the treatment in another department. Clinical data of the children at diagnosis explain *Tab. 1*. Among 43 studied patients, seven developed recurrence, on average 26 months after diagnosis – *Tab. 2*. Children were divided into two subgroups: A-subgroup with relapse and B-subgroup without relapse. The control group included 42 healthy children (the age range from 1.33 to 16.83, median =5.58, 31 males and 10 females).

Therapy

Twenty-seven patients were treated according to the protocol ALL-BFM-90 (for low risk group), six – according to the New York II protocol (for high risk group) and ten – according to the ALL IC-BFM 2002 protocol (3 – standard risk group, 6 – intermediate risk group, 1 – high risk group).

Blood Sampling

Blood samples were collected at diagnosis (1), after induction remission – (after protocol I in the protocols BFM and after protocol of the induction remission, part one of the consolidation of the remission according to the New York II protocol) (2) and after finishing the intensive chemotherapy (3); on average 6-8 months after diagnosis. Serum was stored at -80°C until analysis. The blood samples of the children who had severe infections (fever, elevated CRP) and impairment of liver function (s-aminotransferases as abnormally high levels as 5x the above normal limit) were excluded.

Methods

Anthropometric parameters. Linear growth was measured on a wall-mounted Harpenden stadiometer. The weight was measured and the BMI (body mass index) calculated at all study points. Data were transformed to SDS using Polish reference values [22].

Biochemical measurements. IGF-I analysis was performed after acid–ethanol extraction, by radioimmunoassay (RIA, Biosource Europe S.A., Belgium KIP1589). Inter-assay coefficient of variation was 9.8, 9.6 and 8.1% at 38.8 ng/ml, 160.8 ng/ml and 664 ng/ml respectively.

IGF-II was also determined after acid-ethanol extraction, by IRMA (DSL, Webster, Texas, USA DSL-9100). Inter-assay coefficient of variation was 9.5, 6.3 and 10.4% at 74 ng/ml, 427 ng/ml and 1295 ng/ml respectively.

IGFBP-3 concentration was measured using a commmercially available radioimmunoassay kit (DSL, Webster, Texas, USA DSL--6600). Inter-assay coefficient of variation was 1.9%, 0.5% and 0.6% at 76.9 ng/ml, 21.51 ng/ml and 8.03 ng/ml respectively.

IGFBP-2 concentration was evaluated by RIA (DSL, Webster, Texas, USA DSL-7100). Inter-assay coefficient of variation was 7.4%, 4.5% and 7.2% at 2.7 ng/ml, 13.2 ng/ml and 69.7 ng/ml respectively.

The values of IGF-I, IGF-II, IGFBP-3 and IGFBP-2 were age-adjusted by calculating the standard deviation score (SDS) [23-25].

Statistical Analysis

All values are presented as the mean and SD of the age and sex-independent standard deviation score (SDS). All comparisons were made with age-and sex-matched controls. Statistical procedures were performed using SPSS for windows (STATISTICA 6.0 PL). The statistical difference between the values of two independent groups was tested by the U Mann-Whitney test. Changes in the parameters were assessed for three periods: at diagnosis, after the induction of remission and after intensive chemotherapy. The significance of the changes was analyzed by Friedman, Wilcoxon and ANOVA tests. The correlations were performed using Spearman correlation analysis. The significance was chosen as $p<0.05$.

All the investigations were strictly made in accordance with the guidelines of the medical ethics committee in Białystok.

Results

The values of IGF-I SDS in both subgroups exceeded -2 SD score and rested unchanged during the analysis. Those values were lower comparing to control group – *Tab. 3*. We did not find any differences between mean values of IGF-II SDS in both subgroups at any point of analysis – *Tab. 3*.

Values of IGF-II SDS increased significantly in the group without relapse (B) p=0.001 by ANOVA. In subgroup A the values did not differ statistically at any the time of the observation. In comparison to control group (0.57±1.04), we found lower IGF-II SDS at diagnosis in subgroup B (p=0.009) – *Tab. 3*. In other points of analysis there were no differences between control and the subgroups.

Table 1. **Hematologic parameters of the patients at diagnosis**

	immunophenotype	Sex F/M	age	initial leukocyte (x109/l)	blast count (x109/l)	Hb (G/l)	Karyotype of blasts
1.	ALL-common	M	4.56	2.80	0.28	8.1	normal
2.	ALL-common*	M	14.40	33.90	27.12	11.8	normal
3.	ALL-common	F	4.40	32.70	25.06	7.9	normal
4.	ALL-common	M	2.00	4.90	0.29	9.1	normal
5.	ALL-common	M	5.00	11.00	3.30	13.2	abnormal**
6.	ALL-common*	F	6.40	80.00	72.00	7.4	normal
7.	ALL-common	M	3.00	540.00	507.60	8	normal
8.	ALL-common	M	8.08	4.80	0.96	7.9	normal
9.	ALL-common*	M	2.24	3.50	3.50	6.8	normal
10.	ALL-common	M	3.56	7.50	1.27	11.2	normal
11.	ALL-common	M	13.72	1.60	0.00	7.7	normal
12.	ALL-common	F	3.00	7.00	1.40	8.4	normal
13.	ALL-common*	M	5.80	79.80	71.80	9.1	t (9;22)
14.	ALL-common	M	2.75	1.70	0.51	8.9	normal
15.	ALL-common	M	13.16	1.40	0.00	5.7	normal
16.	ALL-common	M	3.16	30.60	18.48	10.2	normal
17.	ALL-common*	F	4.64	9.10	0.91	7.2	normal
18.	ALL-common	M	10.16	2.80	0.84	9.8	normal
19.	ALL-common*	M	5.80	56.00	45.36	6.8	normal
20.	ALL-common	F	3.91	2.70	0.54	10.2	normal
21.	ALL-common	M	4.40	11.50	9.43	3.6	normal
22.	ALL-common	M	1.56	3.10	0.31	7	normal
23.	ALL-common	M	16.00	2.70	0.00	8.4	normal
24.	ALL-common	F	4.83	6.60	2.54	9.3	normal
25.	ALL-common	F	12.58	2.40	0.38	9	normal
26.	ALL-common	F	14.33	2.30	2.02	10.4	normal
27.	ALL-common	M	2.83	8.70	4.52	10.3	normal
28.	ALL-common	M	2.75	26.50	24.38	7.1	normal
29.	ALL-common	M	6.50	6.50	1.43	9.8	normal
30.	ALL-common	M	4.66	5.60	1.51	6.7	normal
31.	ALL-common	M	4.33	30.90	28.42	7.8	normal
32.	ALL-common	M	2.33	5.10	2.28	3.3	normal
33.	ALL-common	M	4.60	31.10	7.46	5.5	normal
34.	ALL-common	F	1.75	9.90	6.33	7	normal
35.	ALL-common	M	6.08	4.90	2.05	8.9	normal
36.	ALL-common	F	2.91	23.90	5.97	4.9	normal
37.	ALL-common	M	14.16	3.40	0.13	8.3	hyperdiploidia
38.	ALL-common + coexpression CD15*	F	13.64	9.80	9.80	9.9	normal
39.	ALL-common + coexpression CD33	F	13.40	12.30	8.65	8.9	normal
40.	ALL-pre T	M	6.750	107.50	80.00	3.3	47XY+ mar[3]
41.	ALL-T	M	7.40	66.80	37.07	12	normal
42.	ALL-T	F	2.75	6.00	0.00	6	normal
43.	ALL-T	M	7.58	1.200	0.48	6.7	t(8;10), t(7:14)

* – children with relapse; ** – 44-46,XY,del(1)(q42),del(3)(q32),del(5) (?p11),?del(7)(?q31?q34),r(?),-19,-21[cp19]/46,XY[20]

Table 2. **Characteristics of patients with relapse**

F/M	relapse	time after diagnosis (month)	program of treatment
F	early bone marrow	21	New York II
M	early bone marrow	28	New York II
F	early bone marrow	26	BFM-90
M	early bone marrow	30	BFM-90
F	late mixed (bone marrow+CNS)	40	BFM-90
M	very early testicular	7	BFM-90
M	late mixed (bone marrow and testicular)	30	New York II

We observed significant increase of the values of IGFBP-3 SDS in both subgroups; in group A p=0.005 by ANOVA and in subgroup B p=0.00035 by ANOVA. After induction remission the mean values of IGFBP-3 SDS were lower in subgroup A, than in subgroup B, p=0.01. The mean value of IGFBP-3 SDS in control group differed statistically from group A at diagnosis (p=0.006) and after induction remission (p=0.01). After the end of intensive chemotherapy we did not find the differences between subgroup A and control. In subgroup B, IGFBP-3 SDS values were lower than in control group only at diagnosis (p=0.009).

At diagnosis and after induction remission the mean values of IGFBP-2 SDS did not differ significantly between subgroup A and subgroup B (p=0.95, p=0.98 respectively). However, after the end of intensive chemotherapy we observed tendency to lower values of IGFBP-2 SDS in subgroup B comparing to subgroup A (p=0.06) – *Tab. 3*. The values of IGFBP-2 SDS declined significantly during the observation in subgroup B (p=0.02 by ANOVA) whereas in subgroup A they stayed unchanged (p=0.84 by ANOVA). Those values were higher than in control group at diagnosis and after induction remission. However, after the end of intensive chemotherapy we found the difference between control and subgroup A (p=0.001) but there was not difference between control and subgroup B (p=0.77).

We did not find the differences in IGF-I, IGF-II, IGFBP-3 and IGFBP-2 expressed as SD score at any point of analysis between the group of children with initial leukocytosis >20 000 /mm^3 and <20 000/mm^3.

We observed the correlation between IGF-I SDS and IGF-II SDS in control group (r=0.38 p=0.01), and similarly in subgroup B: at diagnosis (1) r=0.41 p=0.01 and after induction remission (2) r=0.42 p=0.01. We did not find such correlation at any point of analysis in subgroup A. We found the correlation between IGF-I SDS and IGFBP-3 SDS in subgroup A at diagnosis (r=0.75 p=0.04) and after induction remission (r=0.94 p=0.004) similarly to control (r=0.53 p=0.0003) but there was no such correlations in subgroup B – *Tab. 4*. There was no correlation between IGF-I SDS and IGFBP-2 SDS at any stage of analysis. The correlation between IGF-II SDS and IGFBP-3 SDS in subgroup without relapse were similar to control r=0.44 p=0.008 (1), ns (2) and r=0.46 p=0.008 (3), whereas in patients with relapse it was not observed. We did not find any correlation between IGF-II SDS and IGFBP-2 SDS in analyzed subgroups, whereas in control it was observed – *Tab. 4*. We found the negative correlation between IGFBP-2 SDS and hemoglobin (-0.57 p=0.0001), between IGFPB-2 SDS and total protein at the time of diagnosis -0.45 p=0.003 and after induction remission -0.46 p=0.03. However, in our study we did not observe any corelation between initial leucocytosis and platelets and count of blast cells.

Discussion

The amount of leucocytosis (>20 000/mm^3), the age of the child and genetic changes within the karyotype of the lymphoblasts at the time of diagnosis are recognised as the risks of ALL relapse. There are other factors of relapse such as the response

Table 3. **Values of the components the IGF-system, expressed as SDS (subgroup A, B and C-control): 1 – at diagnosis, 2 – after induction remission, 3 – after intensive chemotherapy, p – difference between subgroup A and subgroup B; p*difference between control and subgroup A – p<0.05, p**difference between control and subgroup B – p<0.05**

		A n	mean	range	standard deviation	B n	mean	range	standard deviation	p	C n	mean	range	standard deviation	p*,**
IGF-I SDS	1	7	-2.10	-2.79 - (-1.18)	0.61	36	-2.02	-4.59 - 1.10	1.15	ns					
	2	7	-2.16	-3.13 - (-0.98)	0.77	27	-2.08	-4.47 - 1.14	1.43	ns	41	-0.31	-2.93 - 12.01	2.50	p*, **
	3	7	-2.24	-3.62 - 0.03	1.12	31	-1.60	-3.96 - 1.58	1.10	ns					p*, **
IGF-II SDS	1	7	0.30	-1.66 - 4.31	2.08	36	-0.19	-2.85 - 2.62	1.33	ns					p**
	2	7	0.30	-1.19 - 1.07	0.84	27	0.40	-1.90 - 3.76	1.48	ns	41	0.57	-1.33 - 3.17	1.04	ns
	3	7	0.09	-1.94 - 2.90	1.81	31	0.77	-3.16 - 6.09	1.77	ns					ns
IGFBP-3 SDS	1	7	-0.78	-1.98 -1.80	1.29	36	-0.08	-4.50 - 7.79	2.29	ns					p*, **
	2	7	-0.74	-1.80 - 0.20	0.83	27	1.13	-1.53 - 4.53	1.72	p=0.01	41	0.92	-1.99 - 6.39	1.72	p*
	3	7	1.12	-1.54 - 5.80	2.41	31	1.96	-1.26 - 11.28	2.72	ns					ns
IGFBP-2 SDS	1	7	3.87	1.32 - 6.15	1.86	36	3.92	-3.69 - 8.44	2.50	ns					
	2	7	3.68	2.64 - 5.41	0.99	27	3.45	0.22 - 5.84	1.25	ns	41	1.34	-2.03 - 4.31	1.37	p*, ** p*, **
	3	7	3.52	1.54 - 5.36	1.26	31	2.15	-1.22 - 5.89	1.84	p=0.06					p*

Table 4. **Correlation between components of the IGF-system: 1 – at diagnosis, 2 – after induction of remission, 3 – after intensive chemotherapy, c – control**

corelation r Spearman		IGF-II SDS	IGFBP-3 SDS	IGFBP-2 SDS
IGF-I SDS	1	0.37 p=0.01	ns	ns
	2	0.44 p=0.01	ns	ns
	3	ns	ns	ns
	c	0.38 p=0.01	0.53 p=0.0003	-0.56 p=0.0001
IGF-II SDS	1		0.43 p=0.004	ns
	2		ns	-0.41 p=0.01
	3		0.47 p=0.002	ns
	c		0.46 p=0.002	-0.40 p=0.008
IGFBP-3 SDS	1			ns
	2			-0.38 p=0.03
	3			ns
	c			ns

to induction therapy and the presence of minimal residual disease in the 12th week of chemotherapy treatment. Despite appropriate allocation of children to therapeutic programs, there is a group of children in which the relapse of the disease may occur. Unfortunately, the treatment of the second attack of the disease involves a high risk of failure. This is why the efforts of the researchers within this area are concentrated upon investigating unfavourable factors affecting prognosis, in order to intervene early enough to prevent a relapse of the disease.

During the current study, the relapse occurred in seven children (out of 43). Only three out of seven were classified into the high risk group, based on the classical risk factors of relapse and received more intensive chemotherapy treatment. The remaining four were treated with protocol ALL BFM-90 for the low risk group.

In both of the subgroups – with and without relapse, a significant increase of the level of IGFBP-3 SDS was observed in subsequent phases of the study. In the subgroup without relapse it was observed a significant increase of IGF-II SDS, whereas in the subgroup with the relapse, mean values of insulin-like growth factors (IGF-I SDS and IGF-II SDS) as well as IGFBP-2 SDS remained relatively constant throughout the time of the analysis (therefore no significant changes were observed).

No statistically significant differences in the mean values of IGF-I SDS, IGF-II SDS were observed between the subgroups at any stage of the investigation. The subgroup which relapsed displayed higher mean values of IGFBP-2 SDS in comparison to the group without relapse, following intensive chemotherapy p=0.06. It should be noted that the mean values of IGFBP-2 SDS, both at diagnosis and after the induction of remission were nearly identical in both subgroups p(1)=0.95 and p(2)=0.98. The presence of significant differences between both subgroups in the mean values of IGFBP-2 SDS was noted only after the end of intensive chemotherapy. Therefore, the high level of IGFBP-2 SDS at the end of intensive treatment might be an independent risk factor for the relapse of the disease in children with ALL.

Mohnike et al. found that the values of IGFBP-2 SDS normalise in children with ALL in remission [26]. Vorwerk et al. observed that the expression of the IGFBP-2 gene in mononuclear cells in children with ALL is the same at the time of diagnosis and during the 33rd day of the treatment (i.e. at the time of remission). In children who later experienced a relapse of the disease, there was a higher proportion of cells expressing the IGFBP-2 gene at the time of diagnosis, compared to the rest of the children. Vorwerk et al. investigated the expression of the following genes: IGF-I, IGF-II, IGF-IR, IGF-IIR, IGFBP-1 to 5, IGFBP-rP1 and IGFBP-rP2. They found that the significant differences in IGFBP-2 and IGFBP-3 gene expression between the group which relapsed and the remaining children [27].

Wex et al. showed a positive correlation between the expression of the IGFBP-2 gene in mononuclear cells obtained from children with ALL (from bone marrow or peripheral blood) and the concentration of IGFBP-2 in the serum. They concluded that higher concentration of IGFBP-2 in the serum was related to its abnormally high production by neoplastic cells [28]. Dawczynski et al. observed relapse after a blood marrow transplantation in AML-patients with increase of IGFBP-2 at day 100 after a blood marrow transplantation. The authors suggest the high possibility of relapse and poor outcome in patients with IGFBP-2 higher then 4.5 SDS [29].

It has also been noted that the concentration of IGFBP-2 in the blood serum is correlated with the stage of the disease and can serve as a marker for reccurrence of solid tumours. In patients with ovarian cancer, in whom a continuously increased level of IGFBP-2 was observed during post-operative treatment, a relapse of the disease subsequently occurred [30]. Likewise, in patients with colorectal cancer, high values of IGFBP-2 correlated with recurrence and degree of dissemination of the disease [31].

We found strong negative correlation between IGFBP-2 SDS and hemoglobin (g/L) at diagnosis (r=-0.57, p=0.0001). It indicates that IGFBP-2 level is proportional to degree of anemia at diagnosis due to elimination of precursors of normal erythropoiesis by leukemic clone. The high level of IGFBP-2, observed during fetal development, might suggest that in patients with ALL the increase of IGFBP-2 is connected with proliferation of early hematopoietic progenitors [29,32]. The decrease of IGFBP-2 values during the treatment, described in malignant disease by other authors, indicates their role in leukemogenesis [26,30].

Petridou et al. found that high levels of IGFBP-3 at the time of diagnosis of ALL in children decrease the risk of death from the disease [33]. Vorwerk et al. showed that high values of

IGFBP-2 and low values of IGFBP-3 at the time of diagnosis increase the risk of relapse [34].

In the current study, at diagnosis the mean levels of IGFBP-3 SDS were lower in both analyzed subgroups comparing to control. However, these values rose during the treatment. Similarly, Mohnike et al. found that low values of IGFBP-3 at diagnosis increased after induction remission [26]. It has suggested that increased IGFBP-3 proteolysis might be responsible for the enhanced growth of IGFBP-2 – over expressing tumors *in vivo* [18]. In our study, in the group with relapse, the increase of IGFBP-3 SDS was less intensive. After induction remission, we found the difference in IGFBP-3 SDS between the group with and without relapse. Dawczynski et al. observed the increase of IGFBP-2 with simultaneous decrease of serum IGF-I and IGFBP-3 100 days after bone marrow transplantation [29]. In our opinion, the differences in correlations between IGF-I SDS and IGFBP-3 SDS, IGF-II SDS, in the group with and without relapse, indicate different mechanisms of regulation and their possible role in leukemogenesis. It is suggested that IGFBP-3 has antiproliferative and proapoptotic action.

Values of IGFBP-2 rested elevated for the group which relapsed throughout the whole analysis. Following the end of intensive chemotherapy, they were higher in comparison to the group without relapse, in which they decreased by 41%. It may suggest the local production of IGFBP-2 by residual blasts. The small group of children with relapse might perhaps prevent the formulation of definite conclusions and these findings therefore require verification with a larger group. Increased values of IGFBP-2 in children may be a risk factor for relapse.

Conclusion

The differences in the levels of insulin-like growth factors and their binding proteins between healthy children and those with acute lymphoblastic leukemia at the time of diagnosis, as well as the changes observed during therapy and in particular differences in the group of patients who relapsed, suggest the involvement of IGFs and IGFBPs in the process of leukemogenesis. The continuously increased values of IGFBP-2 after the end of intensive chemotherapy in the group of children who subsequently experience a relapse of the disease, suggest that high values of IGFBP-2 might constitute a prognostic factor in ALL. However, this requires verification with a larger group of children.

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Reactive oxygen and nitrogen species in the course of B-CLL

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Abstract

Purpose: The study objective was to investigate the production of NO, cGMP and superoxide anion radical by neutrophils, and to examine alterations in serum or plasma total nitric oxide, MDA and cGMP levels in B-CLL patients.

Material and methods: PMNs were isolated from 20 patients with B-CLL. Total nitrite was measured in cell supernatants and serum by Griess method. The generation of superoxide anion radical by cells was estimated using cytochrom-c reduction test. The cGMP level in cell supernatants and plasma was assessed by ELISA kit whereas serum MDA level using a spectrophotometric assay by Guege and Aus.

Results: The PMNs in B-CLL patients were characterised by impaired NO generation and enhanced cGMP production. Contrary to the control group, no significant effect was found of rhIL-15 and rhIL-18 on the release of these mediators by PMNs. Superoxide anion radical release by PMNs was decrease. Serum MDA and plasma cGMP levels were elevated in B-CLL patients as compared to the controls.

Conclusions: The reduced production of nitric oxide and superoxide anion radicals by PMNs in B-CLL may impair the cytotoxic effect of neutrophils on leukaemic B cells. Low secretion of nitric oxide by PMNs and high levels of cGMP in PMN supernatants suggest that activation of guanyl cyclase (sGC) in these patients may occur in the presence of agents other than nitric oxide. Moreover, the findings indicate the enhancement of lipid peroxidation with the progression of the neoplastic process in B-CLL patients.

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Introduction

Chronic B cell lymphocytic leukaemia (B-CLL) is a neoplastic disease characterised by proliferation and accumulation of B cells arrested in the early phase of cell division [1,2].

Disturbances can be observed in cellular and humoral defence mechanisms. These changes may also affect non-specific response cells – neutrophils. Recent reports seem to indicate a significant role of these cells, particularly in the early phase of the antitumour response [3].

Activated neutrophils have the potential to destroy cancer cells through a direct cytotoxic action. In the vicinity of cancer cells, a rapid rise is observed in oxygen metabolism of neutrophils, resulting in the formation of numerous reactive oxygen species (ROS), such as superoxide anion radical, hydroxyl radical, hydrogen peroxide and nitric oxide (NO). Reactive oxygen and nitrogen species exert an effect on cell membrane lipids and organelles, by changing their fluidity, signal transmission, cellular transport and accelerating cell apoptosis [3-5].

Nitric oxide is produced during the conversion of L-arginine to L-citrulline, the reaction being catalysed by NO synthase (NOS). Induced NOS (iNOS), controlled by a series of cytokines, is found in neutrophils (PMNs) [5,6]. Previously, we observed enhanced expression of iNOS in PMNs from healthy persons under the influence of rhIL-15 and rhIL-18 [7].

Cyclic guanosine monophosphate (cGMP) is an indicator of the amount of generated NO and has been known as a "secondary transmitter" of information in the cell, activated by nitric oxide [8-10]. Lipid oxidation by nitric oxide and by proteinated form of superoxide anion radical, i.e. hydrogen peroxide (hydroperoxy radical), results in the production of toxic malondialdehyde (MDA), another "secondary transmitter" with a significant role in neoplastic promotion [11,12].

The study objective was to investigate the production of NO, cGMP and superoxide anion radical by neutrophils, and to examine alterations in serum or plasma total nitric oxide, MDA and cGMP levels in patients with chronic B cell lymphocytic leukaemia.

Material

Twenty patients with B-CLL hospitalized in the Department of Haematology, Medical University of Białystok (13 men and 7 women), mean age 58, were recruited for the study. Ten patients had stage I B-CLL and the remaining ten were in stage III in the Rai classification.

Stage I of inactive disease was referred to as the initial phase due to higher detectability than in stage 0. In Rai stage III, the disease is advanced, but patients are in better general condition than in stage IV.

The diagnosis was based on clinical observation, peripheral blood morphology tests, bone marrow punctate, trepanobiopsy, lymph node biopsy and cytochemical examinations. A flow cytometer EPIX XL (Coulter, USA) was used to identify immunophenotypes of leukaemia cells. The monoclonal antibody panels of CD19 and CD20 for B cells and CD3, CD7 and CD8 for T cells were applied to differentiate between these cells.

All the study patients underwent the investigations prior to treatment with cytostatics and corticosteroids. The patients who had accompanying acute inflammatory bacterial, viral, mycotic or allergic conditions were excluded from the study.

The control group consisted of 15 healthy age-matched subjects (6 women and 9 men).

Methods

Cells were isolated from heparinized (10 U/ml) whole blood by Gradisol G gradient 1.115 g/ml (Polfa) by Zemman et al. [13]. Plasma was also obtained from heparinized (10 U/ml) whole blood. Serum was received from whole blood without additives.

Polymorphonuclear cells (PMNs) were suspended in the culture medium (HBSS) to provide $5x10^6$ cells/ml and the cells were incubated in flat-bottomed 96-well plates (Microtest III-Falcon) for 4 h at 37ºC in a humidified incubator with 5% CO_2 (NUAIRETM). rhIL-15 (50 ng/ml; R&D) and/or rhIL-18 (50 ng/ml; R&D Systems) were tested to stimulate secretion by PMN.

Determination of total nitric oxide (NO_3^-/NO_2^-) concentration in cell cultures and serum

Nitric oxide produced in cells in the presence of superoxide anion radical is rapidly converted to nitrate and nitrite (NO_3^-, NO_2^-). Nitrate and nitrite are stable final products of NO metabolism and may be used as indirect markers of NO presence. Total NO concentration is commonly determined as a sum of nitrate and nitrite concentrations. NO production by PMN was determined using an indirect method based on measurement of NO_2^- ion concentration in culture supernatants and serum according to Griess's reaction. In the samples analyzed, nitrates were reduced to nitrites in the presence of cadmium, and then converted to nitric acid that gave a colour reaction with Griess's reagent [14]. NO_2^- ion concentrations were determined by spectrophotometric analysis at λ=540 nm with reference to a standard curve.

Analysis of generation of superoxide anion radical by PMN using cytochrom-c reduction test

"Oxygen burst" in neutrophils was explored by detecting the production of O_2^-* by these cells according to Mc Cord's method, in Bhuyan's modification [15], which utilizes differences in light absorbance between solutions containing non-reduced and reduced cytochrome-c. Cytochrome-c does not permeate through the plasmic membrane and thus its reduction by superoxide anion radical in PMN supernatants indicates O_2^- * release outside of the cell.

Cytochrome-c solution in phosphate buffer (KH2PO4/K2HPO4), pH=7.8, containing 0.1 mM EDTA, was added to two parallel samples with isolated neutrophils. Cytochrome-c concentration was 15 mg/ml. Superoxide dismutase (SOD), 5000 U/ml activity, was added to the reference sample, while buffer to the study sample. Next, after addition of LPS (10 μg/ml) to both test-tubes, the samples were incubated at 37ºC, and then absorbance was read at λ=550 nm in the presence of deionized water. The result was presented as nontitrated F index expressed by the absorbance ratio of reference sample to reduced sample.

$$F = \frac{\text{Abs Cyt-c +SOD}}{\text{Abs Cyt-c}}$$

Determination of cGMP concentration in the PMN supernatants and plasma

The cGMP level in the cell supernatants and plasma was assessed using ELISA kit (R&D Systems).

Determination of MDA concentration in serum

MDA level in serum was assessed using a spectrophotometric assay by Guege and Aus [16].

Statistical evaluation

The results obtained were analyzed statistically using Microsoft Excel spreadsheet and Statistica 5.1 suite. Data are expressed as mean ±standard deviation (SD). Normal distribution of data was assessed by the Kolmogorov-Smirnov test. Since the data were not normally distributed, U-Mann-Whitney nonparametric tests for unrelated results were used to compare differences between the groups. A p value of <0.05 was accepted as statistically significant.

Results

1. Concentration of total nitric oxide (NO_3^-/NO_2^-) in PMN supernatants of B-CLL patients

In stage I and III B-CLL patients, both unstimulated and stimulated PMNs were found to release smaller amounts of NO as compared to the control group (*Tab. 1*).

Total NO levels in unstimulated PMN supernatants were significantly higher in stage III patients, as compared to stage

***Table 1*. Concentrations of total nitric oxide (NO_3^-/NO_2^-) in PMN supernatants of B-CLL patients**

PMN	Concentrations of total NO (NO_3^-/NO_2^-) (μM/5x10^6 cells/ml)		
	Control subjects n=15	Patients in stage I n=10	Patients in stage III n=10
	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD
Unstimulated	20.87±3.05	14.15*±2.86	16.8^{b}*±3.1
LPS stimulated	30.01^{a}±5.25	14.69*±2.72	19.33^{ab} *±3.25
rhIL-15 stimulated	24.19^{a}±3.16	15.75*±3.7	17.66*±4.01
rhIL-18 stimulated	26.45^{a}±3.44	15.4*±2.17	17.76*±4.32

* – statistical differences as compared to control subjects ($p<0.05$); ^{a} – statistical differences between unstimulated and stimulated cells ($p<0.05$); ^{b} – statistical differences between patients in stage I and patients in stage III ($p<0.05$)

***Table 2*. Generation of superoxide anion radical by neutrophils in B-CLL patients**

PMN	Control subjects n=15	Patients in stage I n=10	Patients in stage III n=10
	$\bar{x}$±SD	$\bar{x}$±SD	$\bar{x}$±SD
Unstimulated	1.27±0.23	0.67*±0.08	0.78*^{b}±0.05
LPS stimulated	2.91^{a}±0.97	0.91*±0.39	0.82*±0.35
rhIL-15 stimulated	2.69^{a}±1.21	0.88*±0.29	0.95*±0.48
rhIL-18 stimulated	2.78^{a}±0.97	0.77*±0.24	0.97*±0.36

* – statistical differences as compared to control subjects ($p<0.05$); ^{b} – statistical differences between patients in stage I and patients in stage III ($p<0.05$)

I patients. PMN stimulation with LPS in stage III led to higher production of NO as compared to unstimulated cells in stage I patients (*Tab. 1*).

No differences were observed in total NO levels between unstimulated and rhIL-15 and rhIL-18 stimulated PMNs both in stage I and III B-CLL.

2. Concentration of total nitric oxide (NO_3^-/NO_2^-) in the serum of B-CLL patients

The analysis of total serum NO levels in B-CLL patients (14.29 μM±1.4) showed no changes as compared to the control group (14.26 μM±1.56; $p>0.05$).

Total serum NO concentrations in stage III patients was higher in comparison to stage I patients (15.7 μM±1.8; 12.88 μM ±1.4; $p<0.05$).

No correlation was observed between total NO levels in unstimulated PMN supernatants and serum concentration in B-CLL patients.

3. Generation of superoxide anion radical by neutrophils in B-CLL patients

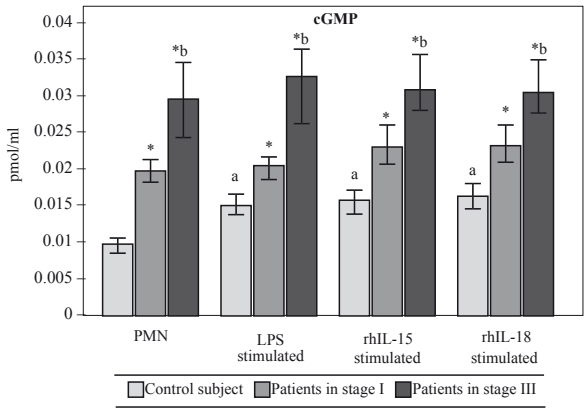
The ability of neutrophils to generate superoxide anion radical was found to be much lower in both B-CLL groups as compared to the control group (*Tab. 2*).

In stage III patients, PMNs had greater potential to produce superoxide anion radical in comparison with stage I patients (*Tab. 2*).

4. Concentrations of cGMP in PMN supernatants and in the plasma of B-CLL patients

In all patients, cGMP levels were higher than in control subjects. Moreover, in stage III, leukocytes released more cGMP than in stage I (*Fig. 1*).

***Figure 1*. Concentrations of cGMP in PMN supernatants of B-CLL patients**

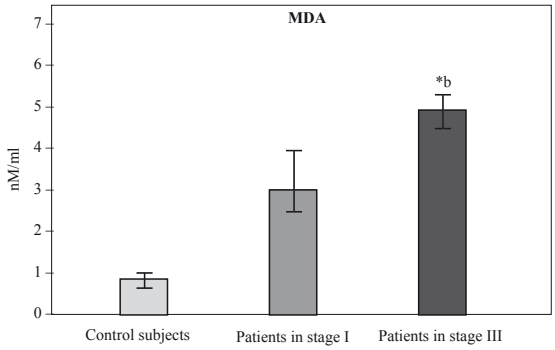


* – statistical differences as compared to control subjects ($p<0.05$); a – statistical differences between unstimulated and stimulated cells ($p<0.05$); b – statistical differences between patients in stage I and patients in stage III ($p<0.05$)

However, unlike in control subjects, cGMP levels in LPS, rhIL-15 or rhIL-18 stimulated leukocyte supernatants in stage I and III patients were not significantly higher as compared to unstimulated cells (*Fig. 1*).

Higher plasma cGMP levels were found in stage I and III patients (0.46 pmol/ml±0.14 and 0.46 pmol/ml±0.17 respectively) in comparison with the control values (0.27 pmol/ml±0.09).

Figure 2. **Concentrations of MDA in the serum of B-CLL patients**



* – statistical differences as compared to control subjects ($p<0.05$); b – statistical differences between patients in stage I and patients in stage III ($p<0.05$)

5. Malondialdehyde level (MDA) in the serum of B-CLL patients

Serum MDA levels in stage I and III patients were significantly higher than in the control group (*Fig. 2*). Moreover, serum MDA concentration was found to increase with the disease advancement. It was significantly higher in stage III than in stage I patients.

Discussion

In the current study, we have demonstrated that the production of nitric oxide and superoxide anion radical by peripheral blood neutrophils is impaired in B-CLL patients. This may have a significant implication for these patients. The reduced production of nitric oxide and superoxide anion radical by PMNs of B-CLL patients indicates a reduced cytotoxic effect of neutrophils on cancer cells. There is evidence that low NO concentrations may activate matrix metalloproteinases (MMPs), the enzymes which function as remodelling factors of the extracellular matrix components, enhancing cancer cell growth, migration and invasion [17]. Given the above, the impaired NO production by neutrophils may play an important role in tumour progression in B-CLL patients. Additionally, low levels of NO have been reported to exert angiogenic effects in various tumour models [18]. One the other hand, there are data suggesting that inhibition of the iNOS pathway leads to increased apoptosis of tumour cells *in vitro* [4].

Low concentrations of nitric oxide, undetectable by Griess method applied in the current study, may be due to a rapid reaction with superoxide anion radical resulting in the formation of toxic peroxynitrite. Peroxynitrite exhibits a cytotoxic effect associated with superoxide dismutase nitrolysis, which may eventually lead to disturbances in the reductive state of the cell and promote neoplastic progression [19].

Peroxynitrite may also contribute to the activation of cGMP synthesis in cells, which has been confirmed by our own findings indicating the enhanced synthesis of cyclic guanosine monophosphate by PMNs in B-CLL patients [20].

The process of cGMP synthesis is controlled by various extra- and intracellular mediators, including cytokines [8]. Shindo et al. *in vitro* showed an increase in cGMP production in fibroblasts and myocardial cells under the influence of IL-1β and LPS, but not when exposed to TNF-α, IL-2, IL-6, IL-8 and TGF-β [21]. We observed no significant effect of rhIL-15 and rhIL-18 on cGMP release by PMNs in B-CLL patients. Moreover, high cGMP concentrations in PMN supernatants coexisting with low nitric oxide production by PMNs suggest that guanyl cyclase activation (sGC) in patients with chronic B-CLL is likely to occur in the presence of biologically active agents other than nitric oxide.

Changes in NO released by PMNs can affect its blood serum level. However, total serum NO levels in patients with B-CLL did not differ from the control, but there was difference in NO levels between patients in stage III and in stage I.

Different results have been reported by Bakan et al. who found no significant differences in NO concentrations between stage I and stage III patients [22].

The increased serum NO level may directly affect cell structural components, which is reflected in, e.g., MDA production [22]. We found higher serum MDA levels in B-CLL patients than in control subjects. Elevated serum MDA concentrations observed in stage III patients as compared to stage I indicate enhancement of lipid peroxidation with the progression of the neoplastic process.

High MDA levels despite low serum total nitric oxide concentrations can be explained by the fact that other compounds such as sulfur dioxide, hydroxyl radical, radical cation and hydroperoxyl radical exhibit lipid peroxidation potential. Besides, the method used to estimate MDA concentration, based on reactions with thiobarbituric acid and employed in our study, is nonspecific. Apart from MDA, it also detects such compounds as bilirubin, sialic acid, products of degradation of saccharide and other aldehydes, whose concentration can also change in the course of the neoplastic processs [16].

When elevated, MDA may react with thiol groups and amino acid proteins, lipids, amino saccharides and nitric bases constituting nucleic acids. Modification of physical properties of the cellular membranes by MDA via increased permeability for H+ and other polar substances may lead to changes in the electric potential difference on both sides of the membranes and eventually to the loss of integrity and inhibition of the activity of protein-transporting enzymes [15,16,23].

Similarly, Ghalaut et al. and Oltra et al. observed high serum MDA levels accompanied by reduced activity of antioxidant enzymes, such as superoxide dismutase and catalase [24,25]. However, Devi et al. found no changes in serum MDA in leukaemic patients despite increased production of superoxide anion radical by neutrophils and elevated levels of antioxidant enzymes [26].

The study has revealed that changes in NO in PMN supernatants and in serum, in cGMP in PMN supernatants and in serum MDA are more characteristic of stage III than stage I patients with B-CLL. The above observations seem to suggest that the research on reactive oxygen and nitrogen species and their indirect markers may have significant diagnostic and prognostic implications for the assessment of oxidative processes in patients with B cell lymphocytic leukaemia.

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Predictive value of lymphocytic infiltration and character of invasive margin following total mesorectal excision with sphincter preservation for the high-risk carcinoma of the rectum

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Abstract

Purpose: To assess the prognostic significance of clinico-pathological factors, especially histological parameters of new Jass classification, following sphincter-sparing total mesorectal excision (TME) for high-risk rectal cancer.

Material and methods: Forty-five consecutive patients treated with curative intent in 1998-1999 due to rectal cancer in Dukes stage B and C were studied prospectively. All of them underwent anterior resection with TME technique. Prognostic value was evaluated by the impact on five-year recurrence-free survival (RFS) in uni- and multivariate analysis. Only factors significant in univariate analysis entered the multivariate regression model. P value <0.05 was stated as a significance limit.

Results: Regarding traditional clinico-pathological factors patient age, tumor site, differentiation grade, mucinous histology and the extent of direct tumor penetration did not significantly affect survival rates. Only the lymph nodes status was associated with prognosis with statistical importance (negative vs positive, RFS: 53.8±10.0% vs 26.3±10.4%, respectively). Considering the additional parameters of Jass classification the character of invasive margin of the tumor did not reveal the important predictive value although the lymphocytic tumor infiltration was significantly related to patient outcome (presence vs absence, RFS: 63.6±15.2% vs 37.5±8.7%, respectively). In multivariate analysis the only one statistically important and independent predictive parameter was the lymph nodes status.

Conclusions: Lymph nodes metastases remain the most important prognostic factor after anterior resection with TME for Dukes B and C rectal cancer. From variables included into Jass classification the absence of lymphocytic infiltration of the tumor can be helpful to identify patients with enhanced risk of oncological relapse.

Key words: rectal cancer, anterior resection, total mesorectal excision, lymphocytic infiltration, invasive margin character.

Introduction

Surgery remains the mainstay of treatment for rectal carcinoma. With the development of screening programs, diagnostic tools and stapling devices an anterior resection with sphincter preservation became the preferred option for the most of cases [1]. Moreover, since the introduction of the method of total mesorectal excision (TME) an optimal local control resulting in improved survival can be achieved [2]. In spite of the sub-specialisation, surgical training and advances in operating technique [3] a lot of patients with regional stage of disease have a high risk of cancer recurrence and may benefit from combined-modality therapy [4]. Thus, in the era of TME more individual approach for adjuvant treatment with consideration of numerous predictive factors is postulated [4,5].

In 1987 Jass and co-workers introduced a new prognostic system for rectal cancer considering four variables: tumor penetration through the bowel wall (transmural =1, limited to the bowel wall = 0), lymph-node metastases (>4 involved nodes =2, 1-4=1, negative nodes =0), invasive margin character (infiltrating =1, expanding =0) and peritumoral lymphocytic infiltration (absent =1, present =0). The scores are summated for tumor grouping with the final score: I =0-1, II =2, III =3, IV =4-5 [6]. Authors claimed that new classification was simple to use and was superior to Dukes staging because it placed twice as many patients into groups and provided a more confident prediction of clinical outcome. The independent value of that scoring was noticed in multivariate analysis by some [7-9]. Moreover, prognostic significance of Jass classification even for patients being

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at the same Dukes stage was observed [10-13]. However, other authors reported that traditional Dukes system was of greater prognostic value and was more reproducible than Jass' classification [14-16]. In their opinion the continued use of Dukes staging is, therefore, warranted for prognostic and therapeutic decisions and should be recommended for routine clinical practice [16].

The aim of this study was to estimate the prognostic value of clinical and pathological factors, especially additional histological parameters of Jass classification following anterior resection with TME for rectal cancer patients with high risk of oncological relapse (Dukes stage B/C).

Material and methods

Patients

At the 2nd Department of Surgical Oncology at Lower Silesian Oncology Center seventy-seven consecutive patients with histologically confirmed rectal cancer underwent an anterior resection with sphincter preservation from January 1998 to December 1999. Forty-five of them entered the study fulfilling the inclusion criteria: primary tumor localised maximally 12 cm from the anal verge, absence of distant metastases, lack of intraoperative bowel perforation, absence of macroscopic infiltration of adjacent organs, distal and radial margins microscopically free of cancer infiltration (R0 resection). Time of the follow-up was five years. The data were collected in a prospective manner. Written informed consent was obtained from all the patients.

Surgical treatment

All patients underwent elective surgery with preoperative bowel preparation by means of 4L of polyethylene glycol solution one day before surgery. Prophylactic antibiotics were administered at the anaesthesia induction. Resection of the rectum was strictly performed according to the TME principles with sharp dissection under direct vision of the plane between the parietal and visceral pelvic fascia to the levators level. End to end anastomosis was constructed using double-stapling technique with Proximate TLH transverse device and Proximate ILS circular intraluminal one (Ethicon Endo-Surgery Europe, Norderstedt, Germany). Bowel wash-out was performed using 2% povidone iodine solution.

Adjuvant therapy

Fifteen patients received preoperative five-day radiation 25 Gy (5 x 5 Gy) and postoperative chemotherapy with 5-fluorouracil (325 mg/m^2) and folinic acid (20 mg/m^2) in six courses. For thirty patients combined adjuvant radiochemotherapy (5-fluorouracil + folinic acid and 50.4 Gy radiation: 25 x 1.8 Gy + 5.4 Gy boost) was administered.

Follow-up

Follow-up was scheduled every three months during the first postoperative year and every six months thereafter. Physical examination, blood tests, serum markers, barium enema, endoscopy, chest radiograph and abdominal ultrasound were done. In every supposition of cancer recurrence more precise investigation using endorectal sonography, computed tomography or radioisotope scanning was performed.

Clinical factors

For each patient age and gender were recorded. There were twenty-two females and twenty-three males. Patient age ranged from 37 to 88 years, mean was 60.3, median was 60. Therefore, a level of 60 years as a cut-off point for age analysis was stated. Site of the primary tumor was categorised in two groups: >7 cm and ≤7 cm from the anal verge for separate consideration of the intra- and extraperitoneal tumors.

Pathological features and microscopic evaluation

Microscopic analysis was carried out using formalin-fixed, paraffin-embedded tissue sections routinely hematoxylin-eosin stained and assessed at a x 200 and 400 magnification. Stage parameters were analyzed considering the extent of direct tumor spread (beyond the bowel wall or not) and regional lymph nodes status (presence or absence of metastases). Patients were divided into two groups depending on differentiation grade: well/moderately and poorly differentiated. Adenocarcinomas with mucin histology (more than 50% of the tumor volume composed of mucin) were distinctly evaluated from non-mucinous ones. The character of invasive margin (expanding or infiltrating) and lymphocytic infiltration (conspicuous or little/absent) were assessed strictly according to criteria originally described by Jass et al. [6], as following below. Tumor margin was defined as the transition zone between the periphery of the tumor and normal rectal tissue. Considering the character of invasive margin tumors were divided into three categories: circumscribed, intermediate and diffusely infiltrating and then the circumscribed and intermediate were defined as expanding. Conspicuous lymphocytic infiltration of the tumor was recognized when the loose inflammatory lamina including lymphocytes at the deepest point of tumor penetration was present. *Tab. 1* shows patients characteristics and factors analyzed in this study.

Statistical analysis

Statistical analysis was performed using software package StatisticaTM ver. 5.0. All clinical and pathological variables were considered in univariate analysis. To examine the impact of individual parameters on long-term outcome, five-year recurrence-free survival analysis was used. Recurrence-free survival was calculated according to the Kaplan-Meier method. Survivals were compared by the F Cox test using $P<0.05$ as significance limit. Variables significant in univariate analysis were entered into Cox's proportional hazards regression model to evaluate them in multivariate analysis as independent factors.

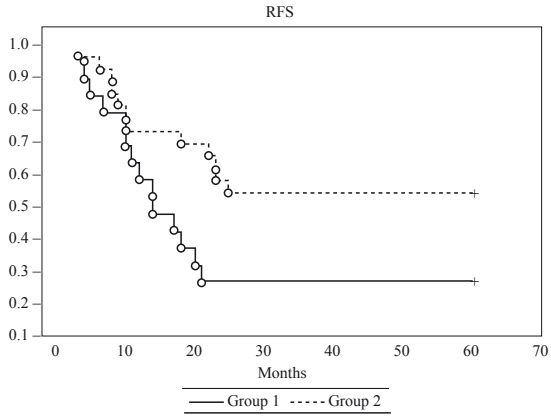
Results

Postoperative mortality was not noticed. Survival rates were enhanced for the female patients not older than 60 years, with well or moderately differentiated cancers sited >7 cm from the anal verge and without tumor penetration beyond the

***Table 1*. Prognostic value of clinico-pathological factors in univariate analysis**

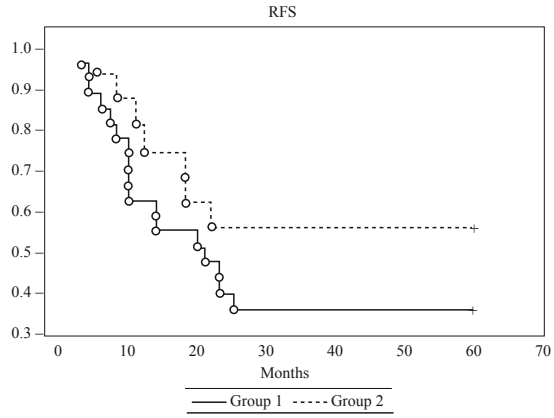
Parameter		n	Survival	P
Patient	≤ 60 years	19	52.6±11.8	
age	> 60 years	26	34.6±9.5	0.260
Patient	Female	22	45.5±10.9	
gender	Male	23	39.1±10.4	0.367
Tumor	> 7 cm	9	55.6±17.6	
location	≤ 7 cm	36	38.9±8.2	0.287
Differentiation	I/II	26	44.0±10.1	
grade	III	19	42.1±11.6	0.376
Mucine	Absent	38	42.9±20.2	
secretion	Present	7	42.1±8.1	0.864
Lymph-node	Negative	26	53.8±10.0	
status	Positive	19	26.3±10.4	0.025
Penetration beyond	Absent	6	43.6±8.0	
the bowel wall	Present	39	33.3±21.1	0.702
Invasive margin	Expanding	18	56.3±12.8	
character	Infiltrating	27	37.0±9.5	0.094
Lymphocytic	Present	13	63.6±15.2	
infiltration	Absent	32	37.5±8.7	0.034

***Figure 1*. Impact of lymph-node metastases on survival**



RFS: recurrence-free survival; Group 1: lymph-node metastases positive; Group 2: negative

***Figure 2*. Impact of invasive margin character on survival**



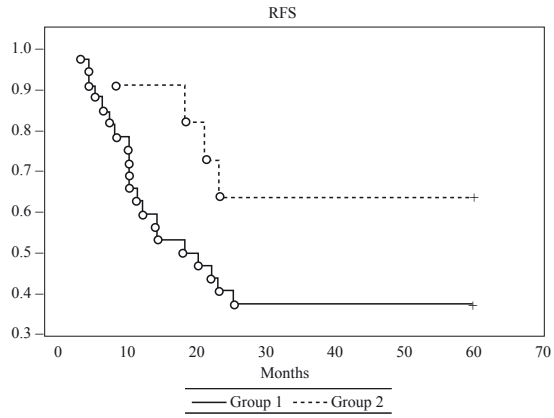
RFS: recurrence-free survival; Group 1: infitrative margin; Group 2: expanding margin

bowel wall (*Tab. 1*). However, the differences were not significant. Survival was decreased for node-positive patients and for patients without lymphocytic tumor infiltration with statistical significance (*Fig. 3* and *Fig. 1*). Considering additional parameters included into Jass classification an improved survival was observed in cases with expanding vs infiltrating tumor margin although with the lack of importance (*Fig. 2*). Multivariate analysis in Cox' proportional hazard regression model revealed the lymph nodes status to be the most important and the only one independent prognostic factor (*Tab. 2*).

Discussion

Dukes and TNM staging of rectal cancer are based on the extent of primary tumor penetration and lymph nodes metastases. In our group of patients the predictive value of the lymph nodes status was more important than the direct tumor spread

***Figure 3*. Impact of lymphocytic infiltration on survival**



RFS: recurrence-free survival; Group 1: absence of lymphocytic infiltration; Group 2: presence of lymphocytic infiltration

Table 2. **Multivariate analysis with Cox regression proportional hazard model**

Parameter	P	Rr	Odds Ratio	95% CI
Lymph-node status	0.045229	2.362942	3.305622	0.835239-13.08265
Lymphocytic infiltration	0.106113	2.229606	2.912445	0.631508-13.43186

Rr – relative risk; CI – confidence interval

which was not significant probably because of analysis of only Dukes B and C cases. Among these patients the absence of tumor penetration beyond the bowel wall with the presence of lymph nodes metastases (Astler-Coller C1 stage; node-negative cases should be regarded as A stage) is a very rare situation. This rarity resulted in statistically non-adequate comparison of six vs thirty-nine patients in our study.

Considering other clinico-pathological factors and histological parameters included in Jass classification we found the statistically important predictive value of conspicuous lymphocytic infiltration of the tumor. That histological feature is well-known to be more frequently associated with early-stage rectal cancers than with those with lymph-node or distant metastases [17]. Tumor stage is a multiparametric variable summarizing some histological features. Jass and co-investigators added two biological parameters to Dukes system: the character of invasive margin which reflects cancer aggressiveness in local spreading and the lymphocytic infiltration of the tumor which pictures patient immune response.

In our study the absence of lymphocytic infiltration significantly influenced decreased recurrence-free survival but it was not independent factor probably because of too small patients number for adequate multivariate analysis. Its independent prognostic value was reported by others [18-21]. Also in the most recent papers low tumor lymphocytic infiltration predicted increased recurrence rate and poor long-term cancer-specific survival [22-26]. In some opinions possible discrepancies of results may be caused by difficult assessment and poor reproducibility for this feature [27]. However, pathological assessment can be significantly improved through the provision of recommended guidelines and gaining the experience [28].

Lymphocytic infiltration is a marker of an effective cell-mediated immune response against the tumor [29]. Some authors suggest the relationship between host immune reaction and the production of angiogenic promoters and thus, with stimulation of tumor vascularity [30]. As the consequence the favourable effect of peritumoral lymphocytic infiltration may interfere with the negative effect of a large vascular surface area within the tumor [31]. On the other hand, host immune response observed as conspicuous lymphocytic infiltration, may prevent metastasis formation [32] and growth of liver metastases [33]. It is more common in the microsatellite instability-high cancers with favourable prognosis [34]. The extent of lymphocytic infiltration correlates with bcl-2 expression, which is known to suppress programmed cell death (apoptosis) [35].

Immune response against rectal cancer is multipathway and multistep mechanism involving different types of lymphocytes, NK cells, dendritic cells and macrophages [27,36,37]. However, the possibility of immunomodulation in cancer patients is intensively investigated [38]. Interferon-alpha can induce cytotoxic T lymphocytes and may elicit long-lasting tumor-specific immunity while interleukin-12 seems to stimulate non-specific killing [39]. Lymphocytic infiltration of the tumor may be suppressed by pentoxifylline [40] and significantly enhanced by preoperative short course of histamine H2 receptor antagonist [41,42]. Those findings are the initial observations but they may be promising perspective for the regulation of immune response against cancer of the rectum.

With the development of combined-modality treatment and the prospect of immunotherapy and gene therapy the identification of markers of cancer behaviour is increasingly needed. The importance of our results is limited by the relatively small sample size. Moreover, neoadjuvant radiotherapy used in one-third of our patients might influence the accuracy of postoperative microscopic evaluation. Despite these disadvantages our results suggest that the lack of conspicuous lymphocytic infiltration of the tumor is related to poor recurrence-free survival of rectal cancer patients. Dismal prognosis was noticed in spite of TME technique and optimal surgical local control. Therefore, lymphocytic infiltration may give additional predictive information for optimal management and follow-up. Potential benefit from more aggressive treatment for these patients should be evaluated in further studies including controlled randomized trials.

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The effects of moderate physical exercise on cardiac hypertrophy in interleukin 6 deficient mice

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Abstract

Purpose: Prolonged physical training leads to compensatory changes in cardiovascular system. One of the most important of them is cardiac hypertrophy. The knowledge, which factors contribute to cardiomyocyte hypertrophy caused by physical exercise is still incomplete. Interleukin 6 (IL6) secreted by contracting skeletal muscles may affect cardiac hypertrophy and remodeling. The aim of the study was to investigate the role of IL6 in exercise induced cardiac hypertrophy.

Material and methods: Female mice lacking functional IL6 gene C57BL6/J$^{IL6-/-tm1Kopf}$ (IL6KO) and age and sex matched controls C57BL6/J (WT) were subjected to 6 week swimming regime. Twenty-four hours after the last training session the mice were sacrificed, hearts were excised and weighed. Two other groups of sex and strain matched mice (9 in each group) not subjected to physical training, were sacrificed and served as controls. Weights of the heart and the left ventricle were related independently to the body weight and the tibia length as measures of hypertrophy. Statistical analysis was performed using multifactorial ANOVA and the Fisher test.

Results: There was significantly higher heart/body weight ratio in both groups of mice which were trained as compared to the respective sedentary animals [$F(3,30)=31.085$ $p<0.001$] There were, however, no significant differences between respective WT and IL6KO groups. Similar relations were found for the left ventricle and also when the weights of the heart and the LV were related to the tibia length.

Conclusion: IL6 is not necessary for cardiac hypertrophy induced by prolonged moderate physical exercise in mice. Additional study is warranted to elucidate this phenomenon.

Key words: interleukin 6, physical exercise, cardiac hypertrophy, mice.

Introduction

Human physiology have evolved to be optimized for repetitive and strenuous physical exercise. Overcrowded world and contemporary western civilization radically diminished the amount of movement performed by individuals. Therefore new epidemics have appeared: type 2 diabetes and obesity. Both of them are strongly related to lacking exercise. Also cardiovascular diseases – the leading cause of death in the developed countries are strongly associated with lacking physical exercise. Prolonged physical training leads to the development of adaptive changes in cardiovascular system and its regulatory mechanisms [1]. Cardiac hypertrophy is vital for meeting increased oxygen demand during strenuous exercise. However, in contrast to the changes occurring due to increased load in pathological conditions like hypertension or heart failure, the physiologic hypertrophy is associated with orchestrated growth of cardiomyocytes, capillaries and connective tissue [2]. Therefore, there is no increase of apoptosis and no increase of fibrosis [2]. Moreover, fetal gene program activation in heart, so typical for any pathological hypertrophy, is also absent after physical training. There is much effort put into research that should dissect molecular mechanisms regulating physiological (during developmental growth, in pregnancy or after exercise) and pathological hypertrophy [3]. Yet, relatively little is known about humoral factor involved in inducing changes in the heart during exercise [4].

Interleukin 6 (IL6) is a prototypical pleiotropic cytokine, involved in inflammatory response, lymphocyte survival and

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***Table 1*. Cardiac hypertrophy parameters in sedentary and swimming mice**

	WT		IL6 KO	
	Sedentary	Swimming	Sedentary	Swimming
Heart weight to body weight ratio (mg/g)	4.2±0.17	4.78±0.31 **	3.99±0.26	4.86±0.12**
LV weight to body weight ratio (mg/g)	3.27±0.2	3.81±0.27 **	3.27±0.21	3.85±0.12 **
Heart weight to tibia length ratio (mg/mm)	5.09±0.38	5.59±0.39 *	4.99±0.42	5.66±0.54 *
LV weight to tibia length ratio (mg/mm)	3.96±0.32	4.45±0.32 *	4.0±0.37	4.49±0.44 *

differentiation, metabolic regulation and cytoprotection [5]. Human studies revealed its association with higher risk of congestive heart failure (CHF), atherosclerosis and worse prognosis after myocardial infarction [5,6]. On the other hand, there are numerous publications presenting protective actions of this cytokine [7]. It has not been fully clarified whether IL6 has positive or detrimental influence on cardiovascular system. Experiments performed on animal models have shown that its transduction pathway is involved in cardiomyocyte hypertrophy [8,9]. There is a strong evidence that this cytokine regulates cardiac lipid metabolism and expression of fatty acid transporters [10]. It is secreted by contracting skeletal muscles and may induce metabolic changes in other organs. IL6 is thought to be responsible for the alterations in insulin sensitivity and anti-inflammatory effects of exercise [11,12]. Due to its abundance and pleiotropic actions some authors call this cytokine even "an exercise factor" [13].

We have undertaken this study in order to investigate whether IL6 deficiency may affect the exercise induced cardiac hypertrophy in mice.

Material and methods

Female mice lacking functional IL6 gene C57BL6/ /J$^{IL6-/-tm1Kopf}$ (IL6KO) and respective wild type C57BL6/J (WT) mice were kept in constant temperature of 22ºC ±1ºC in 12:12 dark-light cycle with constant access to chow and water.

Eight female 8-10 week old mice IL6KO and eight age and sex matched WT controls were subjected to 6 week swimming regime according to previously described protocol [14]. In brief: swimming sessions took place daily on the same time of the day. Mice swam in small tanks (surface cir. 500 cm^2, 18 cm depth) filled with tap water that maintained constant temperature 30-32ºC. The first session lasted 20 minutes and this interval was increased daily by 10 minutes to 90 minutes per session on the eighth day. This session duration was maintained for the remaining 5 weeks. After each session mice were put into clean cages maintained in temperature 27ºC and were allowed to dry. Afterwards the animals returned to their cages. Two groups (IL6KO and WT) of strain and sex matched animals (9 in each group) were handled similarly with the exception that they were put into tanks without water – they were considered as sedentary controls. Twenty-four hours after the last training session mice were sacrificed, hearts were excised and weighed, tibia length was measured. Weights of heart and left ventricle were related independently to body weight and tibia length. All results were multiplied by 1 000 and then presented.

The experimental procedures were carried out according to the European Council Directive of 24 November 1986 (6/609/EEC) and were approved by the Local Ethics Committee in Białystok.

Statistical analysis was performed using multifactorial ANOVA and Fisher post-hoc test.

Results

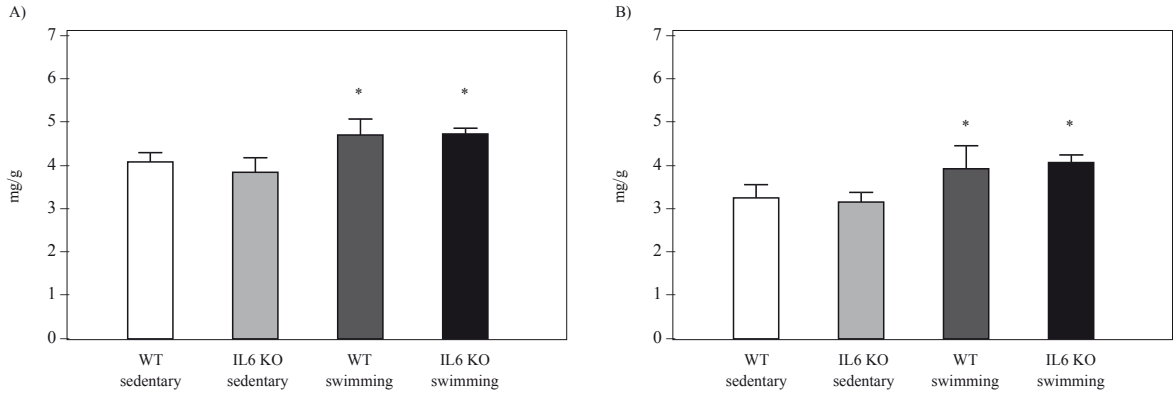
We did not notice any significant differences between sedentary IL6KO and WT animals neither in heart weight to body weight ratio nor in LV weight to body weight ratio. In contrast, mice after 6 week swimming regime presented statistically significant higher heart weight to body weight ratio [ANOVA $F(3,30)=31.085$; $p<0.001$] and LV weight to body weight ratio [ANOVA $F(3,30)=26.105$; $p<0.001$] than their sedentary counterparts (*Tab. 1*). Surprisingly, we did not observe any differences between IL6KO and WT animals neither in basal conditions nor after prolonged physical training (*Fig. 1*).

In order to confirm the abovementioned findings we have related the heart weight and LV weight to the tibia length as a measure independent of changes in fatty tissue encountered in the C57Bl6/J strain. The results of this analysis confirmed earlier findings. The heart weight to tibia length ratio and left ventricle weight to tibia length ratio was significantly higher in mice after 6 week swimming regime than in sedentary controls [ANOVA $F(3,30) = 5.2688$; $p<0.001$ for total heart and $F(3,30)=5.183$; $p<0.001$ for LV]. Further post-hoc comparison made with Fisher post hoc test confirmed that both trained groups (IL6KO and WT) had higher parameters of cardiac hypertrophy than both sedentary groups ($p<0.05$) (*Fig. 2*). Also in this analysis we did not see any significant differences between respective IL6KO and WT animals in any abovementioned parameter.

Discussion

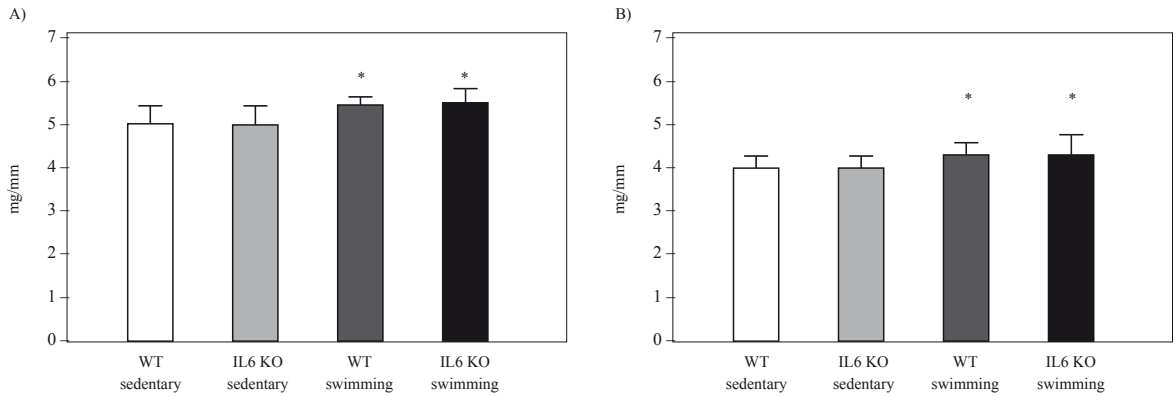
Cardiac hypertrophy is a physiological adaptive mechanism to the increased blood volume or pressure. In order to cope with the increased load late diastolic pressure increases what may

Figure 1. **Heart weight to body weight ratio (A) and left ventricle weight to body weight ratio (B) in interleukin 6 deficient mice (IL6 KO) and in wild type animals (WT) subjected to 6 week swimming regime (swimming) and in respective sedentary controls (sedentary). The difference between groups is statistically significant (ANOVA F(3,30)=31.085 for total heart and F(3,30)=26.105 for LV)**



* p<0.001 vs both sedentary groups (Fisher post hoc test)

Figure 2. **Heart weight to tibia length ratio (A) and left ventricle weight to tibia length ratio (B) in interleukin 6 deficient mice (IL6 KO) and in wild type animals (WT) subjected to 6 week swimming regime (swimming) and in respective sedentary controls (sedentary). The difference between groups is statistically significant (ANOVA F(3,30)=5.2688 for total heart and F(3,30)=5.183 for LV)**



* p<0.05 vs both sedentary groups (Fisher post hoc test)

be associated with the left ventricle (LV) dilation. In such circumstances increasing thickness of the LV walls will decrease the wall tension, hence will diminish oxygen consumption. On the other hand, when LV must develop higher systolic pressure there is also need for more actin-myosin bridges that develop as new sarcomeres arise during hypertrophic growth [15]. There are several physiological conditions that require orchestrated cardiac hypertrophy: developmental growth, pregnancy and nursing associated changes in the cardiovascular system and prolonged physical training [2,16]. In this circumstances a parallel growth of cardiomyocytes, connective tissue and vascular bed occurs, what determines optimal organ function. Interestingly, a different situation occurs when there is hypertrophy caused by pathological states like hypertension, loss of contractile tissue after myocardial infarction or hormonal dysregulation [17]. Then, cardiomyocyte hypertrophy usually is associated with insufficient expansion of the microvasculature and hence increased growth of fibrotic extracellular matrix. The following consequence of the pathological hypertrophy is activation of the fetal gene expression pattern, induction of hypoxia-sensitive genes, activation of apoptotic cardiomyocyte death and finally heart failure [17].

Recently it has been suggested that transduction cascades inducing either type of hypertrophy overlap in some critical points. These include activation of PKC, ERKs, calcineurin, GSK-β3 and GATA-4 dependent mechanisms [3,18]. There is, however, an increasing body of research presenting different factors involved in these two types of cardiac hypertrophy. $G\alpha_q$ and $G\alpha_{11}$ proteins seem to be necessary for the pathologic hypertrophy, as mice harboring their knockout were incapable of increasing heart weight in response to the aortic banding [14,19]. Angiotensin II blockade diminishes pathologic hypertrophy with no effect on physiologic one induced by swimming [20]. There are also factors involved exclusively in physiologic hypertrophy. Phosphoinositide 3-kinase subunit (p110α) and Akt have been shown to be necessary for the exercise induced cardiac hypertrophy and even to counteract heart changes caused by increased pressure [2,3,14]. Precise dissection of the

intracellular mechanisms involved in the physiologic and pathologic hypertrophy is necessary to find the pivotal points where the cell fate is conveyed from physiologic adaptation to pathologic state and in effect apoptosis. With such knowledge we, hopefully, will be able to fix the flawed hypertrophic response and "redirect" it to the physiological pathway in patients with the pathologic conditions like hypertension, valvular heart disease or myocardial infarction. The results of experimental studies have already suggested first answers. The physical training may, to some extent, correct the pathological gene expression profile in hearts of hypertensive animals [21]. This may, at least partially, explain the beneficial effects of exercise in patients with hypertension or heart failure [4].

Interleukin 6 is a cytokine secreted during exercise by a contracting muscle [22]. Its actions may include influence on insulin transduction pathway, carbohydrate metabolism, inflammatory reaction and cognitive processes [23-25]. Some authors have already called this cytokine "an exercise factor" due to its pleiotropic effects during physical training [13]. It has been reported that during exercise IL6 stimulates endogenous glucose production [11,13] and regulates lipid oxidation [26]. This cytokine may also act anti-inflammatory as it attenuates TNFα secretion and stimulates production of IL10 and interleukin 1 receptor antagonist (IL1ra) – two potent anti-inflammatory agents [12,27]. Another interesting study revealed that IL6KO mice present higher myocardial expression of CD36 fatty acid transporter as well as higher concentration of free fatty acids, diacyloglicerols and ceramide [10]. These results suggest that IL6 may decrease the amount of ceramide in heart, an effect that is also evoked by exercise [28].

There is support for the notion that IL6 transduction pathway is closely associated with cardiac hypertrophy [9]. It has been shown that simultaneous overexpression of both subunits of IL6 receptor is sufficient to induce cardiac hypertrophy in mice [29]. On the other hand, IL6 deficiency does not change the weight and dimensions of the left ventricle after myocardial infarction [24]. In this paper we show for the first time that this cytokine is not necessary for exercise induced cardiac hypertrophy as well. The presented data confirmed that after six week swimming regime female mice present higher heart weight to body weight ratio than their sedentary counterparts, regardless of the presence of functional IL6 gene.

One of the limitations of the study is that we have relied only on relatively crude parameters, which the ratios are. Unfortunately, we were not able to perform *in vivo* studies like echocardiographic or magnetic resonance studies. Nevertheless, this preliminary report is the first one to communicate that IL6 deficiency does not change the physiological cardiac hypertrophy in mice. Faldt et al. have shown, that IL6KO mice have lower maximal exercise capacity than the wild type controls [26]. We have chosen a training pattern that utilizes repetitive submaximal exercise with possibly diminished stress for animals. Therefore, we did not encounter animals from our experiment groups that were unable to perform according to protocol. We cannot, however, exclude that there might be differences in hypertrophy when more strenuous exercise is used. Moreover, we have used female animals due to more reproducible swimming induced cardiac hypertrophy [30]. Faldt et al. do not state which sex they used for endurance experiments [26]. Although there were no changes in heart and LV weights we also cannot rule out, that cardiac metabolism during exercise is changed in IL6KO mice.

We can only hypothesize what other factors may take over the function of IL6 and trigger the heart hypertrophy after physical exercise. It has been shown that IL6KO mice present higher expression of other IL6-family cytokines like leukaemia inhibitory factor (LIF) as well as bigger abundance of renin – angiotensin system components [24]. It has been established that both these cascades may affect cardiac hypertrophy. Another possible explanation of the lack of changes in physiological cardiac hypertrophy between IL6KO and WT mice would be the effect of TNFα. A cytokine involved in hypertrophy that is up-regulated in IL6KO mice [27,31]. To support this hypotheses, however, further studies are required.

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Prognostic significance of matrix metalloproteinases type I expression and tumor front parameters in the presence of lymph node micrometastases in carcinoma of the larynx

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Abstract

Purpose: Lymph nodes estimated as pN0 in conventional morphological studies could have focuses of carcinoma cells with a diameter of ≤2 mm referred to as micrometastases (pN+). Matrix metalloproteinases (MMPs) are proteolytic family of endopeptidases which are capable to degrading components of the extracellular matrix and play an important role in cancer invasion and metastases. The aim of this study was to investigate MT1-MMP expression in carcinoma of the larynx and analyze morphological parameters to relate the expression to CKs in pN0 lymph nodes.

Material and methods: To presented the direct correlation between 6 morphological features of tumor front and the probability of micrometastases and prediction of prognosis 22 patients operated for squamous cell carcinoma of the larynx were analyzed. The total score of TFG, chosen clinicomorphological features and grade of matrix metalloproteinase membrane type 1 staining in tumor front were analyzed to predict the presence of micrometastases and prognosis. Immunohistochemical methods with a panel of CKs antigens in lymph nodes and MT1-MMP expression in tumor tissue were performed.

Results: Our study showed that the total morphologic score TFG is very useful in the prediction of micrometastases in patients with laryngeal squamous cell carcinoma. The statistical analysis has revealed a significant correlation between the total TFG score and the presence of cell carcinoma microfocuses in lymph nodes. There was no significant relationship for immunoexpression of MT1-MMP and positive poliCKs stain.

Conclusions: The results of study suggest that extended traditional pathologic evaluation by features from the TFG classification could aid in diagnosis of micrometastases. The positive expression of poliCKs in the pN0 lymph nodes appears to play an important role in determining prognosis in patients with carcinoma of the larynx.

Key words: laryngeal carcinoma, tumor front grading classification, cytokeratin filaments, matrix metalloproteinase membrane type 1, micrometastases.

Introduction

The prognosis of patients, in whom the presence of nodal metastases has not been confirmed in the conventional pathological examination (pN0), is still unclear. The problem is connected with the possibility of the presence of nodal micrometastases – carcinoma foci less than 2 mm in diameter (pN1), which may be the cause of nodal recurrence and shortened survival [1-13]. One of the methods to disclose occult foci of malignant cells within lymph nodes is immunohistochemical analysis of the nodes for the presence of cytokeratin antigens belonging to the family of median filaments (CKs) [1,3,6-9,13].

Matrix metalloproteinases (MMPs) are proteolytic family enzymes of endopeptidases which are capable of degrading extracellular matrix components (ECM), e.g. type I collagen, elastin, fibronectin, gelatin and the basement membrane [14,16]. MMPs including active enzymes such as collagenase, stromelysin and gelatinase [14]. MMPs are inactivated by tissue inhibitors of MMPs (TIMPs) which form complexes with them [15]. MT1-MMP degrades components of the tissue barriers thus could determine the presence of cell carcinoma metastases and microfocuses in lymph nodes [14-23]. Literature reports the prognostic value of the morphological features of the front

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of the primary tumor which determines aggressive malignant invasion and increases the likelihood of micrometastases [6-9,24,25].

The aim of the study was to analyse the morphological features of the primary tumor, its stroma using the classification TFG (Tumor Front Grading) and the expression of MT1--MMP in tumor front to evaluate the relationship between these parameters and cytokeratin antigen expression within the negative lymph nodes, thus attempting to assess the probability of developing micrometastases.

Materials and methods

In study tissue samples obtained from 22 patients (17 men, 5 women; age 58-87 yrs; mean age 63+5 yrs) who had undergone surgery for laryngeal carcinoma at the Department of Laryngology and Laryngeal Oncology of the Medical University in Łódź between the years 1998-1999 were analyzed. All patients underwent total laryngectomy with uni- or bilateral radical or modified neck dissection. The lesions were assessed according to the TNM criteria (TNM Classification of WHO – 2003 – International Classification of Diseases for Oncology). Directly after surgery the material was preserved in a 10% solution of buffered formaldehyde. Samples from the primary tumor designated for microscopic analysis were obtained according to standardized procedure. The depth of invasion was assessed at the point of the deepest invasion of the surrounding tissues. Two samples were obtained from every lymph node after it had been dissected along its greatest diameter. The tissue samples were embedded in paraffin, routinely cut into 4-5 μm sections (at least 3-4 from the primary tumor and each lymph node), attached to glass slides and H&E stained. Pathological analysis was performed according to classification TFG [6,9,25]. We assessed 6 pathological features of the tumor and the type of interaction between the tumor and the surrounding tissues (cytoplasmic differentiation, nuclear differentiation, mitotic figures type and stage of invasion, the presence of lymphocytes invasion). The analysis was performed under a light microscope (magn. 200X, number of mitoses magn. 400X), going by the areas of deepest invasion of the surrounding tissues. Tumor assessment has been presented as the number of scored points. Each factor was graded according to a scale ranging from 1 to 4. Immunohistochemical reactions with policlonal antibodies (NCL-C11, Multi-Cytokeratin 4/5/6/8/10/13/18, RTU-D Novostatin Universal Detection Kit, NCL-L-DAB Liquid DAB Substrate Kit; Novocastra UK) were performed on 2-3 sections obtained from the same lymph node (3-4 μm sections attached to polysin-covered glass slides) in accordance with the manufacturer's directions. For analysis of metalloproteinases expression in tumor front reactions with monoclonal antibodies (Mouse Anti-Human MT1-MMP, Chemicon) were made and immunohistochemical index was used. For analysis of MT1--MMP expression the scale was used: 0 – none, 1 – low expression (<10% positive cells), 2 – medium expression (<50%), 3 – high expression (≥50%). For the sake of pathological analysis of the lymph nodes it was assumed that a micrometastasis is a focus ≤2 mm in diameter. The results of nodal cytokeratin microfilament expression were compared with the morphological features of the tumor and expression of MT1-MMP. In the course of the statistical analysis we applied a Spearman's tests (the significance level was set at $p<0.1$; $R=0.3$) to analyze correlation of chosen features and micrometastases.

Results

In examined group 81.8% cases were diagnosed as advanced cancer involving three regions of the larynx and originating from the supraglottic area. The most numerous group of patients (20/22; 90%) had stage III and stage IV tumors. 16 patients (72.7%) underwent unilateral selective neck dissection, in 2 cases in the form of radical neck dissection. The remaining 6 patients (27.3%) underwent bilateral lymphadenectomy. Pathological analysis of the primary tumor revealed that a majority of the patients presented with squamous cell carcinoma of intermediate differentiation G2 – 12 (54.5%) and distribution of pT feature was: 4 (18.2%) with pT2 tumor, 4 (18.2%) – pT3 and 14 (63.6%) – pT4 tumor. Pathological routine study of H&E stained lymph nodes did not confirm the presence of metastases, however, immunohistochemical evaluation with policlonal anti-CK antibodies confirmed the presence of cancer cells (pN1) within the lymph nodes of 11 (50%) patients. In remaining lymph nodes immunological reactivity was revealed (pN0). The total score obtained in the course of primary tumor assessment acc. to the TFG classification oscillated between 9 and 20 (mean: 15.4 points) and for CKpoli positive tumors and between 12 and 20 (mean: 16.5 points). Distribution of 6 pathological features of the tumor front was estimated. Positive MT1-MMP expression in 15 (68.2%) cases was observed and distribution of matrix metalloproteinases expression acc. to proposed scale was performed (*Fig. 1*, *2*). In study group 12 patients (54.4%) achieved 3-year survival, and 7 (31.8%) 5-year survival. Statistical analysis aimed at the assessment of the correlation of developing nodal micrometastases in relation to the results of pathological analysis revealed the statistical significance for TFG total score ($p<0.09$). Analysis of each morphological feature used for morphologic grading revealed that depth of invasion ($p<0.08$) and number of mitoses per high-power field in cells of laryngeal carcinoma ($p<0.09$) had significant influence on the presence of micrometastases. The statistical analysis showed no significant correlation between expression of matrix metalloproteinases type I in tumor front and the presence of cell carcinoma microfocuses in lymph nodes. Our study showed that the positive policytokeratines stain in the routine pN0 lymph nodes is very useful in prediction of 3- and 5-year survival in patients with laryngeal squamous cell carcinoma as well ($p<0.09$ and $p<0.02$) (*Tab. 1*).

Discussion

On the basis of the several studies performed recently, biologic factors or host-related factors probably play the most important role in determining the eventual disease outcome and maybe thus the presence of occult micrometastses

***Figure 1*. Positive expression of policytokeratines in lymph node (magn. 200X)**

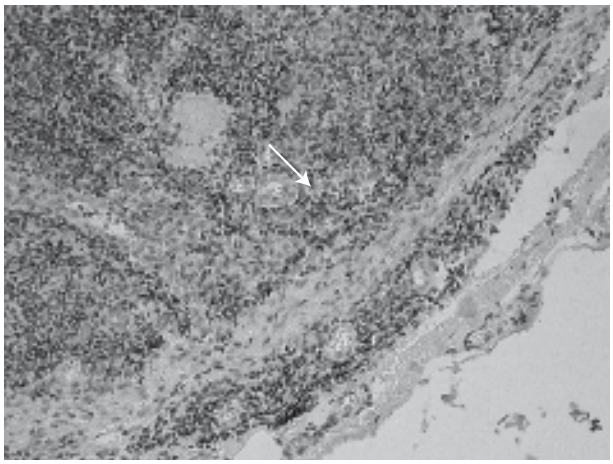

***Figure 2*. Positive expression of matrix metalloproteinases type I (MT1-MMP) in tumor front of laryngeal carcinoma (magn. 200X)**

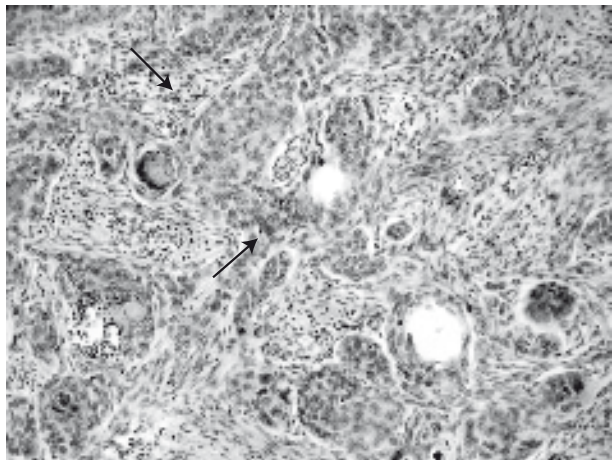

***Table 1*. Analysis of correlation positive expression for CKs (micrometastses) and chosen features of tumor front, MT1-MMp expression and survival**

Feature	R Spearman's	p [<0.1]	t(N-2)
cT	-0.39	0.06	-1.93
cN	-0.39	0.06	-1.93
Number of nodes	-0.41	0.05	-2.06
G	-0.12	0.57	-0.57
TFG	0.36	0.09	1.77
MT1-MMP expression	-0.09	0.73	-0.34
Nuclear differentiation	-0.11	0.60	-0.52
Number of mitoses	0.36	0.09	1.73
Mode of invasion	0.32	0.14	1.52
Stage of invasion (depth)	0.37	0.08	1.81
Plasmalymphocytic invasion	0.23	0.29	1.08
3-year survival	-0.36	0.09	-1.75
5-year survival	-0.48	0.02	-2.50

Statistically significant correlation (R=0.3; p<0.1) was marked with bold faced type

in lymph nodes [1-13]. TFG is the technique, which assesses the dynamics of the tumor growth and provides multifactorial morphologic information about the carcinoma tissue. This classification includes assorted pathological features of the tumor and the types of interactions between the cancerous tissue and the front of the tumor [6-9,24,25]. The results presented here, obtained from 22 laryngeal cancer patients confirm the value of this classification in prognosis of the development of nodal micrometastases. A high total score relates to an increased probability of the presence of foci of single cancer cells within lymph nodes which were originally, in the course of routine assessment, pronounced non-metastatic (pN0). Important factors influencing the probability of nodal micrometastases, and affecting patient prognosis, include the depth of laryngeal wall invasion [6,8,9,24]. Tumors characterized by diffuse invasion with small foci of malignancy were found to be more commonly associated with treatment failure and nodal recurrence. Our studies have shown the value of the depth of invasion for assessing the probability of the presence of occult foci of cancer cells within the lymph nodes. While studying the prognostic factors of laryngeal cancer, pathologists also assess the interactions between the tumor and the surrounding tissues, however, there exist no reports as to the independent prognostic value of these morphological features. Nevertheless, they begin to appear as elements of numerous classifications and scores for the assessment of the primary tumor [9,24,25]. Our own studies performed on the postoperative material obtained from the patients which were aimed at assessing the correlation between the total score acc. to the modified classification of Anneroth, Batsakis and Luna and risk of micrometastses have shown that the achieved score relates to prognosis. A more detailed morphological assessment of laryngeal cancer specimens based on this classification might improve the diagnosis of nodal micrometastases. The risk of nodal micrometastases in laryngeal cancer increases with a high total Anneroth, Batsakis and Luna score (>16 points), diffuse cancerous invasion with single cells and deep invasion of the laryngeal wall involving the cartilages [6,8]. Many authors have undertaken multifactorial morphologic valuation of the tumor in squamous cell carcinoma of other sites introduced similar conclusion [26].

Matrix metalloproteinases type I (MT1-MMP) play an important role in destruction of ECM thus effect on tumor

genesis, angiogenesis and presence of nodal metastases in carcinomas with epithelial and non-epithelial origin [17,18]. Many studies have analyzed the incidence of micrometastases that were not detectable in pN0 lymph nodes with using routine methods in various types of carcinomas. Micrometastases were found in 21-100% cases [10,19,20]. A lot of authors confirmed the meaning of positive expression of MMPs enzymes family in tumor stroma as important indicator of neoplasm invasive growth and prognostic factor of nodal metastases [17,19,21,22]. Neverthelees findings confirmed the role of metalloproteinases expression as prognostic parameter of metastases in lymph nodes are still unclear. Imanishi et al. [18] analyzed a large group of patients suffered from squamous cell head and neck carcinomas confirmed connection MT1-MMP expression with nodal stage. Shimada et al. [23] introduced similar observation. The authors investigated expression of MMP-1,2,3,7,8,9 and 13 in stroma of squamous cell carcinoma of the oral cavity and noted significant increasing risk of nodal metastases in positive group of neoplasm. Dependence the micrometastases presence on expression level of MMPs in carcinomas was confirmed in other study [16,21-23]. Many studies have analyzed the incidence of micrometastases undetectable in pN0 lymph nodes and not disclosed significant connection of the positive matrix metalloproteinase expression in tumor stroma with pN feature [16,19]. Gorogh et al. [27] analyzed the activity of MMPs and TIMPs and noted that MMP-1,9,10 positive expression did not correlate with the presence of the foci of carcinoma cells in lymph nodes. Kawata et al. [16] noted no prognostic significance of MMP-2 expression in head and neck carcinomas for pN stage.

Lymph nodes pathologically defined as negative may contain micrometastases and patients with such neoplastic cells may have a lower rate of disease-free survival than genuinely lymph node-negative patients [10]. Therefore tumors classified as pN0 by routine methods could be reclassified more correctly as pN1 when the lymph nodes are examined immunohistochemically by means of antibody cocktail for cytokeratines. It is important for estimate the prognosis for each patient. In this study the presence of micrometastases was significantly correlated with 3- and 5-year survival and the percentage was 36,4% and 9,1% for patients with poliCKs-positive lymph nodes. Experience with micrometastses in other tumor types confirmed our conclusions [1,13,28,29]. Lee et al. [1] demonstrated that the percentage of 5-year survival in patients with squamous cell carcinoma of the stomach and corrected pN0 (49%) is significantly lower than in pN- group (76%). Ishida et al. [13] indicated that the presence of micrometastases as indispensable factor determining the prognosis of gastric carcinoma patients. Every authors no confirmed such conclusions. Glickman et al. [11] did not support that the presence of lymph nodes micrometastases in adenocarcinoma and squamous cell carcinoma of the esophagus is an independent poor prognostic factor. Although in study group the follow-up of the patients was only 2 years and it could not be indifferent for the results.

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Concentration of TGF-β1 in the supernatant of peripheral blood mononuclear cells cultures from patients with early disseminated and chronic Lyme borreliosis

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Abstract

Purpose: The aberrant inflammatory response is probably involved in the pathogenesis of chronic Lyme borreliosis, including chronic Lyme arthritis and neuroborreliosis. Transforming growth factor-beta 1 (TGF-β1) is an important anti-inflammatory and immunomodulatory cytokine and its deficient synthesis is linked to exaggerated inflammation and immune response.

Material and methods: Peripheral blood mononuclear cells (PBMC) from 25 patients with Lyme borreliosis and 6 controls were incubated for 7 days with suspension of *Borrelia afzeli*, *B. garinii* and *B. burgdorferi* sensu stricto spirochetes. TGF-β1 concentration in culture supernatants was measured with ELISA. Results were analyzed according to disease duration (group I – chronic borreliosis, n=20; group II – early borreliosis, n=5) and clinical form (LA – arthritis, NB – neuroborreliosis).

Results: TGF-β1 concentration was increased in supernatants of PBMC cultures of patients with early neuroborreliosis, in comparison with chronic borreliosis and controls. In chronic, but not in early borreliosis, there was a tendency for decrease of TGF-β1 synthesis under stimulation with *B. burgdorferi* spirochetes.

Conclusions: Impaired synthesis of TGF-β1 by mononuclear cells seems to be present in patients with chronic forms of Lyme borreliosis when compared to those with early stage of the disease. It may be a factor contributing to the persistence of inadequate inflammatory response in patients in whom chronic form of the disease develops.

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Introduction

Lyme borreliosis is a chronic and multiform infectious disease caused by a spirochete *Borrelia burgdorferi* sensu lato (*B. burgdorferi* s.l.) transmitted to humans from animal reservoir by *Ixodes* sp. tick. Three clinical stages: localized, early disseminated and chronic infection are distinguished in the course of the disease, with lesions typically developing within skin (erythema migrans, acrodermatitis chronica atrophicans), musculoskeletal system (Lyme arthritis) and central and peripheral nervous system (neuroborreliosis) [1,2]. Arthritis symptoms range from brief attracts of arthralgia, through intermittent episodes of arthritis to chronic arthritis and/or persistent musculoskeletal pain. Central nervous system involvement may manifest itself early as comparatively mild meningitis or in chronic form as a progressive encephalopathy or leukoencephalitis, the later presenting with focal demyelination and some clinical resemblance to sclerosis multiplex [2,3]. Response to antibiotic therapy tends to be good in early and more variable and generally poorer in later stage of the disease, with some patients exhibiting treatment-resistant symptoms, pathogenesis of which has not been fully explained [2,3]. *B. burgdorferi* s.l. expresses surface lipoproteins with potent pro-inflammatory properties, however, it does neither produce toxins nor damage the tissues directly, and spirochete number within affected tissue is relatively low with regard to the severity of the lesions. In some patients with chronic Lyme arthritis symptoms persist even after *B. burgdorferi* s.l. DNA becomes undetectable within the lesions [4]. Development of the chronic, antibiotic-resistant form of the disease is associated with certain types of major histocompatibility complex (MHC) molecules and some features of humoral and cellular response against *B. burgdorferi* s.l., especially with strong response to OspA lipoprotein [4-6].

These observations led to assumption that it is not active infection, but rather inability to control inflammatory and immune reaction against *B. burgdorferi* s.l., or even autoimmunity triggered by the infection, which contribute to the pathogenesis of chronic Lyme arthritis, and possibly also chronic neuroborreliosis [2,5-8]. However, the mechanisms of autoimmunity remain enigmatic, and the role of proposed self-antigens has not been confirmed [5].

TGF-β1 is a pleiotropic cytokine with a wide range of anti-inflammatory and immunosuppressive effects, antagonistic towards a wide array of pro-inflammatory cytokines. It plays unique role in ensuring control and proper termination of inflammatory reaction, and its deficient synthesis has been observed in autoimmune disorders [9-14]. As such, TGF-β1 impaired synthesis could be possibly involved in the pathogenesis of the chronic Lyme borreliosis, but so far data on its role in this disease has been few.

The inflammatory infiltrates in Lyme borreliosis are typically mononuclear, with presence of lymphocytes specifically recognizing *B. burgdorferi* s.l., and the generalized character of the infection is reflected by the changes in peripheral blood lymphocyte subpopulations as well [8,15-17]. In this study, we used peripheral blood mononuclear cells (PBMC) as an indicator of immune and inflammatory processes undergoing in the disease lesion, measuring concentrations of TGF-β1 in the supernatant of PBMC cultures from patients with early disseminated as well as chronic Lyme borreliosis, incubated with *B. burgdorferi* s.l. spirochetes, with an aim of detecting a possible impairment of TGF-β1 synthesis.

Material and methods

Patients

The study group consisted of 25 patients with Lyme borreliosis (6 women and 19 men) aged from 24 to 62 years ($\bar{x}$±SD=46.6±9.2), hospitalized in years 2004-2005 in the Department of Infectious Diseases and Neuroinfections of the Medical University of Białystok. Patients were qualified for the study according to diagnosis made by a treating physician and documented in a medical record. All patients reported tick bites or frequent exposure to bites in endemic areas and had clinical symptoms suggestive of Lyme borreliosis. All patients had antibodies against *B. burgdorferi* s.l. detected in serum. Basic laboratory tests (ESR, leukocyte count) showed no signs of acute inflammatory response. Dependent on clinical features, other examinations (rheumatoid factor detection and other serological tests, radiological examination of the joints) were performed to exclude other possible etiologies. Lyme arthritis (LA) was diagnosed in patients with musculoskeletal pain, especially affecting the large joints of the extremities. Neuroborreliosis (NB) was diagnosed on the basis of the abnormalities in the neurological examination, inflammatory changes in cerebrospinal fluid (CSF) examination and presence of antibodies against *Borrelia burgdorferi* s.l. in the CSF. Twenty patients with the history of complaints persisting or recurring for more than 6 months (in 18 patients – more than a year, maximum – 8 years) constituted the group of chronic borreliosis (group I) – 4 women and 16 men, mean age – 48.4±8.4 years. They all had been at least once treated with doxycycline and/or III generation cephalosporin, typically with a temporary improvement, after which ailments recurred. Early disseminated borreliosis was diagnosed in 5 patients (2 women and 3 men, aged 39.8±10.1) with symptoms present for less than 6 months and anti-*B. burgdorferi* s.l. IgM class antibodies detectable in serum (group II). Diagnosis of LA was established in 21 patients: 19 from group I and 2 from group II, and NB in 9 patients, including 6 in the group I and 3 in the group II (5 patients, all from the group I, had both symptoms of LA and neuroborreliosis). The control group consisted of 6 patients (3 women and 3 men, aged 41.7±11.6), without suspicion of Lyme borreliosis or after it was excluded, all without detectable antibodies against *B. burgdorferi* s.l in serum and without any features of acute inflammatory response. All participants gave a conscious consent and a study design was approved by the Ethical Committee of the Medical University of Białystok.

Detection of anti-B. burgdorferi s.l. antibodies

Antibodies against *Borrelia burgdorferi* s.l. were detected in a sample taken on EDTA with ELISA, using a kit from Biomedica (Boston, USA), according to manufacturer's instructions. The kit included panel of following recombinant antigens: *B. burgdorferi* s.l. p21 (OspC), *Borrelia garinii* p41 and *Borrelia afzelii* p41 to detect IgM and p21, *B. garinii* p41, *B. afzelii* p41, p18 and p100 to detect IgG. The results were expressed in BBU/ml (Biomedica Borrelia units/ml), with >11 BBU/ml considered as positive. The same diagnostic kit was used for antibody detection in CSF.

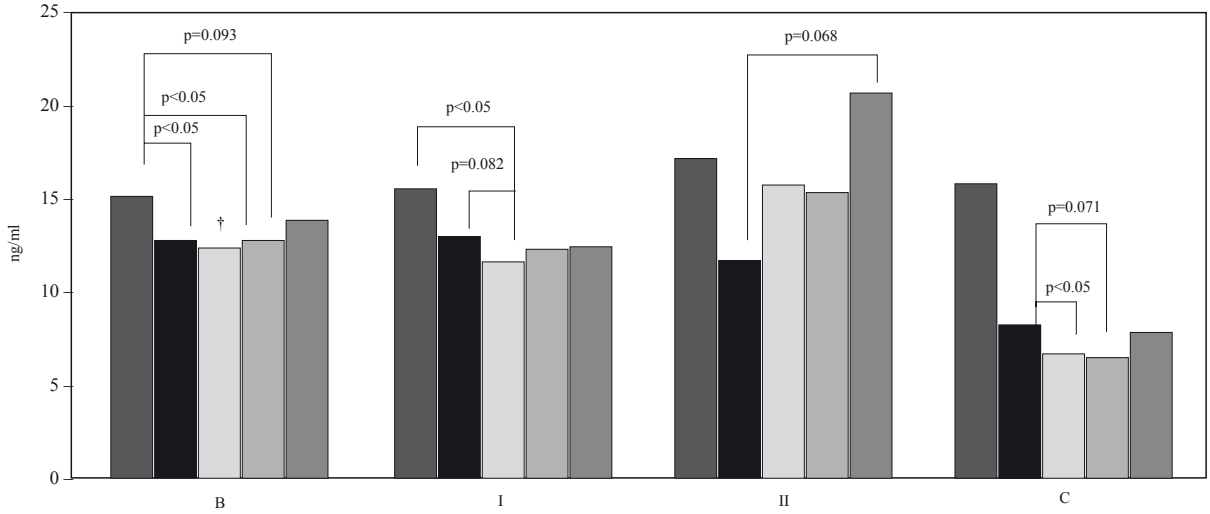
Cell culture

The samples were obtained before the start or during the first week of antibiotic therapy. Ten ml of venous blood was taken to heparnised tubes and further processed within 30 min. PBMC were isolated by centrifugation in Gradisol G medium (Aqua Medica, Poland) at 400 g for 30 min. After centrifugation PBMC were suspended in the culture medium RPMI 1640 (Biomed, Poland) and rinsed again. Then they were resuspended in the concentration of 2 x 10^6 cells/mL in RPMI 1640 with addition of 10% inactivated bovine serum, streptomycin and penicillin. The suspension of 10^5 cells/well was incubated in the 24-well plate Nunclon Multidishes (Nunc Brand Products, Denmark), in 5% CO_2, at 37°C, with the culture medium alone as a negative control, culture medium and phytohemagglutinin (Biomed, Poland) as a positive control and suspension of spirochetes *B. afzelii* VS 461 (ATCC 51567) (B.a.), *B. garinii* 20047 (ATCC 51383) (B.g.) and *B. burgdorferi* s.s. B31 (ATCC 35210) (B.ss.) at the concentration of 10^8 cells/well. Culture conditions and time were based on our previous experiments and selected to enable maximum PBMC response to antigenic stimulation [16]. After 7-day incubation, the culture was centrifuged and the supernatant was frozen and stored in -70°C until being tested.

TGF-β1 detection

In the supernatant, TGF-β1 concentration was determined by ELISA, using the kit from Bender MedSystems (Vienna,

Figure 1. **Mean concentration of TGF-β1 in the supernatant of the culture of PBMC from patients with Lyme borreliosis and from controls. B – patients with borreliosis in total (n=25), I – patients with chronic borreliosis (n=20), II – patients with early disseminated borreliosis (n=5), C – controls (n=6). Concentrations were determined in the supernatant obtained after 7-day culture. Bars from the left to the right in each group, represent, respectively: no stimulation, stimulation with phytohemagglutinine and stimulation with antigens of *Borrelia afzelii*, *Borrelia garinii* and *Borrelia burgdorferi* sensu stricto (see: Material and methods). Significant differences between the cultures within the groups are marked on the chart. † – borderline difference (p=0.079) in comparison with controls**



Austria), following the manufacturer's instructions. The detection limit of the test according to the manufacturer was 0.06 ng/ml and the standard curve covered the concentration range from 0.47 to 30 ng/ml.

Statistical analysis

Results were analyzed in the group of patients with Lyme borreliosis as a whole and in the subgroups defined on the basis of the duration of the disease (chronic borreliosis – I, early – II) and clinical form (LA and NB). There were only two patients with early LA, so this group was not included in the analysis. LA and NB, as well as chronic LA and chronic NB groups were not compared directly with one another, since they overlapped due to coexistence of articular and neurological symptoms in some patients. Analysis was performed with SSST software. The Mann-Whitney test was used to compare groups. Comparisons between the cultures within groups were performed using t test for dependent samples. $p<0.05$ was regarded as significant and $p<0.1$ as a borderline significance.

Results

Results for the group of patients with Lyme borreliosis as a whole, group I, II and controls, with statistical interpretation, are presented in *Fig. 1*. Analogous data for neuroborreliosis patients are shown in *Fig. 2*. The results for patients with LA and chronic LA did not differ from those found in group I and are not presented separately.

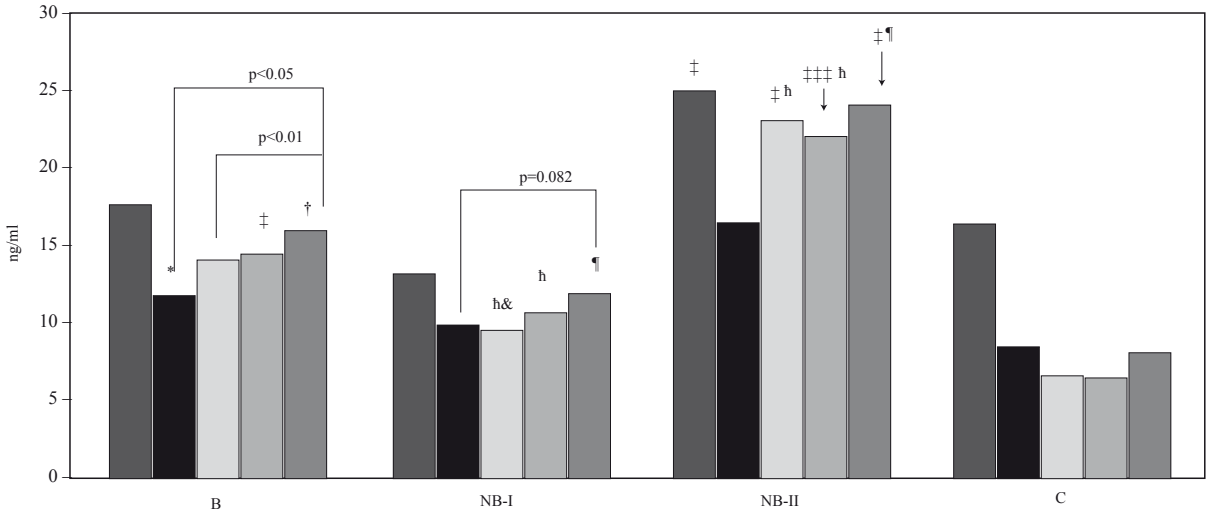
The mean concentration of TGF-β1 in the supernatant tended to decrease in the cultures stimulated with phytohemagglutinin in comparison with unstimulated ones. In the antigen-stimulated cultures in controls and group I, there was a tendency for TGF-β1 concentration decrease to values lower even than at phytohemagglutinin stimulation. In the NB group and in group II, such a tendency was not present, and concentrations in the antigen-stimulated cultures tended to be higher than with the phytohemagglutin stimulation, which was statistically significant for B.ss. stimulation.

TGF-β1 concentrations showed a tendency to increase in patients with borreliosis in comparison with controls, however the difference was statistically significant only in the NB group, especially in early neuroborreliosis patients (NB-II), in which TGF-β1 concentrations were significantly higher than in controls both under antigenic stimulation and without stimulation. In NB-II the concentration of TGF-β1 in antigen-stimulated cultures was also significantly or with borderline significance higher than in chronic neuroborreliosis (NB-I) and Lyme arthritis groups (LA).

Discussion

TGF-β1 is a potent anti-inflammatory cytokine, released by most of the cell types, especially by leukocyte populations, including activated macrophages and lymphocytes [18,19]. It is released in latent form, which requires further activation to exert its biologic effects. Increased synthesis of TGF-β1 in the final stages of inflammatory and immune response establishes anti-inflammatory local environment and promotes tissue repair, by stimulating release of other anti-inflammatory factors, counteracting pro-inflammatory cytokines, inhibiting functions of B and T lymphocytes and finally stimulating local fibrosis and angiogenesis [11,14,18,19]. In animal models of inflammation and autoimmunity, TGF-β1 appears to be a factor essential for termination of the inflammation and resolution of inflammatory infiltrate, both in Th1 and Th2-dependent type of inflammatory response [9,10,13]. Administration of TGF-β1 has been shown to prevent or ameliorate symptoms and histological features of inflammation in animal model of sclerosis

***Figure 2*. Mean concentration of TGF-β1 in the supernatant of the culture of PBMC from patients with neuroborreliosis and from controls. NB – patients with neuroborreliosis in total (n=9), NB-I – patients with chronic neuroborreliosis (n=6), NB-II – patients with early neuroborreliosis (n=3), C – controls (n=6). Concentrations were determined in the supernatant taken after 7-day culture. Bars from the left to the right within each group represent, respectively: no stimulation, stimulation with phytohemagglutinine and stimulation with antigens of *Borrrelia afzelii*, *Borrelia garinii* and *Borrelia burgdorferi* sensu stricte (see: Material and methods). & – borderline difference in comparison with lack of stimulation (p=0.068), * – significant difference in comparison with lack of stimulation (p<0.05), other differences between cultures are marked directly on the chart; † – borderline difference (p=0.086) in comparison with controls; ‡ – significant difference in comparison with controls, p<0.05; ‡‡‡ – highly significant difference in comparison with controls, p<0.001; ¶ – borderline difference between patients with early and chronic neuroborreliosis, p=0.059; ħ – significant difference between patients with early and chronic neuroborreliosis, p<0.05**



multiplex, as well as in acute and chronic arthritis [9,20,21]. Mokhtarian et al. observed that peripheral blood lymphocytes from patients with sclerosis multiplex synthesize decreased levels of TGF-β in culture, which coincided with increased synthesis of pro-inflammatory cytokines [12]. Moreover, the defective production of TGF-β correlated with clinical activity of the disease [12]. As the inability to properly control and terminate inflammatory response seems to underlie pathogenesis of chronic Lyme borreliosis, impaired synthesis of TGF-β1 might be of importance in this group of patients as well.

To our knowledge, the only study assessing the TGF-β1 in human Lyme borreliosis was conducted by Widhe et al., who measured serum concentrations of TGF-β1 in patients with erythema migrans (early, localized stage of Lyme borreliosis) and in the early and late stage of neuroborreliosis. The authors monitored the patients for several months and checked for correlation of the early TGF-β1 levels with further clinical course of the disease. The serum concentration of TGF-β1 in early borreliosis appeared to be higher in patients with a favorable course leading to recovery in comparison with those in whom borreliosis finally developed into the chronic stage [21]. In the same study, analogous observation was made for cerebrospinal fluid concentration of tumor necrosis factor-a (TNF-α), a strong pro-inflammatory factor. According to the authors, the synthesis of TNF-α in the early stage of the disease is a sign of a potent inflammatory response leading to eradication of spirochetes, while a high level of TGF-β1 is a marker of the effectiveness of an anti-inflammatory reaction, preventing prolonged inflammation and further tissue damage [22]. High levels of TGF-β1 were also observed in the sera of patients with erythema migrans and persisted for several months after successful treatment, which supports the importance of this immunoregulatory factor in recovery from Lyme borreliosis [22].

In our previous studies we assessed concentration of transforming growth factor-β1 (TGF-β1) in sera of patients with different clinical forms of Lyme borreliosis, observing weakly increased TGF-β1 concentration in Lyme arthritis after antibiotic treatment in comparison with controls [23]. No change in TGF-β1 concentration was observed in patients with erythema migrans or neuroborreliosis. Afterwards, we measured TGF-β1 concentration in the supernatant of *B. burgdorferi* s.l. antigens-stimulated culture of peripheral blood mononuclear cells (PBMC) obtained from a small group of 10 patients with chronic Lyme arthritis, finding no significant differences in comparison with unstimulated cultures and cultures of PBMC from healthy persons [24].

In our present study, concentrations of TGF-β1 in the PBMC culture supernatant from patients with chronic, treatment resistant Lyme borreliosis, tended to decrease in presence of *B. burgdorferi* s.l. spirochetes. This phenomenon suggests development of pro-inflammatory environment in response to borrelial antigens in this group of patients. In patients with early infection concentration of TGF-β1 did not decrease in the presence of *B. burgdorferi* s.l. antigens and was generally higher in comparison with patients with chronic borreliosis and controls. This group of patients was small and not all clinical forms of Lyme borreliosis were equally represented, especially we were not able to include sufficient number of patients with early Lyme arthritis into the study, which makes the results more difficult to interpret, however, some differences seem apparent. The increase of TGF-β1 under stimulation with *B. burgdorferi* s. l. in early neuroborreliosis, statistically significant in spite of

a small number of patients in this group, is especially of note. It is paralleled by the clinical difference between this subacute, antibiotic-responding form of Lyme borreliosis, and late, poorly responding to treatment manifestations present in other patients, in whom analogous TGF-β1 response was apparently absent. This observations are in accordance with the interpretation of serum measurements in erythema migrans and neuroborreliosis as presented by Widhe et al. [22]. This finding could be further confirmed by studying TGF-β1 synthesis by mononuclear cells directly isolated from the disease lesion, especially from synovial fluid or synovium of patients with Lyme arthritis.

The results suggest a shift of the balance towards the pro--inflammatory response in patients with chronic, treatment resistant Lyme borreliosis. An ability to control an inflammatory and immune response against *Borrelia burgdorferi* s.l. spirochetes, present in an early stage, may be impaired in patients in whom Lyme borreliosis changed to a chronic form and impaired synthesis of TGF-β1 by patients' mononuclear cells in response to *B. burgdorferi* s.l. seems to be involved in the pathogenesis of this phenomenon.

Acknowledgments

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Prevalence of *Chlamydia trachomatis* infection in women with cervical lesions

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Abstract

Purpose: The study objective was to evaluate the prevalence of *Chlamydia trachomatis* (*C. trachomatis*) infection in women with and without pathological lesions in the uterine cervix.

Material and methods: A total of 120 patients, aged 15-57 (mean age 29), recruited for the study, were referred by gynaecological clinics in the Podlasie province. Gynaecological examinations confirmed cervicitis accompanied by erosions in 75 patients (group I) and cervicitis alone in 45 women (group II). The comparative group (control) consisted of 35 women, aged 16-48 years (mean age 29), who had no clinical symptoms or pathological lesions in the cervix.

Direct immunofluorescence tests (MicroTrack, Syva) or polymerase chain reaction assays (PCR, Roche) were used to detect *C. trachomatis* infection in cervical samples. Anti-chlamydial IgG antibodies in the serum were determined using an immunoenzymatic assay (*C. trachomatis* IgG, EIA medac).

Two-frequencies test was used for the statistical analysis of results. P values of <0.05 were considered statistically significant.

Results: In the direct tests, *C. trachomatis* infection was found in group I in 9/75 women (12.2%), in group II in 9/45 (20%) and in the comparative group in 1/35 (2.9%) (group I vs control p>0.1252; group II vs control p<0.025). IgG specific antibodies were detected in group I in 17/49 patients (34.7%), in group II in 5/18 (27.8%) and in the comparative group in 2/35 (5.7%) women (group I vs control p<0.0022; group II vs control p<0.0319).

Conclusions: Our results show a higher prevalence of *C. trachomatis* infection in female patients with cervical lesions as compared to unaffected women, thus suggesting that diagnostic tests for *C. trachomatis* infection should be included in the screening programs for women.

Key words: *Chlamydia trachomatis*, cervicitis, cervical erosion, anti-chlamydial trachomatis antibodies.

Introduction

Chlamydia trachomatis (*C. trachomatis*) according to CDC (Centre for Disease Control and Prevention in Atlanta) is one of the most frequently detected sexually transmitted bacterial pathogens [1]. Chlamydial infections in women have major epidemiological and clinical significance, and are usually asymptomatic (in up to 80% of all cases) [2]. The most common clinical manifestation of *C. trachomatis* infection in women is cervicitis, being associated with the affinity of the chlamydial pathogen for epithelial cells. In the literature, this ailment is usually called "mucopurulent cervicitis" and characterized by congestion of the vaginal part of the uterine cervix, which bleeds easily, and by the presence of mucopurulent secretion from the cervical canal [3]. The relationship between *C. trachomatis* infection and cervicitis was first described by Dunlop et al. in 1966 [4].

Recently, a relationship has been observed between exposure to *C. trachomatis* infection and subsequent development of cervical precancerous or cancerous lesions [5-8].

The aim of the current study was to evaluate the incidence of *C. trachomatis* infection in women with and without pathological lesions in the uterine cervix.

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Figure 1. **The result of direct diagnostics in women with pathological cervical changes and in comparative group**

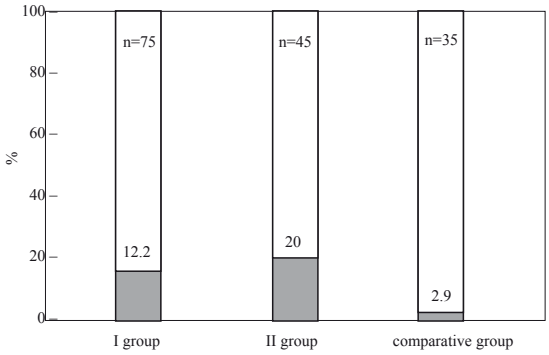


Figure 2. **The result of serological studies in women with pathological cervical changes and in comparative group**

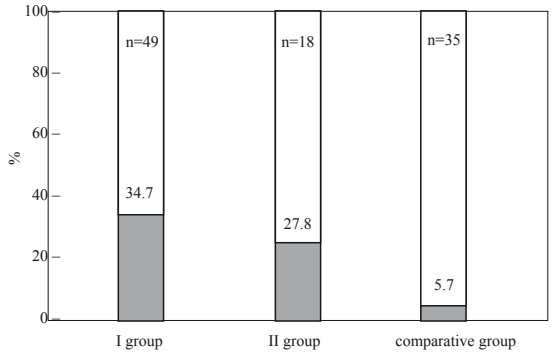
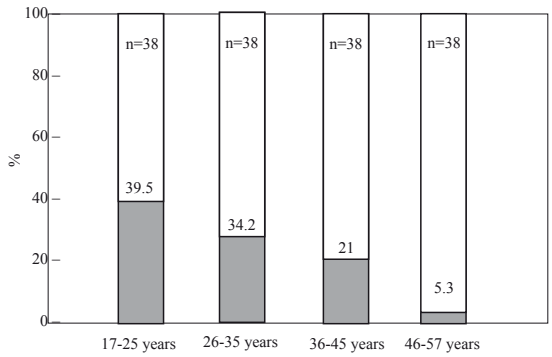


Figure 3. **Prevalence of *C. trachomatis* infection in age groups**



Material and methods

The study involved 120 women, aged 17-57 (mean age 29), who were referred to the Centre for STD Research and Diagnostics in Białystok by gynaecological clinics in the Podlasie province. All the patients came with a previously established gynecological diagnosis to undergo testing for *C. trachomatis* infection. Among them, 75 had cervicitis with erosion (group I) and 45 had cervicitis alone (group II). None of the patients had received antibiotic treatment during the preceding 3 months. Mucopurulent vaginal discharge was the predominant clinical symptom.

The comparative group consisted of 35 women aged 16-48 (mean age 29), with no clinical symptoms or pathological lesions in the cervix.

Endocervical swabs and blood samples were obtained for analysis. Direct testing methods were used in all the patients and serological tests were performed in 49 women from group I and in 18 patients from group II.

Direct immunofluorescence tests (MicroTrack, Syva) or polymerase chain reaction assays (PCR, Roche) were performed to detect *C. trachomatis* antigen and/or genetic material. Immunoenzymatic assays (Chlamydia IgG EIA, medac) were used to detect specific IgG antibodies against *C. trachomatis*. According to the manufacturer's instruction, the ISR value of >1.1 was considered positive.

Additionally, we evaluated the incidence of *C. trachomatis* infection according to the patients' age, analyzed data concerning contraceptive methods used by each patient and referred to demographic data, i.e. place of residence and education.

Two-frequencies test was used for the statistical analysis of results. P values of <0.05 were considered statistically significant.

Results

In group I (patients with cervicitis accompanied by erosion), *C. trachomatis* infection was detected by direct methods in 9/75 women (12.2%), in group II (cervicitis without erosion) in 9/45 (20%). In the comparative group, *C. trachomatis* was found in 1/ 35 (2.9%). The results are shown in *Fig. 1*.

Positive results of IgG anti-*C. trachomatis* specific antibodies were detected in 17/49 women (34.7%) in group I and in 5/18 (27.8%) in group II (p>0.5958). In the comparative group, specific antibodies were present only in 2/35 cases (5.7%) (group I vs control p<0.0022; group II vs control p<0.0319). Serology results in women with cervical lesions (group I and II) and in the comparative group are shown in *Fig. 2*.

The percentage of chlamydia-positive patients was the highest in the age group of 17-25 (39.5%), while the lowest in the under – 57 (5.3%) – *Fig. 3*.

Most of the women with chlamydial infection were city dwellers (92.1%-35/38), and only 7.9% (3/38) lived in the country. In this group, 31.6% of patients (12/38) had secondary education, 28.9% (11/38) had technical education, 26.3% (10/38) had higher education and 13.2% (5/38) primary education. Also in this group, 23.7% (9/38) gave a history of taking hormonal contraceptives. None of the patients used barrier contraception methods such as intrauterine devices or condoms.

Discussion

The role of *C. trachomatis* infection in cervical lesions has been the focus of numerous researches. Literature data show high incidence rates of chlamydial infection in women with cervicitis alone (20-40%) [9] and with cervicitis accompanied by erosion (50- 80%) [10,11]. Using either the direct or serological tests we found no statistically significant differences between group I and group II.

However, in our study a higher percentage of patients with cervical lesions were *C. trachomatis*-positive, as compared to

the control. In the direct test, *C. trachomatis* was detected in 12.2% of the patients in group I, in 20% in group II and in 2.9% in the comparative group. Similar results were reported by Qian, who found *C. trachomatis* infection in 13.4% of women with cervical erosion, the rate being substantially higher than in lesions-free patients [12]. We found statistically significant differences between group II and the comparative group in the direct tests for *C. trachomatis*, but not between group I and the control.

In the literature, *C. trachomatis* IgG antibodies in patients with symptoms of cervicitis were detected in 30-40% of patients [13,14], which is consistent with our findings. The slightly lower rate of positive results in our study as compared to earlier reports of other authors might be due to differences in the quality of diagnostic methods, which are currently more specific.

Recent scientific reports have indicated a possible role of *C. trachomatis* infection in the development of neoplasia as well as cervical carcinoma. Some authors reported a high percentage of chlamydia-positive patients with previously detected HPV infection (47.7-65.7%) [7,15]. Anttila et al. point at the role of *C. trachomatis* infection as an independent factor in the development of dysplasia and cervical carcinoma [5].

C. trachomatis infections are most commonly detected in women under 25 years of age [8,16]. We found as many as 39.5% of chlamydia-positive patients in the age group of 17-25.

A literature survey suggests that the use of hormonal contraceptive methods increases the risk of *C. trachomatis* infection [17,18]. Hormonal contraception promotes sexual activity and frequent changes of sexual partners, thus leading to cervical ectopy. In our study, among the *C. trachomatis*-positive patients only 23.7% used oral contraceptive pills, as most of them were married or had one sexual partner.

As revealed by demographic analysis, the majority of women with chlamydial infection originate from towns and have higher education. This is probably associated with better availability of diagnostic procedures and easier access to information concerning sexually transmitted infections in larger towns.

Conclusions

1. *C. trachomatis* infection is more common in women with pathological cervical lesions as compared to those without.
2. Our results show the necessity to include screening for chlamydial infection in the prophylactic schemes for women.
3. No statistically significant differences were found in the prevalence of *C. trachomatis* infection between women having and not having cervical erosions.

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Opinions of gynaecologists on prenatal diagnostics in first/second trimester and abortion – ethical aspect

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Abstract

Purpose: The aim of the study was the assessment of the influence of ethics or the lack of medical ethics on everyday gynaecological practice, particularly the usefulness and purpose of detecting genetic irregularities in the first and second trimester and abortions.

Material and methods: A sample of 164 gynaecological doctors was encompassed by the study. A questionnaire survey was applied as an independent empirical procedure on the basis of the theory of attitudes and the following questionnaires: WCQ (The Ways of Coping Questionnaire) – Folkman, Lazarus, Dukiel – Scheier & Weintraub, as the authors own adaptation of that instrument for the requirements of the study.

Results: In response to the question on the purpose of performing prenatal diagnostics in detecting genetic irregularities in the first and second trimester – 35% of physicians were against such diagnostics if it served abortion, 60% supported the test even if in consequence an abortion was carried out, whereas 5% had no stance on the matter. The problem of physicians' approach to abortion for so-called "social reasons" was also studied. Over half, as many as 51% of physicians were against abortion in any form whatsoever, including pharmacological abortions; 45% agreed to abortion and 4% had no opinion. The veracity of ethical motivations was also measured: approx. 4%, refrained from expressing their support of either position; 29% stated that a physician, although they do not perform abortions themselves, should indicate other possibilities of performing the abortion and as many as 67% thought that the indication of a place or a person who performs abortions is obvious.

Conclusions: The results of the survey indicate the differences in the attitudes of physicians towards the diagnosis of prenatal tests, especially the ones revealing genetic defects and lethal disease. There are two ambivalent patterns of behaviour: one group of physicians opt for delivering every child without any exceptions, but the other one is for destroying deformed foetuses.

Key words: opinions of ginaecologists, prenatal diagnostics, abortion, ethical aspect.

Introduction

A physician gynaecologist-obstetrician, due to the specificity and nature of the work that involves accompanying the development of new life and its birth while at the same time being aware of the situation threatening that life in the form of abortion, the use of contraceptives as early abortive measures and performing experiments on conceived embryos – regardless of the existing legislation – very often has to make a clear decision in support of life or against the conceived human life [1-9]. The aim of the study was the assessment of the influence of ethics or the lack of medical ethics on everyday gynaecological practice. Physicians were posed with questions from the field of prenatal diagnostics, particularly the usefulness and purpose of detecting genetic irregularities in the first and second trimester, they were also asked about abortion for so-called 'social reasons' and whether the physician who does not carry out abortions should indicate a place that does.

Material and methods

A total of 164 gynaecologists with first (30%) and second (53%) degree specialisation were encompassed by the study and partly (17%) during the course of their specialisation

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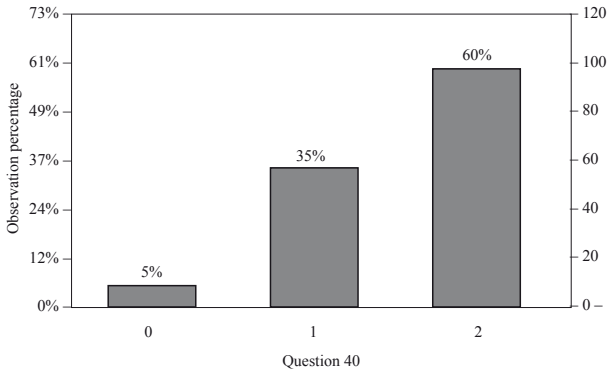
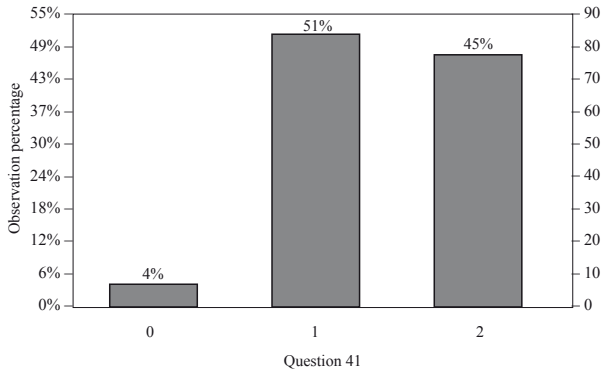
Figure 1. **Opinions of physicians on prenatal diagnostics**



0 – no opinion; 1 – negative opinion, if the test is to serve abortion and positive if it is not for abortion purposes; 2 – positive opinion also if an abortion is to be carried out

Figure 2. **Opinions of gynaecologists on abortion for so-called "social reasons"**



0 – no opinion; 1 – negative opinion regarding abortion; 2 – positive opinion regarding abortion

according to the new regulations. The study was conducted during the years 2002-2006. The physicians were usually studied during various types of courses. Specialist training, conventions and different types of conferences lasting several days created favourable conditions to standardise the conditions of the conducted research. The anonymous survey was and carried out in Polish. It was given to respondents, who were supposed to complete them at home and bring them back in the sealed envelopes. The questioned placed the envelopes in the boxes personally. 221 copies of survey were distributed, 35 were not returned, 22 were incomplete, so only 164 were qualified. A questionnaire survey was applied as an independent empirical procedure on the basis of the theory of attitudes and the following questionnaires: WCQ (The Ways of Coping Questionnaire) – Folkman, Lazarus, Dukiel – Scheier & Weintraub, as the authors own adaptation of that instrument for the requirements of the study [10-12]. WCQ – The Ways of Coping Questionnaire stirred a lot of interest in Poland. The following three consecutive adaptations are testimony to this: the adaptation performed under the supervision of Wrześniewski, by Łosiak and by the Heszen – Niejodek team [13-15].

Results

In response to the question on the purpose of performing prenatal diagnostics in detecting genetic irregularities in the first and second trimester – 35% of physicians were against such diagnostics if it served abortion ("served" in this context means "enable"), 60% supported the test even if in consequence an abortion was carried out, whereas 5% had no stance on the matter (*Fig. 1*).

Physicians who were against abortion (35%) justified their standing (in the commentary to the questionnaire) in the following manner:

"I am against if the defect is lethal, the situation will be solved in any way; one should simply teach parents to accept a handicapped child and to look for a place for the child in society; if the prenatal diagnostics are to serve carrying out an abortion – I am definitely against; if the genetic testing are to serve the selection of damaged fetuses in order to encompass pregnant women with greater care – I am in support of the testing, but if the tests will only serve abortions, I am against; yes – for the purpose of further care over the mother and fetus/ /child, no – for abortion purposes; I am in support of prenatal testing due to the possibility of intrauterine therapy of certain fetus illnesses; I have seen complications after such tests many a time that have, for instance, ended in a miscarriage, e.g.: in the case of a 37-year-old primipara when the results of the tests turned out to be correct".

Different opinion of physicians (60%) who clearly opted for prenatal testing even if it resulted in an abortion are the following:

"In Poland, prenatal diagnostics has no substantiation for what to do if there is a defect and the pregnancy cannot be terminated, the patient is left for twenty weeks to let her think it over but nothing will come of it anyway; if we are taking care of the psyche of the patient then tests are imperative as one can always have an abortion; I always encourage my patients to undergo testing, prenatal testing should always be performed for medical reasons regardless of the consent of the patient; I am in support of prenatal testing if the patient knows what to do if a defect is discovered in the child".

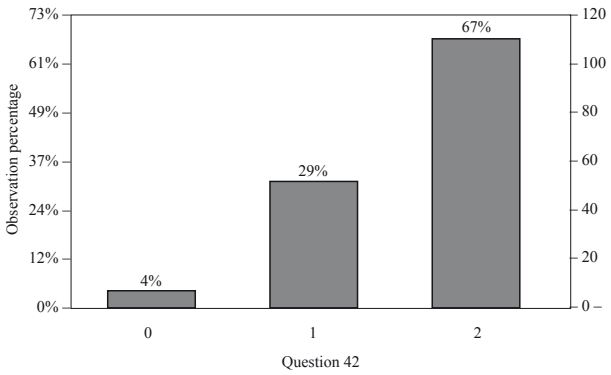
The problem of physicians' approach to abortion for so-called "social reasons" was also studied (*Fig. 2*). Over half, as many as 51% of physicians were against abortion in any form whatsoever, including pharmacological abortions; 45% agreed to abortion and 4% had no opinion. The gynaecologists who were defending life (51%) presented their stance (in the commentary to the questionnaire) among others in the following manner:

"I am against in the situation when infertility is becoming a social problem, abortion is pointless and a crime; it should not take place; there are no such indications, I hope that it will never happen, so many families cannot have children; I have no opinion on the matter but I don't perform them".

The viewpoints of physicians (45%) who permit abortion are as follows:

"the decision should be the work of the patient and the physician; this is the best solution as women have abortions illegally

***Figure 3*. Ethical dilemmas connected with indicating a place for the abortion by physicians who do not perform abortions for ethical reasons**



0 – no opinion; 1 – they should not indicate; 2 – they should indicate

anyway; I am for it, I believe that before the abortion a long talk is necessary with the physician or a consultation with a psychologist spread out in time; it should be admissible; I am "for", although I think that it is murder; I support it along with sterilisation procedure if the patient wishes to do so; I can't judge because I don't know how I would behave; it always has to be the decision of the person concerned; there would be no problem if contraception was cheap; I don't carry out abortions on healthy fetuses; everyone understands it in their own conscience".

The veracity of ethical motivations was also measured by posing the following question: "According to you, should a physician who does not perform abortions for ethical reasons indicate the possibility of performing the abortion somewhere else?" Not many gynaecologists, approx. 4%, refrained from expressing their support of either position; 29% a physician stated that do not perform abortions themselves and should not indicate other possibilities of performing the abortion and as many as 67% thought that the indication of a place or a person who performs abortions is obvious (*Fig. 3*).

Here are some examples of statements of physicians who are against indicating a place that performs abortions:
"the refusal to perform an abortion by a physician may influence the decision as to the abortion itself; I am definitely against; No, I would not like to be forced to do this; I do not indicate the possibility of performing it somewhere else but I tell them that I will always help lead the pregnancy and help solve any problems that are connected with it; indicating other places would be an inconsistency between one's beliefs and one's actions; No, the patient will find a place herself; No, but rather to explain why she should not do it; this is a trick question".

Those who were in support of indicating a possibility of performing the abortion somewhere else asserted their views in the following manner:
"Yes, of course but only in cases that are encompassed by the Act; there is no other option, the law requires a physician to do this; they should – abortion is down to the decision of the woman; it's down to him/her (the gynaecologist)".

Discussion

In everyday gynaecological practice the problem lies, above all, in the relationship of general principles with concrete actions [16-22]. The gynaecology environment is the creator of a specific morality within society by means of their concrete actions. However, specific actions, particularly those referring to human life and specifically to its beginning do not necessarily stem from ethical principles.

The studied gynaecologists who expressed their viewpoint on prenatal diagnostics in order to detect genetic irregularities in the first and second trimester of pregnancy present a specific morality (*Fig. 1*). Support in 60% of prenatal testing (even if it resulted in abortion) may signify the presence of relativism and a reductionist vision of the human being.

According to Fijałkowski [3], the acceptance and performance of a holistic value system leads to a respect for every human being (also from the moment of conception) and to the preservation of human dignity. According to the Author, shows traits of ethical relativism and conditions the phenomenon of adapting actions and motivations to one-sided subjective views that are often contradictory to universal medical law like the Hippocratic Oath which serve the true good of the human being.

Emphasising the purpose and benefits of prenatal diagnostic tests, we must admit that such tests are also used for unethical behaviour such as eliminating disease by destroying the foetus. In reference to the ethical issues, it seems to be reasonable to present main canons of ethics, which medical ethos derives from. Among various ethical opinions and standpoints there are some alternatives to abortion.

Dangel & Dangel [23] report on the significance of perinatal palliative care in the case of terminally ill fetuses. Paediatric palliative care appears to be a very possible option of conduct and an alternative to the termination of pregnancy. It constitutes a form of support and assistance for families that do not consider abortion and it also protects the child from refractory and at the same time ineffective treatment. This type of perinatal care over terminally ill fetuses is a special help for gynaecologists directed solely and exclusively at terminating a pregnancy. This type of solution may inspire attitudes that support life among physicians experiencing ethical dilemmas connected with obstetric failure, particularly the diagnosis of a defect in the child, including lethal defects. The paediatric palliative care performed in the Warsaw children's hospice for instance, is an active and holistic approach that encompasses the physical, emotional, social and spiritual elements that raises the quality of life of the child and provides support for its relatives. It involves the treatment of painful symptoms, carries relief for families and provides the necessary care during the process of dying as well as bereavement support.

The opinions of gynaecologists on abortion for so-called "social reasons" (*Fig. 2*) revealed that as many as 45% support this type of procedure which eliminates conceived children. The fact that the question did not touch on possible controversies around the so-called "medical reasons", which according to certain Authors do not exist [2,3,6-8] in contemporary medicine, but concerned the social conditions that are susceptible

to change even during the prenatal or postnatal period of the child's development, gives a lot to think about. According to numerous Authors [3,6,7,24,25] who are concerned with the interpretation of the point of view of medical deontology, there can be no indications for destroying of a human being, particularly committed by a physician whose main ethical duty is to save, safeguard and protect human life. Thus, Bilikiewicz [1] protests: "I absolutely cannot consent to the fact that abortion is a medical procedure, the term 'medical indication', therefore, has to be replaced with some other term".

The veracity of a physician's intentions was revealed by the question concerning the possibility of indicating a different place that performs abortions by the physician that does not perform it for ethical reasons (*Fig. 3*). As many as 67% of gynaecologists considered that it was obvious that a place or person that does perform abortions should be indicated.

According to Wojtyła [9], moral perfection is the "main and central act of human nature", to which every person is invited, particularly the physician that fosters human life from the moment of conception. Pursuant to the Medical Code of Ethics [4]: "the most important ethical imperative of a physician is the good of the patient". This wording, according to Meissner [5], intends to present the human being as the highest value constituting the criterion for the ethical judgement of a physician's action. He explains further that it is good for the patient that a physician "serves the sick patient with his/her medical skills in order to protect their life and care for their health with the utmost respect for their goods".

Conclusions

The results of the survey indicate the differences in the attitudes of physicians towards the diagnosis of prenatal tests, especially the ones revealing genetic defects and lethal disease. There are two ambivalent patterns of behaviour: one group of physicians opt for delivering every child without any exceptions, but the other one is for destroying deformed foetuses.

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Activity of lysosomal exoglycosidases in saliva of patients with HIV infection

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Abstract

Purpose: The aim of this work was to evaluate the influence of HIV infection on the catabolism of glycoconjugates in the oral cavity, by determination of the activity of lysosomal exoglycosidases in mixed saliva.

Method: The specific activities of the following exoglycosidases were tested: N-acetyl-β-hexosaminidase (HEX), its isoenzymes A (HEX-A) and B (HEX-B), α-mannosidase (MAN), β-galactosidase (GAL) and α-fucosidase (FUC).

Result: A significant increase of activity of HEX-A, GAL and FUC, and a significant decrease of the activity of HEX-B was found, but no significant changes in the HEX and MAN activity we noted.

Conclusion: Our results indicate that following HIV infection, there is probably an increased rate of catabolism of glycoconjugates in saliva resulting from changes in the proportions of the activity of isoenzymes A and B of N-acetyl-β-hexosaminidase, β-galactosidase and α-fucosidase. An increase of HEX-A activity can implicate the beginning of neoplastic changes developing in the oral cavity.

Key words: HIV, human saliva, lysosomal exoglycosidases.

Introduction

Glycoproteins and glycolipids form an integral part of the membranes of cells lining the oral cavity, and together with proteoglycans are present in teeth and the intercellular matrix of gingival's connective tissue [1]. Glycoproteins are also components of viral envelopes e.g. HIV [2]. Biosynthesis of host and viral glycoproteins take place in the endoplasmatic reticulum and Golgi apparatus [3], by concerted action of sugar transferases and glycosidases [4]. Degradation the oligosaccharide chains of glycoconjugates is performed by aminohydrolases, endoglycosidases and lysosomal exoglycosidases [5,6]. Inside lysosome glycoproteins are broken down by a combined action of proteases and exoglycosidases: neuraminidase (sialidase), N-acetyl-β-hexosaminidase (HEX), β-glucuronidase, β-galactosidase, α-fucosidase and α-mannosidase, which release neuraminic (sialic) acid, N-acetyl-hexosamines (N-acetylglucosamine and N-acetylgalactosamine), glucuronic acid, galactose, fucose and mannose, from non-reducing ends of oligosaccharide chains, respectively [7]. N-acetyl-β-D-hexosaminidase (HEX) and its isoenzymes A (HEX-A) and B (HEX-B) are most active of lysosomal exoglycosidases [8]. Isoenzyme A of N-acetyl-β-D-hexosaminidase is thermolabile and N-acetyl-β-D-hexosaminidase B is thermostabile form of HEX. As HIV infection is followed by neoplastic changes [9], we were interested in early diagnosis of transition to neoplasms, by exploiting significant differences in activity of HEX isoenzymes between normal and neoplastic tissues. An increase in HEX-A activity in comparison to HEX-B was observed by Borzym-Kluczyk et al. [10] in the renal tissue, serum and urine patients with renal cancer, Eden et al. [11] in acute undifferentiated leukaemia and Gil-Martin et al. [12] in human gastric adenocarcinoma. In inflammatory processes changes in proportion between HEX-A and HEX-B are not significant [13-15]. In healthy people, the activity of lysosomal exoglycosidases in periodontal tissues is low, but sufficient to maintain a steady state of glycoconjugates metabolism [6].

It has been estimated that worldwide about 14,000 people are infected each day by type 1 human immunodeficiency virus (HIV-1). The WHO estimated that 39.4 mln people all over the world were suffering from AIDS at the end of 2004

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[16]. However, therapy is limited by the number of drugs currently available. The drugs present on the market act in different ways; some pharmaceuticals act against the HIV virus, by blocking entrance to the host cells. One target which is involved in blocking viral entry into host cells are glucosidases. They has been recently explored because the biosynthesis of oligosaccharide chains of viral envelope glycoproteins depends on the activity of glucosidases and sugar transferases, which are also involved in the biosynthesis of glycoproteins responsible for the transport of viral particles from the cell and in adherence to and infection of new cells [17]. Hart et al. studied the effects of inhibitors of endoplasmatic reticulum glucosidases and Golgi mannosidase as well as neuraminidase on the interaction between HIV and mannose-binding lectin, a C-type lectin component of the human innate immune system, which binds to the gp120 envelope glycoprotein of HIV-1 [18]. The activities of β-hexosaminidase (using a 4-methylumbelliferyl-β-N--acetylglucopyranoside substrate) and of α-mannosidase and β-mannosidaseand were studied by Costanzi et al. who found that the activities of these enzymes were significantly higher in the serum of patients at the C3 stage of disease than in controls. No significant differences were observed in the activity of beta-glucuronidase or β-galactosidase [19].

HIV infection can coexist with periodontitis and neoplasms in the oral cavity [9,20,21].

Bacteria of dental plaque, the so-called bacteria of "red complex" (*Porphyromonas gingivalis*, *Taneralla forsythensis*, *Treponema denticola*) participating in the pathology of periodontitis. The chronic periodontitis is accompanied by an accumulation of neutrophils, macrophages and lymphocytes which take part in destruction of periodontal tissues. The increase in activity of proteolytic enzymes and enzymes degrading glycoconjugates which intensifies the destruction of connective tissue and bone in alveolar processes may cause increase in activity of lysosomal enzymes in serum and saliva [13].

Saliva is used for immunological and biochemical diagnosis and prognosis of oral changes in type I diabetes [22], diseases of the periodontium [23,24], and in Sjogren's syndrome, as it is easily accessible and a collection is a non-invasive procedure. Therefore, the aim of the present work is to evaluate the influence of the HIV infection on the catabolism of glycoconjugates in oral cavity by determination of the activity of lysosomal exoglycosidases in saliva from HIV patients.

Material and methods

The samples were 3-5 ml of mixed saliva collected from 68 patients by a spitting method without mechanical and chemical stimulation, not earlier than 1 hour and not later than 3 hours after a meal.

Control group (C) – 34 healthy patients, aged 21-48, extracted teeth – 3.35, need for extraction – 0.35, need for restorative and surgical treatment – 4.98. Any drugs were taken at least 8 hours before investigation.

Study group (HIV) – 34 HIV infected patients, aged 20-53, extracted teeth – 9.59 (CD 4 >500 – 6.25/8 patients; 200-500 – 9.19/27 patients; <200 – 12.64/14 patients), need for extraction – 2.10 (CD 4 >500 – 3.00/8 patients; 200-500 – 1.93/27 patients; <200 – 2.07/14 patients), need for restorative and surgical treatment – 11.18 (CD 4 >500 – 13.00/8 patients; 200-500 – 11.07/27 patients; <200 – 10.43/14 patients).

***Table 1*. The activity of HEX, HEX-A, HEX-B, GAL, FUCMAN (μKat/kg protein) and concentration of protein (mg/ml) in saliva of the HIV patients**

The activity of enzymes μKat/kg protein (±SD)			
Control HIV			
HEX	mean	19.286±3.24	19.302±4.08
	p	~	
HEX A	mean	7.525±2.86	15.102±4.06
	p	0.047	
HEX B	mean	11.968±3.74	7.368±2.01
	p	0.000182	
GAL	mean	1.589±0.28	20.154±5.87
	p	0.00002	
FUC	mean	1.7131±0.75	1.925±0.57
	p	0.00115	
MAN	mean	2.423±0.97	2.384±1.24
	p	~	
Concentration of protein (mg/ml)			
protein	mean	1.9764±0.13	1.7614±0.19
	p	0.000683	

The collected saliva was centrifuged at 12,000 x g for 30 minutes at 4ºC. The resulting supernatant was divided into 100 μl portions and stored at -80ºC.

Determination of the activity (μKat/kg of protein) of N-acetyl-β-hexosaminidase (HEX), thermolabile isoenzyme A (HEX-A), thermostabile isoenzyme B (HEX-B), α-mannosidase (MAN), β-galactosidase (GAL) and α-fucosidase (FUC) in supernatants, was performed according to Zwierz et al. method [25]. The protein content (mg/ml) was determined by the Lowry method with bovine serum albumin as a standard [26]. All determinations were performed in duplicate.

The results were analyzed by a Statistica 6.0 StatSoft (Cracow, Poland) according to ANOVA and a post-hoc test (test NIR). The statistical significance of differences was regarded to be $p<0.05$.

This study was performed with the content of the Bioethical Commission of the Medical University of Białystok.

Results

Tab. 1 shows that no changes in the activity of HEX in saliva of patients with HIV infection (in comparison to activity of HEX in saliva of control group) was noted. However, the activity of thermolabile HEX-A in the HIV patients' saliva is over two times higher than the activity of HEX-A in the saliva of control group while the thermostabile HEX-B is 1.6 times lower in saliva of the HIV infected patients than in the saliva of control group. The activity of α-galactosidase and the α-fucosidase in the saliva of HIV infected patients is statistically higher than the activity of these enzymes in the saliva of the control group. The activity of α-mannosidase in the saliva of HIV infected patients does not differ significantly from the activity

***Table 2*. Activity of exoglycosidases calculated per volume of received saliva (μKat/kg protein/ml)**

The activity of enzymes μKat/kg protein/ml			
		Control	HIV
HEX	mean	6.248	5.939
HEX A	mean	1.835	3.107
HEX B	mean	2.3936	1.479
GAL	mean	0.496	5.447
FUC	mean	0.361	0.458
MAN	mean	0.502	0.599

of α-mannosidase in saliva of the control group. The mean concentration of the proteins in the saliva of HIV infected patients presents significant decrease in comparison to concentration of proteins in saliva of control group. *Tab. 2* shows activity of exoglycosidases calculated per volume of received saliva.

Discussion

It is established that HIV infects cells possessing the receptor CD 4, and in the case of lymphocytes deficient in CD 4, also through other receptors, such as the mannose or galactose receptors. The major determinant of viral tropism is at the entry level. This occurs only if the appropriate coreceptor is present. Entry of HIV-1 into its CD4+ target cells requires fusion/entry cofactors. Recently, the seven-transmembrane, G protein-coupled chemokine receptors CXCR4 and CCRS have been identified as cofactors for fusion and entry of T cell (T)3-tropic and macrophage (M)-tropic strains of HIV-1, respectively, into CD4+ cells [27-32].

CCR5 is the major coreceptor for HIV transmission in vivo. However, while CD4-positive cells obtained from CCR5-negative individuals are resistant to infection by viruses that require this coreceptor, they are readily infectable by viruses which use CXCR4 receptor [33,34]. In the literature we have not found any data on the influence of HIV infection on enzymes in saliva, except for those enzymes involved in innate immunity (lactoferrin, lysozyme, peroxidase) [35].

The aim of the present work was to evaluate the activity of lysosomal exoglycosidases in the saliva of HIV patients as indicators of glycoconjugate catabolism. Exoglycosidases [6] together with aminohydrolases and endoglycosidases take part in degradation of glycoconjugates [36]. Glycoconjugates are either proteins or lipids to which saccharide chains of different lengths are attached. The glycoconjugates (proteoglycans and glycolipids) function as receptors. Glycoproteins function both as receptors and transporters [37] on the surface of cellular membranes. Proteoglycans and glycoproteins are the main constituents of the intracellular matrix, where they form an intricate three-dimensional network responsible for proper hydratation, regulation of the activity of secreted proteins and exchange of the products of metabolism. Catabolism of glycoconjugates is connected with maintaining the balance between degradation of old and synthesis of new molecules. Exoglycosidases remove monosaccharides from the non-reducing end of oligo- or polysaccharide chains of glycoconjugates, by hydrolysis of glycosidic bonds.

We estimated the activity of N-acetyl-β-hexosaminidase and its isoenzymes (thermolabile isoenzyme A and thermostabile isoenzyme B), β-galactosidase, α-fucosidase and α-mannosidase, in the saliva of HIV infected patients. No significant differences were found between the activity of HEX and α-MAN in the saliva of HIV infected patients in comparison to control group. However, we noticed a significant increase in the activity of HEX-A, β-GAL and α-FUC, and a significant decrease in the activity of HEX-B in the saliva of HIV infected patients. Dramatical increase in GAL activity in saliva may be a result of intensive degradation of all glycoconjugates: glycoproteins, glycolipids and proteoglycans, as galactose is component of oligosaccharide chains of glycoproteins (especially salivary) [38], glycolipids [39] and glycosaminoglycans [40]. The lack of information in the literature concerning the activity of exoglycosidases in saliva of HIV infected patients, did not allow us any comparison of our results with data of other authors. Reports of increased activity of lysosomal exoglycosidases and salivary enzymes in the saliva of patients with periodontitis have been published [41-44]. In our study drug users HIV positive patients were included persons who did not care much about oral hygiene. We expected that an increase in the activity of lysosomal exoglycosidases could be found in HIV positive patients, because of presence of periodontal disease caused by poor oral hygiene. The lack of changes in the activity of HEX, the most characteristic enzyme in human tissue inflammation, could be explained by a low number of teeth and a low number of periodontal pockets. We conclude that within so low number of periodontal pockets even with existing inflammatory process differences in activity of salivary lysosomal exoglycosidases as compared to control could not be detected. The different number of teeth in groups is not very important in this study because volunteers included to control group (higher number of teeth) were healthy without periodontal disease. However, an increase in HEX-A activity as shown in our study, and in the light of previous research [10-12,45], can implicate the beginning of neoplastic changes developing in the oral cavity. An increase in GAL and FUC activity can implicate increase in catabolic process of glycoconjugates which is the sign of tissues destruction.

It has been reported that lymphocytes and macrophages are the sources of lysosomal exoglycosidases in saliva [43]. The way in which HIV infection changes the activity of exoglycosidases and the influence of the activity of exoglycosidases on HIV infection is still unknown. It is known, however, that the receptors for HIV are glycoproteins, but not which part of the HIV envelope oligosaccharide chains, if any, binds to the receptor on the surface of sensitive cells. It may be proposed that exoglycosidases removing appropriate sugars from non-reducing end of oligosaccharide chains can modify the possibility and strength of binding the envelope of HIV to cellular receptors, by exposing suitable oligosaccharide structures on the surface of sensitive cells. Thus the exoglycosidases can influence the docking of the HIV virus to the cell receptor.

HIV infection may induce T cell apoptosis through indirect mechanisms, including activation-induced cell death and autologous infected cell-mediated killing. The death of the cell by apoptosis or necrosis is preceded by the damage of cellular membranes. Lysosomal membranes also undergo this process

which result in release of their content to the cellular environment. Damage to lysosomal membranes of salivary glands may increase the release of exoglycosidases to saliva and change their activity. The release of the content of lysosomal granules to the extracellular matrix, crevicular fluid and saliva is responsible for destruction of periodontal tissue, associated with HIV infection [21,46,47]. The changes which we observed in the activity of lysosomal exoglycosidases in saliva from infected patients may result from any of the following causes:

– mutations of the sequences of DNA coding lysosomal exoglycosidases,

– disorders in biosynthesis of the polypeptide chains for lysosomal exoglycosidases,

– the influence of virus on chaperones which results in incorrect folding,

– degradation of exoglycosidases in endoplasmatic reticulum and Golgi apparatus,

– changes in activity of glycosylotransferases in membranes of endoplasmatic reticulum and Golgi apparatus damaged by HIV, which synthesis the oligosaccharide chains of lysosomal exoglycosidases. We have no data on concerning HIV influence on the activity or the structure of glycosyltransferases, or on the influence of HIV on the endoplasmatic reticulum and Golgi apparatus,

– disturbances of intracellular transport of exoglycosidases, by influence on the Man-6-P receptor or GGA proteins. This hypothesis is particularly interesting because HIV has affinity for the mannose receptor. HIV binding to the mannose receptor may block binding of Man-6-P of the oligosaccharide chain of lysosomal exoglycosidases to its receptor and this may trap exoglycosidases in the trans Golgi compartment.

Conclusion

Our results indicate that HIV and bacterial infections probably increase the catabolism of glycoconjugates in saliva by changing the relative activity of N-acetyl-β-glucosaminidase isoenzymes A and B as well as the activity of β-galactosidase and α-fucosidase.

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Smoking habit and gastritis histology

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Abstract

Purpose: Long-term cigarette smoking may increase the risk of digestive tract pathologies, however, what is the influence smoking habit on gastric mucosa histology is still poorly elicited. The aim of the study was to compare histological evaluation of gastritis in smoker and non-smoker groups.

Material and methods: A total of 236 patients of various *H. pylori* status (109 infected, 127 non-infected), clinical diagnosis (107 duodenal ulcer disease, 129 dyspepsia), and smoking habit (92 smokers, 144 non-smokers) were included. Subjects were classified as smokers if they smoked 5 or more cigarettes per day for at least 3 years. A histological examination of endoscopically obtained samples was performed by two experienced pathomorphologists blinded to the diagnoses and smoking habit. Microscopic slices of the gastric mucosa were stained with hematoxylin-eosin and Giemsa. Apart from histological diagnosis, *H. pylori* status was additionally confirmed by an urease test (CLO-test) at least in one of two gastric locations (antrum or corpus).

Results: In the *H. pylori* infected population, *H. pylori* density, neutrophils, and mononuclear cells infiltration in the gastric corpus mucosa were lower in smokers than non-smokers, while in the antrum the differences were not significant. In the non-infected population, no significant differences in neutrophils and mononuclear cells infiltration between smokers and non-smokers were found.

Conclusions: Since the significant differences in studied parameters of chronic gastritis between smokers and non-smokers were found in the corpus mucosa of *H. pylori* infected subjects, smoking should be taken into account when a histological evaluation of the gastric mucosa in the *H. pylori* infected population is performed.

Key words: gastritis, *Helicobacter pylori*, smoking.

Introduction

Clinical and experimental studies have shown that smoking exerts many different effects in living organisms, and most of them are unprofitable. Poisoned components of cigarette smoke reached the stomach either through the circulation or through the gastrointestinal tract, usually within swallowed saliva [1,2]. The mechanism of the action of cigarette smoke constituents on the stomach is not well defined, however, many factors, such as increased synthesis of reactive oxygen species, endothelin 1, disturbences in microcirculation, increased duodeno-gastric reflux, and delayed gastric emptying, should be considered [3-7]. Although chronic exposure to cigarette smoke in *Helicobacter pylori* infected patients enhances the risk of gastric mucosal atrophy and intestinal metaplasia [8], the histological gastritis characterized by *H. pylori* density, neutrophils, and mononuclear cells infiltration (Sydney classification of gastritis [9]) in relation to smoking habit has not been systematically studied. The aim of the present study was to compare histological gastritis severity in smokers and non-smokers with duodenal ulcer disease and dyspepsia.

Material and methods

Patients

The study was performed in 236 patients (both sexes) with various *H. pylori* status (109 infected, 127 non-infected) and clinical diagnosis (107 with duodenal ulcer disease, 129 with

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***Table 1*. Characteristics of the study groups**

	Duodenal ulcer disease		Dyspepsia	
	H. pylori (+)	*H. pylori* (-)	*H. pylori* (+)	*H. pylori* (-)
Total number	45	62	64	65
Age, years (mean ± S.D.)	39.3±9.7	50.4±11.3	52.6±12.3	50.4±14.4
Gender (M/F)	30/15	53/9	27/37	34/31
Cigarette smoking				
None (%)	12 (26.7)	30 (48.4)	44 (68.8)	50 (76.9)
<5/day (%)	1 (2.2)	2 (3.2)	3 (4.7)	2 (3.1)
5-9/day (%)	3 (6.7)	2 (3.2)	1 (1.6)	3 (4.6)
10-19/day (%)	12 (26.7)	13 (21.0)	7 (10.9)	5 (7.7)
20 or more/day (%)	17 (37.8)	15 (24.2)	9 (14.1)	5 (7.7)

dyspepsia). Smokers and non-smokers represent 92 and 144 subjects, respectively (*Tab. 1*). Patients with dyspepsia (pain and/or discomfort in the upper abdomen lasting for at least three months) showed no abnormalities in the gastroscopy. In patients with duodenal ulcer not related to non-steroidal anti-inflammatory drugs (NSAIDs) treatment but infected with *H. pylori*, an ulcer niche 0.5-1.0 cm was present in the duodenal bulb. All patients with duodenal ulcer disease, not currently infected with *H. pylori* and without the ulcer niche, had documented histories of duodenal ulcers not related to NSAIDs treatment. They also underwent eradication therapy at least 36 months before. Subjects with emerge symptoms and abnormal results in basic laboratory tests as well as with the gastric mucosal atrophy and/or intestinal metaplasia in microscopic examination were not included.

Participants that smoked 5 or more cigarettes per day were classified as smokers. Subjects who did not smoke at all were classified as non-smokers. Only those with unchanged smoking habit, according to used criteria for at least 3 years, were included. Patients who were obligated to take any drugs, permanently or sporadically for at least two weeks before undergoing gastroscopy, were excluded. Similarly, subjects using any anti-acid drug, Misopostol, antibiotics, NSAIDs within 4 weeks, or alcoholic beverages within 7 days before the gastroscopy examination were excluded as well.

Endoscopy

Gastroscopy examinations were performed with a gastroscope, GIF V2 or Q145 (Olympus), during which mucosa samples were taken from the prepyloric and corpus region for an urease test (CLO-test) and histological examinations from each side. Separate biopsies were taken from the antrum and corpus to search for possible histological differences in these two locations.

Helicobacter pylori testing

CLO-test was conducted according to Marshall et al. method [10]. Sensitivity and specificity of this test according to a histology examination was 98.1% and 90.2%, respectively. The test was accepted as positive if a 24 hours room temperature incubaction changed its color from orange to pink. Endoscopically taken samples of gastric mucosa were placed in buffered formalin and subjected to standard histological procedures. Subjects classified as infected had positive results in both tests. Subjects classified as non-infected had negative results in both tests. In the case of participants successfully treated for *H. pylori*, the inclusion was possible if eradication had been performed at least 36 month earlier.

Histological study

The examination of endoscopically taken specimens was performed by two experienced pathomorphologists who were blinded to the diagnoses and smoking habit. In the case of inconsistency between pathologists, the final diagnoses were established by joint evaluations of the slide. Endoscopic slices of the gastric mucosa were stained with hematoxylin-eosin and additionally with Giemsa. Gastritis was graded on the basis of a four step scale (0-3), including: 1) *H. pylori* density; 2) activity (neutrophils infiltration); and 3) inflammation (mononuclear cells infiltration) [9]. They were scored as follows:

H. pylori density

(0): no *H. pylori* present

(1): single bacterium or their groups covering up to 1/3 of the gastric mucosa surface

(2): groups of bacteria covering up to 2/3 of the gastric mucosa surface

(3): groups of bacteria covering the whole gastric mucosa surface;

Activity

(0): no neutrophils present

(1): single neutrophil found in the limited number of fields

(2): neutrophils found in the lamina propria

(3): neutrophils found in the lamina propria, gastric glands and epithelium;

Inflammation

(0): no mononuclear cells present

(1): mononuclear cells found in the upper one third of the mucosa or dispersed mildly in the whole mocosa

(2): mononuclear cells found in the upper half of the mucosa or dispersed moderately in the whole mucosa

(3): dense infiltrate of mononuclear cells found within the whole mucosa.

Statistical analysis

The results (means ±S.D or medium and range) were subjected to statistical analysis using Mann-Whitney U-test; significant differences were accepted at $p<0.05$.

***Table 2*. The influence of smoking habit on histology of the gastric mucosa in *Helicobacter pylori* infected subjects**

	Dyspepsia			Duodenal ulcer			Total		
	smoking (+)	smoking (-)		smoking (+)	smoking (-)		smoking (+)	smoking (-)	
	(n=17)	(n=47)	p	(n=32)	(n=13)	p	(n=49)	(n=60)	p
colonisation									
antrum	3 (1-3)	2 (1-3)	NS	3 (1-3)	3 (1-3)	NS	3 (1-3)	3 (1-3)	NS
corpus	1 (0-3)	2 (1-3)	<0.001	1 (0-3)	2 (1-3)	NS	1 (0-3)	2 (1-3)	<0.001
p	<0.001	<0.01		<0.001	NS		<0.001	<0.01	
activity									
antrum	3 (1-3)	3 (1-3)	NS	3 (2-3)	3 (1-3)	NS	3 (1-3)	3 (1-3)	NS
corpus	2 (0-3)	2 (1-3)	<0.005	2 (0-3)	2 (2-3)	NS	2 (0-3)	2 (1-3)	<0.005
p	<0.01	<0.001		<0.001	NS		<0.001	<0.01	
inflammation									
antrum	3 (1-3)	3 (1-3)	NS	3 (2-3)	3 (1-3)	NS	3 (1-3)	3 (1-3)	NS
corpus	1 (0-3)	2 (1-3)	<0.01	1 (0-3)	2 (1-3)	NS	1 (0-3)	2 (1-3)	<0.05
p	p <0.001	<0.001		<0.001	<0.01		<0.001	<0.001	

***Table 3*. The influence of smoking habit on histology of the gastric mucosa of *Helicobacter pylori* non-infected subjects**

	Dyspepsia			Duodenal ulcer			Total		
	smoking (+)	smoking (-)		smoking (+)	smoking (-)		smoking (+)	smoking (-)	
	(n=13)	(n=52)	p	(n=30)	(n=32)	p	(n=43)	(n=84)	p
activity									
antrum	1 (0-2)	0 (0-3)	NS	1 (0-2)	0 (0-1)	NS	1 (0-2)	0 (0-3)	NS
corpus	0 (0-2)	0 (0-2)	NS	0 (0-1)	0 (0-1)	NS	0 (0-2)	0 (0-2)	NS
p	<0.01	<0.05		<0.001	<0.001		<0.001	<0.001	
inflammation									
antrum	1 (0-2)	1 (0-2)	NS	1 (0-2)	0 (0-2)	NS	1 (0-2)	0 (0-2)	NS
corpus	0 (0-1)	0 (0-3)	NS	0 (0-1)	0 (0-1)	NS	0 (0-1)	0 (0-3)	NS
p	<0.001	<0.01		<0.001	<0.001		<0.001	<0.01	

The local Ethical Committee approved the study and all subjects gave informed consent.

Results

H. pylori infected population

In patients with dyspepsia, irrespectively of smoking habit, a significantly higher scores for *H. pylori* density, neutrophils, and mononuclear cells infiltration were found in the antrum compared to the corpus. With regards to smokers and non-smokers, the differences of studied parameters were found only in the corpus mucosa; *H. pylori* density, activity, and inflammation were higher in non-smokers than smokers (*Tab. 2*).

In duodenal ulcer patients, the differences within studied parameters were not significant between smoker and non-smoker groups, both in the antrum and in the corpus (*Tab. 2*). However, when smokers and non-smokers were analyzed separately, only the smokers exhibited higher scores for *H. pylori* density, neutrophils and mononuclear cells infiltration in the antrum versus the corpus. In non-smokers the differences were not significant; however, the number of subjects in this group was small. In joint analysis of subjects with dyspepsia and duodenal ulcer, the differences between smokers and non-smokers were the same as in the group with dyspepsia; although, the differences between the antrum and the corpus were more pronounced in smokers than non-smokers.

H. pylori non-infected population

In patients with dyspepsia and duodenal ulcer disease, the differences in activity and inflammation between smokers and non-smokers were not significant in both separate and joint analysis of these two clinical groups, regardless of the stomach location (antrum, corpus) (*Tab. 3*). Moreover, apart from the clinical diagnosis, there was a significant difference in studied parameters of gastritis between the antrum and the corpus of both smokers and non-smokers.

Discussion

Despite the evidence on the association between smoking habit and atrophic/metaplastic lesions within gastric mucosa [8,11], there was no data on the influence of smoking on histological gastritis characterized by the intensity of *H. pylori* infection, neutrophils, and mononuclear cells infiltration. The results of the current study have shown that smoking influences colonization of *H. pylori* within the stomach and modifies the mucosal distribution of neutrophils and mononuclear cells infiltrates. The mechanism of these changes is not clear. Cigarette

smoke contains about 4000 different compounds of various biological effects. These compounds when act separately or more frequently in combination can cause significant changes in histological image of the mucosa. Also, gender and age may be of relevance. However, since the two clinical groups were not comparable, most ulcer disease patients were men and smokers while patients with dyspepsia women and non-smokers, additionally, duodenal ulcer patients infected with *H. pylori* were younger than remaining, the correlation of histological findings with gender and age has not been made.

The gastric response to a chronic *H. pylori* infection is characterized by the infiltration of polymorpho-cells and mononuclear-cells into the mucosa, and an inflammatory response is a specific reaction to the presence of this bacterium. After the eradication of *H. pylori*, the inflammatory cells population decreases significantly [12]. According to our data in duodenal ulcer patients, the histological changes within the gastric mucosa decrease within a few years after *H. pylori* eradication. Observed severity of gastritis was also lower in *H. pylori* negative than positive subjects with dyspepsia, regardless of smoking habits. We do not know how long-time patients with dyspepsia were *H. pylori* free or how many of them eradicated spontaneously within the last three years. However, as spontaneous eradication from the stomach is rare [13] and currently non-infected subjects with dyspepsia had never been eradicated (patients' report), we can assume that no more than a few *H. pylori* negative subjects with dyspepsia could have the spontaneous eradication within the last few years, and thus the relevant error was small.

Formerly, short time lasting exposition to cigarette smoke did not cause the gastric mucosal accumulation of inflammatory cells, while potentiated ethanol- and indomethacin-induced them [14,15]. We have found that in *H. pylori* positive smokers, histological changes are predominantly located in the antrum; while in non-smokers, they are both in the antrum and corpus. The significant differences in studied parameters of chronic gastritis between smokers and non-smokers were found in the corpus mucosa of *H. pylori* infected subjects (separate analysis of patients with dyspepsia and joint analysis of two clinical groups). This would mean that smoking influences gastritis histology mainly in *H. pylori* infected population and additionally in one stomach location.

Smoking is a known risk factor for duodenal ulcer development in the *H. pylori* infected population [16,17]. The pattern of gastritis correlates strongly with this disease; the antral-predominat gastritis is most frequently recognized. The results of our study provides evidence that not only duodenal ulcer disease is related to the observed pattern of gastritis. The antral-predominat gastritis was also found in subjects with dyspepsia, and this observation is in line with recently published data of Western populations [18]. According to these data, the pattern of gastritis may not be related to the clinical diagnosis but more likely to some other factors, e.g., *H. pylori* infection. It is known that the severity of gastritis is positively associated with the intensity of *H. pylori* infections and is higher in the antrum than in the corpus [19]. Interestingly, the results of our study demonstrates that in non-infected subjects, histological gastrits is also more severe in the antrum than in the corpus, and this is more pronounced in smokers than non-smokers. Therefore, one can speculate that *H. pylori* infection may only cause quantitative shifts in microscopic image of chronic gastritis.

It is unclear why in smokers *H. pylori* colonization is larger in the antrum than in the corpus but this fact may have some clinical implications. We know from earlier reports that in *H. pylori* infected smokers, the risk of gastric carcinoma refers predominantly to prepyloric region [20]. Thus, the combined action of two factors, i.e., cigarette smoke components and *H. pylori* presence over a relatively long period, may predispose to neoplastic transformation, making the association of antral carcinoma with smoking and *H. pylori* infection not incidental.

Although the current study is limited to two clinical groups and the number of analyzed patients is relatively small, we conclude that smoking should be taken into account when histological evaluation of the gastric mucosa in *H. pylori* infected population is performed.

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Diagnostic difficulties during combined multichannel intraluminal impedance and pH monitoring in patients with esophagitis or Barrett's esophagus

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Abstract

Gastroesophageal reflux disease (GERD) is one of the most common esophageal diseases in developed countries. It is widely believed that GERD symptoms are caused by acid refluxate within the esophagus, so ambulatory 24 hour pH-monitoring became the gold standard in detecting gastroesophageal reflux. Traditional ambulatory pH monitoring is unable to detect a gastroesophageal reflux with pH>4. The introduction of multichannel intraluminal impedance and pH (MII-pH) brought new possibilities in detecting GERD. In this technique impedance identifies reflux episode whereas pH sensor further characterizes it as either acid (pH<4) or non-acid (pH≥4). This is a great progress in diagnosing GERD but MII has also some imperfections related to pathological changes in the esophageal mucosa such as esophagitis or Barrett oesophagus, which are connecting with a very low baseline impedance values. Changes in the esophageal mucosa may also impair the esophageal motility and esophageal transit leading to some fluid retention in the esophagus. It should be stressed that very low impedance baseline creates a difficulty in interpreting the MII-pH study. In such a case it might be almost impossible to interpret the study as the interpreter does not see characteristic drop in impedance progressing either orally (reflux episode) or swallow but only almost flat impedance lines. Therefore, future studies are needed to further evaluate this problem.

Key words: gastroesophageal reflux disease, combined multichannel intraluminal impedance, esophagitis, Barrett esophagus, pH-metry.

Gastroesophageal reflux disease (GERD) is one of the most common esophageal diseases in developed countries. It is a condition in which gastric contens reflux into the oesophgaus and provoke symptoms, complications and impairs quality of life. Typical GERD symptoms are: heartburn, regurgitation, pain in supraabdominal area, nausea or belching. Atypical symptoms, connecting with extraoesophageal manifestations of GERD are: reflux disease related asthma, chronic cough and laryngitis, but both typical and atypical GERD symptoms can impair quality of life. The pathogenesis of reflux disease is multifactorial, connecting also with insufficiency of antireflux barrier, especially lower oesophageal sphincter (LES) pressure abnormalities or LES transient relaxations (tLESR). It was commonly believed that symptoms attributed to GERD were caused by acid refluxate (pH<4) occurring in the esophagus. Therefore proton pump inhibitors (PPI) were considered the drugs of choice in the pharmacologic therapy of GERD [1-3].

Other factors contributing to the pathophysiology of reflux disease include hiatal hernia, impaired esophageal clearance, delayed gastric emptying and impaired mucosal defensive factors. It has been suggested that hiatal hernia is promoting LES dysfunctions. An impaired esophageal clearance is responsible for prolonged acid exposure of the esophageal mucosa and delayed gastric emptying results in gastric destension which may significantly increase the rate of tLESR corresponding with higher incidence of postprandial refluxes. Finally, the mucosal defensive factors play an important role against development of reflux disease by neutralizing the backdiffusion of hydrogen ion into the esophageal tissue [1,2].

Typical GERD symptoms occur every day in about 5-10% of population in the developed countries and once a week even in 20% of population. Incidence of GERD increases with the

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age, social status, dietary habits, lifestyles and many other factors [1,2]. One of the most serious complications of GERD is esophagitis, with different severity, according to most common endoscopic graduation (most frequent in use is Los Angeles scale) and Barrett's esophagus. The widely accepted definition of Barrett`s esophagus, according to American College of Gastroenterology is: an oesophagus in which any portion of the normal squamous lining has been replaced by a metaplastic columnar epithelium which is visible macroscopically. In order to make a positive diagnosis of Barrett`s esophagus, a segment of columnar metaplasia of any length must be visible endoscopically above the oesophagogastric junction and confirmed or corroborated histologically. There is a need to specifically define the columnar metaplasia which carries a risk of malignant transformation and implications regarding surveillance [3].

The ambulatory 24 hour pH-monitoring became the gold standard in detecting gastroesophageal reflux, because it was widely believed that GERD symptoms were caused by acid refluxate within the esophagus, however, it was shown that there was a subset of patients who despite adequate gastric acid suppression still experienced GERD symptoms [4-7].

It has been suggested that symptoms occurring despite adequate gastric acid suppression might be caused by reflux with a pH greater than 4 [4]. Traditional ambulatory pH monitoring is unable to detect a gastroesophageal reflux with pH>4. However, some authors proposed esophageal pH≥7 as an indirect marker of alkaline reflux during ambulatory pH monitoring [5], but on the other hand several studies have shown that increased production of saliva or bicarbonate secreted by esophageal submucosal glands may increase esophageal pH in the absence of reflux and confound measurements [6-11]. Other authors claim that pH monitoring can still detect gastroesophageal reflux when esophageal pH remains above 4 but with accompanying definite fall greater than one pH unit [12].

The introduction of multichannel intraluminal impedance and pH brought new possibilities in detecting gastroesophageal reflux. Multichannel intraluminal impedance evaluates the direction of bolus movement and is determined by multiple impedance measuring segments placed within the esophagus. In this technique impedance identifies a reflux episode whereas pH sensor further characterizes it as either acid (pH<4) or non-acid (pH≥4). Reflux episode detected by impedance is defined as a retrograde bolus movement progressing from the most distal esophageal measuring site to at least the second distal esophageal measuring site. Swallow in turn is defined as an antegrade bolus movement progressing from the proximal esophageal measuring site to the distal esophageal measuring sites. In the absence of the bolus within the esophagus, the impedance is determined by the electrical conductivity of the esophageal lining [13].

Intraluminal impedance (expressed in Ohms) depends on changes in resistance to alternating current between two metal electrodes produced by the presence of bolus inside the esophageal lumen. Refluxed contents are characterized by high conductivity, which is the inverse of impedance what makes possible practical qualitative analysis of refluxate. For instance, the conductivity of air is almost zero and then impedance increases compared with baseline, whereas the conductivity of liquid is much higher and the impedance curve decreases remarkably. If we use the combination of impedance and traditional pH-metry we can detect both acid and non-acid liquid reflux episodes. From a clinical point of view, it might be useful for identifying the number and percent times of gas, acid and non-acid reflux episodes, it may improve the yield of symptom index, it may allow to evaluate the reasons for poor response of reflux symptoms to proton pomp inhibitors and to know the proximal extent of reflux events in patients with atypical symptoms [13-16].

Recent studies in adults and children suggested that combined multichannel intraluminal impedance and pH measurement has the potential to become the new "gold standard" for gastroesophageal reflux testing [17] and has the potential to become a useful clinical tool to assess ongoing reflux in patients on acid-supression therapy [18]. A recent multicenter study from the U.S. observed that among patients presenting with symptoms related to GERD despite gastric acid suppressive therapy, 37% of symptomatic patients had a positive symptom index with non-acid reflux whereas 11% of symptomatic patients had a positive symptom index with acid reflux [19]. These data were further supported by a recent multicenter study from Europe which observed that among symptomatic patients receiving PPI, 33% had a positive symptom index with non-acid reflux, 5% with acid reflux and another 5% with both acid and non-acid reflux [20]. In the group of symptomatic patients studied off PPI therapy, 10.8% had a positive symptom index with non-acid reflux, 32.4% with acid reflux and 13.% with both acid and non-acid reflux [20]. Therefore, it was shown that GERD symptoms might be caused by non-acid reflux in patients on or off PPI therapy [20].

This is a great progress in diagnosing GERD but MII has also some imperfections related to pathological changes in the esophageal mucosa such as esophagitis or Barrett`s esophagus. These changes are very likely to cause that baseline impedance values are very low and detection of the bolus movement in the esophagus is very difficult. In addition, changes in the esophageal mucosa may also impair the esophageal motility and esophageal transit leading to some fluid retention in the esophagus. A recent study by Domingues et al. [21] observed significantly lower postdeglutitive impedance values among GERD patients with mild-esophagitis than healthy controls indicating presence of bolus residues in the distal esophagus. Another study observed that patients with ineffective esophageal motility (IEM) had low baseline impedance values in the distal esophagus which were likely caused by some level of fluid retention within the esophagus and possibly inflamed esophageal mucosa [22]. In that study the distal baseline impedance values found in patients with IEM were comparable with those found in patients with achalasia or scleroderma [22]. The authors claimed that the low distal esophageal impedance values in patients with IEM may also reflect the inflammation within esophageal mucosa due to gastroesophageal reflux [22].

There are no further data regarding the difficulties of interpretation of MII-pH tracings in patients with very low impedance baseline which are very likely to occur in patients with abnormal esophageal mucosa (Barrett's esophagus or esophag-

titis). It should be stressed that very low impedance baseline creates a difficulty in interpreting the MII-pH study. In such case it might be almost impossible to interpret the study as the interpreter does not see characteristic drop in impedance progressing orally (reflux episode) but only almost flat impedance lines throughout the length of entire tracing.

During MII-pH monitoring the beginning of a reflux episode is defined as sequential 50% decrease in impedance baseline value beginning at the most distal recording site and reaching at least the second most distal recording site. The baseline impedance value used to determine a 50% decrease is the average impedance baseline in a 5-second interval preceding the reflux episode. The end of a reflux episode in turn is defined as sequential increase in impedance to at least 50% of baseline value. Therefore, in case of low impedance baseline values in the distal esophagus it is very difficult or almost impossible to notice the impedance detected reflux episodes.

Future studies are needed to further evaluate the use of MII-pH in patients with low values of impedance baseline.

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24-hour esophageal pH monitoring in children with pathological acid gastroesophageal reflux: primary and secondary to food allergy

Part I

Intraesophageal pH values in *distal* channel; preliminary study and control studies – after 1, 2, 4 and 9 years of clinical observation as well as dietary and pharmacological treatment

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Abstract

Purpose: Among 264 children suspected of GERD, acid gastroesophageal reflux (GER) was confirmed in 138 children on the basis of 24-hour pH monitoring.

Aims of the study: Comparative analysis of parameters of 24-hour intraesophageal pH monitoring (in distal channel – above cardia) in children with acid GER: primary and secondary to cow milk allergy and/or other food allergy (CMA/FA) diagnosed; comparison of examined values of pH monitoring parameters with regard to duration of the disease (preliminary study and prospective studies – after 1, 2, 4 and 9 years of clinical observation and/or conservative treatment).

Material and methods: 264 children suspected of GERD, of both sexes (140 boys – 53.0% and 124 girls – 47.0%), aged: 1.5-102 months; x=20.78±17.23 months, were enrolled in the study. In order to differentiate acid GER: primary from secondary to CMA/FA in 138 (52.3%) children with GERD immunoallergological tests were performed. Positive result of oral food challenge test confirmed the allergy being the cause of GER.

138 children with pathological acid GER were qualified into two groups: 1 and 2. Group 1 – 76 patients (55.1%), aged: 4-102 months; x=25.2±27.28 months, with pathological primary GER. Group 2 – 62 patients (44.9%), aged: 4-74 months, mean age x=21.53±17.79 months, with pathological GER secondary to CMA/FA.

Results: Significant differentiation of the mean values of these parameters between preliminary study and control studies within groups was shown in the case of: number of episodes of acid GER and episodes of acid GER lasting more than 5 minutes, duration of the longest episode of acid GER, acid GER index: total and supine (distal channel).

Statistical significance ($p<0.05$) was higher in group 1, especially during prospective clinical observation and/or conservative treatment. At the same time significant differentiation of the mean values of: number of episodes of acid GER and episodes of acid GER lasting more than 5 minutes and mean values of acid GER index: total and supine was shown between the groups. Statistical significance ($p<0.05$) was higher in group 2.

Coclusions: The preliminary study of examined children confirmed that values of pH monitoring in distal channel were comparable and did not contribute to differentiation of GER into primary (group 1) and secondary (group 2). During prospective clinical observation and/or clinical treatment the intensity of reflux in these groups was assessed on the basis of the number of episodes of acid GER and episodes of acid GER lasting more than 5 minutes in distal channel. Acid GER index: total and supine appeared to be important diagnostic parameter but only after the first year of dietary and pharmacological treatment.

Key words: children; GER: primary, secondary; CMA/FA; 24-hour esophageal pH monitoring; oral food challenge test.

Introduction

Among 264 children suspected of gastroesophageal reflux disease (GERD), acid gastroesophageal reflux (GER) was confirmed in 138 (52.3%) children on the basis of 24-hour intraesophageal pH monitoring [1-7]. In order to differentiate primary GER from GER secondary to cow milk allergy and/or other food allergy (CMA/FA), a complex differential diagnosis was performed on the basis of various examinations, e.g. immunoallergological tests [8-14]. Positive result of oral food challenge test (open or blind study) confirmed allergy being the cause of GER [13]. Positive result of oral food challenge test was crucial to assign children into study groups: 1 and 2,

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and these children underwent further prospective observation. Qualification of children to further comparative studies resulted from abnormalities of pH monitoring and clinical symptoms.

Aims of the study:

– comparative analysis of parameters of 24-hour intraesophageal pH monitoring with dual-channel probe (in distal channel – above cardia) in children with acid GER: primary and secondary to CMA/FA diagnosed,

– comparison of examined values of pH monitoring parameters with regard to duration of the disease (preliminary study and prospective studies – after 1, 2, 4 and 9 years of clinical observation and/or conservative treatment).

Material and methods

264 children suspected of GERD, of both sexes (140 boys – 53.0% and 124 girls – 47.0%), aged 1.5 months to 102 months, mean age x=20.78±17.23 months, were enrolled in the study. According to anamnesis these children had various ailments of gastrointestinal tract reported in family histories.

1. 24-hour intraesophageal pH monitoring

The study was performed with dual-channel, antimony pH monitoring probe that measures intraesophageal pH in distal and proximal channel, and Digitraper MK III device, Synectics Medical, registering these values.

pH monitoring probe 2.1 mm diameter, was placed into esophagus of examined child through one of the nostrils and pharynx and distal channel (2) was situated 3-5 cm above upper edge of lower esophageal sphincter (LES).

Proximal channel (1) was 10, 15 or 20 cm above LES, depending on the length of esophagus.

To localize the probe Strobel's mathematical mode was used, and if difficulties in localizing occurred, radiological or manometric control of LES was performed [15-17].

Computer assessment of numerical measurements obtained from both channels: distal and proximal, concerned following pH monitoring parameters:

– number of episodes of acid GER (intraesophageal pH below 4.0),

– number of episodes of acid GER lasting more than 5 minutes (so-called "long episodes"),

– reflux index (RI), i.e. percentage of time that the pH is below 4.0 within 24-hour intraesophageal pH monitoring.

Pathological acid GER was diagnosed on the basis of ESPGHAN diagnostic criteria [1,2].

In children below 2 years of age results of esophageal pH monitoring were juxtaposed against borderline values summarized by Vandenplas et al. [3,4] and another authors [5,6].

In older children (above 2 years of age) the borderline values at qualitative and quantitative assessment of pathological GER in both channels were [1,2,18-20]:
total number of episodes of acid GER (pH<4.0/24 hrs) =50; number of episodes of acid GER lasting more than 5 minutes ≤2, percentage of time that the pH is below 4.0 (%) – acid GER index total =5.0%: percentage of time that the pH is below 4.0 (%) – acid GER index in supine position =2.5%.

Pathological acid GER was diagnosed in 138 children of the examined group.

2. Differential diagnosis of pathological acid GER

A. In order to differentiate pathological GER in 138 children with GERD into primary (idiopathic) and secondary – triggered off or aggravated by CMA/FA, the following immunoallergologic tests were performed [11,13,21,22].

* Skin tests

Prick tests

• with12 native food allergens, i.e. fresh (cow's milk, soya, of hen's egg white, hen's egg yolk, chicken's meat, beef, wheat flour, peanuts, bananas, fish, orange, white sesame);

• with 9 commercial inhalant allergens (SmithKline Beecham – USA)(house dust mites, grass , trees, bushes and weeds pollens, dog's fur, cat's fur, mixed feathers, wool).

71 out of 138 children, of different age, with pathological acid GER and 32 children with CMA/FA exclusively, underwent these test once in order to confirm or exclude the ability of early IgE-dependent hypersensitivity to food allergens and/or inhalant allergens (atopic factor influence and or cross reactions) to trigger off symptoms observed.

Results of control tests were the point of reference in assessment of reaction to allergens.

The diameter of blister ≥3 mm assessed after 15-20 minutes of allergen placement was concerned a positive result of skin Prick tests, compared to negative result of negative control.

* Eosinophilia

One-time assessment of relative eosinophilia in full blood count and its analysis were performed in 138 children with pathological acid GER and in 32 children with CMA/FA exclusively. Improper percentage value of eosinophilia, determined in full blood count, was >5%.

* Total IgE concentration (c IgE) in serum – assessed with Fluoro-Fast method (3M Diagnostic Systems, USA).

One-time assessment of serum IgE concentration was done in 170 children – 138 with acid GER and 32 with CMA/FA exclusively. Serum c IgE concentration >50 IU/ml was considered as elevated in examined children.

Taking into consideration restricted specificity of one-time measurement of total IgE in diagnosis of atopy, this test was performed together with determination of specific Ig in this particular class for selected food allergens.

* Qualitative and quantitative assessment of specific IgE against food allergens (a-s IgE) and inhalant allergens (i-s IgE) with Fluro-Fast method (3M Diagnostic Systems, USA).

Assay of allergen specific Ig concentration in examined children enabled confirmation of IgE-dependent pathomechanism of food allergy and determination of food allergens.

These tests appeared to be helpful in cases where tests cannot be performed or their results are doubtful, due to various reasons.

103 patients suspected of allergy, with positive Prick tests results (food allergens and/or inhalant allergens and increased total serum IgE concentration) underwent qualitative and quantitative assessment of a-s IgE and i-s IgE.

***Table 1*. General characteristics of 138 children with primary GER and GER secondary to CMA/FA, including age**

Examined groups	Sex	Number of patients		Age of patients			
				4-16 months		16-102 months	
		N	[%]	N	[%]	N	[%]
Group 1 Primary GER N=76	Boys	39	28.3	23	16.7	16	11.6
	Girls	37	26.8	21	15.2	16	11.6
	TOTAL	76	55.1	44	31.9	32	23.2
Group 2 GER secondary to CMA/FA N=62	Boys	33	23.9	16	11.6	17	12.3
	Girls	29	21.0	14	10.1	15	10.9
	TOTAL	62	44.9	30	21.7	32	23.2
TOTAL		138	100.0	74	53.6	64	46.4

Positive results of specific IgE were:

a) a-s IgE against cow milk proteins, hen's egg white, hen's egg yolk, soy, fish, orange;

b) i-s IgE against grass, trees, bushes and weeds pollens, house dust mites and cat's fur, assayed in serum – presence supported in class ≥2-5.

B. Oral food challenge test [10,12,13,21,23].

Open or blind oral food challenge test (depending on the age of patient) was carried out in order to establish causative relationship between food and clinical symptoms, regardless of pathogenetic mechanisms of allergy (IgE-dependent or IgE-non-dependent) [8,13].

The first stage of diagnostic procedure preceding the beginning of oral food challenge tests was eliminatory diet implementation, lasting 4 weeks in 138 children with acid GER. Diet depended on elimination of the most common food allergens, suspected of triggering off symptoms in examined children.

Eliminatory diet was determined on the basis of information gathered from medical history of past nutrition and the results of additional tests (skin Prick tests, s IgE) [8,13,23].

At the time of study, patients didn't receive or had maximally reduced antillergic and/or antihistaminic medications.

138 children at various age, with pathological acid GER, after eliminatory diet implementation (milk-free and/or hypoallergic diet) underwent 204 biological oral food challenge tests; 138 (67.6%) with cow's milk and 66 (32.4%) with other food.

In order to establish primary diagnosis, open food challenge test was performed in 104 children (under 3 years of age) and blind food challenge test in 34 children (under 3 years of age) with mainly cow's milk (low-lactose Bebilon, Ovita Nutricia) or with other potentially noxious nutrients [13,23]. Further control challenge tests in 62 children at various age with acid GER secondary to CMA/FA were repeated during 9-year clinical observation and/or conservative treatment:

- after 1 year (open study in 33 children or blind study in 29 children),
- after 2 years (blind study in 47 children),
- after 4 and 9 years of treatment (blind study in 8 children).

Every time child spent 1-3 days at hospital (Laboratory of Allergy Diagnostics, of IIIrd Department of Pediatrics)

Time of appearance of biological reaction in examined child was counted from the last food challenge up to 48-72 hours after intake of specific nutrient in native, blind form.

Every patient examined received every day observation chart for reporting intensity of clinical manifestation.

In case of cow's milk allergy or soy milk allergy and/or other food allergy the time of challenge test lasted 4 weeks.

The first challenge test was performed at the time of enrollment, further challenge tests after 1, 2, 4, 9 years of treatment with eliminatory diet. Long-lasting clinical observation was conducted to determine the acquisition of children's tolerance towards previously noxious nutrient.

Positive result of food challenge test and/or positive results of immunoallergological tests enabled to qualify a selected 62 children into group 2 – children with GER secondary to FA.

C. In order to establish the cause of secondary GER, different from food allergy, the results of additional tests performed in these children were analyzed, i.e. chest X-ray and upper gastrointestinal tract X-ray with barium swallow, X-ray or CT of sinuses (exclusively in school children).

In some patients it was necessary to analyze; full blood count, sedimentation rate, CRP, ASO and protein fraction pattern, IgA, IgM, IgG, *Helicobacter pylori* antibodies of the IgG class, iron level in order to confirm or exclude infectious cause of the symptoms presented.

Bacteriological examinations were performed in some children (blood, urine, faeces, bile, pharyngeal and nasal excretion) and metabolic screening by assaying lactic acid, ammonia, acid-base balance parameters in blood. Pilocarpine test (chlorine concentration in perspiration) was performed to exclude cystic fibrosis [9,22,24,25].

3. Assignment of children into groups

Taking into consideration esophageal pH monitoring results, complex differential diagnosis, oral food challenge tests and nutrition analysis in 264 children, pathological acid GER was confirmed in 138 of them (52.3%). These children were assigned into group 1 and group 2 (*Tab. 1*).

Group 1 – 76 patients (55.1%), of both sexes (39 boys – 28.3%, 37 girls – 26.8%), aged 4-102 months (mean age x=25.2 ±27.28 months), with pathological primary GER.

Group 2 – 62 patients (44.9%), of both sexes (33 boys – 23.9%, 29 girls – 21.0%), aged 4-74 months (mean age x=21.53 ±17.79 months), with pathological GER secondary to CMA/FA.

***Table 2*. Values of selected parameter of 24-hour esophageal pH monitoring in 138 children with pathological primary GER and GER secondary to CMA/FA, before and during treatment and/or clinical observation (prospective study)**

Groups of examined children with specific ailment N=138	pH monitoring parameter – duration of the longest episode of acid GER (minutes) Distal channel Range of values; mean (X); standard deviation (± SD); statistical significance (p); number of patients (N)				
	Before treatment (0)	Treatment and/or clinical observation in progress			
		after 1 year	after 2 years	after 4 years	after 9 years
Group 1 Primary GER	5.50 – 37.80 17.45±8.21 (76)	2.80 – 30.20 10.21±6.54 (76)	3.80 – 24.60 7.81±6.06 (46)	7.20 – 17.00 11.41±2.96 (12)	3.80 – 5.00 4.66±0.44 (12)
Statistical significance within the groups (p)	0 – 1, p=0.0001; 0 – 2, p=0.0001; 0 – 4, p=0.0022; 0 – 9, p=0.0022; 1 – 2, p=0.0001; 1 – 4, p=0.0022; 1 – 9, p=0.0022; 2 – 4, p=0.0022; 2 – 9, p=0.0022; 4 – 9, p=0.0022				
Group 2 GER + CMA/FA	8.50 – 41.50 14.61±7.68 (62)	3.20 – 30.20 10.03±5.83 (62)	3.30 – 27.60 6.23±4.89 (47)	8.20 – 16.30 10.19±2.71 (8)	3.90 – 9.80 5.28±1.86 (8)
Statistical significance within the groups (p)	0 – 1, p=0.0001; 0 – 2, p=0.0001; 0 – 4, p=0.0117; 0 – 9, p=0.0117; 1 – 2, p=0.0001; 1 – 4, p=0.0117; 1 – 9, p=0.0117; 2 – 4, p=0.0117; 2 – 9, p=0.0117; 4 – 9, p=0.0117				
Statistical significance between the groups (p)	ns	ns	ns	ns	ns

Statistical analysis

The statistical analysis of the results comprised arithmetical mean, standard deviation, minimal and maximal values and median – for measurable features and quantitative percentage distribution for qualitative features.

To compare the groups, features compatible with normal distribution, assessed with Kolomogorov compatibility test, were assessed together with the post hoc Bonferroni one-way analysis of variance. Features non-compatible with the distribution underwent Kruskal-Wallis test followed with Mann-Whitney test. T-Student pair test or Wilcoxon matched pairs test, respectively were used for the comparison between the two tests within each group at time interval. Statistical significance was $p<0.05$. Calculations were performed with the help of statistical package SPSS'12.0 PL.

Results

Prospective analysis of values of parameters measured during 24-hour intraesophageal pH monitoring with dual-channel probe (distal channel) was performed in 138 children. Assessment concerned preliminary study and control studies (during clinical observation and/or conservative treatment). 76 children had acid primary GER (group 1) and 62 children GER secondary to CMA/FA.

pH monitoring parameters were defined as follows:
in 76 children before treatment (preliminary study) and after 1 year of treatment, in 46 children – after 2 years, and in 12 children after 4 and 9 years of clinical observation and/or dietary and pharmacological treatment (group 1) and in 62 children – before treatment (preliminary examination) and after 1 year, in 47 children after 2 years, and in 8 children after 4 and 9 years of clinical observation and treatment (group 2).

The analysis is presented in *Fig. 1-4* and in *Tab. 2* (distal channel – above cardia).

*** according to number of episodes of acid GER (pH<4) (*Fig. 1*)**

In children with primary GER (group 1) mean values of measured parameter, before administration of treatment, x=75.68±25.61, were similar to x=74.60±16.02 in children with GER and CMA/FA (group 2).

During clinical observation and/or treatment mean values in group 1 were decreasing and accounted for x=46.75±22.88 after 1 year; 35.20±13.00 after 2 years; 41.08±4.44 and 26.33±4.42 after 4 and 9 years, respectively.

In children with GER and CMA/FA (group 2) during clinical observation and/or treatment a downward tendency of number of episodes of acid GER measured before treatment administration (x=74.60±16.02) was observed. Its mean values made x=57.52±21.71 after 1 year; 45.49±15.99 after 2 years; 49.25±7.21 and 27.00±1.69 after 4 and 9 years, respectively. Mean number of episodes of acid GER, measured in distal channel in both groups (1 and 2) revealed significant differentiation within the groups, between preliminary study (0) and control studies. Statistical significance was higher in group 1, especially during prospective clinical observation and treatment.

During clinical observation and treatment, differentiation of mean number of episodes of acid GER between study groups (1 and 2) was observed. Statistical significance was higher in group 2 after 1 year than after 4 years.

**** according to the number of episodes of acid GER (pH<4), lasting >5 minute (*Fig. 2*)**

In children with primary GER (group 1) mean values of measured parameter, before treatment administration,

Figure 1. **Prospective comparative analysis of number of episodes of acid GER between groups: 1 and 2 (distal channel)**

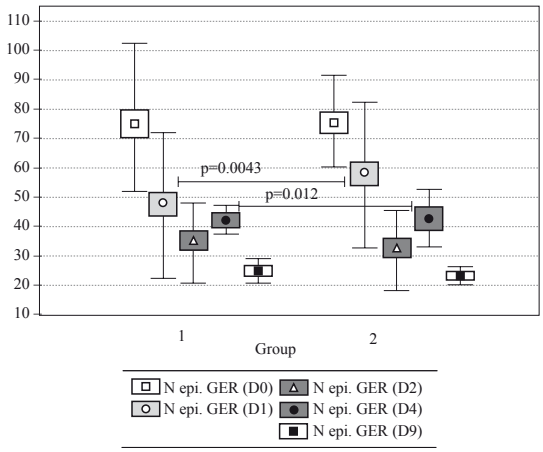


Figure 2. **Prospective comparative analysis of number of episodes of acid GER (pH<4) lasting >5 minutes between groups: 1 and 2 (distal channel)**

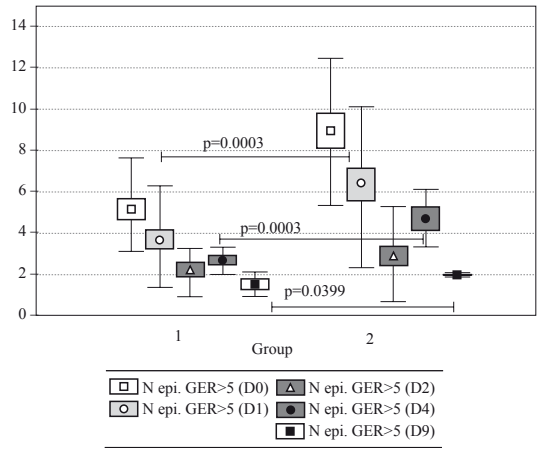


Figure 3. **Prospective comparative analysis of total acid GER index between groups: 1 and 2 (distal channel)**

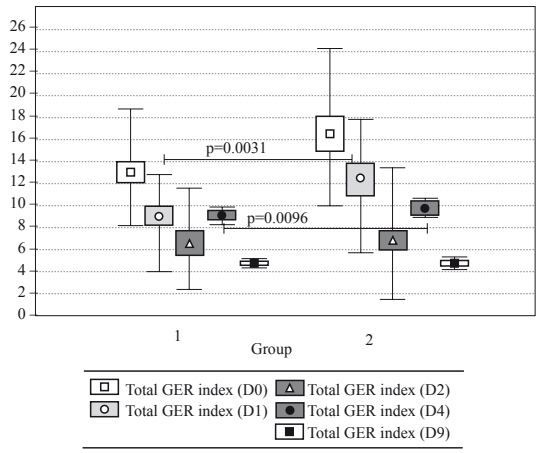
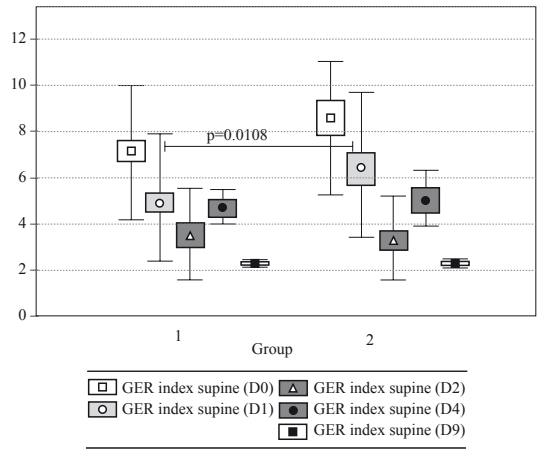


Figure 4. **Prospective comparative analysis of acid GER index (supine) between groups: 1 and 2 (distal channel)**



x=5.17±1.95 were lower than the values x=8.87±3.64 in children with GER and CMA/FA (group 2).

During clinical observation and/or treatment in group 1 mean values were decreasing and accounted for x=3.55±2.17 after 1 year; 2.17±1.18 after 2 years; 2.75±0.62 and 1.58±0.51 after 4 and 9 year, respectively.

In children with GER and CMA/FA (group 2) a downward tendency of mean number of episodes of acid GER lasting >5 minutes, measured before treatment (x=8.87±3.64) was observed. During clinical observation and/or treatment mean values accounted for x=5.87±3.75 after 1 year; 2.89±2.33 after 2 years; 4.88±0.99 and 2.00±0.00 after 4 and 9 years, respectively.

Mean number of episodes of acid GER lasting >5 minutes, measured in distal channel in both groups (1 and 2) constituted significant differentiation within groups between preliminary study (0) and control studies. Statistical significance was higher in group 1, especially during prospective clinical observation and treatment.

During clinical observation and treatment, differentiation of mean number of episodes of acid GER lasting >5 minutes between study groups (1 and 2) was confirmed. Statistical significance was higher in group 2, especially after 1 year and 4 years than after 9 years.

***** according to duration of the longest episode of acid GER (minutes) (*Tab. 2*)**

In children with primary GER (group 1), mean values of the parameter measured before treatment administration x=17.45±8.21 were higher than the values in children with GER and CMA/FA x=14.61±7.68 (group 2).

During clinical observation and/or treatment in group 1, mean values were decreasing and accounted for x=10.21±6.54 after 1 year; 7.81±6.06 after 2 years; 11.41±2.96 and 4.66±0.44 after 4 and 9 years.

A downward tendency of mean value of duration of the longest episode of acid GER, measured before treatment (x=14.61±7.68) was observed in children with GER and CMA/FA.

During clinical observation and/or treatment mean values accounted for x=10.03±5.83 after 1 year; 6.23±4.89 after 2 years; 10.19±2.71 and 5.28±1.86 after 4 and 9 years, respec-

tively. Mean values of time of the longest episode of acid GER measured in distal channel in both groups (1 and 2) were significantly different within the groups, between preliminary examination (0) and control examinations. Statistical significance was higher in group 1, especially during prospective clinical observation and treatment.

During clinical observation and treatment there was no differentiation of mean values of duration of the longest episode of acid GER between study groups (1 and 2). Statistical significance was comparable in both groups: 1 and 2, in particular years.

****** according to total acid GER index (%) (*Fig. 3*)**

In children with primary GER (group 1), before administration of treatment, mean values of measured parameter x=13.42±5.52 were lower than the values x=17.17±6.96 in children with GER and CMA/FA (group 2).

During clinical observation and/or treatment in group 1 mean values were decreasing and accounted for x=8.71±4.67 after 1 year; 6.99±4.24 after 2 years; 8.78±0.74 i 4.76±0.23 after 4 and 9 years, respectively.

In children with GER and CMA/FA (group 2) a downward tendency of mean values of total acid RI, measured before treatment (x=17.17±6.96) was observed.

During clinical observation and/or treatment mean values constituted x=11.83±5.85 after 1 year; 6.93±5.34 after 2 years; 10.21±1.12 i 4.59±0.24 after 4 and 9 years, respectively.

Mean values of total acid RI, measured in distal channel, in both groups (1 and 2) revealed significant differentiation within the groups, between preliminary examination (0) and control examinations. Statistical significance was higher in group 1, especially in prospective clinical observation and treatment.

During clinical observation and treatment differentiation of mean values of total acid RI between study groups (1 and 2) was observed. Statistical significance was higher in group 2, especially after 1 year than after 4 years.

******* according to acid RI, supine (%) (*Fig. 4*)**

In children with primary GER (group 1), before treatment administration mean values of measured parameter x=6.96±2.64 were lower than values x=7.67±2.87 in children with GER and CMA/FA (group 2).

During clinical observation and/or treatment in group 1 mean values decreased and accounted for x=4.82±2.84 after 1 year; 3.46±2.06 after 2 years; 4.59±0.86 i 2.40±0.18 after 4 and 9 years, respectively.

In children with GER and CMA/FA a tendency of mean value of acid RI, in supine position, measured before treatment administration was observed (x=7.67±2.87).

During clinical observation and/or treatment mean values constituted x=5.92±2.81 after 1 year; 3.18±1.88 after 2 years; 5.06±0.92 and 2.40±0.09 after 4 and 9 years respectively. Mean values of acid RI, in supine position, measured in distal channel in both groups (1 and 2) revealed significant differentiation within the groups, between preliminary examination (0) and control examinations.

Statistical significance was higher in group 1, especially during prospective clinical observation and treatment.

During clinical observation and treatment differentiation of mean values of acid RI, in supine position, between study groups (1 and 2) was seen. Statistical significance was higher in group 2, only after 1 year of treatment.

Discussion

Of 264 children with symptoms suggestive of GERD and various diseases of gastrointestinal system in family history, 138 children (52.3%) had pathological acid GER confirmed on the basis of 24-hour esophageal pH monitoring [1-7] and GERD diagnosed.

The results of immunoallergological tests and positive outcome of oral food challenge test [8-14,23] were pivotal in determining the cause of the disease, and acid GER was differentiated into primary (76 – 55.1%, group 1) and secondary to CMA/FA (62 – 44.9%, group 2) in 138 children.

Children with pathological acid GER diagnosed were included in a prospective study. The qualification of children was consequent upon the anomalies of pH monitoring recording and clinical manifestation. Both age and sex of 138 examined children of both study groups at diagnosis did not reveal statistically significant differences.

It was assumed that the higher positioning of the sensor of electrode in esophagus during 24-hour pH monitoring with dual-channel probe, the less number of shorter lasting reflux episodes were recorded. Total time of reflux is therefore shortened, which results from better efficiency of mechanism responsible for neutralizing gastric content retrograding into esophagus and the ability of esophagus to self-purification [5-7,19].

In both study groups (primary GER and secondary GER), the comparative analysis of mean values of pH monitoring parameters measured on the basis of 24-hour intraesophageal pH monitoring in distal lead was carried out. The measurements were done in preliminary study and during prospective clinical observation and conservative treatment.

The analysis showed significant differentiation of mean values between preliminary study and control study within groups in the case of: number of episodes of acid GER and episodes of acid GER lasting more than 5 minutes, duration of the longest episode of acid GER and acid GER index: total and supine. Statistical significance was higher in group with GER secondary to CMA/FA, especially during prospective clinical observation and treatment.

Differentiation of mean number of episodes of acid GER and episodes of acid GER lasting more than 5 minutes was shown during clinical observation and treatment.

Statistical significance of mean values of parameters mentioned was higher in group of GER secondary to CMA/FA after 1 year and 4 years.

Differentiation of mean values of total acid GER index within the groups was also statistically significant in this group after 1 year and 4 years. Similar mean values of supine acid GER index differed within the groups and were of higher statistical significance in group with GER secondary to CMA/FA but only after the first year of clinical observation and treatment. This is attributable to fast reduction of intensity of supine

GER at rest, especially during night sleep due to improvement of mechanisms responsible for neutralization and self-purification of esophagus under dietary and pharmacological treatment [12,14,19,21,22,25].

In the summary of the aforementioned comparative analysis, significantly higher values of pH monitoring parameters measured were shown during prospective studies, excluding duration of the longest episode of acid GER in children with GER secondary to CMA/FA in comparison with children with primary GER. Results obtained in patients with GER, in a study group with allergy are attributable to more intense to disorder of gastroesophageal junction arguably due to coexistent allergization of the upper gastrointestinal tract with noxious nutrient or food from children's regular diet [5-7,12,21,22,25].

Conclusions

In conclusion it was stated that values of pH monitoring parameters in distal channel in children at diagnosis were comparable, and results enabled to differentiate acid GER into primary and secondary.

During prospective clinical observation and/or conservative treatment the intensity of reflux in study groups was assessed on the basis of the results of episodes of acid GER and episodes of acid GER lasting more than 5 minutes in distal channel.

Acid GER index: total and supine appeared to be important diagnostic parameter but only after the first year of dietary and pharmacological treatment. It was therefore determined that GER secondary to CMA/FA was more intense than primary GER at that time.

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24-hour esophageal pH monitoring in children with pathological acid gastroesophageal reflux: primary and secondary to food allergy

Part II
Intraesophageal pH values in proximal channel; preliminary study and control studies – after 1, 2, 4 and 9 years of clinical observation as well as dietary and pharmacological treatment

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Abstract

Purpose: Among 264 children suspected of GERD, acid GER was confirmed in 138 children on the basis of 24-hour pH monitoring.

Aims of the study: Comparative analysis of parameters of 24-hour intraesophageal pH monitoring with dual-channel probe (in proximal channel) in children with acid GER: primary and secondary to cow milk allergy and/or other food allergy (CMA/FA) diagnosed; comparison of examined values of pH monitoring parameters with regard to duration of the disease (preliminary study and prospective studies – after 1, 2, 4 and 9 years of clinical observation and/or conservative treatment).

Material and methods: 264 children suspected of GERD, aged: 1.5-102 months; x=20.78±17.23 months, were enrolled in a study. In order to differentiate acid primary GER from GER secondary to CMA/FA in 138 (52.3%) children with GERD immunoallergological tests were performed. Positive result of oral food challenge test confirmed the allergy being the cause of GER.

138 children with pathological acid GER were qualified into two groups: 1 and 2.

Group 1 – 76 patients (55.1%), aged: 4-102 months; x=25.2±27.28 months, with pathological primary GER. Group 2 – 62 patients (44.9%), aged: 4-74 months; x=21.53±17.79 months, with pathological GER secondary to CMA/FA.

Results: Significant differentiation of the mean values of these parameters between preliminary study and control studies within groups was shown in the case of: number of episodes of acid GER and duration of the longest episode of acid GER, acid GER index: total and supine (proximal channel). Statistical significance ($p<0.05$) was higher in group 1, especially during prospective clinical observation and/or conservative treatment. At the same time significant differentiation of the mean values of: number of episodes of acid GER and episodes of acid GER lasting more than 5 minutes and mean values of acid GER index: total and supine was shown between the groups. Statistical significance ($p<0.05$) was higher in group 2.

Conclusions: The preliminary study of examined children confirmed that values of pH monitoring in proximal channel were comparable to those in distal channel and did not contribute to differentiation of GER into primary and secondary. During prospective clinical observation and/or clinical treatment, on the basis of consecutive measurements, especially the number of episodes of acid GER and episodes of acid GER lasting more than 5 minutes, and also supine acid GER index it was stated that GER secondary to CMA/FA was of wider extent (higher) in comparison with primary GER in these patients.

Key words: children; GER: primary, secondary; CMA/FA; 24-hour esophageal pH monitoring; oral food challenge test.

Introduction

Among 264 children suspected of gastroesophageal reflux disease (GERD), acid gastroesophageal reflux (GER) was confirmed in 138 (52.3%) children on the basis of 24-hour intraesophageal pH monitoring [1-7].

Aims of the study

– comparative analysis of parameters of 24-hour intraesophageal pH-monitoring with dual-channel probe (in proximal channel) in children with acid GER: primary and secondary to CMA/FA diagnosed,

– comparison of examined values of pH monitoring parameters with regard to duration of the disease (preliminary study

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Table 1. **Values of selected parameter of 24-hour esophageal pH monitoring in 138 children with pathological primary GER and GER secondary to CMA/FA, before and during treatment and/or clinical observation (prospective study)**

Groups of examined children with specific ailment N= 138	pH monitoring parameter – number of episodes of acid GER (pH<4) Proximal channel Range of values; mean (X); standard deviation (± SD); statistical significance (p); number of patients (N)				
	Before treatment (0)	Treatment and/or clinical observation in progress			
		after 1 year	after 2 years	after 4 years	after 9 years
Group 1 Primary GER	31.00 – 107.00 61.45±20.43 (76)	10.00 – 78.00 34.13±16.71 (76)	10.00 – 49.00 22.17±12.40 (46)	24.00 – 37.00 31.00±3.77 (12)	10.00 – 19.00 15.00±3.02 (12)
Statistical significance within the groups (p)	0 – 1, p=0.0001; 0 – 2, p=0.0001; 0 – 4, p=0.0054; 0 – 9, p=0.0022; 1 – 2, p=0.0001; 1 – 4, p=0.0022; 1 – 9, p=0.0022; 2 – 4, p=0.0022; 2 – 9, p=0.0022; 4 – 9, p=0.0022				
Group 2 GER+ CMA/FA	32.00 – 93.00 62.48±14.67 (62)	19.00 – 79.00 45.60±16.70 (62)	21.00 – 65.00 30.11±10.58 (47)	36.00 – 49.00 41.13±4.29 (8)	21.00 – 27.00 23.50±1.77 (8)
Statistical significance within the groups (p)	0 – 1, p=0.0001; 0 – 2, p=0.0001; 0 – 4, p=0.0117; 0 – 9, p=0.0117; 1 – 2, p=0.0001; 1 – 4, p=0.0117; 1 – 9, p=0.0117; 2 – 4, p=0.0117; 2 – 9, p=0.0117; 4 – 9, p=0.0117				
Statistical significance between the groups (p)	ns	p=0.0001	p=0.0001	p=0.0003	p=0.0002

and prospective studies – after 1, 2, 4 and 9 years of clinical observation and/or conservative treatment).

Detailed diagnostic procedure is presented in 'Material and methods' section, Part I of the study [8-25].

Assignment of children into study groups

Taking into consideration esophageal pH-monitoring results, complex differential diagnostics, oral food challenge tests and nutrition analysis in 264 children, pathological acid GER was confirmed in 138 of them (52.3%). These children were assigned into group 1 and group 2.

Group 1 – 76 patients (55.1%), of both sexes (39 boys – 28.3%, 37 girls – 26.8%), aged 4-102 months (mean age x=25.2±27.28 months), with pathological primary GER.

Group 2 – 62 patients (44.9%), of both sexes (33 boys – 23.9%, 29 girls – 21.0%), aged 4-74 months (mean age x=21.53±17.79 months), with pathological GER secondary to CMA/FA.

Statistical analysis

The statistical analysis of the results comprised arithmetical mean, standard deviation, minimal and maximal values and median – for measurable features and quantitative percentage distribution for qualitative features.

To compare the groups, features compatible with normal distribution, assessed with Kolomogorov compatibility test, were assessed together with the post hoc Bonferroni one-way analysis of variance. Features non-compatible with the distribution underwent Kruskal-Wallis test followed with Mann-Whitney test. T-Student pair test or Wilcoxon matched pairs test, respectively were used for the comparison between the two tests within each group at time interval. Statistical significance was $p<0.05$. Calculations were performed with the help of statistical package SPSS'12.0 PL.

Results

Prospective analysis of values of parameters measured during 24-hour intraesophageal pH monitoring with dual-channel probe (proximal channel) was performed in 138 children. Assessment concerned preliminary study and control studies (during clinical observation and/or conservative treatment). 76 children had acid primary GER (group 1) and 62 children GER secondary to CMA/FA (group 2).

pH-monitoring parameters were defined as follows:

in 76 children before treatment (preliminary examination) and after 1 year of treatment, in 46 children – after 2 years, and in 12 children after 4 and 9 years of clinical observation and/or dietary and pharmacological treatment (group 1) and in 62 children – before treatment (preliminary examination) and after 1 year, in 47 children after 2 years and in 8 children after 4 and 9 years of clinical observation and treatment (group 2).

The analysis is presented in tables (proximal channel; *Tab. 1-5*).

*** according to number of episodes of acid GER (pH<4) (*Tab. 1*)**

In children with primary GER (group 1) mean values of parameter measured before treatment x=61.45±20.43 were similar to the values x=62.48±14.67 in children with GER and CMA/FA (group 2).

During clinical observation and/or treatment mean values in group 1 were decreasing and accounted for x= 34.13±16.71 after 1 year; 22.17±12.40 after 2 years; 31.00±3.77 and 15.00±3.02 after 4 and 9 years, respectively.

In children with GER and CMA/FA (group 2) during clinical observation and/or treatment, a downward tendency of number of episodes of acid GER measured before treatment administration (x=62.48±14.67) was observed. Its mean values

***Table 2*. Values of selected parameter of 24-hour esophageal pH monitoring in 138 children with pathological primary GER and GER secondary to CMA/FA before and during treatment and/or clinical observation (prospective study).**

Groups of examined children with specific ailment N=138	pH monitoring parameter – number of episodes of acid GER lasting >5mins (pH<4) Proximal channel Range of values; mean (X); standard deviation (± SD); statistical significance (p); number of patients (N)				
	Before treatment (0)	Treatment and/or clinical observation in progress			
		after 1 year	after 2 years	after 4 years	after 9 years
Group 1 Primary GER	1.00 – 7.00 3.96±1.37 (76)	0.00 – 6.0 2.62±1.60 (76)	0.00 – 4.00 1.37±1.10 (46)	1.00 – 3.00 2.08±0.79 (12)	1.00 – 2.00 1.17±0.39 (12)
Statistical significance within the groups (p)	0 – 1, p=0.0001; 0 – 2, p=0.0001; 0 – 4, p=0.0209; 0 – 9, p=0.0033; 1 – 2, p=0.0001; 1 – 4, p=0.0022; 1 – 9, p=0.0022; 2 – 4, p=0.0277; 2 – 9, p=0.0022; 4 – 9, p=0.0117				
Group 2 GER + CMA/FA	1.00 – 15.00 5.87±3.64 (62)	0.00 – 12.00 4.24±3.09 (62)	0.00 – 8.00 2.40 ±1.94 (47)	3.00 – 6.00 4.38±1.19 (8)	1.00 – 2.00 1.75±0.46 (8)
Statistical significance within the groups (p)	0 – 1, p=0.0001; 0 – 2, p=0.0001; 0 – 4, p=0.0117; 0 – 9, p=0.0117; 1 – 2, p=0.0001; 1 – 4, p=0.0117; 1 – 9, p=0.0117; 2 – 4, p=0.0117; 2 – 9, p=0.0117; 4 – 9, p=0.0117				
Statistical significance between the groups (p)	ns	p=0.0039	p=0.0046	p=0.0005	p=0.011

made x=45.60±16.70 after 1 year; 30.11±10.58 after 2 years; 41.13±4.29 and 23.50±1.77 after 4 and 9 years, respectively. Mean number of episodes of acid GER, measured in proximal channel in both groups (1 and 2) revealed significant differentiation within the groups, between preliminary study (0) and control studies. Statistical significance was higher in group 1, especially during prospective clinical observation and treatment.

During clinical observation and treatment, differentiation of mean number of episodes of acid GER between study groups (1 and 2) was observed. Statistical significance was higher in group 2 after 1, 2, 4 and 9 years.

**** according to the number of episodes of acid GER (pH<4), lasting >5minutes (*Tab. 2*)**

In children with primary GER (group 1) mean values of measured parameter, before treatment administration, x=3.96±1.37 were lower than the values x=5.87±3.64 in children with GER and CMA/FA (group 2).

During clinical observation and/or treatment in group 1 mean values were decreasing and accounted for x=2.62±1.60 after 1 year; 1.37±1.10 after 2 years; 2.08±0.79 and 1.17±0.39 after 4 and 9 year, respectively.

In children with GER and CMA/FA (group 2) downward tendency of mean number of episodes of acid GER lasting >5 minutes, measured before treatment (x=5.87±3.64) was observed. During clinical observation and/or treatment mean values accounted for x=4.24±3.09 after 1 year; 2.40±1.94 after 2 years; 4.38±1.19 and 1.75±0.46 after 4 and 9 years, respectively.

Mean number of episodes of acid GER lasting > 5 minutes, measured in proximal channel in both groups (1 and 2) constituted significant differentiation within the groups between preliminary study (0) and control studies. Statistical significance was higher in group 1, especially during prospective clinical observation and treatment (exception: higher significance in group 2 between preliminary study and control study – after 4 years and between control studies: after 2 and 4 years).

During clinical observation and treatment, differentiation of mean number of episodes of acid GER lasting >5 minutes between study groups (1 and 2) was observed.

Statistical significance was higher in group 2, the highest after 4 years and the lowest after 9 years.

***** according to duration of the longest episode of acid GER (minutes) (*Tab. 3*)**

In children with primary GER (group 1), mean values of the parameter measured before treatment administration x=12.91±5.14 were higher than the values in children with GER and CMA/FA x=9.51±3.78 (group 2).

During clinical observation and/or treatment in group 1 mean values were decreasing and accounted for x=7.61±4.80 after 1 year; 5.32±3.96 after 2 years; 7.90±1.09 and 3.96±0.73 after 4 and 9 years.

A downward tendency of mean value of duration of the longest episode of acid GER, measured before treatment (x=9.51±3.78) was observed in children with GERD and CMA/FA (group 2).

During clinical observation and/or treatment mean values accounted for x=7.17±3.40 after 1 year; 4.61±2.68 after 2 years; 7.85±1.11 and 3.90±0.37 after 4 and 9 years, respectively. Mean values of duration of the longest episode of acid GER measured in proximal channel in both groups (1 and 2) were significantly different within the groups, between preliminary examination (0) and control examinations. Statistical significance was higher in group 1, especially during prospective clinical observation and treatment.

During clinical observation and treatment there was no dif-

***Table 3*. Values of selected parameter of 24-hour esophageal pH monitoring in 138 children with pathological primary GER and GER secondary to CMA/FA before and during treatment and/or clinical observation (prospective study)**

Groups of examined children with specific ailment N=138	pH monitoring parameter – duration of the longest episode of acid GER (mins) Proximal channel Range of values; mean (X); standard deviation (± SD); statistical significance (p); number of patients (N)				
	Before treatment (0)	Treatment and/or clinical observation in progress			
		after 1 year	after 2 years	pafter 4 years	after 9 years
Group 1 Primary GER	5.50 – 24.10 12.91±5.14 (76)	1.20 – 21.00 7.61±4.80 (76)	1.10 – 16.90 5.32±3.96 (46)	6.60 – 9.90 7.90±1.09 (12)	2.60 – 5.00 3.96 ±0.73 (12)
Statistical significance within the groups (p)	0 – 1, p=0.0001; 0 – 2, p=0.0001; 0 – 4, p=0.0022; 0 – 9, p=0.0022; 1 – 2, p=0.0001; 1 – 4, p=0.0022; 1 – 9, p=0.0022; 2 – 4, p=0.0022; 2 – 9, p=0.0022; 4 – 9, p=0.0022				
Group 2 GER + CMA/FA	5.60 – 21.50 9.51±3.78 (62)	2.30 – 19.30 7.17±3.40 (62)	2.20 – 14.70 4.61±2.68 (47)	6.30 – 9.30 7.85±1.11 (8)	3.50 – 4.50 3.90±0.37 (8)
Statistical significance within the groups (p)	0 – 1, p=0.0001; 0 – 2, p=0.0001; 0 – 4, p=0.0117; 0 – 9, p=0.0117; 1 – 2, p=0.0001; 1 – 4, p=0.0117; 1 – 9, p=0.0117; 2 – 4, p=0.0117; 2 – 9, p=0.0117; 4 – 9, p=0.0117				
Statistical significance between the groups (p)	ns	ns	ns	ns	ns

***Table 4*. Values of selected parameter of 24-hour esophageal pH monitoring in 138 children with pathological primary GER and GER secondary to CMA/FA before and during treatment and/or clinical observation (prospective study)**

Groups of examined children with specific ailment N=138	pH monitoring parameter – total acid GER index (%) Proximal channel Range of values; mean (X); standard deviation (± SD); statistical significance (p); number of patients (N)				
	Before treatment (0)	Treatment and/or clinical observation			
		after 1 year	after 2 years	After 4 years	after 9 years
Group 1 Primary GER	5.20 – 20.20 11.26±4.18 (76)	2.70 – 16.40 7.28±3.78 (76)	2.90 – 14.10 6.48±4.92 (46)	6.40 – 8.80 7.36±0.66 (12)	3.20 – 5.00 4.11±0.55 (12)
Statistical significance within the groups (p)	0 – 1, p=0.0001; 0 – 2, p=0.0001; 0 – 4, p=0.0022; 0 – 9, p=0.0022; 1 – 2, p=0.0001; 1 – 4, p=0.0022; 1 – 9, p=0.0022; 2 – 4, p=0.0022; 2 – 9, p=0.0022; 4 – 9, p=0.0022				
Group 2 GER + CMA/FA	5.20 – 19.20 10.47±3.80 (62)	3.00 – 14.40 7.99±3.13 (62)	2.90 – 11.90 4.84±2.47 (47)	6.00 – 8.50 7.48±0.88 (8)	3.30 – 4.30 3.81±0.41 (8)
Statistical significance within the groups (p)	0 – 1, p=0.0001; 0 – 2, p=0.0001; 0 – 4, p=0.0117; 0 – 9, p=0.0117; 1 – 2, p=0.0001; 1 – 4, p=0.0117; 1 – 9, p=0.0117; 2 – 4, p=0.0117; 2 – 9, p=0.0117; 4 – 9, p=0.0117				
Statistical significance between the groups (p)	ns	ns	ns	ns	ns

ferentiation of mean values of duration of the longest episode of acid GER between study groups (group 1 and 2). Statistical significance was comparable in both groups: 1 and 2, in particular years.

****** according to total acid GER index (%) (*Tab. 4*)**

In children with primary GER (group 1), before administration of treatment, mean values of measured parameter x=11.26±4.18 were lower than the values x=10.47±3.80 in children with GER and CMA/FA (group 2).

During clinical observation and/or treatment in group 1 mean values were decreasing and accounted for x=7.28±3.78 after 1 year; 6.48±4.92 after 2 years; 7.36±0.66 and 4.11±0.55 after 4 and 9 years, respectively.

In children with GER and CMA/FA (group 2) a downward tendency of mean values of total acid reflux index (RI), measured before treatment (x=10.47±3.80) was observed.

During clinical observation and/or treatment mean values constituted x=7.99±3.13 after 1 year; 4.84±2.47 after 2 years; 7.48±0.88 and 3.81±0.41 after 4 and 9 years, respectively.

Mean values of total acid RI, measured in proximal channel, in both groups (1 and 2) revealed significant differentiation within the groups, between preliminary examination (0) and control examinations. Statistical significance was higher in

***Table 5*. Values of selected parameter of 24-hour esophageal pH monitoring in 138 children with pathological primary GER and GER secondary to CMA/FA before and during treatment and/or clinical observation (prospective study)**

Groups of examined children with specific ailment N=138	pH monitoring parameter – acid GER index (supine position) (%) Proximal channel Range of values; mean (X); standard deviation (± SD); statistical significance (p); number of patients (N)				
	Before treatment (0)	Treatment andor clinical observation in progress			
		after 1 year	after 2 years	After 4 years	after 9 years
Group 1 Primary GER	2.70 – 13.80 6.41±2.64 (76)	1.00 – 12.10 4.07±2.59 (76)	1.30 – 8.00 3.03±2.02 (46)	2.90 – 5.10 3.93±0.53 (12)	1.80 – 2.50 2.13±0.19 (12)
Statistical significance within the groups (p)	0 – 1, p=0.0001; 0 – 2, p=0.0001; 0 – 4, p=0.0022; 0 – 9, p=0.0022; 1 – 2, p=0.0001; 1 – 4, p=0.0022; 1 – 9, p=0.0022; 2 – 4, p=0.0022; 2 – 9, p=0.0022; 4 – 9, p=0.0022				
Group 2 GER + CMA/FA	3.30 – 15.00 7.16±2.76 (62)	1.80 – 11.50 5.35±2.59 (62)	1.70 – 8.30 2.85±1.78 (47)	3.50 – 5.50 4.60±0.70 (8)	1.80 – 2.50 2.16±0.24 (8)
Statistical significance within the groups (p)	0 – 1, p=0.0001; 0 – 2, p=0.0001; 0 – 4, p=0.0117; 0 – 9, p=0.0117; 1 – 2, p=0.0001; 1 – 4, p=0.0117; 1 – 9, p=0.0117; 2 – 4, p=0.0117; 2 – 9, p=0.0117; 4 – 9, p=0.0117				
Statistical significance between the groups (p)	ns	p=0.0011	ns	p=0.0637	ns

group 1, especially in prospective clinical observation and treatment.

During clinical observation and treatment, differentiation of mean values of total acid RI between study groups (1 and 2) was observed. Statistical significance was comparable in both groups 1 and 2, in particular years.

******* according to acid RI, supine position (%) (*Tab. 5*)**

In children with primary GER (group 1), before treatment administration mean values of measured parameter x=6.41±2.64 were slightly lower than values x=7.16±2.76 in children with GER and CMA/FA (group 2).

During clinical observation and/or treatment on group 1 mean values decreased and accounted for x=4.07±2.59 after 1 year; 3.03±2.02 after 2 years; 3.93±0.53 and 2.13±0.19 after 4 and 9 years, respectively.

In children with GER and CMA/FA a downward tendency of mean value of acid RI, in supine position, measured before treatment administration was observed (x=7.16±2.76).

During clinical observation and/or treatment, mean values constituted x=5.35±2.59 after 1 year; 2.85±1.78 after 2 years; 4.60±0.70 and 2.16±0.24 after 4 and 9 years respectively. Mean values of acid RI, supine measured in proximal channel in both groups (1 and 2) revealed significant differentiation within the groups, between preliminary examination (0) and control examinations.

Statistical significance was higher in group 1, especially during prospective clinical observation and treatment.

During clinical observation and treatment differentiation of mean values of acid RI, in supine position was statistically significant between study groups (1 and 2). Statistical significance was higher in group 2, only after 1 year of treatment.

Discussion

The comparative analysis of mean values of pH monitoring parameters recorded in 24-hour intraesophageal pH monitoring in proximal channel of both study groups: children with primary GER and children with GER secondary to CMA/FA was conducted [1-7].

The measurements were done before the treatment and during prospective clinical observation and treatment.

The analysis showed statistically significant differentiation of mean values of episodes of acid GER and episodes of acid GER lasting more than 5 minutes, the longest episode of acid GER duration and acid GER index (total and supine) between preliminary study and control study. Statistical significance of these differences was higher in group 1, especially during prospective, long-term clinical observation.

This is attributable to higher effectiveness of classical antireflux treatment rather than combined treatment (antiallergic and antireflux) in eliminating the results of reflux and the causative and pathogenic role of food allergy in secondary GER [9,10,14,21,26-28].

Statistically significant mean value of episodes of acid GER lasting more than 5 minutes, higher in group 2 in preliminary study and control study after 4 years and in control studies after 2 and 4 years of clinical observation and treatment seems to be an exception.

The higher number of episodes of GER lasting more than 5 minutes constituted a characteristic feature of GER secondary to FA.

During clinical observation differentiation of mean number of episodes of acid GER and episodes of acid GER lasting more than 5 minutes, especially after 4 years was shown. Statistical significance of mean values mentioned was higher in group with GER secondary to CMA/FA.

In the case of acid GER index only supine mean values were differentiated within the groups and had higher statistical significance in group 2, exclusively after the first year of clinical observation and treatment.

The results may suggest that the reduction of the intensity of supine GER, especially during night sleep is due to improvement of mechanism responsible for neutralization and self-purification of esophagus under dietary and pharmacological treatment [5,7,10,14,19,21,22].

The comparative analysis of examined pH monitoring parameters measured in proximal channel showed significantly higher mean values of episodes of acid GER and acid GER lasting more than 5 minutes and supine acid GER index in children with GER secondary to CMA/FA than in children with primary GER during the study.

The results obtained in patients of group 2 are attributable to more clearly expressed dissociation of motor activity of gastroesophageal junction, which could be the result of coexistent allergization of upper gastrointestinal system triggered off by noxious nutrient from the paients' diet [12,14,21,22,25].

During 24-hour esophageal pH monitoring with dual-channel probe it was assumed that the higher the positioning of the sensor of the electrode the lower number of short-term reflux episodes.

It was also assumed that the total reflux time is shortened, which results from the better efficiency of the mechanism responsible for neutralisation pH gastric content and the ability of esophagus to self-purification [5,7,19,20].

The results of our studies do not confirm the stated hypothesis completely because the mean values of analyzed pH monitoring parameters in proximal channel were not lower (not statistically significant) than in distal part of esophagus in children with GERD of both study groups with GERD. The results are comparable with pH-metric esophageal results obtained by Cucchiara et al. [19] and another authors [5,6,20].

The percentage values of the number of episodes of acid GER registered in preliminary study and after 1 year and 9 years of treatment accounted for 81%, 73% and 57% in proximal channel, respectively (group 1) and 84%, 79% and 87% in distal channel, respectively (group 2).

The number of episodes of acid GER lasting more than 5 minutes recorded in proximal channel accounted for 76% to 74% (group 1) and 66%, 72% and 87% (group 2) in distal channel, in preliminary study and control studies.

Also the duration of the longest episode of acid GER recorded in proximal channel reached 74% and 75% (group 1) and in distal channel 65%, 71% and 74% (group 2), respectively in preliminary study and control studies.

Total acid GER index recorded in proximal channel accounted for 84% and 86% (group 1), whereas in distal channel 61%, 67% and 83% (group 2), in preliminary study and control studies after 1 year and 9 years of treatment, respectively.

Supine acid GER index recorded in proximal channel made 92%, 84% and 89% (group 1) and 93% and 90% (group 2) in distal channel, in preliminary study and control studies.

During prospective studies the gradual tendency of mean values of pH monitoring parameters in both channels to return to normal values. Although the reflux was diminishing in consecutive pH recordings in examined children with primary and secondary GER, mean values of parameters were comparable and did not show significant difference between both channels (distal and proximal).

In both groups the values of pH monitoring parameters obtained in proximal channel constituted more than 50% of the values obtained in distal channel. This may appear due to considerable range of reflux (high reflux), persisting regardless of the normalisation of pathological pH monitoring recording under antireflux or combined treatment (antireflux and antiallergic) [5,7,10,14,19,21,22].

On the basis of the results obtained in preliminary study and control studies in these groups there were no significant quantitative differences in episodes of acid GER reaching both distal and proximal channel, regardless of the age of the children.

The preliminary study in children with primary GER showed that mean values of pH monitoring parameters measured in proximal channel were similar.

However, the control pH monitoring in proximal channel showed that mean values of episodes of acid GER and episodes of acid GER lasting more than 5 minutes during clinical observation and/or treatment, similarly to mean values of supine acid GER index – only after the first year of observation were significantly higher in group with GER secondary to FA than in group with primary GER ($p<0.05$).

The high GER, reaching proximal esophagus is important in children of both groups, but in children with atypical symptoms, especially of respiratory tract (latent reflux) may suggest microaspiration of gastric content into bronchial tree [5-7,9].

In children below 3 years of age, who had recurrent inflammations of upper respiratory tract reported, latent GER was confirmed with pH esophageal monitoring with single-channel probe in 56% and 57% of other gastroenterological centers in Poland, respectively [29,30].

On the basis of 24-hour esophageal pH monitoring with dual-channel probe in children with symptoms outside the gastrointestinal tract, in the same age group, in own studies, the percentage of high GER in both study groups was reported accounting for 77.4% and 88.3%, respectively.

The results of own studies contribute to intensity of acid GER reaching distal and proximal esophagus, and mean values of pH monitoring parameters in proximal and distal channel show statistical significance between the groups, especially during the prospective clinical observation and administered treatment.

The comparable mean values of duration of the longest episode of acid GER in both channels, supine acid GER index in distal channel and total acid GER index in proximal channel constitute the exception.

This differentiation of examined pH monitoring parameters between the groups could be important in predicting who of the examined children is at risk of primary GER and who is at risk of GER secondary to CMA/FA.

Conclusions

In conclusion, the values of all pH monitoring parameters in proximal channel recorded during the preliminary study (before treatment) were comparable (similarly to distal channel) and

did not serve as a source to differentiate GER into primary and secondary to CMA/FA.

During clinical observation and/or treatment of the patients the dynamics of acid GER, especially its range, and its intensity was assessed at control studies, in proximal channel.

The results of control studies, especially the number of episodes of acid GER and episodes of GER lasting more than 5 minutes, and supine acid GER index it showed that the range of GER secondary to CMA/FA was higher than primary GER range in these patients.

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Is acid gastroesophageal reflux in children with ALTE etiopathogenetic factor of life threatening symptoms?

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Abstract

Purpose: Commonly described multiorgan manifestation of acid GER: primary and secondary to cow's milk allergy and/or other food (CMA/FA) sometimes coexists with ALTE (Apparent Life Threatening Events) syndrome symptoms. Among these symptoms are apnea, cyanosis, pallor, hypotonia, non-epileptic seizures, consciousness disorders and bradycardia.

Materials and methods: 264 children aged: 4-102 months (x=20.78±17.23 months) of both sexes, with symptoms suggestive of GER were enrolled into study. 8 children (4.8%) aged up to 2 years (x=10.00±2.78 months) of both sexes with symptoms suggestive of ALTE were selected from the group. 24-hour esophageal pH monitoring was used for acid GER diagnosis in these children.

X-ray of esophagus with barium swallow was performed in order to evaluate the height of GER in infants. Immunoallergologic tests were performed in order to differentiate acid GER: primary and secondary to food allergy in these children.

Aims: 1. Assessment of the prevalence of acid GER in children with symptoms suggestive of ALTE,

2. Clinical evaluation of symptoms in children with ALTE and acid GER,

3. Assessment of efficacy of conservative treatment in children with reflux and ALTE symptoms,

4. Natural regression of the disease in children with ALTE

Results: From among 264 examined children who underwent 24-hour esophageal pH monitoring acid GER was confirmed in 170 (64.4%), and ALTE in 8 (4.8%). The causative role of primary acid GER in children with ALTE regarded to 4 children (50.0%) and GER secondary concerned 4 remaining children (50.0%). Mean number of ALTE episodes that appeared before admission to the hospital was similar in both study groups. The presence of typical reflux symptoms in 5 (62.5%) out of 8 children with ALTE symptoms on the basis of primary or secondary acid GER is significant. Mean value of total acid GER index in a subgroup of children with primary GER constituted x=11.13±1.45 and was not statistically significant in comparison with mean value x=12.13±1.30 of a parameter measured in a subgroup of children with secondary GER. The most common clinical manifestation was apnea and it was of identical prevalence in both study subgroups. Analysis of clinical differentiation of the course of ALTE in children with primary and secondary acid GER under conservative therapy was performed. Under this therapy, gradual regression of ALTE symptoms was achieved in all (8/100.0%) patients, with a tendency to longer time of improvement in children with secondary GER. Typical and atypical symptoms of GER receded in a subgroup with primary GER and were alleviated in a subgroup with secondary GER. In the second half year of clinical observation aggravation of reflux and ALTE symptoms was observed in subgroups. In the second year of clinical observation various typical and atypical symptoms of GER were observed in both subgroups. All these malaises during this period coexisted with ALTE symptoms. In the third year of clinical observation in both subgroups ALTE symptoms connected with acid GER were not observed.

Conclusion: Primary and secondary GER were defined as the causative factors of ALTE in 8 (4.8%) examined infants.

Key words: Acid GER, ALTE, food allergy, infants, youngest children.

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Table 1. **Research methods (by Wasilewska et al.) [17]**

Basic laboratory tests	Standard diagnostic procedures
1. laboratory parameters of inflammation	1. medical history
2. bacteriological examination	2. clinical examination (disorders)
3. titer of antiviral antibodies	3. cardiological examination + ECG/ ECHO
4. laboratory biochemical-metabolic screening	4. neurological examination + EEG
5. acid-base balance parameters (i.e. partial pressure of O_2 and CO_2, blood saturation)	5. ophthalmological examination
6. chest X-ray postero-anterior and lateran	6. ultrasound scan of abdominal cavity (esophageal gastric junction)
	7. transfontanellar ultrasound scan
	8. 24-hour intraesophageal pH monitoring
	9. X-ray of upper gastrointestinal tract with barium swallow
	10. immunoallergological examination (skin tests with food and inhalant allergens i.e. Prick tests, total and specific IgE, oral food challenge test
	11. polysomnography – not performed due to lack of informed parental consent or psychomotoric hypersensitivity in these children

Introduction

Acid gastroesophageal reflux (GER) in infants and children has various clinical manifestation. Commonly described multiorgan manifestation of GER is sometimes accompanied by life threatening symptoms defined as ALTE syndrome (Apparent Life Threatening Events). The most typical symptoms of ALTE syndrome are: breathing disorders (apnea); with apnea or independent of it: change in skin colour (cyanosis or pallor), muscle tension disorders (hypotonia or non-epileptic seizures), gagging, choking and salivation, consciousness disorders and arrhythmias (bradycardia) [1-8]. Intensity of these symptoms may be variable and symptoms may recede spontaneously or as a result of administered treatment. These symptoms are nonspecific and may appear in many other disorders during childhood, i.e. infections or metabolic, neurological, cardiovascular disorders etc. Diagnosis of ALTE syndrome requires differential diagnosis due to various causes of the syndrome [2,9].

Acid GER is considered to be one of the causes of ALTE. However, opinions on coexistence of symptoms and etiopathogenetic interconnections between these disorders are still divergent [10-14]. Also food allergy, especially cow's milk allergy could be a causative factor of GER (secondary GER) but hardly ever a direct cause of general ailments described in the course of ALTE [15,16].

Aims of the study

1. Assessment of the prevalence of acid GER in children suspected of ALTE,

2. Clinical evaluation of symptoms in children with ALTE and acid GER,

3. Assessment of efficacy of conservative treatment in children with reflux and ALTE symptoms,

4. Natural regression of the disease in children with ALTE and coexistent GER (3-year clinical observation).

Materials and methods

264 children aged 4 to 102 months (mean age x=20.78±17.23 months), of both sexes with symptoms suggestive of gastroesophageal reflux (GER) were enrolled in a study. These children were admitted to the III Department of Pediatrics of the Medical University of Bialystok. A subgroup of 8 children up to 2 years of age (mean age x=10.00±2.78 months), of both sexes with symptoms suggesting ALTE was selected. Diagnosis of ALTE was determined on a basis of differential diagnosis (*Tab. 1*), excluding infections of respiratory tract/urinary tract/digestive tract etc., infections (bacterial/viral), neurological disorders, cardiovascular disorders/anomalies, metabolic disorders (lactic acidosis, with or without hypercholesterolemia), adverse drug and food reactions [17].

24-hour esophageal pH monitoring was used for diagnosis of GER. The result of the examination was always related to clinical manifestation of the disease in examined children. The following parameters of pH monitoring were examined: the number of acid GER episodes (intraesophageal pH below 4.0), the number of acid GER episodes lasting more than 5 minutes, total acid GER index (RI), i.e. percentage of time with intraesophageal pH below 4.0.

Qualitative and quantitative evaluation of gastroesophageal reflux was performed together with the basic pH parameter i.e. total acid GER index (RI) – percentage of time with pH below 4.0 (%).

The results of esophageal pH monitoring in infants were related to borderline values collected by Vandenplas, et al. [18-20]. The borderline value for total acid GER index was ≤9%.

A chest X-ray with barium swallow was performed in order to rule out anatomical anomalies of the upper gastrointestinal tract, sometimes coexisting with tracheal, bronchial or diaphragm disorders and to establish height of GER in infants.

The range of GER was defined on the basis of Mc Cauley Grading System of the intensity of reflux [21].

The diagnostic and therapeutic algorithm was used in order to differentiate primary GER and GER secondary to cow's milk allergy and/or other food (CMA/FA) in these children [9].

This algorithm includes results of immunoallergologic tests, i.e. skin tests with food and inhalant allergens (Prick tests), total serum IgE and specific IgE, peripheral blood eosi-

***Table 2*. Analysis of selected children with ALTE and acid GER: primary and secondary to food allergy (FA)**

Children with ALTE	Total	Children with primary GER	Children with secondary GER
Number of children	8	4	4
Sex: girls/boys	3/5	1/3	2/2
Age at diagnosis (months; mean value)*	10.00±2.78	12.00±2.58*	8.00±0.82*
Gestational age (weeks; mean value)	36.7	36.0	37.0
Body mass at birth (grams; mean value)	2760	2820	2700
Pregnancy pathology/perilabour period	3/8	1/4	2/4
Presence of reflux symptoms (vomiting, regurgitation)	5/8	3/4	2/4
ALTE (mean no. of episodes before hospital admission)	3.5	3.25	3.75
Abnormalities in physical examination (at hospital admission, after ALTE episode)	4/8	0/4	4/4

* p=0.03

nophilia and oral food challenge test with a potentially noxious nutrient [9,22,23].

In children up to 2 years of age an open provocative test with cow's milk and/or other noxious nutrient (determined with the help of medical history) were performed [22].

Information about the ALTE episodes gathered from parents was compared with the type and intensity of reflux symptoms according to the agreed score system (scope 1-4 pts):

1 – symptoms of mild intensity occurring episodically

2 – symptoms of moderate intensity occurring episodically

3 – symptoms of considerable intensity occurring episodically

4 – symptoms of high intensity occurring daily.

Patients with reflux symptoms of variable degree of manifestation required temporary (few days) or periodically repeated (4-6 weeks) treatment; and they were taken under constant clinical care. The aim of this procedure was to establish the efficacy of treatment and prevention of recurrence of ALTE episodes.

In order to diagnose pathological primary and secondary acid GER in examined children 2 versions of comprehensive treatment were administered [16,24-26]:

1) antireflux treatment exclusively (in children with primary GER)

a) Stage I – positional treatment (postural) + antacids and protective drugs + parental education,

b) Stage II – Stage I + prokinetics,

c) Stage III – Stage II + histaminic receptor (H2) antagonists and/or proton pomp inhibitors;

2) combined treatment – antiallergic and antireflux (in children with GER secondary to food allergy)

a) variant I – antiallergic treatment (dietetic treatment – elimination diet, antiallergic treatment) and antireflux treatment (Stage I),

b) variant II – antiallergic and antireflux treatment (Stage II or III).

Duration of treatment was dependent on the clinical manifestation and the degree of intensity of the disease and also on the efficacy of the treatment implemented. Efficacy of exclusive antireflux treatment or combined treatment was assessed on the basis of the intensity of symptoms (resolution or alleviation). Esophageal pH monitoring or oral food challenge test (GER secondary to food allergy) were performed periodically to verify diagnosis of acid GER.

In order to standardize the assessment of treatment efficacy the following agreed classification has been implemented:

resolution of symptoms	(Res)
alleviation of symptoms=improvement	(I)
no improvement	(NI)
aggravation	(A)
symptoms of variable intensity, appearing periodically = recurrences	(Rec)

The study was approved by local Bioethical Committee of the Medical University of Białystok and informed parental consent was obtained from parents of examined children.

Any statistical comparison was not reliable consequent upon insufficient number of children with ALTE syndrome examined in both subgroups.

Results

Among 264 examined children 24-hour esophageal pH monitoring confirmed pathological acid GER in 170 (64.4%) and ALTE in 8 (4.8%). Simultaneously upper gastrointestinal X-ray with barium swallow was performed in children with ALTE symptoms which confirmed high reflux. The causative role of primary acid GER in ALTE was confirmed in 4 children (50.0%) and GER secondary to cow's milk allergy in remaining 4 children (50.0%). The analysis of selected children with ALTE and primary and secondary GER is presented in *Tab. 2*. Presentation of age differentiation (statistically significant; p=0.03) between both subgroups at diagnosis seemed to be important. Children with primary GER were older than children with GER secondary to CMA/FA. Mean number of ALTE episodes that occurred before hospital admission was comparable in both study groups.

Clinical manifestation of the disease revealed typical reflux symptoms in 5 children (62.5%) and lack of these symptoms in 3 (37.5%) out of 8 children with ALTE symptoms on the basis of primary or secondary GER. Mean number of ALTE episodes that occurred before hospital admission was comparable in both study groups. Typical reflux symptoms in 5 (62.5%) and lack of these symptoms in 3 (37.5%) children out of 8 with ALTE symptoms on the basis of primary or secondary GER are highly significant. The clinical examination of children with symptoms of ALTE

***Table 3*. Comparative analysis of the results of tests confirming acid GER in examined children with ALTE**

Examinations confirming acid GER in children with ALTE							
24-hour intraesophageal pH monitoring Percentage of time with pH<4.0 (total GER index %)				X-ray of upper gastrointestinal tract Mc Cauley classification of grading [21]			
Inicials	Children with primary GER	Inicials	Children with secondary GER	Inicials	Children with primary GER	Inicials	Children with secondary GER
K.P.	9.7	K.K.	10.3	K.P.	III^0	K.K.	IV^0
B.J.	11.2	B.M.	12.5	B.J.	III^0	B.M.	IV^0
L.A.	10.5	S.G.	12.3	L.A.	IV^0	S.G.	III/IV^0
A.W.	13.1	M.M.	13.4	A.W.	III^0/IV^0	M.M.	IV^0
X	11.125±1.45	X	12.125±1.30	X	3.37±0.48	X	3.87±0.25
p=ns				p=ns			

***Table 4*. Analysis of clinical manifestation of ALTE in children with acid GER: primary and secondary to food allergy (FA)**

Clinical manifestation of ALTE in examined children	Children examined – total [N=8]	Type of disorder	
		Primary GER [N=4]	Secondary GER [N=4]
Apnea	8	4	4
Consciousness disorder	6	2	4
Hypotonia	5	2	3
Pallor	4	1	3
Cyanosis	3	1	2
Bradycardia	2	1	1
Non-epileptic seizures	1	-	1

and GER did not reveal any anomalies after ALTE episode. Confirmation of diagnosis of acid GER and assessment of its range in examined children with ALTE are presented in *Tab. 3*.

Pathologic acid GER was diagnosed on the basis of values of total acid GER index registered. Mean value of this parameter obtained in a subgroup of children with primary GER was x=11.13±1.45 and was lower than mean value of the examined parameter obtained in a subgroup of children with GER secondary to CMA/FA x=12.13±1.30 (difference not statistically significant). The range of GER in selected children was considered high due to approved radiological Mc Cauley's classification [21].

Mean value of height degree of barium reflux in a subgroup of children with primary GER was x=3.37±0.48 and was lower than a mean value of reflux in a subgroup of children with GER secondary to CMA/FA x=3.87±0.25 (difference not statistically significant).

Clinical manifestation of ALTE in children with primary and secondary GER (*Tab. 4*) revealed that the most common symptom was apnea appearing with the same prevalence in both study groups. Relatively less common were consciousness disorder, hypotonia, pallor, cyanosis – and these were more common in a subgroup with secondary GER, whereas bradycardia was a very rare symptom in both study groups. Similarly non-epileptic seizures were observed exclusively in a subgroup with secondary GER.

Symptoms aforementioned, which are typical for ALTE, were predominant in a subgroup of the youngest children with secondary GER. In a subgroup of children with secondary GER, ALTE symptoms were accompanied by allergy symptoms: skin lesions, mucous lesions in oral cavity (aphthae), rhinitis, chronic diarrhoea with mucus or eosinophilia in faeces.

Characteristics of clinical manifestation in children with ALTE and GER at admission to hospital (diagnosis, medical history) is presented in *Tab. 5*.

The prevalence of typical reflux symptoms was demonstrated in 5 children (62.5%) and such symptoms were not observed in 3 children (37.5%) with acid GER who had atypical clinical manifestation. All these ailments appeared in combination with ALTE symptoms. Among typical reflux symptoms vomiting was more intense in a subgroup of children with secondary GER and regurgitation prevailed in a subgroup of children with primary GER.

The less common symptoms were: food refusal, swallowing and belching in children with secondary acid GER and failure to thrive and choking in children with primary GER.

Among the atypical symptoms of reflux (out of gastrointestinal tract) anxiety and postprandial crying and/or night cough and coryza were observed in children with secondary GER, and anxiety and/or crying during day and night sleep only in 1 child with primary GER.

Analysis of clinical differentiation of the course of ALTE in children with primary GER and GER secondary to CMA/FA under administered treatment was performed. Two endpoints were taken into account: the time of basic treatment completion and the first year of 3-year-clinical observation (*Tab. 6*).

Two types of treatment were administered: antireflux treatment and combined treatment – antiallergic and antireflux. The time of antireflux treatment in a subgroup of children with primary GER lasted from 6 up to 24 weeks (mean time x=15.0±7.75) and was shorter than combined treatment in children with secondary GER which lasted 12 to 36 weeks (mean time x=21.0±11.49), (difference statistically not significant). Gradual resolution of ALTE symptoms was achieved in all children (8/100.0%), with a tendency to longer period of obtaining it in children with sec-

***Table 5*. Clinical symptoms of ALTE (data from patients' medical history) in children with acid GER: primary and secondary to food allergy (FA) – at diagnosis**

Children with ALTE					Clinical manifestation of ALTE						
No	Age at diagnosis (months)	Sex	Cause	Clinical symptoms of GER	Apnea	Consciousness disorder	Hypotonia	Pallor	Cyanosis	Bradycardia	Non-epileptic seizures
1.	7	M [♂]	Secondary GER	vomiting 2-3 x per day, swallowing, food refusal	+	+	+	+	-	-	-
2.	8	F [♀]		anxiety/postprandial crying, food refusal	+	+	+	+	+	-	-
3.	8	M [♂]		swallowing, anxiety and night cough.	+	+	+	+	-	-	-
4.	9	F [♀]		vomiting 3-4 x per day, belching, appetite loss, coryza.	+	+	-	-	+	+	+
5.	9	M [♂]	Primary GER	regurgitation 5-6 x per day/vomiting 2-3 x per day	+	-	+	+	-	-	-
6.	11	M [♂]		regurgitation/choking 3-5 x per day	+	-	-	-	+	-	-
7.	13	F [♀]		regurgitation/vomiting 2-3 x per day, failure to thrive	+	+	-	-	-	+	-
8.	15	M [♂]		anxiety/crying during sleep every day	+	+	+	-	-	-	-

***Table 6*. Clinical observation of children with acid GER and ALTE (1st year after the completion of treatment)**

Children with ALTE														
No.	Age at diagnosis (months)	Cause	Conservative treatment				Age after treatment (months)		Assessment of treatment efficacy in the 1st year after treatment completion (weeks)					
			Antireflux		Combined				0-6	6-12	13-24	25-36	37-48	
			receiving/ not receiving	Duration (weeks)	receiving/ not receivng	Duration (weeks)								
1.	7	GER secondary to FA	-	-	+	12	10	x=13.2±3.6	Rec(2)	I(1)	Res	Rec(2)	I(1)	Res
2.	8		-	-	+	24	14		Rec(2)	NI(2)	I(1)	A(4)	Res	Rec(2)
3.	8		-	-	+	12	11		Res	Res	Rec(3)	I(2)	I(1)	Res
4.	9		-	-	+	36	18		Rec(2)	NI(2)	A(4)	I(3)	I(1)	
						x=21.0±11.49								
5.	9	Primary GER	+	6	-	-	10.5	x=16.0±4.3	Res	Res	Rec(2)	NI(2)	I(1)	Res
6.	11		+	18	-	-	15.5		Res	Res	Rec(3)	I(2)	A(4)	Res
7.	13		+	12	-	-	17		Res	Res	Res	Rec(2)	NI(2)	Res
8.	15		+	24	-	-	21		Rec(2)	I(1)	A(3)	NI(3)	I(1)	
				x=15.0±7.75										

ondary GER. However, regression of typical and atypical reflux symptoms was observed in 4 children with primary GER and their significant alleviation in 4 children with secondary GER.

The efficacy of administered treatment was assessed after first year of observation and longer time of absence of reflux ailments and ALTE symptoms (first 6 months: 12-24 weeks) was revealed in a subgroup of children with primary GER, in comparison with a subgroup of children with secondary GER. At that time the lack of clinical recovery or a tendency to recurrence of symptoms was observed in the discussed group.

In the second half year (25-48 weeks) of clinical observation the intensification of clinical manifestation of reflux symptoms and ALTE was observed in children of both subgroups. These children required periodical intensive treatment.The assessment of clinical examination of children with primary and secondary acid GER in a consecutive years of clinical observation are presented in *Tab. 7* (Part I and II).

In the second year of clinical observation paroxysmal abdominal pain, vomiting, and saliva swallowing were observed among typical reflux symptoms. Less common were regurgitation and belching appearing in a subgroup of children with secondary GER.

Table 7. Further clinical observation of children with acid GER and ALTE syndrome (following years). Part I.

Children with acid GER and ALTE									
No.	Age after 1 year of clinical observation (months)	Cause	2nd year of clinical observation		Age after 2 years of clinical observation (months)	3rd year of clinical observation		Age after 3 years of clinical observation (months)
			Reflux symptoms	ALTE symptoms		Reflux symptoms	ALTE symptoms	
1.	22	GER secondary to FA	Rec vomiting 1-2 x per day and abdominal pains – 2-3 days (4 episodes every 5-6 days)	Rec apnea, pallor, hypotonia and consciousness disorder 2 episodes every 5-6 days (after 8 days of open provocation test to cow's milk)	34	Rec swallowing, hiccough, morning bad breath – 14-21 days (2 episodes every 12-18 weeks)	Res	46
2.	26	GER secondary to FA	Rec vomiting/regurgitation, swallowing, hawking 2-3 x per day – 3-4 days (6 episodes every 10-14 days)	Rec apnea, pallor, hypotonia and consciousness disorder (3 episodes every 14 days)	38	Rec hawking and hoarsness lasting 3-4 weeks, receding after combined treatment (3 episodes every 5-7 weeks)	Res	50
3.	23	GER secondary to FA	Rec night cough and wheezing breath; hoarsness – 2-3 weeks (4 episodes every 4-6 weeks)	Rec apnea, short-term (≤1min.) consciousness disorder, pallor, hypotonia, and excessive salivation (5 episodes every 4-6 weeks)	35	Rec night and postprandial cough, hoarsness lasting 2-3 weeks (3 episodes every 8-9 weeks)	Res	47
4.	30	GER secondary to FA	Rec paroxysmal abdominal pains, belching and swallowing – 10-14 days (3 episodes every 4-6 weeks)	Rec apnea, cyanosis, consciousness disorder and non-epileptic seizures (2 episodes)	42	Rec pain/heartburn, hoarsness after night and hawking – 5-7 days (3 episodes every 4-10 weeks)	Res	54
	x=25.25±3.59				x=37.25±3.59			x=49.25±3.59

Table 7. **Further clinical observation of children with acid GER and ALTE syndrome (following years). Part II**

Children with acid GER and ALTE									
No.	Age after 1 year of clinical observation (months)	Cause	2nd year of clinical observation		Age after 2 years of clinical observation (months)	3rd year of clinical observation		Age after 3 years of clinical observation (months)
			Reflux symptoms	ALTE symptoms		Reflux symptoms	ALTE symptoms	
5.	22	Primary GER	Rec regurgitation 3-5x per day, anxiety/crying during sleep and feeding – 5-8 days (3 episodes every 4- 5 weeks)	Rec apnea, hypotonia, pallor (3 episodes every 4-5 weeks)	34	Res	Res	46.5
6.	27	Primary GER	Rec belching and swallowing 3-4 x per day, lasting 3-4 weeks (2 episodes every 4-5 weeks)	Rec apnea and cyanosis at exacerbation of reflux symptoms (2 episodes)	39.5	Res	Res	51.5
7.	29	Primary GER	A vomiting 1-2 x per day, regurgitation and heartburn – 10-14 days (3 episodes every 8-10 weeks)	Rec apnea and bradycardia at exacerbation of reflux symptoms (1 episode)	21	Res	Res	53
8.	33	Primary GER	Rec vomiting, anxiety/postprandial and during sleep crying, appetite loss – 2 weeks (4 episodes every 7-8 weeks)	Rec apnea, hypotonia and consciousness disorder at exacerbation of reflux symptoms (2 episodes)	45	Rec abdominal pain, chronic hoarsness and appetite loss, lasting 7-10 days (4 episodes every 4-6 weeks)	Res	57
	x=27.75±4.57				x=34.87±10.28			x= 52.00±4.33

In a subgroup of children with primary GER vomiting and regurgitation, and also occasional swallowing, belching, regurgitation of stomach contents into the esophagus and heartburn or appetite loss should be mentioned. Atypical symptoms such as night cough, wheezing breath, hoarsness, and hawking were observed with a similar prevalence in 2 children with secondary GER, whereas anxiety and/or crying during feeding and during sleep in 2 children with primary GER. All these ailments still coexisted with ALTE symptoms during the period of clinical observation.

In children with secondary GER apnea with short consciousness disorder (≤1 min.) was a dominant symptom, whereas pallor and hypotonia were less common and cyanosis and non-epileptic seizures appeared occasionally. In all children with primary GER apnea with other occasional ALTE symptoms such as hypotonia, pallor or cyanosis, bradycardia or consciousness disorders were observed. After 2 years of clinical observation mean age of children with GER secondary to CMA/FA was x=37.25 months ±3.59 and was not statistically significant with regard to mean age of children with primary GER which accounted for x=34.87 months ±10.28. In the third year of clinical observation no ALTE symptoms connected with acid GER were observed in both study subgroups of children with primary and secondary GER. Foetor ex ore, saliva swallowing, abdominal pain and heartburn were observed among typical reflux symptoms of similar prevalence in children with secondary GER. Within atypical reflux hoarsness was a dominant symptom, and hawking, night and/or postprandial cough were less common.

In the third year of clinical observation in a subgroup of children with primary GER periodical abdominal pains, appetite loss and hoarsness of recurrent character were observed in 1 child only. At the completion of 3-year clinical observation mean age of children with secondary GER was x=49.25 months ±3.59 and was similar to mean age of children with primary GER which was x=52.00 months ±4.33 (not statistically significant).

Discussion

In 3-year clinical observation chronic or recurrent reflux and/or ALTE symptoms of variable intensity were observed especially in children with secondary GER, regardless of periodically administered treatment. This tendency was presumably the result of aggravation of gastrointestinal allergy to various allergens (food or inhalant) [16,24,25].

To prove cause-and-effect relationship between acid GER symptoms and ALTE episodes it is required to perform 24-hour esophageal pH monitoring and assess function of the following systems: nervous system, respiratory and cardiovascular system. It is also necessary to prove that ALTE symptoms remain with the time correlation with inappropriate acid esophageal pH<4.0. Nevertheless, these correlations have not been reliably proved in a clinical practice yet.

Nowadays polysomnography is used instead of standard esophageal pH monitoring [14,27]. On the basis of implemented antireflux treatment or combined treatment these correlations can be indirectly presupposed. Simultaneous registration of esophageal pH and polysomnography in the youngest healthy children showed that acid GER is a physiological process in this group, especially intense in a REM (rapid eye movement) phase of sleep [14]. As a physiological process it does not trigger of symptoms of a disease but may be the cause of awakenings of the youngest children. Together with age and development of nervous system and sleep-vigil cycle, and reduction of percentage of REM phase, physiological acid GER gradually decreases [14,27].

Only older infants with acid GER proved with pH monitoring were enrolled in a study.

Therefore divergent opinions on the ethiopathogenesis of ALTE with acid GER could at least partially be the effect of different methods of the performed examinations.

Crucial methodological suggestion could be performance of 24-hour intraesophageal pH-monitoring, which is a standard diagnostic procedure in acid GER in children with ALTE symptoms [18-20].

Kahn et al. did not show the time relationship between episodes of drop in pH in esophagus and apnea and bradycardia in infants with ALTE [13]. However, in the discussion part of the study the possibility of obtaining false negative results due to reflux of gastric contents of low acidity (pH>4.0) or neutral (pH=5-7) is mentioned, which may appear especially after feeding and is not reliably registered by pH monitoring probe [13].

There is also lack of unambiguous confirmation of time relationship between pH drop in esophagus and symptoms of reflux and ALTE in the youngest children, in both subgroups. On the other hand the time relationship between the onset of reflux symptoms (typical and atypical) and apnea and consciousness disorder, and other ALTE symptoms, usually within 30 up to 60 minutes after feeding and/or in a supine position, during sleep was proved in a clinical practice.

Taking into consideration methodological representations aforementioned, these authors do not exclude the existence of acid GER in children with ALTE especially in infants with obstructive apnea during sleep. In case of doubts they suggest administration of antireflux treatment as a verifying diagnostic and therapeutic test [13].

Significant methodological recommendation should be 24-hour intraesophageal pH monitoring approved as a standard procedure in a diagnosis of acid GER in children with ALTE [17-20].

Graff et al. showed uselessness of short-term monitoring techniques: 1 hour Tuttle test or 4 hour polisomnographic recording with a simultaneous esophageal pH-registration as a diagnostic method of acid GER in infants with ALTE [10]. Sensitivity of these tests with regard to 12 hour monitoring was 55% and 82%, respectively [10].

On the basis of reports aforementioned it could be thought that chest X-ray of upper gastrointestinal tract with barium swallow is not an optimal procedure in diagnosis of GER [10].

This procedure was performed in own studies in the youngest children, in order to rule out anatomical anomalies of this part of gastrointestinal tract and at the same time to assess the height of regurgitation of gastric contents into esophagus.

The presence of symptoms from the gastrointestinal tract

typical for acid GER (vomiting, regurgitation, etc.), which were present in 62.5% examined children supports the necessity of reflux diagnostics, regardless of the absence of abnormalities in clinical examination after ALTE episode.

Despite the fact that 24-hour esophageal pH monitoring has been considered as the diagnostic gold standard, it only enables to reveal changes of pH<4.0, i.e. acid GER. This procedure does not allow to discover non-acid GER, in which pH of esophageal contents is between >4-6.8 (neutral pH) or above 7.0 (alkaline bile GER) [18-20,28].

The reliable diagnostic method is electric impedance measurement (Multichannel Intraluminal Impedance – MII) combined with pH-monitoring enabling to diagnose reflux regardless of pH (acid and non-acid GER) [29].

The prevalence of recurrent vomiting or regurgitation in children with ALTE syndrome constitutes 60-70%, and at the same time in 40-80% of cases inappropriate pH monitoring recording is ascertained.

Clinical reports regard ALTE episodes that are triggered of by backward food movement into oral part of pharynx or aspiration of gastric contents.

Intense vomiting or regurgitation correlated with prolonged apneas (>20 sec.) and also with shorter apneas connected with bradycardia, but majority of long lasting apneas was connected with regurgitation in these patients [1,2,27].

Studies regarding simultaneous esophageal pH monitoring, heart rate, chest movements and nasal flow showed that GER could precede apnea [2,27,30].

In selected children who experienced ALTE episodes it was proved that hydrochloric acid infusion into esophagus triggers of obstructive apnea or reducing blood saturation, which suggests that ALTE episodes on the basis of acid GER may appear due to stimulation of chemoreceptors of pharynx, esophagus and larynx with subsequent contraction [2,3,7].

Despite previous reports that GER may lead to apnea, examinations of children after ALTE episode did not prove actual time correlation between esophagus acidity and apnea or bradycardia [2,3,7,31].

With few studies presenting occasional correlation between acid GER with short-term mixed central apneas (5-15 sec.), in all those patients apneas with no correlation with GER were observed, which could suggest primary disorder of breathing regulation [2,3,7,31].

The most convincing cause-and-effect relationship between GER an apnea episodes on the mixed basis or due to defect of respiratory tract permeability was shown in infants, who experienced those apneas during vigil phase in a supine position and during the first hour after feeding [29,30]. This interdependence can be confirmed with polisomnography. At the same time the lack of evidence that ALTE episode and polisomnography results enable anticipation which infant is at risk of ALTE episode in the future [27,30,31].

In patients with frequent ALTE episodes, with uncertain role of GER, 24-hour esophageal pH monitoring may facilitate determination of time correlation between acid GER and ALTE. In order to provide proper interpretation of the 24-hour esophageal pH monitoring, registration of heart rate, chest impedance, nasal flow and oxygen saturation should be performed simultaneously, which together enable to observe apnea as a result of respiratory tract obstruction (polysomnography recording, electroencephalography recording) [27,30].

Some evidence suggest that better response to antiallergic treatment and/or antireflux treatment may be observed in infants with ALTE and GER of different ethiology in the case of very intense vomiting or regurgitation at ALTE episode and if the episode occurs while child is awake or manifests as obstructive apnea [25,32,33].

In order to reduce the intensity of vomiting and to inhibit acid GER it is advisable to opt out classical method of antireflux treatment [26].

Combined antiallergic and antireflux treatment are taken into consideration only in the case of inefficacy of previous treatment and also if the allergic pathogenesis of antireflux and ALTE symptoms of severe course is confirmed [16,24,25,33]. The comparison of efficacy of both treatments was performed in own studies.

Conclusions

1. Both primary and secondary acid GER have been recognized as causative factors of ALTE in 8 examined children (4.8%).

2. The results obtained justify the necessity of implementation of 24-hour esophageal pH monitoring and immunoallergologic test or polisomnography in diagnostic procedure in children with ALTE syndromes.

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Exocrine pancreatic function in biliary tract pathology treated with the endoscopic methods

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Abstract

Purpose: Incidence of pancreatic exocrine insufficiency in biliary pathology is estimated for about 30%. The objective was to assess pancreatic exocrine function in biliary tract pathology (cholelithiasis, strictures) before and after endoscopic treatment.

Patients and methods: Twenty-eight patients with choledocholithiasis and its complications (19F/9M; aging 31-90 years, median: 69 years) were evaluated. Fecal elastase 1 concentration was measured using ELISA, before, early, and 6-8 weeks after endoscopic treatment. The inflammatory response of pancreas to the treatment was also assessed.

Results: Initial fecal elastase 1 concentration in patients (median 454 μg/g) was not significantly different as compared to the control (median 357 μg/g). Nine patients (32%) had low fecal elastase 1 concentration (below 250 μg/g) and out of them 6 had the concentration below 200 μg/g, suggesting impairment of exocrine pancreatic function. Endoscopic treatment was successful in 82% of patients. Pancreatic inflammatory response was noted only in one patient. After 6-8 weeks fecal elastase 1 concentration in the whole group of patients did not significantly change in comparison to the initial level. However, out of 9 patients with initially low fecal elastase 1 concentration (median 191 μg/g) at least in 6 pancreatic function improved (median 310 μg/g), $P<0.001$.

Conclusion: One third of the patients with biliary pathology had a low fecal elastase 1 concentrations, suggesting pancreatic dysfunction. In at least 2/3 of these patients successful endoscopic treatment of biliary pathology resulted in the significant increase of fecal elastase 1 concentration. Therefore, an additional positive effect of such treatment in some patients, could be an improvement of the exocrine pancreatic dysfunction.

Key words: exocrine pancreatic function, elastase 1, choledocholithiasis, endoscopic treatment.

Introduction

Choledocholithiasis occurs in 10-18% of patients with cholecystolithiasis [1] and may lead to cholangitis and postinflammatory stenosis. Stenosis of bile ducts can also be iatrogenic, e.g. formed after surgery [2]. Close anatomical vicinity and functional relationship suggest that diseases of bile ducts and pathological processes in the pancreas may exert a mutual effect on each other. Correlation of bile duct pathologies with chronic inflammatory pancreatic lesions are relatively little known. According to Misra et al. [3], abnormal pancreatograms were found in 48% patients with gallstones, of whom 16% had changes suggesting chronic pancreatitis (CP). Hardt et al. [4] observed features of CP in pancreatograms of 77% of patients with a history of gallstones and in 47% without the disease among patients submitted to endoscopic retrograde cholangiopancreatography (ERCP). Reduced concentration of fecal elastase 1 was more common in patients with a history of gallstones. Lamarque et al. [5] observed a reverse correlation, i.e. increased incidence of cholelithiasis in CP patients. However, some reports appear to negate the increased incidence rate of pancreatic pathology in cholelithiasis or etiological relationship between them [6]. Thus, the frequency and severity of pancreatic lesions, with a potential organ dysfunction accompanying bile duct disorders, still remain to be explained.

ERCP is a major diagnostic and therapeutic procedure used in pathologies of bile ducts and pancreas. Its diagnostic merits are associated with a simultaneously performed treatment

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for bile and pancreatic ducts, including sphincterotomy of the sphincter of Oddi, bile duct stone removal and insertion of prosthesis to dilate stenosis. The therapeutic efficacy of endoscopic procedures reaches 80-90% for bile duct stone removal and 89% for cancers that impair bile outflow [7,8]. The effect of endoscopic treatment for bile duct pathologies on a potential impairment of exocrine pancreatic function has not been elucidated yet.

A number of methods have been used to assess pancreatic secretion. In recent years, determination of fecal elastase 1 has become a commonly accepted standard. Elastase 1 is a pancreas-specific enzyme that does not undergo degradation during the intestinal passage [9]. Its concentration remains stable during therapeutic supplementation with pancreatic enzymes. The test is non-invasive and correlates well with those using secretin and cholecystokinin [10]. Its specificity reaches 93% while sensitivity is higher than that of the fecal chymotrypsin test [11], the respiratory test using triacylglycerols labeled with carbon 13C [12] or the para-aminobenzoic acid test [13].

Moreover, serum elastase 1 can be determined to evaluate acute pancreatitis (AP). In a study by Katsanos et al. [14], increase in serum elastase 1 level two hours after ERCP was found to be 100% specific in predicting acute pancreatitis (AP).

The current study objective was to assess exocrine pancreatic function based on the determination of fecal elastase 1 in patients with bile duct pathology (stones, stenosis) and to evaluate any immediate or distant in time changes in this function following endoscopic treatment of this pathology in the context of other laboratory tests used to control the disease and its treatment.

Patients and methods

Patients

Patients planned for ERCP were recruited for the study, except for those with suspected tumor of the head of the pancreas or bile duct carcinoma. The presence of bile duct pathology was established based on patients' history, physical examination, ultrasonography, features of cholestasis and/or cholangitis.

Twenty-eight patients (19 women, 9 men, aged 31-90 years, median 69 years) treated in the Department of Gastroenterology and Internal Medicine, Medical University of Białystok, were investigated. In 24 of them ERCP was indicated due to suspected choledocholithiasis and in 4 due to postsurgical stenosis of common bile duct (CBD). Four patients (2 with gallstones and 2 with CBD stenosis) had cholangitis. Sixteen patients showed a past history of cholecystectomy (12 with gallstones and all with CBD stenosis). Four suffered from type 2 diabetes (all with gallstones). An elevated bilirubin level was noted in 14/28 patients (50%).

All ERCP procedures were performed using an endoscope TJF-145 Olympus. Sphincterotomy was conducted with a sphincterotome KD-301Q-0330, stones were removed by means of balloons type B7-2Q, basket Dormia type FG-22Q-1 and lithotriptors type BML-3Q-1. Plastic prostheses (10F in diameter) were inserted into CBD, if indicated.

The study design

The concentrations of fecal elastase 1 as a measure of exocrine pancreatic function were estimated three times: before (determination 1) and twice after ERCP, combined with therapeutic procedure. Determination 2, to assess an immediate effect of the treatment was done in the first bowel movement, usually at the 1st to 3rd day following ERCP. Determination 3, to evaluate a long-term effect of the procedure was completed after 6-8 weeks.

Other measurements included determination of serum elastase 1 concentration [14], routine laboratory tests for the activity of α-amylase in serum and urine, serum bilirubin, alkaline phosphatase (ALP), alanine aminotransferase (ALT), aspartate aminotranferase (AST) and leucocytosis. Serum was collected prior to ERCP (determination 1), on day 2 (determination 2) and 6-8 weeks after ERCP combined with therapeutic procedure (determination 3).

Elastase 1 was determined by ELISA using two monoclonal antibodies which bind to strictly defined epitopes of the enzyme (ScheBo Tech kit).

Control group

Ten healthy volunteers (6 women, 4 men, aged 27-75, median 56) were recruited in order to determine normal fecal and serum elastase 1 levels.

The protocol was accepted by the local Bioethics Committee. Patients gave their written informed consent to participate in the study.

Statistical analysis

Statistical analysis was carried out using the Statistica 6.0 program. The Kolgomorov-Smirnov test was applied to examine the normality of distribution of variables. As normal distribution was not confirmed, the U Mann-Whitney test was applied to assess statistically significant differences between the study and control group. The Wilcoxon test (sequence within pairs) was performed to assess the respective parameter in time. The values were presented as median as well as 25 and 75 percentile. The correlation and regression coefficients were calculated according to Pearson. $P \leq 0.05$ was considered statistically significant.

Results

Endoscopic procedures

Bile duct cannulation during ERCP was successful in 26/28 patients (92%). Twenty-three patients underwent sphincterotomy, three had sphincterotomy performed earlier (2 endoscopic, 1 surgical). CBD stenoses were balloon-dilated and prostheses were inserted. Bile duct stones, macroscopically visible in 19 patients, were evacuated using a Dormia basket and a balloon, and in 4 cases mechanical lithotripsy was carried out. In total, the procedure was fully successful in 23 patients (82%). Three patients developed complications: bleedings from the papilla of Vater in 2 cases (7%), which were treated endoscopically with adrenaline solution during the same procedure, and mild AP in one case.

***Table 1*. Biochemical parameters of the patients with bile duct pathology. Medians (in brackets 25th and 75th percentile) are reported**

Parameter	Measurement 1, before endoscopic procedure (n=28)	Measurement 2, early# after procedure (n=25)	Measurement 3, 6-8 weeks after procedure (n=24)
Fecal elastase 1 (μg/g)	454 (224, 572)	405 (319, 540)	448 (286, 515)
Serum elastase 1 (ng/mL)	1.13 (0.83, 1.42)	1.07 (0.87, 1.63)	1.09 (0.89, 1.49)
Serum α-amylase (IU/L)	40 (30, 46)	43 (31, 57)	40 (36, 52)
Urine α-amylase (IU/L)	140 (63, 229)	202 (129, 313) * P<0.05	Not determined
Serum bilirubin (mg/dL)	1.33 (0.95, 3.87)	1.55 (1.06, 2.74)	0.97 (0.78, 1.14) ** P< 0.001 *** P< 0.001
Alkaline phosphatase (IU/L)	261 (149, 361)	224 (138, 321) * P<0.05	103 (92, 120) *** P< 0.001
AST (IU/L)	59 (25, 115)	54 (28, 105)	32 (24, 42) ** P< 0.005 *** P< 0.01
ALT (IU/L)	83 (24, 204)	68 (21, 126)	36 (23, 44) **P< 0.001 *** P< 0.005
White blood cell count (x10^3/μL)	7.76 (5.60, 9.41)	6.79 (5.23, 8.30)	5.86 (4.95, 7.49) *** P< 0.05

fecal elastase 1-3 days (the first bowel movement), other parameters in the next day after endoscopic procedure; * (difference between 1 and 2 analysis); ** (difference between 2 and 3 analysis); *** (difference between 1 and 3 analysis)

Complete laboratory tests were done in 28 patients (100%) prior to the procedure (determination 1); in 25 at determination 2 (89%) and in 24 at determination 3 (86%); the remaining patients did not come for a check-up.

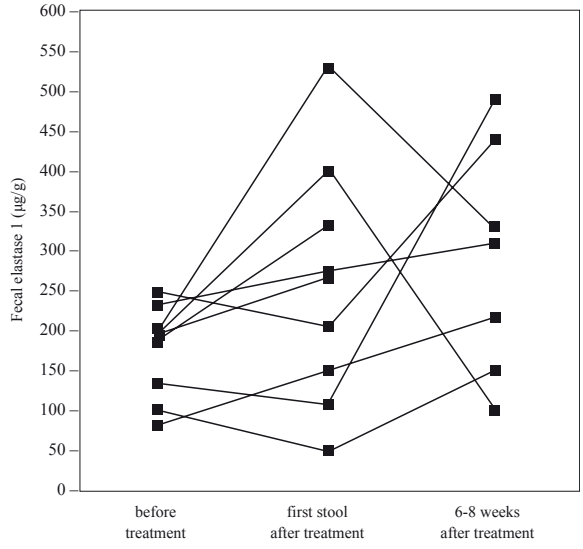
Fecal elastase 1

In the whole group, fecal elastase 1 concentration prior to the procedure (median 454 μg/g feces; 25 and 75 percentile, 224 and 572 μg/g, respectively) did not differ significantly from the control values (median 357 μg/g; 284 and 391μg/g). However, in 6 patients (21%) fecal elastase 1 level below 200 μg/g was noted. In other 3 cases, the concentrations ranged between 200 and 250 μg/g. In total, concentrations below 250 μg/g (median 191; 128 and 201 μg/g) were found in 9 patients (32%). It is noteworthy that a slightly higher levels were found in a group of 9 patients suffering from jaundice (median 467; 293 and 571 μg/g); however, in comparison with the control the difference was not statistically significant.

In the first stool following the procedure, elastase 1 concentration was reduced in 12 patients (by 103 μg/g on average), and increased in 13 (by 115 μg/g on average). The changes were not correlated with those noted in serum bilirubin. The overall concentration in the whole group of patients did not differ statistically significantly as compared to determination 1 (median 405 μg/g; 319-540 μg/g).

At determination 3, 6-8 weeks after endoscopic procedure, fecal elastase 1 level (median 448 μg/g; 286-515 μg/g) did not differ statistically significantly as compared to the 1st and 2nd measurement. However, in 6 from 9 patients (67%) with lowered elastase 1 level prior the procedure its level increased (median 310; 179 and 381 μg/g). In one patient (with unsuccessful removal of stones from bile ducts after sphincterotomy), a distant check-up revealed a further decrease in elastase 1. Two patients did not come for the long-term check-up, but in both of them fecal elastase 1 was higher at determination 2 than at baseline. Moreover, in one patient with normal fecal elastase at baseline, the concentration was below the norm 6-8 weeks after the procedure (*Tab. 1*, *Fig. 1*).

***Figure 1*. Fecal elastase 1 concentration in the patients with its initial low value: before and after endoscopic treatment of bile duct pathology**



Serum elastase 1

The median value of serum elastase 1 in the control group was 1.35 ng/mL; (percentile 25 and 75; 1.02 and 1.95). Baseline serum elastase 1 in the group of patients was 1.13 ng/mL (0.83 and 1.42) and did not differ significantly as compared to the control.

One day after the procedure, serum elastase 1 did not differ statistically from the values noted at determination 1 – median 1.07 (0.87 and 1.63) ng/mL, slightly exceeding the norm suggested by the kit producer only in sole patient with mild AP. Serum elastase 1 on day 2 strongly correlated with the simultaneously determined activities of α-amylases in serum and urine (r=0.67 and r=0.66), AST (r=0.77) and ALT (r=0.61). All the correlations were statistically significant (P≤0.001). Moreover, elastase 1 on day 2 showed a weak but statistically significant correlation with the activity of alkaline phosphatase (r=0.41, P<0.05). After 6-8 weeks, the median of serum elastase 1 was 1.09 ng/mL (0.89 and 1.49) (with no significant differences between determination 1 and 2), (*Tab. 1*).

Activity of α-amylase

Serum activities of α-amylase did not differ significantly between all the three determinations. After the procedure, four patients developed transitory hyperamylasemia. In one of them the activity of α-amylase was 5-times the norm. The median of α-amylase activity in urine at determination 2 (202 IU/L; 129 and 313 IU/L) was higher than at determination 1 (140 IU/L; 63 and 229 IU/L), (P<0.05), (*Tab. 1*).

Bilirubin concentration

The median of baseline bilirubin was 1.33 mg/dL (0.95 and 3.87), while its level on day 2 increased to 1.55 mg/dL (1.06 and 2.74). After 6-8 weeks, the concentration decreased to 0.97 mg/dL (0.78 and 1.14), as compared to the laboratory norm below 1.2 mg/dL. The elevated bilirubin levels prior to endoscopic procedure were observed with similar frequency both in patients with low and normal fecal levels of elastase 1 (4/9 patients with low and 10/19 with normal level). The differences between determination 2 and 3 and between 1 and 3 were statistically significant (P<0.001), (*Tab. 1*).

Activity of alkaline phosphatase

The activity of alkaline phosphatase (norm 45-123 IU/L) was reduced from 261 IU/L (percentile 25 and 75, respectively, 149 and 361 IU/L) prior to the procedure to 224 IU/L (138 and 321 IU/L) on day 2 (P<0.05) and to 103 IU/L (92 and 120 IU/L) 6-8 weeks after the procedure (P<0.001), (*Tab.1*).

Activity of aminotransferase

The activities of AST and ALT were as follows: 59 IU/L (25 and 115 IU/L) and 83 IU/L (24 and 204 IU/L) at determination 1, 54 IU/L (28 and 105 IU/L) and 68 IU/L (21 and 126 IU/L) at determination 2; 32 IU/L (24 and 42 IU/L) and 36 IU/L (23 and 44 IU/L) at determination 3 (norm 5-50 IU/L). The differences were statistically significant between determination 1 and 3 (P<0.05 for AST and P<0.005 for ALT) and between determination 2 and 3 (P<0.005 for AST and P<0.001 for ALT), (*Tab. 1*).

Leucocytosis

No significant differences were observed between the values of leucocytosis before the procedure (median $7.76\times10^3/\mu L$; 5.6 and $9.41\times10^3/\mu L$) and a day after the procedure (median $6.79\times10^3/\mu L$; 5.23 and $8.30\times10^3/\mu L$). However, 6-8 weeks later a statistically significant decrease was noted in comparison to baseline (median $5.86\times10^3/\mu L$; 4.95 and $7.49\times10^3/\mu L$; p<0.05), (*Tab. 1*).

Discussion

Cholelithiasis is the most common pathology within the biliary ducts, affecting 15% of the population (1). In 10-18% of patients, cholecystolithiasis is accompanied by bile duct stones. Choledocholithiasis may induce biliary colic and cause life-threatening complications, such as mechanical jaundice, cholangitis with postinflammatory stenoses, secondary biliary cirrhosis with portal hypertension and acute pancreatitis. It is therefore suggested that every case of choledocholithiasis should undergo treatment in order to prevent potential complications [1,15]. The effect of cholepathies on the exocrine function of the pancreas still remains a subject of controversy [3-6].

In all our patients, ERCP was performed with the purpose to treat biliary duct pathology. The efficacy of such procedures as cannulation (92%), stone removal or stenosis correction (82%) did not differ from the world statistics [7]. Once the indications had been established, sphincterotomy of the sphincter of Oddi was performed in the patients who had never undergone this procedure before. Although sphincterotomy increases the risk of complications, it has also positive effects. It has been reported that spincterotomy may lead to the evacuation of stones or biliary sludge in 55% of patients without visible pathological changes in cholangiogram [16]. According to some data, even up to 84% of stones in biliary colic and 55% in mechanical jaundice will spontaneously pass into the duodenum and are thus not detected [17]. In our study, the presence of macroscopically visible gallstones was confirmed in 19/24 patients (79%).

For 200 μg/g accepted as the borderline norm, determination of elastase 1 in feces exhibits 100% sensitivity for severe and moderate exocrine pancreatic insufficiency as well as 25-63% sensitivity for mild insufficiency [10,11]. Hamwi et al. [18] suggest that the borderline value can be + 25% of the recommended concentration of this enzyme due to its possible fluctuations in various stool samples from one patient.

Considering normal fecal elastase 1 levels to be over 250 μg/g, we found abnormal values in 9/28 patients (32%), including 6 (21%) with the concentrations below 200 μg/g, which may suggest moderate or severe exocrine pancreatic insufficiency. A similar tendency was observed by Hardt et al. [4], who noted reduced concentrations of elastase 1 in 30.8% of patients suffering from gallstones.

Pancreatic secretion depends on a number of factors. A reduction in fecal elastase 1 in patients with choledocholithiasis might be caused by mechanical obstruction in the outflow of pancreatic juice with bile. However, elevated bilirubin levels

were observed with similar frequency both in patients with low and normal fecal levels of elastase 1. Moreover, the mean fecal elastase 1 concentration was the highest in jaundice patients. Even though the difference was not statistically significant, its direction was consistent with *in vitro* observations and some animal studies, in which cholestasis led to an increase in basal and cholecystokinin-stimulated pancreatic secretion [19]. It thus seems that disturbances of pancreatic juice outflow are not the only factor responsible for the reduction in fecal elastase 1 in patients with bile duct pathology.

Removal of stones from biliary ducts is only the evacuation of the final product of complex pathological processes. Choledocholithiasis recurs in approximately 10% of patients within 6 years after surgery [20]. It cannot be excluded that not only gallstones but also various factors promoting this pathology may affect pancreatic function. Inflammatory reaction, fibrosis and formation of stenosis at the level of papilla of Vater caused by macroscopically visible gallstones or by even more common microlithiasis are the likely pathogenetic link in CP. Stenosis may lead to mild obstructive pancreatitis and cause a gradual loss of endocrine and exocrine function of the organ [4,21]. Elimination of stenosis should theoretically improve the exocrine function of the pancreas.

In the current study, fecal elastase 1 remained stable in all the three determinations. Although in the respective patients the levels of fecal elastase 1 changed in comparison to baseline values, there was a strong statistically significant correlation between determination 1 and 2 as well as 1 and 3, which suggests certain individual stability of secretion. Endoscopic treatment of biliary duct pathologies did not significantly change the exocrine function of the pancreas in the overall study population. However, in 6/9 patients whose baseline fecal elastase 1 levels were found to be low, an increase was noted at determination 3, and in 4 out of these 6 the values were normalized. In two out of these 9 patients, fecal elastase 1 reached normal values in the first stool after endoscopic treatment, but they did not come for a check-up 6-8 weeks later for further assessment. In one patient (with unsuccessful removal of stones from bile ducts after sphincterotomy) a further decrease of fecal elastase 1 level was noted during long-term check out.

The current findings seem to indicate a relationship between endoscopic treatment of biliary pathology and improvement of exocrine function of the pancreas. Our results correspond to those reported by Ewald et al. [22], who observed an increase in fecal elastase 1 in patients after endoscopic sphincterotomy performed due to dysfunction of the sphincter of Oddi. The increase was most significant in patients with pathologically low baseline levels of elastase 1. Also Doubilet et al. [23] showed improvement or even regeneration of exocrine function of the pancreas after surgical sphincterotomy.

After the procedure, four patients developed transitory hyperamylasemia. Although on day 2 serum elastase 1 correlated strongly with the increase in serum and urine activities of α-amylase, its concentration slightly exceeded the norm suggested by the kit producer just in one patient with mild AP, according to Cotton`s et al. [24] criteria.

Katsanos [14] observed higher specificity but lower sensitivity of serum elastase 1 in the detection of AP after ERCP. Our findings did not indicate on any prevalence of serum elastase 1 as compared to α-amylase in the detection of AP after ERCP.

Conclusions

No significant exocrine pancreatic dysfunction determined as fecal elastase 1 concentration was found in the overall study group of patients with biliary duct pathology, as compared to the control. However, in 32% of cases fecal elastase 1 level was below 250 μg/g, which suggests exocrine insufficiency of the pancreas. Endoscopic treatment of biliary duct pathology resulted in a significant reduction in cholestase parameters, but did not affect the mean elastase 1 concentration in the whole group. Nevertheless, at least 2/3 of patients who prior to the procedure had fecal elastase 1 below 250 μg/g showed its increased levels 6-8 weeks following endoscopic treatment. This may suggest improvement in pancreatic secretion due to endoscopic treatment for biliary duct disorder and its complications.

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Bone pain in dialysis patients is not associated with bone mineral density but with serum concentration of small uremic toxins

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Abstract

Purpose: Abnormalities in bone mineral density (BMD) are frequent disorder in dialysis patients. In our study we checked if such clinical symptom as bone pain may be associated with BMD.

Patients and methods: The study was performed in 30 dialysis patients. They were divided according to declared or not declared bone pain in any localization. The group with bone pain (n=10) included 7 women and 3 men, age 57.4±16.2 years, dialysis vintage 19.3, 6.5-45.5 months. The group without bone pain (n=20) consisted of 11 women and 9 men, age 55.5±18.9 years, dialysis vintage 20.5, 6.3-59.6 months. BMD was assessed by dual-energy x-ray absorptiometry in femoral neck (N) and lumbar spine from the second to the fourth lumbar vertebra (L2-L4). Routine clinical and laboratory parameters were evaluated and compared in both groups.

Results: The group with bone pain had higher serum concentrations of phosphate (6.2±1.4 mg/dl vs 4.9±1.1 mg/dl, p=0.012) and urea (136.0±37.4 mg/dl vs 111.3±23.5 mg/dl, p=0.035) than the group without bone pain. After adjustment of results to gender, age and dialysis vintage these differences remained significant, additionally the group with bone pain had higher serum creatinine concentration than the group without bone pain (9.5±2.4 mg/dl vs 7.5±2.9 mg/dl, p=0.009). There were no statistically significant differences between groups in BMD measured in N and L2-L4.

Conclusion: Our results suggest that bone pain in dialysis patients is associated rather with serum concentration of small uremic toxins than with BMD.

Key words: bone mineral density, bone pain, dialysis, uremic toxins.

Introduction

Abnormalities in bone mineral density (BMD) are well known disorder in dialysis patients [1-7]. They are frequently accompanied by signs and symptoms (bone pain, bone fractures), which diminish quality of life in affected patients.

Although pain has been appreciated as a problem for end-stage renal disease patients for more than 20 years, few studies exist on this subject [8]. It was shown that pain is present in 21-50% of hemodialyzed patients and is the important determinant of their quality of life as well as is associated with depression [9,10]. Bone pain in the lower back, pelvis, rib cage areas, or long bones of upper and lower limbs, which is made worse by movement, is frequent complaint in uremia. The pain may come on gradually or fluctuate over a period of weeks, or it may develop suddenly, associated with bone fracture. In the study of Weisbord et al. [10] bone or joint pain was shown in at least 50% of cases and reached over 3 scores in Likert Scale Score, similarly like muscle cramps. According to questionnaire studies of Lichodziejewska-Niemierko [11] nephrologists in Poland suspect that pain is present in 4-7 dialyzed patients of every 10 patients, and 13.6% of nephrologists assume that 8-10 patients of 10 ones complain of pain. Moreover, 72.7% of nephrologists suspect that pain should be described by 4-7 scores in VAS 0-10 scale.

In our study we checked if in dialysis patients bone pain may be associated with BMD or other routinely evaluated parameters.

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***Table 1*. The demographic and clinical characteristics of examined patients with and without bone pain**

Parameter	Patients with bone pain (n=10)	Patients without bone pain (n=20)	p value
Gender	7 women, 3 men	11 women, 9 men	0.693
Age (years)	57.4±16.2	55.5±18.9	0.786
Dialysis modality	9 PD, 1 HD	17 PD, 3 HD	0.849
Dialysis vintage (months)	19.3 (6.5-45.5)	20.5 (6.3-59.6)	0.975

HD – hemodialysis; PD – peritoneal dialysis

***Table 2*. Results of bone mineral density (BMD) obtained in examined patients with and without bone pain**

Parameter	the group with bone pain	the group without bone pain	p value
Femoral neck			
BMD (g/cm^2)	0.830±0.172	0.806±0.139	0.681
T-score	-1.43 (-3.25-2.37)	-1.57 (-4.06-1.15)	0.315
BMD as % peak bone mass	87.6±23.3	80.0±15.7	0.297
Z-score	-0.65 (-2.36-2.97)	-0.77 (-2.36-2.01)	0.384
BMD as % age norm	97.6±24.3	90.4±14.5	0.328
Lumbar spine			
BMD (g/cm^2)	1.067±0.226	1.078±0.282	0.917
T-score	-0.33 (-3.95-1.73)	-1.25 (-3.13-4.07)	0.538
BMD as % peak bone mass	92.1±18.9	90.5±20.9	0.837
Z-score	0.54 (-4.41-2.14)	-0.73 (-1.85-4.49)	0.409
BMD as % age norm	100.2±24.3	95.5±19.5	0.577

Patients and methods

The study was performed in 30 stable patients in stage 5 of chronic kidney disease: 26 persons were treated with peritoneal dialysis (PD) and 4 – with hemodialysis (HD). The underlying disorders leading to end-stage renal failure were chronic tubulointerstitial nephritis (8 cases), diabetic nephropathy (7 cases), chronic glomerulonephritis (5 cases), polycystic kidney disease (4 cases), hypertensive nephropathy (1 case), obstructive nephropathy (1 case). In 4 cases a reason for end-stage renal disease remained unknown.

All patients were asked by the younger investigator about feeling pain in limbs, spine, pelvis, rib cage areas and their answers were evaluated and qualified by both investigators together with analysis of medical histories in order to check patients' pain complaints over time documented in the written form. Uremic patients, who declared bone pain at least 4 times and occurrence of pain was documented at least two times by a physician on routine visit during a year preceding the study, were included to the group of patients with bone pain.

Data on demographic and clinical characteristics of patients with bone pain and without it are presented in the *Tab. 1*.

BMD was examined by dual-energy x-ray absorptiometry (DEXA), which is a reference method to measure bone mass in various skeletal sites and to assess fracture risk [12]. Assessment of bone mass was performed in two sites: femoral neck – N and lumbar spine from the second to the fourth lumbar vertebra – L2-L4. Simultaneously the following parameters were evaluated: serum concentration of intact parathyroid hormone (iPTH), total calcium, ionized calcium, inorganic phosphate, urea, creatinine and uric acid, serum activity of total alkaline phosphatase, blood pH, serum markers of inflammation (C-reactive protein – CRP, ferritin), bioimpedance records of body composition (total body water, extracellular water, intracellular mass, lean body mass, fat body mass) as well as blood/ /serum parameters (hemoglobin, total protein, albumin, total cholesterol) and anthropometric markers (waist circumference, hip circumference, body mass index) of nutritional state. Laboratory markers were determined using standard methods.

Results are expressed as mean and one standard deviation, or as median and range. The distribution of variables was assessed using the Kolmogorov-Smirnow test. Serum concentrations of CRP and iPTH were not normally distributed in both groups. Patients with bone pain additionally did not show the normal distribution of total cholesterol, albumin and total alkaline phosphatase, whereas patients without bone pain did not have the normal distribution of total calcium, volume of extracellular water as well as T-score and Z-score for BMD in L2-L4.

The results were compared with adjustment for gender, age, dialysis modality and dialysis duration to eliminate their possible influences on significance of differences in evaluated variables as associated with bone pain. Our previous studies showed associations between BMD and both age and gender of examined patients [13]. In multiple regression analysis ANCOVA methodology was used. A p value below 0.05 was considered as statistically significant.

Results

The examined groups did not show significant differences (p values >0.05) in gender distribution, age, dialysis modality and dialysis vintage (*Tab. 1*).

BMD assessed in the femoral neck and the lumbar spine (L2-L4) was not statistically significant (p values >0.05) in the examined groups (*Tab. 2*).

***Figure 1*. Serum concentration of inorganic phosphate in dialyzed patients with and without bone pain**

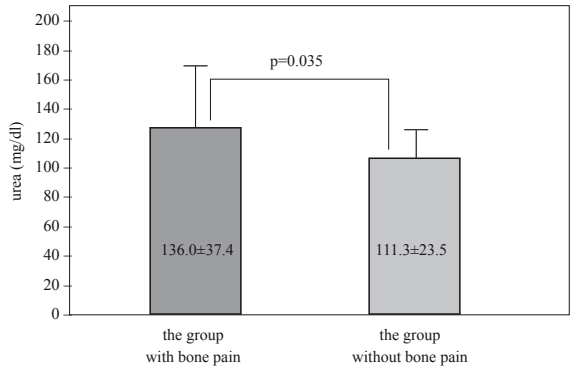


***Figure 2*. Serum concentration of urea in dialyzed patients with and without bone pain**

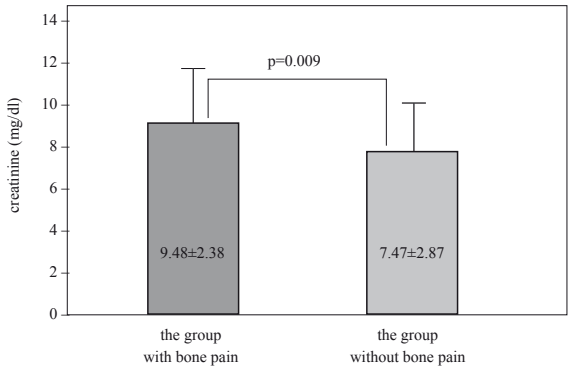
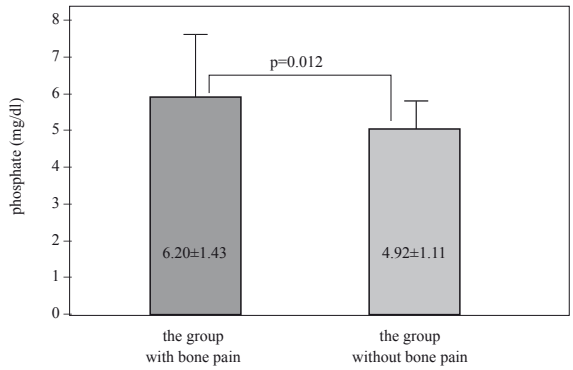


***Figure 3*. Serum concentration of creatinine in dialyzed patients with and without bone pain**



In univariate analysis, the group with bone pain had significantly higher serum concentrations of inorganic phosphate (p=0.012, *Fig. 1*) and urea (p=0.035, *Fig. 2*) than the group without bone pain. In multivariate analysis, after adjustment of results for gender, age and dialysis vintage these differences remained significant. Moreover, the group with bone pain revealed higher serum creatinine concentration than the group without bone pain (p=0.009, *Fig. 3*). Other examined parameters were not significantly different (p values >0.05) between both groups (*Tab. 3*).

Discussion

Pain is a subjective complaint, difficult for quantitative evaluation. Bone pain is usually attributed to bone disease, also to renal osteodystrophy [14] and osteoporosis [15,16]. The latter disorder may be successfully diagnosed by evaluation of bone mineral density (BMD) [12]. However, in our study, patients suffering from bone pain did not show significant differences in BMD. Moreover, also serum parameters closely related to

***Table 3*. Selected results obtained in examined patients with and without bone pain**

Parameter	Patients with bone pain	Patients without bone pain	p value
iPTH (pg/ml)	192 (14.9-1967)	242 (12.3-913)	0.660
Total calcium (mg/dl)	9.06±1.22	8.87±0.67	0.843
Ionized calcium (mg/dl)	4.19±0.79	4.32±0.53	0.625
ALP (IU/l)	82.8±47.1	83.7±27.9	0.235
Uric acid (mg/dl)	5.78±0.55	5.91±0.88	0.662
Blood pH	7.37±0.05	7.38±0.05	0.773
CRP (mg/l)	1.70 (0.00-10.46)	0.80 (0.00-12.20)	0.367
Ferritin (ng/ml)	366±220	369±249	0.977
TBW (l)	39.5±9.2	37.7±6.8	0.542
ECW (l)	17.1±3.6	16.7±4.6	0.644
ICW (l)	22.4±6.0	20.7±3.5	0.340
LBM (kg)	52.2±12.7	48.9±9.0	0.423
FBM (kg)	23.1±8.6	21.3±7.3	0.555
BMI (kg/m^2)	28.5±6.3	25.7±4.6	0.171
Waist circumference (cm)	98.1±14.7	95.8±12.2	0.656
Hip circumference (cm)	104±13	100±9	0.333
Hemoglobin (g/dl)	10.9±1.5	11.7±0.8	0.051
Total protein (g/l)	70.3±6.8	68.7±5.9	0.500
Albumin (g/l)	34.6±5.5	36.1±4.2	0.281
Total cholesterol (mg/dl)	208±55	212±43	0.823

ALP – alkaline phosphatase; BMI – body mass index; CRP – C-reactive protein; ECW – extracellular water; FBM – fat body mass; ICW – intracellular water; LBM – lean body mass; PTH – intact parathyroid hormone; TBW – total body water

bone metabolism (iPTH, total and ionized calcium, alkaline phosphatase) were not different between the examined groups.

Pain is one of the main components of inflammatory state. Laboratory markers of inflammation – increased serum concentrations of CRP and ferritin and decreased serum level of albumin – were similar in both groups. It may indicate that bone pain cannot be related to differences in expression in inflammatory state frequently seen in dialyzed patients [17-19].

Obese people frequently complain of pain in the lumbosacral spine, hips, knees, and ankles because obesity/overweight is frequently associated with joint pathology (osteoarthrosis), which leads not only to joint pain, but also to bone pain [20]. In our study, patients suffering from bone pain showed, however, similar anthropometric markers of nutritional state, bioimpedance records and laboratory indices of nutrition.

Uremic toxicity, evaluated by measurements of serum concentrations of small molecules (urea, creatinine, uric acid, phosphate), was more pronounced in dialysis patients who complained of bone pain. Our observation indicating association of bone pain and uremic toxicity is in agreement with results of Unruh et al. [21] showing that the high dose hemodialysis intervention was accompanied by significantly less pain in treated patients. Intensive HD treatment obviously caused lower uremic toxicity.

It cannot be excluded that patients with more advanced uremia are especially sensitive for pain signals, or such signals are released easier in the uremic environment. High or low serum concentrations of uremic solutes can be harmful. Serum level of inorganic phosphate is a factor related to bone metabolism, and also to less adequate dialysis treatment, like higher serum concentrations of urea and creatinine. Our patients with bone pain had higher serum level of phosphate than patients without it, but acute deficiency of phosphate also leads to bone pain [22]. Factors directly contributing to development of bone pain are not classified. Thus, we can only identify associations between serum concentrations of urea, creatinine and phosphate and bone pain, but causality cannot be confirmed.

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Counteraction against obesity – is it possible?

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Abstract

The obesity epidemic is one of the most serious public health problems across many countries. In Poland more than half of the adult population has excessive body weight, while approx. 20% are obese. 15-20% of children and adolescents suffer from excessive body weight, while 4% of them are obese. Moreover, the number of overweight or obese children is growing alarmingly.

Obesity can lead to many serious health consequences. Though the most serious disorders are cardiovascular diseases, diabetes type 2 and some cancers. In the nearest future diseases related to obesity will probably become the main cause of death in many countries. This may lead to shorter average life expectancy. The treatment costs of obesity and related diseases are constantly increasing.

The most important preventive measure aiming at curbing the effects of obesity involves lifestyle change, including a change in diet and physical activity. The best results should be obtained by multifaceted programmes, which cover activities aiming at the improvement of both diet and physical activity.

Due to the spread of the obesity epidemic, the countries of WHO European Region signed the European Charter on Counteracting Obesity, in which they declared their commitment to combat obesity.

Activities aiming at combating obesity in Poland should be closely connected with the implementation of the National Programme for the Prevention of Overweight, Obesity and Non-Communicable Diseases through Diet and Improved Physical Activity, which will be implemented in 2007-2016.

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Introduction

Obesity is a worldwide large epidemiological problem. In many countries the growing obesity epidemic can be observed for many years. This epidemic causes health deterioration and hence, increases health care costs.

At the same time in many countries overweight and obesity prevention programmes are being implemented. Most of these programmes did not bring the desired results, especially on the scale of the whole population. However, there are also examples of prophylactic programmes implemented for a given population, which resulted in a lower overweight and obesity incidence due to the improvement in diet and increased physical activity.

The effectiveness of obesity prevention methods depends on many factors. Education directed toward whole population, especially to families, children and adolescents at schools, is an important element of these methods. Research institutions and local governments with the help of national authorities should develop their implementation.

The alarming situation as far as obesity and its consequences are concerned, resulted in great commitment of the WHO and the European Commission to counteract obesity. One of the more important initiatives was the one developed by WHO, expressed in the Global Strategy on Diet, Physical Activity and Health, adopted on the 57th World Health Assembly in Geneva on May 2004 [1]. The WHO European Region Ministers of Health signed the European Charter on Counteracting Obesity in November 2006 [2]. The signatory states declared full commitment to counteracting obesity, at the same time calling to enhance the measures in this field, adjust them to local conditions and to search for innovations and initiate new research, which could improve the effectiveness of the policy.

Overweight and obesity prevalence in Poland and other countries

The results from research conducted in the last few years indicate a high prevalence of overweight and obesity in Poland. According to the data based on a country-wide representative research, conducted under the following programmes: "Household Food Consumption and Anthropometric Survey" [3], NATPOL PLUS [4] and WOBASZ [5], excessive body weight concerns approx. 60% of adult men and approx. 50% of women. The incidence of overweight among men is estimated to be 39-40% and 28-29% among women, while the percentage of obese men and women is 16-21% and 19-22% respectively.

There are less overweight and obese children and adolescents than adults. According to the data of the "Household Food Consumption and Anthropometric Survey" [6] programme, 16% of boys and 11% of girls are overweight – overweight evaluated on the basis of international cut off points proposed by Cole et al. [7] – and 4% of boys and almost 3.5% of girls are obese.

The prevalence of overweight and obesity changes with age. The youngest age groups of children most often have excessive body weight. As children get older, the incidence of excessive body weight falls, especially as far as obesity incidence is concerned [3,6]. Also among young adults the prevalence of obesity is not high. Overweight is far more common, especially among men. The percentage of persons being overweight or obese rises clearly with age [3,4]. In advanced age, excessive body weight, including obesity, is far more common in women than in men. Moreover, some women, mainly after 40, suffer giant obesity, while men rarely do [3-5].

Abdominal obesity concerns to a larger degree women than men [4-6]. According to the ATP III guidelines [8] from 2001, abdominal obesity prevalence among men is estimated to be 16-28% and among women – 35-40%. The estimation basing on the IDF criteria [9] for the European population from 2005 indicated that the risk of complications related to fat tissue accumulation close to the stomach is more frequent and concerns approx. 39% and 56% women. The incidence of abdominal obesity increases to a large extent with age [6].

In the 1990's, the percentage of overweight or obese persons increased, both as far as children and adolescents and adults are concerned [3,10,11]. However, in the last few years, a turn in this unfavorable tendency is observed among women. Among men the prevalence of excessive body weight is still going up to a lower degree [3-5,12].

Similarly as in Poland, overweight and obesity has become a serious problem in many other countries. The countries with the greatest overweight and obesity prevalence in Europe are: Malta, Greece, UK and Belarus, where the percentage of people above the age of 15 with excessive body weight is 60-70%. In USA overweight and obese persons amount to 73-76% of the population above the age of 15 [13].

Health and economic consequences of overweight and obesity

Predictions concerning health consequences of obesity are very worrying. Obesity will probably become the main cause of death. Moreover, obesity may even outstrip smoking. In 2000 in USA 16.6% of deaths was the result of diseases related to overweight or obesity [14]. It should be mentioned that this number increased by 2% in comparison to 1990.

Persons with BMI>30 have between 50% to 100% higher risk of premature death than persons with BMI=20-25. It is estimated that obesity is the cause of 300,000 premature deaths each year [15-17].

A very worrying phenomenon of falling life expectancy may be observed in many countries. This is the result of the growing prevalence of obesity. It is assumed that in the following two decades in many countries (USA, UK) the average life expectancy will fall [18], if the epidemiological situation does not change.

The main causes of premature deaths among obese persons are cardiovascular diseases (CVD): ischaemic heart disease, hypertension, congestive cardiac failure. The risk of cardiac infarction among women with BMI>29 is three times higher than among women with the normal BMI [19,20].

Obesity is connected with disorders which raise the atherosclerotic disease incidence risk, and therefore also CVD incidence. First of all, lipid disorders, i.e. hyperlipidemia, should be mentioned here. In the state of obesity, hyperlipidemia is characterised by an increased level of triglycerides (hypertriglyceridenemia) and a lower level of HDL (High Density Lipoproteins) [21].

An obese person, especially a person with hypertension and/or hypertriglyceridenemia, is more prone to have greater blood coagulability, and thus more prone to have blood clots. Such an inclination is very unfavorable as it increases the cardial infarction risk and the cerebral stroke risk [22].

The results of the epidemiological investigations indicate that obese persons suffer hypertension more often than persons with the normal body weight [23]. Women are especially vulnerable – they have a four times higher risk of developing diastolic hypertension than women with normal body weight [24]. It has been observed that young and obese people are more prone to develop hypertension and suffer cerebral stroke than their slim peers [25].

It happens often that persons with stomach obesity suffer the so-called metabolic syndrome X. The crucial phenomenon within the metabolic syndrome X is the so-called insulin resistance [26]. This expression means that tissues, to which insulin travels, are resistant to insulin in order to help utilize glucose by their cells – these tissues recognize weakly or not at all the signal connected with the presence of insulin. At first, our body breaks this resistance by increasingly secreting insulin. As a result, hyperinsulinemia takes places. Hyperinsulinemia is in itself unfavorable, as it creates favorable conditions for the development of hypertension and fat management disorders. With time the increased level of insulin is not able to break insulin resistance and the level of sugar in blood increases. In the end, the pancreas cells are exhausted, insulin is no longer secreted

and diabetes develops. It should be stressed that the reduction in body weight makes the tissues sensitive to the insulin activity and may turn the above-described course of events [27].

Obesity increases the risk of diabetes type 2 by 3-7 times. A person with BMI>35 has a 20 times higher risk of developing diabetes, than a person with the normal BMI [28]. Obesity followed by diabetes considerably increases the risk of cardiovascular complications and death caused by them [29]. It should be stressed that the main element of treating diabetes type 2 is the reduction in body weight [30]. In persons with no tolerance to glucose, which is a pre-diabetes state, the reduction in body weight by 5 kg and keeping that weight, decreases the risk of developing diabetes considerably (by 74%) [31].

Obesity is also linked to risk of cancer incidence. Forward-looking investigations of Cancer Prevention Study I, conducted in USA, covering 750 thousand men and women, indicated that death rate of obese persons caused by some type of cancers is higher than in case of persons with the normal body weight [32].

The mechanism of relation between overweight and higher cancer risk remains vague [33]. One of the available hypotheses explains that obesity is connected with a bigger number of cells and with more frequent division of them. This in turn increases the risk of the creation of abnormal cells. According to this theory, the excess of calories consumed in childhood favors the developed of big organs with a big number of cells. However, excessive consumption during adulthood causes intensified division of cell mucous membrane. In the case of the so-called hormone dependent cancer (cervical cancer), the impact of the excess of oestrogens and the extended exposure to them, due to the release of them from fat tissue, is taken into consideration.

Among other health disorders related to obesity, osteoarthritis, gallstones, obstructive sleep apnea syndrome, reproductive system diseases and psychosociological diseases should be mentioned.

An increasing incidence of overweight and obesity is also unfavorable from the economic point of view. It is estimated that health care costs for an overweight or obese person are 44% higher than those for a person with normal weight [34].

Overweight and obesity are a great burden to the health care budget of each country. In USA the costs connected with the treatment of overweight or obese persons make for almost $ 100 billion annually [15,35]. In UK the total costs incurred by overweight and obese persons are estimated to be 6 billion pounds [36]. On the basis of data gathered in different countries the direct costs related to obesity are estimated to amount from 1 to 10% of the expenditures spent on health care, depending on country [37].

Therefore, expenditure spent on programmes combating obesity is justified economically. Berkson et al. [38] compared the costs incurred by treating two groups of obese patients in his investigations. In the first group, a programme treating obesity was implemented, while the second one was a control group. After two years the costs of treating the persons in the group in which intervention was made began to fall. After 7 years these costs were lower by 33.8% than in the control group.

To date, the costs of preventing and combating obesity and chronic non-communicable diseases related to obesity in Poland were not estimated in detail. Krzyżanowska-Świniarska [39] estimates that treating obesity and its complications amounts to 21% of the health care budget. This is more than PLN 11 billion.

On the basis of investigation conducted in the Lublin region, it was estimated that the direct costs of treating a million patients amount to PLN 250 million. According to these estimates, the general direct costs incurred by obesity in Poland would amount to cir PLN 3 billion [37].

Methods of obesity prevention and prophylactic programmes

The most important measure aimed at curbing health and economic effects of obesity is the prevention of excessive body weight and reduction in weight of obese people. Of crucial importance is a change in lifestyle, including a change in diet and physical activity.

In order to keep normal body weight, it is necessary to maintain the optimal energy balance – i.e. to maintain a state, in which the energy intake equals the energy expenditure. On the one hand, excessive energy intake should be avoided, while on the other hand it is very important to ensure the energy expenditure at an appropriate level by increasing physical activity.

As far as obesity prevention is concerned, balancing calories intake with the energy expenditure is of crucial importance. However, in treating obesity negative energy balance is vital. This balance is obtained by limiting the energy intake and increasing physical activity. A number of different diets have been developed in order to reduce body weight. They differ in energy value, content of fat, carbohydrate and protein, energy density, the glycemic index and the number and size of food portions [40].

The main determinant of the decrease in body weight is the energy value. There are very low caloric diets (VLCD), which provide <800 kcal/day and low caloric diets (LCD), which contain 800-1500 kcal. The most often used diet is the diet providing 1000-1200 kcal. These diets are comprised of natural food products and are not balanced, i.e. they do not fully cover the need for vitamins and minerals. However, some prepared industrially diets providing less than 800 kcal cover the need for all necessary nutrients. A balanced diet based on natural food products usually provides >1500 kcal/day.

The energy deficit amounting to 500-1000 kcal a day results in a reduction in body weight by 0.5 to 1 kg a week. It should be stressed that American experts recommend to fat intake amounting to 25-35% of the total energy or less, SAFA<7% of the total energy, and carbohydrates in the amount of 50-60% of the total energy [41]. They recommend complex carbohydrates coming from different vegetables, fruit and cereal grains. The proposed amount of fibre is 20-30 g/day. Cholesterol should be consumed in smaller amounts than 200 mg/day.

Apart from bad diets, the most important reason for the growing overweight and obesity is low physical activity. Sedentary lifestyle is viewed as the factor that doubles the risk of the development of non-communicable diseases. On the other hand, increased physical activity has a good effect on

both physical and psychological health and lowers the death rate [42].

Low physical activity among children is caused by watching TV by them. In USA a child in the age group between 2-11 years watches TV 23 hours a week on average [43]. Issuing recommendations to limit TV watching is a key element of obesity prevention – American Academy of Pediatrics Committee on Communication proposes to reduce TV watching to 1-2 hours a day [44].

In order for physical activity to be effective, it must be performed on a regular basis, almost every day and 30 minutes a day at a minimum, though it strongly recommended to exercise even 60 minutes a day [45].

The best place to learn how to lead a healthy lifestyle is the family and the best example for children is that of their parents. As far as children that are not supported by their parents are concerned, the risk of obesity incidence is higher. The research results presented by Lissau et al. [46] indicate that the support of parents is of great importance for childhood obesity prevention. Moreover, these results indicated that children whose parents are not aware of how much sweets their children are consuming, whose parents accept the consumption of great quantities of sweets and who spend much money on such products have a considerable risk of developing obesity during childhood. Parents should remind their children not to eat too many sweets. They should, moreover, develop healthy eating habits of their children and encourage them to perform physical activity.

A very important place in which eating habits are to be developed and physical activity encouraged is the school. European and American pediatric associations put a lot of emphasis on the kind of products, especially beverages, offered at school kiosks. Sweet beverages are the main source of sugar and unnecessary calories in diets for school children. According to American studies, 56 to 85% of children consume one sweet beverage per day at school. The greatest quantities of such beverages are consumed by teenage boys [47]. This habit increases the risk of obesity by 60% [48]. Drinking of sweetened beverages is linked to the development of obesity most probably due to the fact that these are calories consumed in a liquid form, i.e. easily available, but also because these calories are a source of additional energy [49].

National Board of Health in the Netherlands recommends to not place vending machines, through which sweets and sweet beverages are sold at schools and educational facilities for children and adolescents [50]. American Academy of Pediatrics recommends to implement a policy limiting the sale of sweet beverages in school kiosks not only due to the risk of overweight and obesity posed by them, but also due to the fact that they replace milk beverages and this may lead to calcium deficiency and dental caries [51,52].

Of special importance is also the necessity to limit the consumption of fast food, which is a source of additional energy. In the study conducted by Bowman et al. [53], fast food provided additional 187 kcal a day. It should be stressed that additional 100 kcal a day above the level of energy that can be used up, can cause an increase in body weight by 5 kg during one year.

In different countries attempts are made to promote activities preventing overweight and obesity. These activities put an emphasis on a change of environmental conditions [54,55]. They cover measures aimed at increasing physical activity, such as, for example – modifications in buildings to encourage the use of stairs, planning the surrounding area of people's homes in such a way as to encourage walking and jogging, and promoting active means of transportation by constructing safe bicycle lanes. In order to change eating habits, emphasis is placed on appropriate labeling of food products. This in turn will facilitate a good choice of food products and extend the offer of healthy food products in stores, schools and workplace canteens.

Prevention of obesity and overweight conducted among adults proves to be particularly difficult. Measures implemented during researches [56,57] aimed at preventing body weight increase, concentrated mainly on education encouraging adults to change their nutritional habits. However, a long-lasting effect was not obtained in the course of those researches.

As mentioned before the majority of countries including Poland show an alarming tendency to a substantial increase of body weight among children and adolescents. According to the National Association of Pediatric Nurse Practitioners [58] prevention of obesity among children and adolescents is a particularly important issue – without it the present generation of children is facing a shorter life expectancy than their parents.

The best results should be obtained by multifaceted programmes, which cover activities aiming at the improvement of both diet and physical activity. Examples of such programmes conducted in some countries are listed below.

In the state of Georgia the Health Kids' Alliance has initiated a programme in 13 junior high schools aimed at improving fitness and physical activity of students as well as changing their diet [59]. This programme was based on 10 key recommendations of the Centres for Disease Control and Prevention (CDC) designed to help children develop and maintain healthy nutritional habits and increased physical activity. Provisional evaluation shows positive results, however, a longer perspective is necessary to provide an opinion on programme effectiveness.

"Girls on the Run" [60], conducted in all US states, is a programme for improving physical activity through running, walking and dancing. The programme offers preteen girls (8-12 years) two hours of activities per week for 12 weeks. Activities are run in different time periods (before, during and after school) and in different seasons. In the state of Virginia provisional evaluation including the self-evaluation of participants, satisfaction with their figure and healthy nutritional habits has been conducted. It has been stated through the participation in the programme all those parameters improved.

Under Kiel Obesity Prevention Study (KOPS) in Germany, which began in 1996, preventive measures have been introduced at home and at school [61]. The most important prediction factors of the prevalence of excessive body weight in the prepuberty period have been: overweight parents, low economic status and high birth weight. Health promotion aimed at students and teachers comprised nutritional education and introducing active breaks between classes. Education on healthy habits was also conducted among families with overweight or obese children or children with the risk of being overweight. One year after implementing intervention measures in schools and at home, impact on their lifestyles and nutritional habits of

***Table 1*. Examples of preventive programmes focusing on overweight and obesity, implemented in different countries among children and evaluation of their effectiveness**

Country and type of intervention	Effects
Austria: PRESTO – nutrition education under school programmes for children aged 10-12 years (pilot research) [64]	Improvement of nutritional awareness, particularly among youth. BMI has not changed.
Crete, Greece: School programme for health education covering children aged 6-12 years [65,66]	Decrease in BMI in the examined group as compared with the reference group, but in both groups the increased percentage of overweight children was observed.
Denmark: Training of families regarding shopping and meal planning [67]	Reduction of body weight in children (2 years of intervention).
Germany: KOPS – eight-year-long intervention in schools, covering children aged 5-7 years at the initial stage [68]	Improvement of nutritional awareness, increase in physical activity, reduced TV watching, reduction in body fat index (skin and fat fold, % of adipose tissue).
Germany: StEP TWO – School programme among children aged 7-9 years [68]	Reduction of BMI growth rate, decrease in the systolic blood pressure.
Israel: Programme covering diet consulting, exercise and learning to change nutritional behaviour [69]	Multi-factor intervention among obese children brought effects in the form of the reduction of body weight, BMI, improvement of fitness, in particular among children, whose parents are not obese.
Great Britain: „Be Smart" – intervention at school and at home covering children aged 5-7 years [70]	Improvement of nutritional awareness, increased fruit and vegetable consumption. No significant changes in the occurrence of overweight.
Great Britain: „MAGIC" – twelve-week programme of increasing physical activity in kindergartens (children aged 3-4-years). Pilot research [71]	Improvement of physical activeness – by 40%. Results referring to changes in body fat – unknown.
Great Britain: „APPLES" – intervention programme among children aged 7-11 years [72,73]	Improvement in nutritional habits in some aspects. No change in physical activity. No change in BMI.

the subjects was observed. An average increase of adipose tissue in one year among overweight children at schools where the studies were conducted was 0.4% as compared to 3.4% among children from control group. The research is still being conducted and the influence of long-term intervention measures is yet to be analysed.

In 1992 the Minister of Health in Singapore initiated a national programme promoting healthy lifestyle, aimed at combating chronic non-communicable diseases like obesity, low physical activity and tobacco smoking [62]. This programme is complementary with the programme of the Minister of Education "Trim and Fit Study" aimed at limiting obesity among children and adolescents and improving their physical fitness. Those activities are intended for various groups – pupils, parents and teachers. Efforts were made to create right conditions in schools to allow and help students to choose healthy habits. Dietary education lies within the limits of formal school programmes. The range of products available in school kiosks is under control. Water dispensers are also installed in schools. Schools with good results receive prizes. Special attention has been paid to overweight and obese students. They take part in special programmes promoting physical exercises, where they are also motivated to choose healthy food products. Obese pupils who require special counseling are referred to a doctor or a dietician. Until 2000 decrease of overweight was observed among children aged 11-12 years from 16% to 14% and among adolescents aged 15-16 years from 15.5% to 13.1%.

The "Copenhagen City Bike Programme" [63] is also noteworthy. Local authorities have created good conditions for using bicycles as the main mean of transport in the entire city. The programme is accompanied by educational activities encouraging all age and professional groups to use bicycles. Hence a clerk heading to work on a bicycle is not a rare sight.

It is worth stressing that among preventive programmes conducted so far in different countries; nine controlled interventional researches among children, which brought positive effects were rewarded. Part of them managed to improve nutritional awareness, part of them showed decrease in overweight or increase of physical activity. *Tab. 1* presents those programmes.

Full evaluation of such programmes is possible not earlier then after several years of intervention actions, therefore in the overweight and obesity prevention it is necessary to develop long-term multi-sectoral preventive programmes implemented on all above levels: family, school, health care, media, governments and industry.

European Charter on Counteracting Obesity

European Charter on Counteracting Obesity has been developed in order to respond to the threat for health, European economies and civilization development in connection with the growing obesity epidemic.

The Charter was adopted and signed by the ministers and delegates of 48 countries of WHO European Region jointly with the Director of WHO Regional Office for Europe in the presence of the European Commissioner for Health and Consumer Protection at the WHO European Ministerial Conference on Counteracting Obesity (Istanbul, Turkey, 15-17 November 2006) [2].

The Charter stresses that the obesity epidemic remains one of the major challenges for public health in the WHO European Region. The tendency of developing obesity among children and adolescents was considered especially alarming, for it may bring a threat of obesity epidemics in next generation and may have an unfavorable impact on economic and social growth. It has been assumed that the increase in obesity epidemics in recent decades relates to the change in social, economic, cultural and physical environment.

The statement that obesity epidemics may be reversed is a very important message. Reduction of epidemic and reversal of trends is the main goal of actions taken in the European Region. All relevant sectors and authorities at different levels should share these actions. Support from the private sector and the media will be of great importance, along with the active participation of the society.

The Charter aims at strengthening works aiming at combating obesity in the whole European Region. It will stimulate and influence policies of respective countries, regulatory work, including legislation and action plans.

Long-term progress monitoring process is needed, since the results in the form of reducing obesity and related diseases will become visible after quite some time. At the WHO European level three-year reports on progress in that respect should be drawn up. First such report should be drawn up in 2010.

Directions for further actions

The Polish response to the WHO initiative expressed in the Global Strategy on Diet, Physical Activity and Health consists in the National Programme for the Prevention of Overweight, Obesity and Non-Communicable Diseases through Diet and Improved Physical Activity, approved by the Minister of Health for implementation in the 2007-2016 period [74]. The Programme implementation is compliant with the European Charter on Counteracting Obesity.

Programme objectives include:

– reducing the prevalence of overweight and obesity in Poland, mainly by improving nutrition and increasing physical activity;

– reduction of disease incidence and mortality caused by chronic non-communicable diseases;

– reduction of expenditure for health protection relating to the treatment of chronic non-communicable diseases, especially obesity and related complications, as well as reducing the economic effects of disability and premature mortality.

Directions of further actions aimed at preventing obesity in Poland should be closely linked with the implementation of the abovementioned Programme. Major tasks of the Programme include:

– implementation of representative, repeated every 5 years, research on the nutritional status, dietary habits, physical activity and general heath state among the Polish population;

– continuous education and skill improvement of the professional groups dealing with overweight and obesity prevention in the society (physicians, nurses, dieticians, employees of the public food control bodies);

– increasing the awareness regarding the importance of proper diet and physical activity in overweight and obesity prevention;

– training of head masters and teachers regarding the importance of proper diet and physical activity in schools;

– activities aiming at increasing physical activity in all age groups;

– cooperation with food industry for the production of food of key importance in overweight and obesity prevention;

– price policy, which will contribute to the choice of food beneficial from the point of view of obesity prevention;

– amendment of legislation referring to food labeling, diet supplements, special-purpose food products;

– support for the development of initiatives, which were already started and verified in local environment and disseminating them to the whole country.

Conclusion

National Programme for the Prevention of Overweight, Obesity and Non-Communicable Diseases through Diet and Improved Physical Activity creates possibilities for reducing obesity epidemic in our country. It seems that only long-term integrated activities in that regard may contribute to the improvement of the unfavorable current situation.

Long perspective of the Programme is of vital importance, for according to estimates, through investing in the improvement of the population's quality of life; even a few percent of persons with incorrect body weight may be influenced each year. World Health Organisation experts believe that the implementation of appropriate prevention programmes will allow for the reduction of obesity epidemics in approx. 10 years [2]. Therefore, the implementation of the Programme is a real chance for counteracting obesity in Poland effectively.

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Dietary intake and body composition of female students in relation with their dieting practices and residential status

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Abstract

Purpose: To examine association of residential conditions and past dieting with current dietary intake and body composition of female pharmacy students.

Participants and methods: The 24-hour recall method was used to evaluate dietary intake of 47 female students. Height, weight and four skinfold thickness were measured to assess body composition. In addition, survey included lifestyle questionnaire to obtain information on type of residence place in time of academic year and about diets continued at least by two weeks. The Mann Whitney U-test and correlation analysis were used.

Results: Current energy and nutrient intake were related to dieting history. Female students with dieting history and living with their parents had significantly lower ($p \leq 0.05$) intake of energy (61.9% RDI), carbohydrate (67.5% RDI), fat (58.7% RDI), phosphorus (111.7% RDI), magnesium (73.4% RDI) and thiamin (72.3% RDI) than those that have never dieted. A significant association ($p \leq 0.05$) between past dieting and current body composition was also found. Compared to students who had never used diets and reside with their parents, students with past dieting behavior indicated significantly greater ($p \leq 0.05$) indices as BMI and % FM.

Conclusions: The high prevalence of non-rationale dieting among young women and dietary inadequacy associated with unhealthy nutritional behavior suggested that more appropriate and systematic educational intervention is needed in this population.

Key words: weight-related behaviors, risky eating patterns, young woman, anthropometric characteristics.

Introduction

Young females are often extremely concerned of weight and body shape. Body weight is both physical and psychological importance to young women and has been very strong associated with self-evaluation [1-3]. Most research suggests that this concern of body is dramatically influenced by mass media which has been implicated in the formation of unrealistically thin body ideals [4]. Moreover, society equates thinness with beauty and attractiveness in women [5]. It is not surprising that feelings of fatness, body dissatisfaction and dieting are common in young women [6,7]. Dieting has also been associated with depression and low self-esteem [3]. Prevalence of obesity steadily increasing in many countries over the world among adult and young people as well, suggesting that a lot of individuals are not successfull at reaching their weight loss goals. According to a recent survey 2-26% young people in Poland are obese depending on age, gender and region of living [8,9]. The very similar situation is observed all over the world [10,11]. The results of many studies have shown that people with a history of dieting were more prone to weight gain than people who had never been on a diet [12,13].

Dieting may lead to inadequate nutrient intake which in the long-term can result in hypotension, osteoporosis and irregular menstruation [14,15]. Most studies have shown that dieting behaviors are often unhealthy, especially among young women, who often diet aggressively in pursuit of ideal body shape. These behaviors may include self-induced vomiting, use of laxatives, fad dieting, skipping meals, smoking cigarettes and using over-the-counter supplements marketed for diet-

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ing [4,8,16,17]. However, some weight-control behaviors are appropriate, even desirable. There may be positive implications if young women consume more fruit, vegetables, whole grain and avoid excessive fat intake and increase exercise [18-20].

Transition to college or university can be frequently a time for initiation and/or continuation of dieting [21]. There are unique experience of female students that may promote dieting, including fear of gaining weight, increased independence, changes of daily schedule, friend and/or peer influence (idealization of "popular" women as thin and pretty) and living away from home [21,22]. This is a time when parents generally have little control or influence on eating behavior of their children [21]. Previous studies have shown very high prevalence of dieting among female students. Dieting is so common that Polivy and Herman suggested that this is "normal" eating for women [23]. Student residence during academic year has also been reported to affect food choices, nutrient intake, dietary practices and activity patterns [24,25].

The purpose of this study was to determine frequency of past dieting in connection with students residence during academic year and examine associations between past diet use and current dietary intake and body composition among female pharmacy students in Bydgoszcz.

Participants and methods

The studied population included 73 fourth-year female students from Faculty of Pharmacy of Collegium Medicum in Bydgoszcz (Poland). All students provided written consent for their participation in the study before data collection. Data were collected during the winter semester of 2005 academic year. Twenty-six female students were excluded from analyses because of missing data, so the final sample size was 47 students. The mean age of students was 22.8±2.4 years. The results are presented as the mean value with standard deviation (x±SD). Research was conducted according to protocols complied with requirements of the Bioethics Committee in Poland and was supported by an internal research grant from Collegium Medicum in Bydgoszcz.

The weekday related dietary intake of subjects was determined by 24-hour recall method according to guidelines of the National Food and Nutrition Institute (Warsaw, Poland) [26]. Detailed description of all supplement, food and beverage with cooking method and brand names consumed during 24 hours before the face-to-face interview were recorded. In addition, to enhance accuracy of the procedure, recalled food items were compared be meal type and estimation of portion size by use of household measure and "Atlas of photographs of food products and dishes" [27]. The microcomputer DOS operated "Dietetyk" software with a currently upgraded database containing Polish food tables [28] was used to calculate of average energy and nutrient intake of participants. Cholesterol and food fiber intake was compared with WHO recommendation (300 mg and 25 g respectively) [30]. The results were compared with the Polish nutritional norm at the safe level (RDI) for adult in age of 19 – 26 years and moderate physical activity [29]. Moderate physical activity was chosen because it was the most common activity level among female students (85 % of participants). Physical activity level was assessed using a questionnaire and was rated as active (female students who everyday intensive physical activity /min. 20 minutes per day/), moderate active (female students who declared 3 times per week intensive physical activity /min. 20 minutes per day/) and sedentary (female students who indicate no physical activity).

Measurements of height, weight and four skinfolds thickness (triceps, biceps, subscapular and suprailiac) values were used for calculation of body mass index (BMI, kg/m^2) and percentage of fat mass (% FM, %) [31]. These antropometric data were determined using, respectively, height scale (RADWAG, Poland), digital electronic weighing scale (RADWAG, Poland) and electronic skinfold caliper (SKYNDEX I, USA). Three consecutive measurements of skinfolds thickness were performed and mean values were reported (see *Tab. 1*). The Durnin and Womersley [32] formula was applied to calculate total body fat. Shoes and top coverings (coat and jacket) were removed preceding body composition measurements.

Information regarding past dieting which was continued permanently at least by two consecutive weeks in any time in the past and data concerning residence type during the academic year was obtained using the home designed, pilot tested and validated purpose designed lifestyle questionnaire. The participants were classified into two categories according their type of residence place during academic year: 1) residing with family and 2) residing in dormitory or rented flat.

The relationship between type of residence and past diets was verified using the Mann Whitney U-test and correlation analysis with the significance level $p \leq 0.05$. The statistical analyses were carried out by the software STATISTICA PL v. 6.1 (Stat-Soft, Inc., Tulsa, OK, USA).

Results

Among the total of 47 female students, 47% resided in family home with their parents and 53% lived in dormitory or rented flat during academic year (*Tab. 1*). It was observed that 28% female students residing in dormitory or rented flat have been on diet which was ever continued at least two consecutive weeks in the past. On the contrary, among students living in family home dieting was more common, i.e. dieting history was reported by 50% of them. More than 60% of total participants indicated that they wanted to lose weight. A sizable percentage of students indicated misperception in their body weight status, 21% described themselves as overweight when, in fact, only about 6% of students were actually overweight (data not shown).

BMI, percentage body fat mass (% FM), and biceps skinfold thickness were significantly greater for those who had tried dieting and lived in family home (*Tab. 1*).

In general, energy and basic nutrient (protein, total fat and carbohydrate) as well as majority of vitamins and minerals intake was related to dieting history, regardless of students residence (*Tab. 2* and *3*). An inadequate intake of many nutrients in reference to norm at the safe level (RDI) was found in whole studied group of students. Generally, it was observed that stu-

***Table 1*. Anthropometric characteristics (mean ±SD) of pharmacy female students in relation to residential status and declared past dieting**

Parameter	Type of residence					
	Family home N=22 (47%)			Dormitory/rented flat N=25 (53%)		
	Past dieting					
	YES, N=11(50%)	NO, N=11(50%)	r	YES, N=7 (28%)	NO, N=18 (72%)	r
Height, cm	163.6 ± 6.9	170.8 ± 8.5	**0.46***	165.4 ± 5.3	165.4 ± 6.0	-0.02
Weight, kg	61.6 ± 10.7	58.6 ± 10.3	-0.28	58.3 ± 5.8	55.4 ± 5.9	-0.34
BMI, kg/m^2	22.9 ± 3.1a	19.9 ± 1.6a	**-0.56***	21.3 ± 1.7	20.2 ± 1.9	-0.38
% FM	28.3 ± 4.1a	24.8 ± 3.0a	**-0.53***	26.5 ± 2.0	26.2 ± 3.1	-0.06
TSF, Triceps skinfold, mm	19.3 ± 5.8	14.3 ± 2.9	**-0.51***	15.3 ± 2.1	15.3 ± 4.2	-0.08
BSF, Biceps skinfold, mm	15.9 ± 4.9a	10.5 ± 3.2a	**-0.57***	14.3 ± 4.5	13.5 ± 4.2	-0.19
SS, Subscapular skinfold, mm	13.2 ± 3.7	10.8 ± 2.4	-0.36	11.7 ± 2.8	12.3 ± 3.2	0.06
SI, Suprailiac skinfold, mm	9.7 ± 3.0	8.2 ± 3.6	-0.23	9.2 ± 2.1	10.3 ± 3.1	0.19

Notes: SD – standard deviation; a – statistically significant differences ($p \leq 0.05$); r – correlation coefficients; * – statistically significant correlation coefficients ($p \leq 0.05$)

***Table 2*. Macronutrient intake (mean ±SD) of pharmacy female students classified according to residential status and declared past dieting**

Parameter	Type of residence									
	Family home N=22 (47%)					Dormitory/rented flat N=25 (53%)				
	Past dieting declared									
	YES, N=11(50%)		NO, N=11(50%)		r	YES, N=7 (28%)		NO, N=18 (72%)		r
	intake	% RDI	intake	% RDI		intake	% RDI	intake	% RDI	
Energy, (kcal)	1357±365a	61.9±16.7	2061±595a	94.6±271	**0.60***	1483±400	67.4±18.7	1700±463	77.5±21.4	0.20
Protein, (g)	52.9±19.9	66.2±25.0	70.5±28.5	88.2±35.7	0.40	48.0±13.4	60.1±16.7	59.7±157	74.7±19.6	0.32
Carbohydrate, (g)	193.9±46.1a	67.5±16.0	267.4±81.0a	93.0±28.2	**0.50***	197.4±59.3	68.7±20.6	209.6±72.7	72.9±25.3	0.07
Food fiber, (g)	15.9±5.6	—	19.9±9.1	—	0.27	17.9±12.2	—	19.1±5.5	—	0.04
Fat, (g)	41.1±21.3a	58.7±30.4	78.8±39.6a	112.6±56.6	**0.52***	55.7±23.4	79.6±33.5	69.3±25.6	98.9±36.6	0.26
Cholesterol, (mg)	164.2±106.3	—	215.4±93.9	—	0.27	142.8±77.1	—	213.0±94.7	—	0.33
% energy from protein	15.9±5.6	—	13.6±3.0	—	-0.25	13.1±2.4	—	14.2±2.0	—	0.23
% energy from fat	25.9±8.0	—	33.9±10.2	—	0.41	33.2±10.2	—	36.8±8.4	—	0.20
% energy from carbohydrate	58.3±8.3	—	52.5±9.7	—	-0.31	53.7±10.6	—	48.9±8.7	—	-0.25

Notes: SD – standard deviation; a – statistically significant differences ($p \leq 0.05$); r – correlation coefficients between "Intake" data; * – statistically significant correlation coefficients ($p \leq 0.05$)

dents with dieting history had poorer dietary intake than they who never used diet. In case of young women living in family home the estimated mean daily energy intake was statistically significantly lower (61.9% RDI) for students who used diet in the past in comparison to students without dieting history (94.6% RDI) (see *Tab. 2*). It was noticed that – irrespective of residential status – students with dieting history indicated significantly increased percentage of energy provided by fat, accompanied by reduced contribution of carbohydrate related energy intake (*Tab. 2*). In group living with their parents it was noticed that female students who have used diet which was continued at last two successive weeks in any time in the past had significantly lower intake of carbohydrate (67.5% RDI), fat (58.7% RDI), phosphorus (111.7% RDI), magnesium (73.4% RDI) and vitamin B1 (72.3% RDI) (compare *Tab. 2* and *3*). Similar relationship was found for students living in dormitory and/or rented flat. However, irrespective of residence status, for students who ever have been on a diet in the past only intake of phosphorus and vitamin A was excessive or complied with the nutritional recommendation (*Tab. 3*).

It is important to underline the high percentage of female students with inadequate intakes of calcium, copper and iron (see *Tab. 3*). We noticed that – regardless of dieting history – among the female students living without parents intake of calcium and iron was under two-thirds (<66.7%) of Polish RDI norm in case of more than 50% of mentioned subjects. Similar inadequate intake of copper was observed in more than 85% of these students. However, among the subgroup of female students living with their parents and used diet in the past near 73, 91, and 100 percent of them consumed, respectively, iron, calcium and copper below of the 66.7% of Polish RDI's (data not shown).

It was interesting that dietary intake of students with dieting history and residing with parents was poorer than students who have been on diet in the past but living away from family home. The reduced intake of energy, fiber, calcium, magnesium, zinc and vitamin B1 was observed for female students with dieting history and residing in family home (*Tab. 2* and *3*).

Discussion

The present study explored associations between past dieting which was continued at least two successive weeks in any

Table 3. **Micronutrient intake (mean ±SD) of pharmacy female students classified according to residential status and declared past dieting**

Parameter	Type of residence									
	Family home N=22 (47%)					Dormitory/rented flat N=25 (53%)				
	Past dieting declared									
	YES, N=11(50%)		NO, N=11(50%)			YES, N=7 (28%)		NO, N=18 (72%)		
	intake	% RDI	intake	% RDI	r	intake	% RDI	intake	% RDI	r
Calcium, (mg)	439.7±176.3	41.4±17.0	569.6±176.6	53.6±17.5	0.35	572.8±257.8	59.8±36.7	759.4±332.4	69.0±30.2	0.25
Phosphorus, (mg)	870±319a	111.7±43.2	1194±405a	153.1±54.4	0.39	1008±340	134.0±53.2	1122±320	140.3±40.0	0.13
Magnesium, (mg)	205.4±59.9a	73.4±21.4	281.3±83.4a	100.5±2.8	**0.51***	251.2±138.1	89.7±49.3	249.3±72.4	89.0±25.8	-0.04
Iron, (mg)	8.4±2.8	59.9±20.2	10.3±2.7	73.3±19.6	0.36	8.3±3.5	59.6±25.2	9.4±2.5	66.8±17.7	0.15
Zinc, (mg)	6.7±2.4	66.7±23.9	8.6±2.4	85.7±24.1	0.40	7.6±2.8	75.9±28.8	8.3±1.9	83.1±19.0	0.15
Copper, (mg)	0.9±0.3	41.5±12.9	1.3±0.5	57.3±23.1	0.42	0.9±0.4	38.7±19.0	1.1±0.3	46.5±14.6	0.20
Vitamin A, (IU)	630±319	105.1±53.2	916±890	152.8±148.4	0.24	765±944	127.5±157.4	894±735	149.1±122.5	0.06
Thiamin, (mg)	0.63±0.22a	72.3±24.6	1.21±0.40 a	138.3±45.5	**0.72***	0.71±0.28	81.7±31.9	0.84±022	96.1±24.9	0.24
Ryboflavin, (mg)	1.11±0.55	69.5±34.3	1.46±0.57	91.2±35.6	0.31	1.07±0.45	67.2±27.9	1.39±0.38	87.2±23.8	0.36
Niacin, (mg)	12.2±7.2	64.2±37.6	21.1±15.1	111.4±79.6	0.34	8.5±3.8	44.6±20.1	11.7±5.2	61.6±27.3	0.28
Vitamin C, (mg)	44.9±28.7	74.8±47.8	70.7±53.0	122.3±91.8	0.30	32.6±30.5a	54.3±50.8	74.6±41.3a	124.4±68.8	**0.46***

Notes: SD – standard deviation; a – statistically significant differences ($p \leq 0.05$); r – correlation coefficients between "Intake" data; * – statistically significant correlation coefficients ($p \leq 0.05$)

time in the past and current dietary intake. This study found that female students with history of dieting and living with their parents indicated significantly lower energy and nutrient intake. Similarly, Gibbson et al. [15] reported that intake of energy and micronutrient (thiamin, riboflavin, niacin, calcium, iron, zinc) was significantly related with past dieting practice among female high school students from Australia. It was also revealed by Neumark-Sztainer et al. [33] that female adolescent using weight-control behaviors had much lower intakes of energy and micronutrient than non-dieters. Mulvihil et al. [14] and Neumark-Sztainer et al. [34] also reported that total energy intake was inversely related to dietary restriction for female adolescent and woman from, respectively, Great Britain and USA. But these researchers did not find an association with dieting behaviors and reduced micronutrient intake. The possible impact on health should be taken into account as the particular concern of our study was very low intake of calcium, iron and copper in female students who have been on a diet in the past. Moreover, it is important to underline that, regardless of their dieting status, intake of these nutrients, as similarly observed in other countries, tends to be less than dietary recommendation for young women [14,34,35].

Female students with dieting history consumed less fat and more carbohydrate as percentage of total energy, irrespective of their residence type during academic year. Our results were similar with those of Neumark-Sztainer et al. [34], who observed that female dieters had lower intake of energy provided by fat and higher by carbohydrate than non-dieters. Calderon et al. [7] and Malinauskas et al. [22] found that students who had dieting history often eat low fat or fat free foods. Despite the seemingly healthy dietary habits, dieting may be a predictor of weigh gain. It was observed here that students with dieting history had higher BMI, percentage of body fat. Thus, our findings are consistent with those of Provencher et al. [36], who reported that female and male past dieters had higher current BMI than non-dieters. Field et al. [37] observed a relationship between dieting and weight gain. These researches reported that in adolescent females during 3 years of follow-up that dieters gained more weight that non-dieters, even if dieters had lower intake of total energy and percentage of energy from fat. It was also observed by Pasman et al. [38], who reported that female frequent dieters showed significantly more weight regain than less frequent dieters. The one of mechanism through which dieting may lead to overweight is that dieting may be associated with an increase in metabolic efficiency, therefore dieters over time may require fewer energy to maintain their weight [37].

Limitations of this study include small sample size, restricting participants to females only, and the use of 24-hour dietary recall approach. In most of published results considering young woman dieting a dietary intake was estimated using a 4- or 7-day food record or from a food frequency questionnaires [14,15,33-35]. This means that adequate evaluation of reproducibility and validity of mentioned methods [39,40] to demonstrate their performance and utility in monitoring of specific dietary changes related with past dieting in young woman is still the challenge. It should be also noted that results of this preliminary study may not be amenable for generalization, because we have lack of information about possible differences

in eating patterns between students and non-students as well as between pharmacy students and other students. However, beside of these uncertainties, our findings indicate reliable consistency in main conclusions with results of other research considering dieting related behaviors of young woman [14,15,33].

Conclusions

The non-controlled, non-rationale, short-period dieting seems to favor evidences of the long-term or permanent nutritional and health disturbances in female students, irrespective of their residential status. These findings from present study suggested that nutrition education intervention is needed. Especially, future interventions to promote healthy weight management through young women should discourage their reliance on extreme and potentially dangerous weight control methods. Health professional should also be involved in developing and implementing nutritional programs to promote healthful methods to manage weight and maintain adequate nutrient intake as well as regular physical activity among young women. The results of this study provided several ideas that could be considered for intervention to healthful weight management among female students:

– involvement of parents in modeling healthful behavior for their children,

– encourage young women to adapt healthy eating behaviors without focusing on weight loss,

– engage parents, teachers and health professional to provide support to young women when they discuss weight concerns,

– educate female students regarding potential health implications of unhealthful weight management,

– continued recognition of reasons of weight concern and practical help for young women to develop an identity that goes beyond that physical appearance.

Recognizing the restrained value of 24-hour dietary recall method in evaluation of more subtle discrepancies between self-reported and recommended dietary intake of some nutrients [39-42] further extension of present studies is planned to verify and validate findings presented here.

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Markers of pro-inflammatory and pro-thrombotic state in the diagnosis of metabolic syndrome

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Abstract

The metabolic syndrome refers to the clustering of upper body obesity, atherogenic dyslipidemia, insulin resistance and elevated blood pressure. Both, obesity and metabolic syndrome, have the potential to influence on the incidence and severity of cardiovascular disease with serious implications for worldwide health care systems. Obesity plays a central role in the development of insulin resistance and dyslipidemia through the mediation of a pro-inflammatory and pro-thrombotic state. Adipose tissue has been shown to exert important endocrine and immune functions. Pathogenesis of obesity associated metabolic syndrome is mediated by disturbed production and release of biologically active molecules by fat cells and other cells infiltrating fat tissue. In obese subjects synthesis of several bioactive compounds – adipokines and cytokines/chemokines by adipose tissue cells is dysregulated. Those bioactive molecules participate in regulation of apetite and energy homeostasis, lipid metabolism (tumour necrosis factor α – TNF-α), insulin sensitivity (TNF-α, adiponectin, resistin, visfatin) immunity (monocyte chemoattractant protein-1 – MCP-1, TNF-α, IL-6), angiogenesis, blood pressure and hemostasis (plasminogen activator inhibitor – PAI-1). The effects of major pro-/anti-inflammatory and pro-thrombotic adipokines on several physiological processes will be discussed in this review. Also, an evidence-based approach to the laboratory diagnosis and treatment of metabolic syndrome will be presented.

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The metabolic syndrome represents a combined occurrence of atherogenic dyslipidemia, insulin resistance, elevated blood pressure and central adiposity. Pro-inflammatory and pro-thrombotic state contributing to endothelial dysfunction is a common feature of those with metabolic syndrome. Increasing frequency of abdominal obesity, reaching epidemic proportions, enhances the prevalence of metabolic syndrome up to 22% in the adult population of the United States and around 10-15% in Finland [1,2]. Both, obesity and metabolic syndrome, have the potential to influence on the incidence and severity of cardiovascular disease, particularly in the presence of type 2 diabetes mellitus, in men aged over 45 years and women aged 55 years with serious implications for worldwide health care systems.

Metabolic syndrome is defined in various ways. The diagnostic criteria for this disorder have been established by the World Health Organization in 1998, the National Cholesterol Education Program's Adult Treatment Panel III in 2001 and recently by the International Diabetes Federation in 2005. All three definitions are associated with similar risks for cardiovascular diseases and diabetes.

Obesity, particularly in visceral region, is a key component in the development of the metabolic syndrome. It has been found that waist size provides additional information regarding inflammation and insulin resistance. Alterations in visceral fat lipolysis exert direct effects on the liver metabolism. Increased adiposity, leads to greater free fatty acid flux and inhibition of insulin action that may be due to enhanced synthesis and release of TNF-α from fat tissue [3]. In obesity adipose tissue is resistant to insulin which is associated with disturbed glucose metabolism in the muscles and liver. Even mild or moderate degree of obesity with concomitant insulin resistance may be associated with metabolic syndrome. On the other hand, excessive accumulation of abdominal fat may lead to the development of metabolic syndrome independently on degree of insulin resistance.

It is suggested that chronic mild inflammation constitutes an important underlying factor of metabolic syndrome. Pathogenesis of obesity associated metabolic syndrome is mediated by disturbed production of biologically active molecules in fat tissue. In obese subjects synthesis of several bioactive compounds – adipocytokines, by either adipocytes or adipose tissue infiltrated macrophages, is dysregulated; secretion of pro-inflammatory adipocytokines is elevated while that of anti-inflammatory is reduced. Low-grade inflammation constitutes the bridge linking atherosclerosis with metabolic syndrome and is associated with higher risk for acute cardiovascular syndromes.

Adipose tissue has an important endocrine function involved in inflammatory and thrombotic pathways. Fat cells produce and release more than 50 different compounds into the circulation. All identified adipocytokines form a network linking adipose tissue with skeletal muscle, liver, adrenal cortex, brain and sympathetic nervous system. These compounds participate in regulation of apetite and energy homeostasis (leptin), lipid metabolism (TNF-α cholesterol ester transfer protein – CETP, apoE), insulin sensitivity (adiponectin, resistin, visfatin), immunity (IL-1β, IL-6, IL-10, IL-8, MCP-1), angiogenesis (vascular endothelial growth factor – VEGF), vascular function (leptin, resistin, apelin, adiponectin), blood pressure (angiotensinogen), hemostasis (PAI-1) and acute phase reaction (CRP, haptoglobin, SAA) [4].

In obese subjects increased expression of several inflammatory cytokine/chemokine genes in adipose tissue was found that affect its metabolism, insulin signaling and endocrine activity. Those pro-inflammatory mediators have been reported to induce insulin resistance in fat tissue and muscles. Dahlman et al. [5] reported that of several cytokines synthesized in adipose tissue of the obese MCP-1 is the only acting as local factor that recruits monocytes contributing to induction of insulin resistance.

Prospective studies have shown that elevated levels of pro-inflammatory indices like CRP or diminished levels of protective anti-inflammatory marker such as adiponectin are important predictors of the development of type 2 diabetes [6].

Inflammatory state is an important component of wide range of the diseases also those associated with aging. Trayhurn and Wood proposed an explanation to the increasing inflammatory response of fat tissue with developing obesity [7]. The authors suggest that in growing poorly vascularized adipose tissue mass, hypoxia is a critical factor. Expression of some cytokines (leptin), chemokines and angiogenic factors (VEGF) to stimulate vascularization may be induced by hypoxia that has been shown recently in different situations and in adipocyte cultures.

Several adipokines are associated to the immune system and inflammation. In obesity expression, synthesis and release of pro-inflammatory adipokines (TNF-α, IL-6, haptoglobin leptin, resistin) is enhanced but that of anti-inflammatory such as adiponectin is decreased [7]. The inflammation state in obese reflects, at least partly, increased release of inflammatory peptides and proteins such as leptin and PAI-1 from adipose tissue as a major source. However, this is not the case for TNF-α, IL-6, resistin and CRP [8].

Leptin

Leptin, is a hormone with divergent activities. This 16-kD cytokine is produced mainly by adipocytes but also in stomach, ovaries, placenta and vascular cells [7]. Leptin remains a key hormone responsible for the regulation of apetite and energy balance by hypothalamus. Acting as a "starvation signal" is a central factor in the elevation of sympathetic activity found in obese hypertensive patients.

Moreover, it has been reported that leptin increases cellular adhesion molecules and affects vessel wall. Leptin stimulates accumulation of cholesterol in macrophages, activates monocytes and rise pro-inflammatory cytokine (TNF-α, IL-6) release. Leptin has been shown to stimulate production of reactive oxygen species by activated monocytes *in vitro*. This adipokine may act also as angiogenic factor. Leptin exerts pro-thrombotic properties by contributing to arterial thrombosis through a platelet leptin receptor [4].

TNF-α

TNF-α is a multipotential cytokine with several immunologic functions. It is produced and released by adipose tissue, primarily from stromal vascular cells, and it's enhanced expression associated to induction of insulin resistance was reported in obese subjects [7]. In adipose tissue TNF-α is engaged in the stimulation of lipolysis. TNF-α induces endothelium function changes by raising oxidative stress while adiponectin inhibits this factor. TNF-α may activate transcription factor NF-kappa β that leads to increased production of cytokines and adhesion molecules (ICAM-1, VCAM-1) enhancing monocyte adhesion to the vessel wall. A substantial effect of TNF-α on the expression and release of pro-inflammatory adipokines was confirmed, up to now, only by *in vitro* studies. In human adipocytes differentiated in culture TNF-α increased IL-6, MCP-1, nerve growth factor, VEGF while adiponectin, adipsin, haptoglobin and leptin were decreased [9].

Interleukin 6

Interleukin-6 is a cytokine having multiple effects, secreted by immune cells, fibroblasts, endothelial cells, skeletal muscle and in low amounts by fat tissue. Fat cells produce only about 10% of total IL-6 and regional differences has been observed. Visceral adipocytes produce much more IL-6 than from the subcutaneous depot. This inflammatory cytokine is increased in subjects with obesity and insulin resistance and may be regarded as a predictive factor for type 2 diabetes and myocardial infarction [4]. Induction of insulin resistance by IL-6 could be mediated by suppression of insulin receptor signal transduction in hepatocytes.

Adipsin

Adipsin, a serine protease is known to stimulate glucose transport for triglyceride accumulation in fat cells and to inhibit lipolysis [4]. Obese humans have substantially increased blood adipsin concentration but it is not clear whether high concentration reflects increased activity or resistance to adipsin.

Resistin

Resistin was primarily suggested to be a link between obesity and insulin resistance in humans, however, recent human

Table 1. **Interpretation of general and alternative laboratory tests results in the diagnosis of metabolic syndrome (according to http://www.labtestsonline.org)**

Cholesterol	Total cholesterol should be <200 mg/dl (<5 mmol/L); HDL-cholesterol >40 mg/dl (>1.04 mmol/L) in males, >50 mg/dl (>1.29 mmol/L) in females – good; LDL-cholesterol should be <100 mg/dl (2.6 mmol/L)
Triglycerides	44-100 mg/dl (0.5-1.13 mmol/L) – optimal; 100-150 mg/dl (1.13-1.7 mmol/L) – moderate; >150 mg/dl (>1.7 mmol/L) – high
Fasting glucose	70-99 mg/dl(3.9-5.5 mmol/L) – normal; 100-124 mg/dl (5.6-6.9 mmol/L) – indicates pre-diabetes; ≥125 mg/dl (≥7.0 mmol/L) – indicate type 2 diabetes
Oral glucose tolerance test (with 75 g glucose load)	≤140 mg/dl (≤7.8 mmol/L) at 2 hrs – normal glucose tolerance; 140-200 mg/dl (7.8-11.1 mmol/L) at 2 hrs – impaired glucose tolerance; >200 mg/dl (>11.1 mmol/L) twice – indicates type 2 diabetes
Fasting insulin	≤10 IU/ml – optimal; >10 IU/ml – high
CRP (high sensitive method)	<1.0 mg/L – optimal; 1.0-3.0 mg/L – moderate risk; >3.0 mg/L – high risk
Fibrinogen	Results of this test vary greatly with age, sex and test method. Too high and too low results are problematic
Homocysteine	<6 μmol/L – optimal; >9 μmol/L – high
PAI-1	This test is not yet routinely performed

studies failed to confirm this relation [10]. Adipose-derived resistin is probably expressed in monocytes infiltrating adipose tissue [11]. Since it is produced by blood monocytes its pro-inflammatory activity and contribution to development of endothelial dysfunction has been suggested.

Visfatin

Visfatin, recently discovered in the human visceral fat was suggested to play a role in glucose homeostasis through stimulation of the insulin receptor (insulin-mimetic effects). Another effect of visfatin is to promote adipogenesis by influencing on adipocyte precursors differentiation. At present the relation of visfatin to insulin sensitivity is questioned and the contribution of fat tissue to circulating visfatin concentration remains unknown [12,13].

Adiponectin

Adiponectin has been considered as a key regulator of insulin sensitivity and tissue inflammation. This 30-kD protein synthesized exclusively by white and brown adipocytes is present at very high concentrations in the blood. Its level inversely correlates with the amount of body fat that means adiponectin concentration is higher in non-obese than in obese people [6]. Regional difference exists in adiponectin production in humans, omental adipocytes secrete higher amounts than subcutaneous. Adiponectin level may be a predicting factor of diabetes and cardiovascular disease risk.

In the circulation adiponectin exists as varying molecular weight forms. High molecular weight complexes have the predominant action in the liver but in general trimers exert the highest biological activity.

Adiponectin may act as signaling molecule to regulate insulin action in the liver, improves hepatic insulin sensitivity, and in skeletal muscle, increases fuel oxidation. Two adiponectin receptors have been identified: AdipoR1 is highly expressed in skeletal muscle and promotes lipid oxidation, AdipoR2 is mostly expressed in the liver, enhances insulin sensitivity and reduces liver steatosis via increased peroxisome proliferator activated receptor-α (PPAR-α) [14]. PPAR-α is a nuclear transcription factor that regulates expression of genes involved in fatty acids beta-oxidation and regulates energy homeostasis [15].

Adiponectin antagonizes many effects of pro-inflammatory TNF-α by inhibition of NF-kappa β pathway; TNF-α. in turn suppresses adiponectin production.

In type 2 diabetes adiponectin is significantly reduced [16]. It was shown that administration of adiponectin increased glucose uptake by muscles, improved insulin sensitivity and suppressed gluconeogenesis in the liver cells [6].

Protective role of adiponectin within the vascular system results from suppression of the inflammatory processes such as adhesion, proliferation, phagocytosis and deposition of lipids in monocytes.

In obese people increased gene expression of inflammatory and thrombotic cytokines and decreased expression of protective adiponectin has been reported suggesting a close link between abdominal obesity and other underlying risk factors of metabolic syndrome [17].

Recently it has been shown that in obese postmenopausal women visceral adipose tissue volume inversely correlated with leptin and tended to inversely correlate with adiponectin gene expression [17]. Positive relationship between fasting insulin and visceral adipose tissue TNF-α gene expression was observed in the subgroup of non-diabetic women. Additionally, IL-6 gene expression tended to be positively related to fasting insulin in these women. Expression of adiponectin was much lower in obese women with metabolic syndrome than without. These results suggest that enhanced pro-inflammatory cytokine expression in fat tissue links abdominal obesity with its metabolic disturbances.

Apart from impaired glucose tolerance and insulin resistance, dyslipidemia and hypertension a typical feature in metabolic syndrome is a pro-thrombotic state. The metabolic syndrome is frequently diagnosed in patients with venous

thrombosis. Recent study reported the presence of metabolic syndrome in 50% of patients with deep vein thrombosis [18].

The risk of thromboembolism is significantly increased in abdominal obesity that results from activation and changes of coagulation system [19]. This is reflected by enhanced generation of thrombin which converts fibrinogen to fibrin, diminished fibrinolysis and increased platelet aggregation. Increased levels of fibrinogen, factor VII and VIII that leads to hypercoagulability is characteristic of metabolic syndrome. Simultaneously, enhanced production of PAI-1 decrease fibrinolysis.

Abdominal obesity, mainly accumulation of visceral fat, resulting in low-grade inflammation is related to increased fibrinogen levels. Pro-inflammatory state is also associated with increased levels of coagulation factors: tissue factor and factor VII and thus the risk of activation of coagulation cascade [19].

There are few studies in which interrelations between procoagulant factors and anticoagulant proteins were investigated in humans with wide range of body fat. Godsland et al. [20] have found that procoagulant factors VII and X, anticoagulant proteins C and S and PAI-1 correlated directly with total and central body fat but inversely with insulin sensitivity. The authors suggested that procoagulant factors and anticoagulant proteins are the features of the intercorrelated disturbances of the metabolic syndrome.

Also other factors of metabolic syndrome such as: TNF-α and homocysteine has been suggested to contribute to procoagulant state.

Plasminogen activator inhibitor – PAI-1

Fibrin degradation is a process controlled by tissue plasminogen activator – t-PA and PAI-1 balance. Decreased t-PA paralleled by increased plasma level of PAI-1 associated with insulin resistance are common in metabolic syndrome. Chronic inflammation and enhanced lipolysis in adipose tissue, leading to increased free fatty acids – FFA release, stimulate PAI-1 expression and synthesis, decrease conversion of plasminogen to plasmin and in consequence diminish fibrin degradation being the important contributors of hypofibrinolysis.

The relationship between PAI-1 activity, adiponectin and CRP levels, insulin resistance and lipoproteins was studied in overweight and obese women [21]. Interestingly, it was found that, PAI-1 activity inversely correlated with serum adiponectin, independently of the amount of visceral tissue.

The other characteristic feature of metabolic syndrome is endothelial dysfunction often present in insulin resistance and type 2 diabetes mellitus. Excessive lipolysis resulting in chronic elevations of plasma FFA may induce endothelial dysfunction [16]. This is reflected by high levels of markers such as thrombomodulin [19].

Insulin-resistance in obesity and dyslipidemia are associated with excessive platelet activation and aggregation. High levels of VLDL stimulate synthesis of thromboxane A2 – TxA2 in platelets from FFA [19]. TxA2 is known to enhance platelet aggregation.

Laboratory diagnosis of metabolic syndrome

General laboratory tests used in the diagnosis of metabolic syndrome include determination of lipid profile : total cholesterol, LDL-cholesterol, HDL-cholesterol and triglyceride levels, glucose concentration, oral glucose tolerance test and glycated hemoglobin level-HbA1c (*Tab. 1*). Determination of HbA1c was found to be of importance in the diagnosis and prognosis of metabolic syndrome [22,23].

There are other laboratory indicators that may provide additional information and tests that can be used mostly in research setting. Among alternative tests fasting insulin can be measured, however, the values may vary to much to be clinically useful in the diagnosis of metabolic syndrome.

The easiest way to detect a pro-inflammatory state as a part of the cardiac risk assessment is measurement of CRP by a high sensitivity method. If CRP level, measured twice within a few weeks, is above 3 mg/L a pro-inflammatory state is defined. CRP has been confirmed as a clinically important prognostic parameter in the diagnosis of metabolic syndrome [24,25]. The concentration of other inflammatory markers such as IL-6, other adipocytokines or adiponectin are actually assayed only in research setting but in the near future may be very helpful in the diagnosis.

In routine laboratory fibrinogen is used for the assessment of inflammatory proceses and pro-thrombotic state while testing for homocysteine and PAI-1 level is not commonly performed yet. Some evidence-based studies indicated an important role of homocysteine in assessing complications in subjects with metabolic syndrome.

Interestingly, the inflammation state in obese people can be partly reversed after weight loss [7]. Whether improving metabolic syndrome by weight loss and physical exercise is a consequence of changes in adipose tissue cytokine gene expression still needs explanation.

Regular physical activity improves insulin sensitivity and correlates inversely with leptin and mild inflammation (IL-6) in adolescents, independently of fat mass and it's localization [26]. However, the beneficial effects of regular physical exercise on metabolic syndrome features cannot be totally explained by changes in adipokine level.

Therapies addressing the treatment of obesity related disorders should focus primarily on modifying the inflammatory profile. It has been found that glitazones significantly increased serum adiponectin while significantly decreased CRP and resistin level [27]. Also slight increase in HDL-cholesterol and favorable effect on LDL particle size was observed. Thiazolidinediones also significantly diminish plasma CRP levels and increase adiponectin in type 2 diabetics [16]. Treatment with thiazolidinediones may decrease the risk of thrombosis in metabolic syndrome; secretion of PAI-1 stimulated by insulin is suppressed by glitazones.

Fibrates have anti-inflammatory and anti-thrombotic effects in the vessel wall in patients with metabolic syndrome. Statins reduce risk for major cardiovascular disease events even in high risk patients and may be used in combination with fibrates.

In primary prevention of arterial thrombosis, antiplatelet agents like low-dose aspirin may be used in the long-term approach. In patients with risk of atherosclerotic cardiovascular disease aspirin seems to be a good therapeutic possibility.

The better understanding of the molecular actions of adipokines is the key issue to better diagnosis and discovery of

effective therapy. Weight loss and pharmacological treatment leading to decrease of pro-inflammatory, pro-thrombotic adipokine level may prevent the metabolic syndrome and type 2 diabetes and in consequence the development of atherosclerosis complications.

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Multifocal type of pilomatrixoma

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Abstract

Pilomatrixoma is a benign skin neoplasm that arises from hair follicle matrix cells. The skin lesion occurs usually as a solitary tumor and the multifocal types are very rare. Skin changes can be described as a firm to hard, non-painful, oval-shaped tumor that is covered by normal skin. It commonly occurs on a scalp, face, neck and rarely back and extremities. Complete surgical excision with the proper margin is the treatment of choice, what guaranteed the radical therapy of pilomatrixoma.

In this paper case of 16-years-old male patient with many solid tumors in subcutaneous tissue on both arms will be reported. The first skin lesion appeared on the left arm 6 years ago. Clinically the disturbance was diagnosed as an atheroma, and it was excised. One year after surgical procedure the patient observed the appearance of new nodules on both arms. In the therapy surgical excision was performed with histopathological examination of the tissues. Histopathological test has proved the clinical diagnosis of pilomatrixoma.

The case of multifocal pilomatrixoma, which is rarely diagnosed and described in professional literature, will be presented.

Key words: pilomatrixoma, multifocal localization, children, neoplasm.

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Introduction

Pilomatrixoma (*Malherbe and Chenantais, Forbis and Helwig*), also known as a calcifying epithelioma, is a benign skin neoplasm that arises from hair follicle matrix cells [1-3]. It may occur at any age, ranging from children to adults (but rather rarely) [4]. This benign skin neoplasm occurs most often in cases of patients at the age of 20 and younger [5]. There are two main peaks of appearance of pilomatrixoma depending on the age of the patients: below 20 and 50 years of age [5].

This tumor occurs more often in case of women, due to reporting literature the female: male ratio is 2, 4:1 [6], 3:1 [4] or in another reports 2:1 [7].

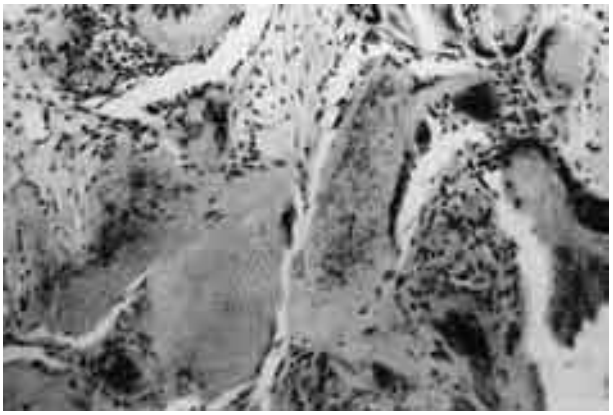
The skin lesion occurs usually as a solitary tumor and the multifocal types are very rare [1]. In some cases pilomatrixoma could coexisted with systemic abnormalities: myotonic dystrophy [8-13], myotonic dystrophy within AIDS [14], internal anomalies in Gardner [15,16], Turner's [17] and Rubinstein-Taybi syndrome [18], that is why the patient with this neoplasm should be carefully examined towards these abnormalities. Skin changes characterized as a firm to hard, non-painful, oval-shaped tumor that is covered by normal skin. The diameter was ranged from several millimeters to several centimeters. The most common localization is the scalp, face, neck and rarely back and extremities [6,19-22]. Complete surgical excision with the proper margin is the treatment of choice, which guaranteed the radical therapy of pilomatrixoma [1,20].

Case report

In the year 2002 a 16-years-old male patient was admitted to the Dermatosurgical Outpatient Clinic in Katowice because of reccurrence of the two skin lesions. Clinically in dermatological examination three asymptomatic, firm, solid tumors in subcutaneous tissues on both arms were proved. The first skin lesion appeared on the left arm 6 years ago. Clinically

Figure 1. **Typical skin lesion on the arm, with cicatrix after surgical excision**

Figure 2. **Histopathological examination of excised nodule (haematoxyline-eosine stain, mag.150x)**



the disturbance was pre-diagnosed as an atheroma, which was excised by a surgeon in the ambulatory at the patient's living area. One year after surgical procedure the patient observed the appearance of new nodules on the left arm and one new on the skin of the right arm. Because of this, the patient came to The Department of Dermatology of Silesian Medical University in Katowice, where pilomatrixoma was recognized.

In dermatological examination three skin lesion were described as a well-circumscribed, firm nodules, oval-shaped, varied in diameter from 0.5 to 1.0 cm, localized on both arms (*Fig. 1*). There was slight pink discoloration of the overlying skin.

The patient has undergone surgical procedure in topical anesthesia with 0,5% solution of xylocaine. Three lesions were excised totally with the healthy margin of the skin with adherent and overlying skin.

In the therapy surgical excision was performed with histopathological examination of the nodules (*Fig. 2*), which showed masses of mummified shadow squamous epithelial cells, focally, with rows of basophilic cells resembling the hair matrix. The surrounding fibrous connective tissue showed prominent resorption including numerous, multinucleated, foreign body type giant cells. No features of osseous metaplasia or calcifications were found. The lesion was diagnosed as plimoatrixoma.

After surgical procedure the patient was treated by neurologist because of peripheral inflammation of the left facial nerve with total improvement.

In additional examination no systemic abnormalities were found. In ophthalmologic consultation normal state and function were described.

The follow-up period was 4 years and no recurrences were found.

Discussion

The presented case of multifocal pilomatrixoma is a rarely diagnosed and described in the professional literature.

The appearance of this neoplasm is almost asymptomatic. In some cases the lesion is associated with pain, inflammation and ulceration [20].

Multiple occurrences of pilomatrixoma is rarely reported in the literature [7,8,11,14,15,23-26] and it is assessed to 3.5% of cases [24]. Mostly appearance of this tumor is associated with familial occurrence [8,11,21,27]. Pilomatrixoma is a well-known pathognomic sign of myotonic dystrophy [8-13]. Pujol, at al. [15] reports that multifocal pilomatrixoma coexists with adenomatous colonic polyps, osteoma of the mandible and ocular-pigmented retinal macules as changes in patients with recognized Gardner syndrome. In the case of our patient no familial and gastrointestinal disturbances were observed. Another rare clinical types of calcifying epithelioma of Mahlerbe are: bullous form [3,28] and perforating type [29-31]. Bertazzoni at al. [32] reported pilomatrixoma with perilesional anetoderma caused by inflammatory processes and lack of elastic fibers.

These neoplasm-involved areas include the scalp, head and the upper extremities [3]. The most affected skin regions are face [4,20], scalp [20,22,33], neck [4,22,33], chest [33] and upper limbs [22,33]. The head, especially the cheek ad preauricular and parotid region are the most common sites – in about 50% [7]. Over 25-30% of present lesions are localized on the skin of the upper limbs.

Most typical clinical picture of pilomatrixoma is occurrence of solitary, small, firm nodule, covered with normal skin, varying in size from 5 to 30 mm [6]. The skin lesion is usually less than 3 cm [4,5]. Pilomatrixomas of atypical large size has been termed giant pilomatrixomas [24,31]. Although pilomatrixoma is a benign skin tumor, in the literature there are reports due to occurrence of pilomatrixoma carcinoma [4,25,34-36]. The surgeons should be aware of the various types of pilomatrixoma with rare occurrence of malignancy [19]. Pilomatrixoma carcinomas usually lead to the metastases to the lungs [34-36], liver [34,36] regional lymph nodes [4,34,35] and brain, heart, pancreas, kidney, adrenal gland, gastric mucosa, skin and bones [35].

Although the pilomatrixoma has its typical clinical picture in many cases the diagnosis is incorrect. In reports of Wells at al. [33] the referring diagnosis was improper in 94% of cases, and the preoperative recognition in 57% was misdiagnosed.

The best-known therapy of the pilomatrixoma is total surgical excision including adherent skin [6,20].

Histopathological picture of pilomatrixoma depends on the stage of the tumor development. There is prevalence of living epithelial elements in early stages, and retrogressive changes in older ones, leading to the formation of foci of mummified epithelium with shadow cells, calcifications, and reactive resorptive response in the connective tissue. The periphery of the basophilic epithelium resembling hair matrix contains viable cells, white central parts undergo mummification. In 15-25% of cases there is osseous metaplasia within the tumor or in its vicinity [37,38].

Conclusions

Despite the typical clinical picture and benign character of pilomatrixoma, the recognition of this dermatological entity may lead to misdiagnosing. Clinically in most cases the lesion occurs like a solitary nodule, but doctors should remember about rare, but existing, multiple localization. Complete surgical excision, including the overlying skin is the treatment of choice.

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Paraneoplastic type of *acanthosis nigricans* in patient with hepatocellular carcinoma

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Abstract

Paraneoplastic *acanthosis nigricans* is connected with malignancies in adults in almost 100% of cases. The typical skin changes include: thickening and hyperpigmentation in typical localization with mucocutaneous involvement.

Purpose: The authors report a case of a malignant type of *acanthosis nigricans* in 42-year old female patient with hepatocellular carcinoma.

Case report: First skin lesions appeared in 2000. The patient died within 22 months (of the first appearance of skin symptoms), because of hepatocellular carcinoma.

Herein we report the clinical picture, skin involvement and diagnostic procedures in acanthosis nigricans.

Conclusions: Paraneoplastic type of *acanthosis nigricans* – in patient with hepatocellular carcinoma is not frequently reported in the literature. In the aspect of clinical occurrence of skin lesions suggesting *acanthosis nigricans* the diagnostics should be focused on internal malignancies

Key words: *acanthosis nigricans*, hepatocellular carcinoma, paraneoplastic syndromes.

Introduction

Acanthosis nigricans (*acanthosis nigricans*, AN) was firstly described by Pollitzer. It is characterized by eruption of many, symmetric, velvety hyperkeratotic lesions with brownish hyperpigmentation. Flexures, skin folds, axills, bend of the elbow, nipples, neck, navel and anogenital regions are predominantly involved.

Acanthosis nigricans appears in the course of the internal malignancies, many systemic disorders, endocrinopathies and dermatological diseases (e.g. atopic dermatitis).

Based on the clinical characteristics *acanthosis nigricans* is divided into 8 types: benign AN, *pseudoacanthosis nigricans* connected with obesity, syndromic AN, paraneoplastic, malignant AN, acral AN, unilateral AN, drug-induced AN and mixed AN. Syndromic type of AN is subdivided into type-A (Hair-AN) with hyperandrogenism, insulin-resistance and typical skin lesions and type-B connected with diabetes and autoimmunological disturbances.

Acanthosis nigricans maligna occurs in three abortive clinical subforms as papillomatosis florida cutis verruciformis, tripe palmar syndrome and Leser-Trèlat sign. They coexist together or appear consecutively after each other.

Material and methods (Case report)

The authors reported a case of a female patient with diagnosed paraneoplastic type of *acanthosis nigricans* at in the course of hepatocellular carcinoma (clarocellular variant G-2).

A female patient, 42 years old, engineer of motorways building. She had taken no drugs and never had been treated because of any other serious disorders. At the beginning the patient was hospitalized in the Dermatological Department of Silesian Medical Academy in Katowice in May 2000. Then, papillomatous, hyperpigmented lesions in folds, neck, abdomen and anus were observed (*Fig. 1*). They were symmetrically and quickly widespread as a papillomatous roughy changes on the skin folds, trunk, hands and anus. No changes abnormalities of oral mucosa, nail plates and hair were found. Body mass index (BMI) was 26. The diagnosis of *acanthosis nigricans* was based on histopatological examination. In laboratory tests:

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Figure 1*. Skin lesions on neck in paraneoplastic type of *acanthosis nigricans

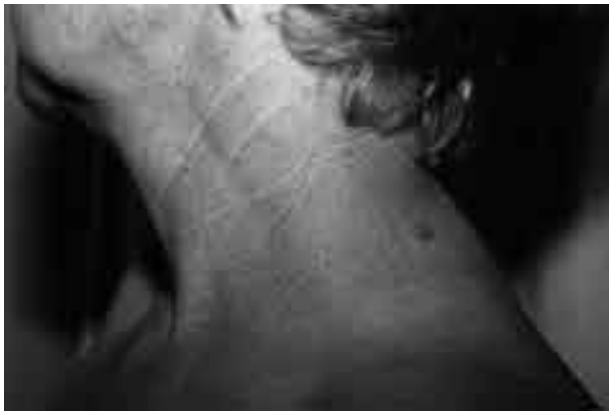

***Figure 2*. Hyperpigmentation with hyperkeratosis of the axil – "dirty skin"**

blood cell count, biochemical parameters in the serum such as electrolytes, uric acid, glucose, aminotransferases, GGTP, LDH, bilirubin, amylase, total protein and electrophoresis were within normal limits. Sedimentation rate – 24/42. X-ray of the chest, upper and lower gastrointestinal tract, and abdominal and thyroid ultrasound examination revealed no neoplastic abnormalities. The patient did not consent for further internal diagnostic tests. Because of skin lesions' progression, within 6 months accompanied by intermittent abdominal pain, the patient was admitted to the Dermatological Department again in October 2000. At this time the dermatological examination revealed skin lesions involving almost all the body. Skin changes were characterized by papillomatous, roughly hyperpigmented and furrowed lesions localized on skin folds, arthral flexures and mucosa of the mouth and anus (*Fig. 2*). The skin of the dorsum of the hands demonstrated features typical for *acanthosis nigricans* mimicked warts. On the plantar and palmar surfaces exceed hyperkeratosis was found. The skin colour of the whole body was brown – *café au lait* lesions. In laboratory findings ESR 36/84, sideropaenia (8.7 mmol/L N: 10-30.8 mmol/L) and elevated liver parameters (GGTP 43 N: 5-24 IU/L, ALP 96 N: 32-92 IU/L, LDH 689 N: 266-519IU/L) were observed. Other biochemical parameters such as glucose, electrolytes, creatinine, urea, uric acid, CPK, bilirubin, aminotransferases, total protein and total blood count cell and electrophoresis were within normal limits. Urine analysis and coagulation parameters were normal. Markers as CEA, CA 19-9 were within normal limits. CA 125 was increased (47.03 U/ml N: 35 U/ml). Chest X-ray was normal. In abdominal ultrasound and in HRCT examination of abdomen a tumor of the left lobe was found. In liver biopsy no atypical cells were shown.

In November 2000 the patient was diagnosed in Gastroenterology Department. Exploratory laparotomy revealed hepatosplenomegaly with 3 cm sized tumor of the left liver lobe infiltrating the round ligament of the liver. On histopathological biopsy of the tumor, hepatocellular carcinoma (clarocellular variant G-2) was diagnosed. In chest X-ray the right-sided presence of two metastases (2 cm diameter each) was shown. The patient died within 22 months of the first appearance of skin symptoms.

Discussion

The malignant type of *acanthosis nigricans* is characterized by its sudden onset, rapid progression, more expressed hyperkeratosis and hyperpigmantation with coexisting pruritus. Pathological lesions are mainly localized on the mucous membranes and once they appear, the diagnosis of malignancy should always be taken into account.

The occurrence of *acanthosis nigricans* in adults is almost in 100% is connected with internal malignancies. The most often proved malignancies are adenocarcinomas of the stomach, neoplasms of the lungs and breasts, carcinoma of the uterine and bladder and sarcomas and haematological proliferation.

Among 247 cases of *acanthosis nigricans*, mostly cancer of the stomach (112), subsequently lungs (20), liver (19), uterus (18), breast (11) and ovaries (9) were determined. Moreover, the coexistence of *acanthosis nigricans* with malignant neoplasms of the liver and biliary ducts such as adenocarcinoma of bile ducts and gallbladder and also liver carcinoma were reported. Acanthosis nigricans has been referred as a sign of liver and bile ducts disturbances, e.g. primary biliary cirrhosis and Wilson's disease.

The occurrence of the abortive clinical forms of *acanthosis nigricans* depends on the type of developing malignancy. This subclinic types are often accompanied by neoplasms of gastrointestinal and respiratory tract.

The frequency of appearance of benign *acanthosis nigricans*, not concerning with malignant neoplasms, has been assesed as 7.1%. In this type involvement of the muscosa is very rare. Both benign and malignant types have similiar histologic features. The velvety surface of the skin lesions is the result of papillomatosis. Therefore only clinical progression with subjective complains can suggest malignant type of *acanthosis nigricans*.

Conclusions

Paraneoplastic type of *acanthosis nigricans* – in patient with hepatocellular carcinoma is not frequently reported in the literature.

In the aspect of clinical occurrence of skin lesions suggesting *acanthosis nigricans* the diagnosis should be focused on internal malignancies. Because of the influence of some causative agents another ethiopathogenetic factors should also be taken into account.

At presented patient other than neoplasmatic processes leading to *acanthosis nigricans* were excluded in additional tests.

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Radial scar of the breast – a confusing lesion

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Abstract

Radial scar is a confusing lesion of the breast which represent a premalignant lesion. It looks like a tubular carcinoma but histologically we can see two rows of cells in tubules. Mammographically there are some typical but not specific signs: 1) the presence of the central radiolucency, 2) the presence of radial long thin spicules, 3) varying appearance in different projection, 4) radiolucent linear structures parallel to spicules, and 5) abscence of palpable lesion or skin changes.

All these signs make the "black star" appearance. Authors reanalyzed 21 from 26 woman with the radial scar diagnosis. Aim of our study was to investigate the different morphologic changes in view of differential diagnosis, frequency and potential prognostic importance of the different lesions. According to our findings we can conclude that the radial scar is unpalpable, subclinical lesion which can be seen on mammography but the final diagnosis is histological.

Key words: radial scar, "black star", sclerosing lesion.

Introduction

Radial scar (RS) is a breast lesion generally 10 mm or less in diameter consistnig of a central fibroelastotic zone from wich tubular structure radiate [1]. It may be two layered or exhibit intraluminal proliferation. It is also a radiological entity [2]. Radial scar, also known as sclerosing lesion has been referred to as several different terms, including sclerosing papillary proliferation, infiltrating epitheliosis and indurative mastopathy [2]. Recent investigation point out that the risk of developing carcinoma is twice higher than in the normal population. Radial scar is a true precursor of malignancy or at last an indicator of an underlying global pathologic process. Radial scars are an independent histologic risk factor for breast carcinoma [3] and in most instances they are incidental findings. It may be in the constellation of hyperplastic, dysplastic and malignant changes. Most of them are visible on sonography [4]. The diagnosis on core biopsy is uncertain [5]. There is no relationship between the presence of carcinoma within radial scars and complex sclerosing lesions [6]. The carcinomas identified in the scars were of variable type and include small and large cell ductal carcinoma in situ, lobular carcinoma in situ, tubular carcinoma and invasive ductal carcinoma (not otherwise specified). On mammographic investigation there are some signs suggestive on radial scar: 1) the presence of a central radiolucency, 2) the presence of radiating long thin spicules, 3) varying appearance in different projections, 4) radiolucent linear structures parallel to the spicules, and 5) the abscence of a palpable lesion or skin changes [2,7]. Long, thin radiating spicules against a background of radiolucent fat create a "black star" appearance [8]. The most often differential diagnosis is between radial scar and tubular carcinoma [9]. It is possible that the CD34 and alpha smooth actin help us to differentiate RS from tubular carcinoma [10].

Radial scar is most often in women between 41 and 60 years old, very rare before the 40th and after the 60th years of age [11,12]. There are central fibroelastotic zone with radial extenstion of tubular structures. These tubular formation has two rows of cells, epithelial and myoepithelial [13-15]. Sometimes epithelial hyperplasia is present. The malignant potential is 2 times greater than in the normal population without radial scar [16,17]. For today contrast enhanced magnetic resonance mammography is the most sensitive presurgical diagnostic method [18].

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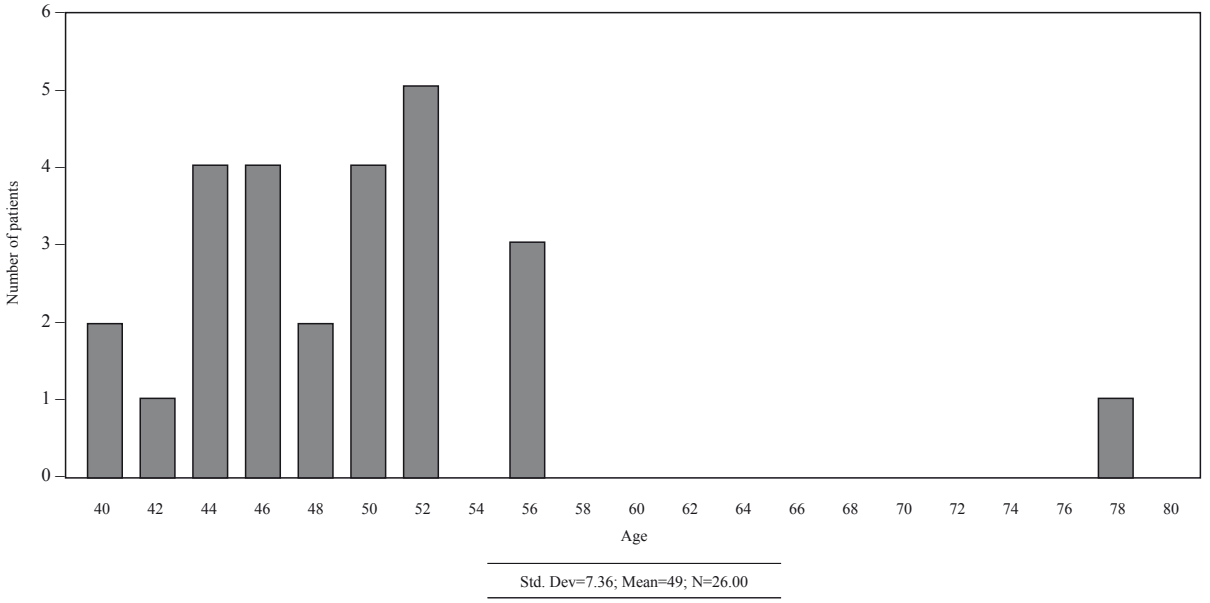
Figure 1. **Age distribution of patients**



Synonyms are: sclerosing complex lesion (bigger than 1 cm), sclerosing papillary proliferation, infiltrating epitheliosis, indurative mastopathy, benign sclerosing ductal proliferation and nonincapsulated sclerosing lesion.

Pathogenesis

Pathogenesis of radial scar is not investigated exactly yet. There are several hypothesis: 1) the lesion is the result of unknown local injury, which results in surrounding fibrosis, 2) the lesion is associate with ducteasia or ductal obliteration, 3) or the lesion is the result of chronic inflammation. A potential differential diagnosis are radiologically detectable morphologic changes due to prior exposure to radiation [19].

In the development of these lesions two phases can be observed: (I) cellular phase which is characterised with many centrally positioned myofibroblasts and (II) or "mature" phase with small number of myofibroblasts and elastic and collagen fibers and distorted parenchyma. On the periphery of a lesion, the number of microvessels is increased but in the central part of the lesion the number of microvessels is decreased. Each suspected lesion has to be investigated further histopathologically.

Radiographic characteristics

Radial scar shows the following characteristics: 1) the central radiolucency, 2) radial distributed spicules, 3) different appearance in different projections, 4) radially lucent linear structures wich are parallel with spicules, 5) the absence of palpable mass or cutaneous changes. The general consensus is that such types of lesion less than 1 cm are radial scar but bigger changes are complex sclerosing lesions.

Pathological findings

Radial scars lesions are look like breast carcinoma because the have creamy-yellow elastotic center. Histologically this is pseudoinfiltrative lesion, whose picture depends on the plane of section and the evolutive stade. Classically there is fibroelastotic center with entraped ducts, with epithelial and myoepithelial cells. Ducts radiate from fibroelastotic center. In surrounding ducts and lobules, different grades of ductal epithelial hyperplasia, ductectasia, adenosis and papillomatosis can be observed. Microcalfications are often present in adenosis and epithelial hyperplasia. Lesions can be solitary, multiple or in clusters. These changes have to be differentiate from the invasive carcinoma, especially from the tubular type. Radial scar can also be a solitary lesion or in associationd with the invasive carcinoma.

Biological characteristics

The main difficulty is the radial scar as an incidental finding. It is a malignant precursor or marker of an increased risk. The relative risk is two times greater than in women without radial scar.

Material and methods

We made retrospective mammographic analyses in 21 from 26 women with diagnosed radial scar. They were 38-77 years old (median 48 years), (*Fig. 1*).

We analysed the basic structure of the breast according to Wolf (N1/P1-predominant lipomatous condition; P1/P2-predominant glandular texture, the presence of radial scar structure – radiolucent center, radial shadow/radiolucence) and calcification. We used χ^2 test for statistic evaluation of the data ($p<0.05$).

Results

Parenchymatous structure was lipomatous, N1/P1 in 5 from 21 woman (23.8%) and glandular, P2/DY in 16 from 21

***Table 1*. Dominant and other lesions**

		All			
		Frequency	**Percent**	**Valid Percent**	**Cumulative Percent**
Valid	DFCnP	2	7.8	8.2	8.2
	DFcP	7	27.0	29.2	37.5
	Hyperplasia	2	7.8	8.3	45.8
	DCIS	5	19.2	20.8	66.7
	LCIS	1	3.8	4.2	70.8
	Adenoma	1	3.8	4.2	75.0
	Duktal carcinoma	1	3.8	4.2	79.2
	Lobular carcinoma	1	3.8	4.2	83.3
	Papilary carcinom	1	3.8	4.2	87.5
	Fibroadenoma	3	11.6	12.5	100.0
	Total	24	92.3	100.0	
	Pure	2	7.7		
Total		26	100.0		

***Table 2*. Microcalcifications**

		Frequency	**Percent**	**Valid Percent**	**Cumulative Percent**
Valid	Yes	6	23.1	100.0	100.0
Missing	System	20	76.9		
Total		26	100.0		

***Table 3*. Dysplasia fibrosa cystica proliferativa**

		Frequency	**Percent**	**Valid Percent**	**Cumulative Percent**
Valid	Yes	19	73.1	73.1	73.1
	No	7	26.9	26.9	100.0
	Total	26	100.0	100.0	

***Table 4*. Dysplasia fibrosa nonproliferativa**

		Frequency	**Percent**	**Valid Percent**	**Cumulative Percent**
Valid	Yes	3	11.5	12.0	12.0
	No	22	84.6	88.0	100.0
	Total	25	96.2	100.0	
Missing	System	1	3.8		
Total		26	100.0		

***Table 5*. Hyperplasia**

		Frequency	**Percent**	**Valid Percent**	**Cumulative Percent**
Valid	Yes	7	27.0	28.0	28.0
	No	18	69.2	72.0	100.0
	Total	25	96.2	100.0	
Missing	System	1	3.8		
Total		26	100.0		

***Table 6*. Adenosis**

		Frequency	**Percent**	**Valid Percent**	**Cumulative Percent**
Valid	Yes	6	23.1	23.1	23.1
	No	20	76.9	76.9	100.0
	Total	26	100.0	100.0	

***Table 7*. Fibroadenoma**

		Frequency	**Percent**	**Valid Percent**	**Cumulative Percent**
Valid	Yes	6	23.1	23.1	23.1
	No	20	76.9	76.9	100.0
	Total	26	100.0	100.0	

(76.2%) woman. Mammographic signs of radial scar were: radiolucent center in 13/21 woman (61.9%), radial lucency (14/21 or 66.7%), radial shadows (16/21 or 76.2%) and planar structure in 17 from 21 woman (81%).

The mean diameter of the change on mamopgraphy was 1.37±0.52 cm. There were no statistic differences between lipomatous and glandular mammographic structure of the breast in view of radiolucent center (p=0.34), radial radiolucency (p=0.44), radial shadows (p=0.66) and planar forms (p=0.30) of radial scar.

There was a statistically important relation between mammographic radiolucent center with radial radiolucency (p=0.006), radial shadows (p=0.001) and planary outlook of the change (p=0.012).

Microcalcifications were found in three cases (14.3%), benign according to morphology (acinar–in ductal epithelial hyperplasia; microcystic/secretory in stromal hyalinisation), and radiologically suspected (pleomorphic in DCIS). All microcalcifications were seen on the periphery of the lesion.

The described results are summarized in *Tab. 1-9* and *Fig. 2-3*.

***Table 8*. In situ carcinoma**

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Yes	10	38.5	38.5	38.5
	No	16	61.5	61.5	100.0
	Total	26	100.0	100.0	

***Table 9*. Invasive carcinoma**

		Frequency	Percent	Valid Percent	Cumulative Percent
Valid	Yes	5	19.2	19.2	19.2
	No	21	80.8	80.8	100.0
	Total	26	100.0	100.0	

***Figure 2*. The relation of benign and malignant findings**

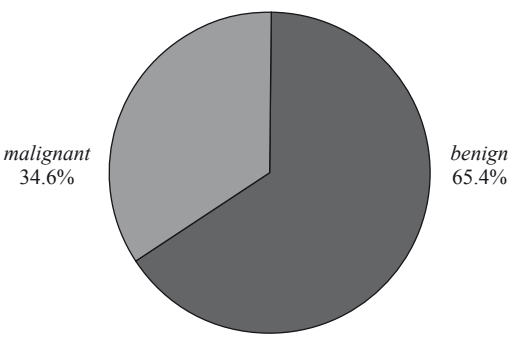
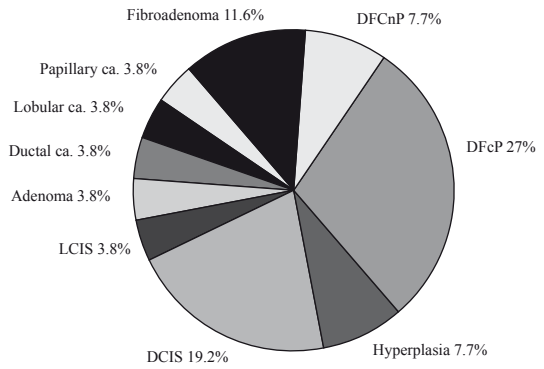


***Figure 3*. The relationship of proliferative changes**



Discussion

Radial scar lesion is usually an unpalpable, subclinical lesion, which can be seen in the mammographic investigation, which points out the importance of mammographic exploration [2].

It is observed in premenopausal women [6]. In our study it was seen almost in glandular but not in lipomatous breast. Our findings point out the sensitivity of mammographic investigation, regardless of mammographic breast structure.

Statistical significance was found in mammographic findings in view of radiolucent center, radial radiolucency, radial shadows and planar forms of radial scar.

Calcifications were found in 3 cases (14.3%) which had benign morphology (acinar with epithelial hyperplasia, microscopic secretion was observed in stromal hyalinisation) and radiological susceptible (pleomorphic with DCIS). All these lesions were found peripheral to the radial scar (mammograpic findings).

According to facts reported in the literature, mammographic findings in radial scars can be different.

For instance radiolucent center is not always present and microcalcification are described as a part of radial scar [15]. In our study, the significant elements are: planar forms, radial shadows, radiolucency and radiolucent center [7]. Calcifications were the mammographic finding in 14.3% of cases and they were found on the periphery of the radial scar.

Beside detecting radial scar on mammography, the differential diagnosis with breast cancer is also very important. The "black star" finding is also typical but not specific for radial scar lesion [8].

In our study a radiolucent center was the least present (61.9%) but statistically significant, accompanied with radial scars, radiolucency and planar forms on mammography. These indicates that the radiologist should point on thes facts beside the diagnosis of a stellate changes. Recent data show the enhanced sensitivity using magnetic resonance imaging in differential diagnosis of small-change lesions [18].

When a possibility of the radial scar lesion should be thought of, the histopathological verification should be achieved [12,14].

We conclude, that the (differential) diagnosis of radial scar by mammography needs to be followed by further investigation, such as cytology or surgical biopsy in order to potentially confirm the suspected diagnosis.

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The selectins E and P in normal labour. A preliminary report on evaluation of their prognostic values for preeclampsia

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Abstract

Purpose: Our question was whether two biochemical markers of preeclampsia, E-selectin and P-selectin, keep their prognostic value in particular stages of labour, or they lose it during labour.

Material and methods: The study group consisted of 36 healthy parturient women who gave physiological births (in control 15 healthy, age-matched women at term). Blood samples were collected in the 1st, 2nd and 3rd stages of labour as well as two hours after placenta expulsion. The levels of soluble E- and P-selectins were measured by immunoenzymatic method (ELISA).

Results: The levels of soluble E- and P-selectins in blood plasma of labouring women were within the similar medians and ranges during the 1st, 2nd and 3rd stages of labour as well as two hours after labour ($p>0.05$).

Conclusion: The natural labour does not influence the level of soluble E-selectin and soluble P-selectin in blood plasma of labouring women, therefore the prognostic values of these markers of preeclampsia are preserved in the time of labour.

Key words: E-selectin, P-selectin, biomarkers of preeclampsia, labour.

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Introduction

Preeclampsia is still one of the leading causes of maternal deaths (haemorrhages, sepsis and preeclampsia) and contributes also negatively to foetal development (intrauterine growth retardation) [1,2]. The markers of preeclampsia facilitate the supervision of preeclamptic women. The following biomarkers are recommended: plasma soluble E-selectin [3] and plasma soluble P-selectin [4], as well as some other adhesive molecules like VCAM-1 (vascular cell adhesion molecule-1), ICAM-1 (intercellular adhesion molecule-1) [5-8] and vWF (von Willebrand factor) [9], and such substances as thrombomodulin, fibronectin and some others [10,11].

E- and P-selectin as well as L-selectin are members of selectin family and members of a large group of adhesion molecules. They all exist in soluble forms in blood plasma, though each is synthesized or stored in different cells: E-selectin in endothelial cells, P-selectin in alpha granules of blood platelets and Weibel-Pallade bodies of endothelial cells, and L-selectin in leukocytes. Under activating conditions (inflammatory and thrombogenic challenges, as well as preeclampsia), the selectins are expressed on cell membrane and thus can initiate interactions between endothelial cells, leukocytes and platelets (tethering and rolling of leukocytes on endothelial cells, adhesion of platelets to leukocytes, weak and firm adhesion followed by extravasal migration of leukocytes).

The selectins show structural similarities and some overlapping functions [12]. In preeclampsia, the elevated level of E-selectin reflects dysfunction of endothelial cells, while P-selectin represents activation of platelets that can be engaged in the transformation of preeclampsia to HELLP (haemolysis, elevated liver enzymes and low platelets) syndrome [13]. L-selectin plays a major role in early embriogenesis [14].

The molecular activity of P-selectin [15-17] has been described in a particularly precise way. Once P-selectin is expressed on cell membrane, it can bind to its ligand – PSGL-1 (P-selectin ligand-1) which at the same time is

expressed on activated endothelial cells and leukocytes. The role of selectins in pathogenesis of preeclampsia is at present still a subject of research and discussion. It is likely that the selectins co-operate with other adhesive molecules including integrins, cadherins, immunoglobulin superfamily members, von Willebrandt factor (vWF) and fibronectin, resulting in necrotic inflammation of vascular wall and disseminated intravascular coagulation (DIC) [18].

Predictive and prognostic values of E- and P-selectins as preeclamptic markers have been studied during pregnancy, but not yet in the course of labour. This is exactly why it is not certain whether the level of these markers do not change during labour. If that were the case then they would lose their predictive value at labor time.

In our hypothesis we assumed that the levels of E- and P-selectins in blood plasma can change (increase or decrease) under the influence of delivery itself.

Material and methods

Patients

The study group consisted of 36 parturient women at term (gestational age: 38.8±.06 weeks) of age 24.3±2.6 years, 20 primiparous and 16 nulliparous with the normal course of pregnancy and labour (we excluded from analysis complicated pregnancy, such as preeclampsia, pregnancy induced hypertension, placenta previa, low lying placenta, prolonged rupture of fetal membranes and intraamniotic infection). They were admitted to the hospital at the beginning of labour (1A stage).

Fifteen healthy women at term, age- and parity-matched with the study group, awaiting on labour in hospital, served as control.

All women were informed about the research and they accepted the sampling of blood. Permission of the Bioethics Committee was also obtained.

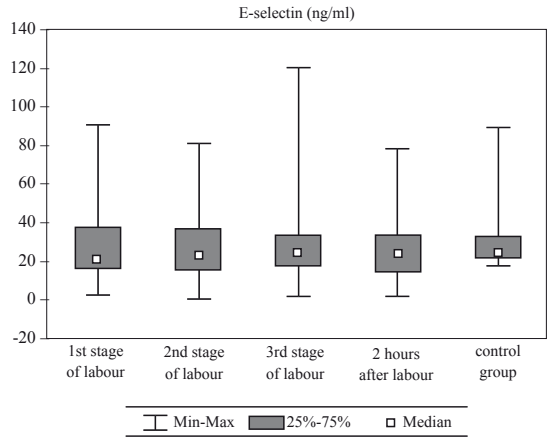
Sampling of the blood

Blood samples were obtained from antecubital vein in the 1st, 2nd and 3rd stages of labour, as well as two hours after placenta expulsion. 3.2% sodium citrate was used as an anticoagulant in the proportion 1:9 (one part of 3.2% sodium citrate, nine parts of the blood). The blood was placed in a plastic test-tube and taken to the lab to be centrifuged (2500 x g, 20 min, +4°C). The plasma was divided into 200 µl portions which were closed tightly and stored for 3-5 weeks at −70°C.

Laboratory measurements

The concentration of soluble selectins E and P was measured by immunoenzymatic method (ELISA), using tests by Bender MedSystems. The manufacturer's instructions were strictly followed. The samples were assayed in bach operations. In detail: 10 µl plasma was used to measure P-selectin, and 90 µl assay buffer was added (procedure in duplicate); 20 µl plasma was used to measure E-selectin and 80 µl sample diluent with added. The interassay and intraassay coefficients of variability were <10%.

Figure 1. **Plasma soluble endothelial selectin (E-selectin) at delivery and post partum**



Statistical analysis

The Kolmogorov-Smirnov test was used to evaluate the data for normality. The data were not normally distributed and thus the medians and corresponding quartiles (lower – Q1, upper – Q3) are given. Statistical analysis was performed with two tests: (I) The ANOVA Friedman's test was used for comparison of the data of labour stages; (II) Non-parametric test of Mann-Whitney was used for evaluation of unpaired data (parturient women vs control group). The significance limit was chosen at p value <0.05.

Results

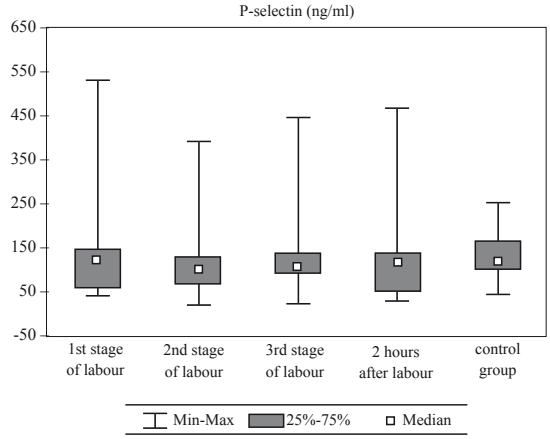
E-selectin: The medians and quartiles (Q1-Q3) of the level of E-selectin in blood plasma of labouring women were as follows: in the 1st stage of labour – 23.50 ng/ml (15.80--38.03 ng/ml), in the 2nd stage – 23.90 ng/ml (16.05--37.25 ng/ml), in the 3rd stage – 24.80 ng/ml (17.30-32.80 ng/m), and two hours after labour – 22.40 ng/ml (11.75-32.00 ng/ml). In control group (non-labouring women at term) the median was 25.67 ng/ml (22.95-33.35 ng/ml). The differences between labouring women and controled women were not significant (p>0.05) (*Fig.1*).

P-selectin: The medians and quartiles (Q1-Q3) of plasma level of P-selectin in blood plasma of labouring women were as follows: in the 1st stage of labour – 117.03 ng/ml (63.66-147.27 ng/ml), in the 2nd stage – 101.90 ng/ml (75.58-127.11 ng/ml), in the 3rd stage – 107.66 ng/ml (91.10-141.51 ng/ml), and two hours after labour – 106.94 ng/ml (47.77-137.19 ng/ml). In the control group (non-labouring women at term) the level was 116.70 ng/ml (91.82-179.00 ng/ml).The differences between labouring women and controled women were not significant (p>0.05) (*Fig. 2*).

Discussion

In our working hypothesis we turned out to be wrong to assume a possibility of change of the level of E- and/or P-selectin in blood plasma under the influence of labour itself. The

***Figure 2*. Plasma soluble platelet selectin (P-selectin) at delivery and post partum**



levels of both molecules were stable in pre-labour time and at delivery as well as two hours after labour. Therefore we have concluded that the labour itself does not infuence the levels of soluble E-selectin and soluble P-selectin in blood plasma. If it is the case, one can believe that these selectins are reliable biomarkers for supervision of preeclamptic patients during labour.

However, different results could be expected, because in the 3rd stage of labour two events take place which could – we thought – influence the level of examined biomarkers of plasma: (I) ablation of placenta which is accompanied by the local destruction of tissues including damage of vascular endothelium, and (II) haemostasis of placental bed, which proceeds with the participation of blood platelets and their activation.

We can not compare our research with that of other authors, as they have not studied either E-selectin or P-selectin during labour, but in pregnancy complicated with preeclampsia. Most often they have studied these two molecules together with other adhesion molecules, like VCAM (vascular cell adhesion molecule) and ICAM (intercellular adhesion molecule) or others. Austgulen et al. [7] found increased levels of ICAM-1, VCAM-1 and E-selectin. Daniel et al. [3], who studied all three selectins, concluded that only the level of E-selectin shows statistically significant increase in preeclampsia. In contrast, Halim et al. [4] found out elevated level of P-selectin, and Chaiworapongsa et al. [5] reported a significant increase both in P-selectin and E-selectin, but decrease in L-selectin. Austgulen et al. [7], Krauss et al. [6] and Kim et al. [8] observed increased levels of VCAM, ICAM and E-selectin, and Besinger et al. [10] increased levels of E-selectin, pregnancy-associated plasma protein A (PAPP-A) and activin A. In pregnancy induced hypertension (PIH) the levels of E-selectin, thrombomodulin and von Willebrand factor (vWF) were also elevated [9].

Among the authors mentioned above, only Austgulen et al. [7] drew attention to the possibility of association of increase of ICAM and VCAM levels with the delivery. No changes were observed at delivery, but it is necessary to emphasize that the examined period of labour was not specified.

A question arises whether other known preeclampsia biomarkers do not lose their predictive values in the time of labour? The answer to that question would have a practical aspect facilitating intrapartal supervision of preeclamptic patients. Thus, it is necessary to do further research of all markers throughout pregnancy and all stages of labour for preeclamptic women.

We consider our present study a preliminary report. We think that similar studies should include a group of preeclamptic women, whose labour will proceed naturally, the ways and powers of nature.

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Evaluation of pulmonary hypertension in COPD patients with diabetes

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Abstract

Purpose: to assess pulmonary hypertension (PH) in patients with chronic obstructive pulmonary disease (COPD) plus diabetes mellitus (DM) using transcutaneous Doppler right jugular venous echo (tDErjv).

Material and methods: We examined 5 groups of patients and control group (30 healthy subjects). The 1st group consisted of 55 COPD in-patients in exacerbation with type II DM (DM2). The 2nd group was formed of 40 in-patients with sole COPD in exacerbation. 30 patients with sole DM2 were included in the 3rd group. The 4th group consisted of 15 COPD in-patients with type I DM (DM1). The 5th group was formed of 18 patients with DM1. The following parameters, using tDErjv, were determined: direction of flow (antegrade or retrograde), velocities of systolic (Sf) and diastolic flows (Df), which are in the strong correlation with the mean pulmonary artery pressure (mPAP). If the flow was biphasic, the ratio of velocities (Df/Sf) and mPAP were calculated.

Results: Antegrade biphasic flow was revealed in all patients. Sf>Df and Df/Sf<1 were detected in the 3rd and control groups. While, in the 1st and 2rd groups Sf<Df (1.14±0.12 and 0.90±0.07 in the 1st and 2rd groups respectively; $p<0.05$ vs control). In the 2nd group patients with Df=Sf the value of the mPAP ~25 mmHg was detected, whereas in the 1st group with Df/Sf>1, this value was >35 mmHg.

Conclusions: PH was more severe in COPD plus DM2 as compared with COPD only. tDErjv allowed determining mPAP in all COPD patients, even with the severe emphysema.

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Key words: chronic obstructive pulmonary disease, diabetes mellitus, standard echocardiography, transcutaneous Doppler sonography, right jugular vein, mean pulmonary artery pressure, pulmonary hypertension.

Introduction

Hypoxic PH is a common and serious complication of COPD as well as an independent factor of the bad prognosis (shorter survival rates) [1-5]. In COPD, PH tends to be of moderate severity and progresses slowly [6]. Right ventricle (RV) function is only mildly impaired with preservation of the cardiac output (CO) [7,8]. Indirect evaluation of mean pulmonary artery pressure (mPAP) in COPD patients by using standard echocardiography (SE) is often difficult due to concomitant severe lung emphysema.

The main morphologic substrate leading to lung pathology in DM is diabetic microangiopathy [9-11]. It is the part of the pathologic process in pulmonary tissue. The lung changes are the same as in diabetic microangiopathy in other organs (kidney, eyes), but it expressiveness is less and these changes develop later than in other organs [12,13].

DM favors hypoxic PH due to negative effects on pulmonary vessels [9,14]. Thus, the abundance of pulmonary microcirculation and connective tissue in lungs makes them often the organ-target in DM [15-18]. Chronic hyperglycemia is the trigger for the development of DM complications. Non-enzyme glycolysis (with the formation of glycated proteins) causes the tissues disorders, including pulmonary vessels (particularly the endothelium of capillaries). The disorders of microcirculation as well as nervous regulation present in DM favor to the changes for the worsening of pulmonary circulation [19-22]. Lungs disorders in COPD proceed across with development of diabetic microangiopathy, which strike first of all the morphological structures, having gas exchange function. This give the base to supposes diabetic pneumopathy progress

Table 1. **Baseline data of patient's groups**

Parameter	Control	COPD+DM2 (1st)	COPD (2nd)	DM2 (3rd)	COPD+DM1 (4th)	DM1 (5th)
Number of patients	30	55	40	30	15	18
males (%) mean age (years) smokers (%)	50 49±5 40	47 55±4 40	58 52±5 75	48 52±3 17	53 50±3 -	56 48±3 22
Mean duration (years) of: COPD DM	-	24±3 7±2	26±3 -	- 7±2	16±3** 13±4***	- 12±3
COPD severity (%) mild moderate severe	-	 36 36 27	 30 40 30	-	 100**	-
Body mass index (kg/m^2	24.0±1.6	31.3±2.0*	32.0±1.8*	32.7±2.0*	27.1±1.3*,***	28.3±1.9*
Glicated hemoglobin (HbA1c) %	4.5±0.4	9.6±1.8*	4.9±0.5	9.3±1.4*	10.6±2.8*	9.4±1.3*
Blood glucose (mmol/L)	5.0±1.7	13.8±3.5*	4.6±2.3	10.6±2.7*,**	15.4±5.2*,***	14.8±4.4*
Subcompensate carbohydrate metabolism (%)	-	36	-	60**		72**
decompensate carbohydrate metabolism (%)	-	64	-	40**	100**,***	38**
Retinopathy I grade, nephropaty I-II grade (%)	-	71	-	57	27**	44
Retinopathy II grade, nephropathy III grade (%)	-	18	-	13	73**, ***	56**
Autonomic neuropathy (%)		18		13	20	22
Neuropathic form of syndrome of diabetic foot (%)	-	51	-	47	80**, ***	44

* p<0.05 vs control; ** p<0.05 vs 1st group; *** p<0.05 vs 3rd group

by analogy with diabetic nephropathy as well as retinopathy [11,19].

A long period of time in COPD evolution with episodes of acute exacerbations leads to the profound disorders of microcirculation [23-25] which play the important role in the development of hypoxic PH in COPD. These disorders in DM become the additional and unfavorable factor in the case of such combined pathology (COPD plus DM). The aggravating disorders of microcirculation become the additional and burden factor which makes worse the present structural and functional disorders of bronchi and lungs in the case of combination of COPD and DM. The combination of COPD with DM2 has been detected in 5-20% of cases [26,27] and proceeds with small symptomatology and recurring course [18,28].

Abnormalities in the cardiac function can occur in the diabetic patients independently of other factors (such as hypertension, coronary atheromatosis) [29]. Metabolic changes within the heart in diabetics associated with hyperglycemia, hyperlipidemia or disinsulinemia appear to contribute to the contractile disorders of myocardium. It is now apparent that the alterations in fatty acids and glucose metabolism in the heart is an important contributing factor to the heart abnormalities in diabetics.

The peculiarities of pulmonary hemodynamics were evaluated earlier only in single COPD patients, but not in the combination of COPD plus DM.

The aim of this study was to assess of PH expressiveness by using tDErjv in COPD patients with acute exacerbation and concomitant DM as compared with sole COPD patients as well as to decide, if it is the evidence linking of PH severity with the same of concomitant DM.

Material and methods

This study was approved by the Human Studies Committee of the Belarusian State Medical University and informed consent has been obtained from the patients. The inclusion criteria for the study were established before the trial and strictly followed. These inclusion criteria were: different severity COPD with FEV1 increasing <15% during bronchodilatating test; duration of DM more than 1 year as well as presence of diabetic complications.

Patients

The 1st group consisted of 55 COPD inpatients (aged 36-60 years) (*Tab. 1*) with an acute exacerbation plus DM2. Mean duration of COPD, DM2 and age were 24.7 and 55 years respectively. Mild, moderate to severe COPD according to consensus "GOLD" [30] had 36%, 36% and 27% of these patients respectively. Current smokers were 40%. Body mass index (BMI) as well as glicated hemoglobin (HbA1c) – the marker of DM compensation for the period of the last 3 months made up 31.3 kg/m^2 and 9.6% respectively. Sixty-four percents of these patients had decompensate stage of carbohydrate metabolism

(mean glucose level was 13.8±3.5 mmol/L) and 36% – subcompensate stage (mean glucose level – 6.4±2.7 mmol/L). We observed the following diabetic complications in these patients: retinopathy I (non-proliferative) and nephropathy I-II (before to the clinical manifestation) grades (71%); retinopathy II (preproliferative) and nephropathy III grades (18%); neuropathic form of syndrome of diabetic foot (51%) and autonomic neuropathy (18%).

The 2nd group was comprised of 40 in-patients comparable according to COPD severity (mean age and COPD duration were 52 and 26 years; BMI – 32.0 kg/m^2) without DM. Current smokers were 75%. The mean levels of HbA1c and glucose made up 4.9±0.5% and 4.6±2.3 mmol/L respectively.

The 3rd group was formed by 30 patients with alone DM2 (mean age and disease duration were 52 and 7 years; BMI – 32.7 kg/m^2). Seventeen percents of these patients were current smokers. Forty percent of these patients had decompensated stage of carbohydrate metabolism (the levels of HbA1c and glucose achieved 9.3% and 10.6 mmol/L respectively). Sixty percents of these patients had subcompensate stage of carbohydrate metabolism (the levels of HbA1c and glucose achieved to 7.0% and 6.3 mmol/L respectively). Diabetic complications were revealed: retinopathy I and nephropathy I-II grades in 57% of these patients; retinopathy II and nephropathy III grades – in 13%; diabetic distal polyneuropathy – in 47%; neurophatic form of diabetic foot syndrome – in 47% and autonomic neuropathy – in 13% of these patients.

The 4th group consisted of 15 COPD in-patients (mean age and disease duration were 50 and 16 years respectively) with DM1 (mean duration – 13 years; BMI – 27.1 kg/m^2) in all patients with decompensated stage of carbohydrate metabolism (mean levels of HbA1c – 10.6±2.8% and glucose – 15.4±5.2 mmol/L; $p<0.05$ vs the 1st group). The small number of these patients was due to the infrequent combination of these two diseases. Diabetic retinopathy of I and nephropathy I-II grades were detected in 27% of these patients.

The 5th group was formed of 18 patients with sole DM1 (mean age and disease duration were 48 and 12 years respectively). Current smokers were 22%. Seventy-two percents of the patients had decompensated stage of carbohydrate metabolism (HbA1c=9.4±1.3%). Diabetic retinopathy and nephropathy I-II stages (preclinical stages) as well as obvious clinical stages of these complications were revealed in 44% and 56% of the patients respectively. Syndrome of diabetic foot and autonomic polyneuropathy were detected in 44% and 22% of these patients. Thus, in COPD patients with DM1 we observed more severe diabetic microangiopathy than in 1st and 3rd groups. We did not include the insulin resistance patients into this study.

Clinical symptoms and signs of mild COPD patients plus DM1 were comparable with the same of mild COPD with concomitant DM2 as well as in patients with sole mild COPD.

Control group was formed of 30 healthy subjects (male – 22, female – 8, mean age – 49 years, BMI – 24.0 kg/m^2; smokers – 40%).

Methods

We performed SE and tDErjv for the examination of pulmonary hemodynamics. SE was made up on apparatus "Siemens Sonoline G 605" (Germany). We detected by SE: maximal and mean blood flow in pulmonary artery (PA), gradient of blood flow and its integral in PA as well as mPAP value (according to the temporal parameters of systolic flow in outlet of right ventricle and formula of Kitabatake et al. [31]; Lg of mPAP= -2.8 AT/ET+2.4; where AT – time of acceleration of blood flow (msec) and ET – ejection time of pulmonary artery; diameter of pulmonary trunk artery (DPTA), stroke volume of right ventricle (SVRV=integral of blood flow in PA$\cdot 2\pi r^2$; where r=1/2 of DPTA) and cardiac output (CO=HR·SVRV).

Direction of flow (antegrade or retrograde), velocities of systolic (Sf) and diastolic flows (Df) (which strongly correlated with the mPAP) were determined by tDErjv. We calculated the ratio of velocities (Df/Sf) (if the flow was biphasic), diameter (mm) of right jugular vein at the moment of maximum inspiration as well as at the end of nonforced expiration and then mPAP according to the diagram of Matsuyama et al. [32].

The collapsing degree of right jugular vein (mm) was detected as difference of this vein diameter at the end of nonforced expiration and at period of maximum inspiration which was divided by the diameter of this vein at the end nonforced expiration and multiplied by 100%. The measurement of interior jugular vein diameter was made at the standard point – isthmus of the thyroid gland. The measurement of the vein diameter was performed in the transverse view from upper inner lager to lower inner lager of the vessel. We made the revision of Doppler angle in all cases, which was predisposed by our ultrasound device. All the patients had sinus rhythm.

The evaluation of diabetic retinopathy grade was made according to classification of Korner and Porta [33] and diabetic nephropaty grade according to Mogensen et al. [34] with determination of glomerular filtration and daily protein loss with urine. The diagnosis of autonomic neuropathy was made on the base of functional tests according to Williams and Pickup [35]. CRP was determined by an immunometric assay ("Diasys" kit).

Statistical analysis

The data are shown as mean ± SD unless otherwise indicated. The paired and unpaired t-test was used to test the significance of baseline characteristics of the 1st-4th groups as well as the treatment effects within the groups and between them. All p values were two tailed. The group comparison used the Student's t-test for quantitative variables and χ^2 method Fisher's exact test for qualitative variables. The χ^2 or Fisher exact test was used to compare categoric variables. The significance level was set at $p\leq 0.05$.

Results

HbA1c>8% and complain of dryness in mouth were detected in 40% of patients with sole DM2 as compared with 64% of patients COPD plus DM2 ($p<0.05$). Polyuria and thirst were revealed in 30% patients with DM2 only vs 56% of patients COPD with DM2 ($p<0.05$). Thus, these symptoms were observed more often in combination of COPD plus DM2 than in sole DM2.

***Table 2*. Acute phase biochemical tests in COPD patients plus diabetes and sole COPD patients**

Patients	N	+CRP (%)	α2-globulins (%)	γ-globulins (%)
COPD+DM2				
mild	20	20	10.6±0.3	15.4±0.6
moderate	20	30*	12.2±0.2*	17.1±0.8*
severe	15	60*, **	14.0±0.4*, **	19.5±0.9*, **
Mild COPD+DM1	15	13●●	9.7±1.4●, ●●	15.2±3.3●, ●●
COPD				
mild	12	25	10.2±0.3	15.0±0.7
moderate	16	25	11.9±0.2▼	16.7±0.6▼
severe	12	50▼, ▼▼	13.7±0.3▼, ▼▼	19.2±0.7▼, ▼▼

* p<0.05 vs mild COPD plus DM2; ** p<0.05 vs moderate COPD plus DM2; ● p<0.05 vs moderate COPD plus DM2; ●● p<0.05 vs severe COPD plus DM2; ▼ p<0.05 vs mild COPD; ▼▼ p<0.05 vs moderate COPD; + CRP – positive C-reactive protein

***Table 3*. Pulmonary hemodynamic parameters according to standard echocardiography**

Group	AT/ET	mPAP (mmHg)
Control (n=30)	0.43±0.02	16.0±2.0
COPD+DM2 (n=45)	0.32±0.04	32.0±3.4*,**,***
COPD (n=34)	0.34±0.03	29.0±3.0*, **
DM2 (n=30)	0.42±0.02	17.0±2.0
COPD+DM1 (n=15)	0.41±0.01	18.0±1.0
DM1 (n=18)	0.42±0.01	17.0±1.0

* p<0.05 vs control; ** p<0.05 vs DM2; *** p<0.05 vs sole COPD

***Table 4*. Pulmonary hemodynamics parameters in different groups according to standard echocardiography**

Parameter	Control	Mild COPD +DM2	Moderate to severe COPD+DM2	Mild COPD	Moderate to severe COPD	Mild COPD +DM1	DM1
DPTA, mm	17.0±1.3	17.5±1.5	20.0±1.4*,**	17.6±1.8	18.6±1.7▼▼	17.6±2.0▼▼	17.5±1.5▼▼
SVRV, mL	72.8±1.5	71.6±1.3	68.5±2.6*,**	71.8±1.6	70.7±1.8▼▼	71.5±1.0▼▼	72.6±1.3▼▼
CO, L/min	5.5±0.1	5.5±0.1	5.5±0.2	5.5±0.2	5.5±0.2	5.5±0.1	5.4±0.1
mPAP, mmHg	16.0±2.0	17.0±1.0	38.0±5.0*,**	17.6±1.1	35.6±2.8▼,▼▼	18.0±1.0▼▼	17.0±1.0▼▼

* p<0.05 vs control; ** p<0.05 vs mild COPD+DM2; ▼ p<0.05 vs mild COPD; ▼▼ p<0.05 vs moderate to severe COPD+DM2

Sixty-four COPD patients with concomitant DM2 were admitted >2 times per year to clinic by ambulance (four times – 25%; three times – 38%) as compared with 48% (p<0.05) patients with sole COPD. Forty-seven of mild COPD patients plus DM1 were admitted to clinic >2 times per year (four times – 13%; three times – 33%) as compared with 17% (p<0.05) of patients with sole mild COPD.

The biochemical acute phase tests of COPD inpatients on admission are presented in *Tab. 2*. As seen from this table, the activity of COPD exacerbation was more expressive in severe COPD patients with concomitant DM2.

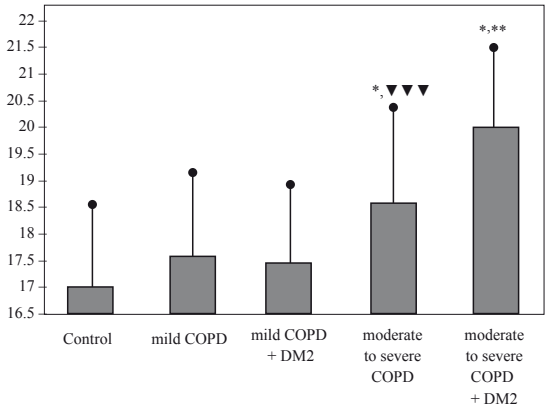
We can not detect mPAP by SE in 10 and 6 patients of the 1st and 2nd groups due to concomitant severe lung emphysema. The level of mPAP was the highest in patients of the 1st group (*Tab. 3*) vs the 2nd group, despite the fact that these groups did not differ according to COPD severity. mPAP was particularly high in moderate to severe COPD patients plus DM2 (*Tab. 4*) as compared with mild COPD plus DM2. The hemodynamic parameters of patients with sole DM1 or DM2 did not differ from the control. The mPAP, DPTA, CO and SVRV in mild COPD patients (with or without DM1-2) were comparable with the control too.

A significant increase of mPAP (by 2.4 and 2.2 times vs the control), DPTA (by 18% and 10% vs the control) and decrease of SVRV (by 6% and 3% vs the control) were noted only in moderate to severe COPD patients with DM2 and without it respectively.

DPTA in moderate to severe COPD patients with concomitant DM2 was larger (p<0.05) than in mild COPD plus DM2 (*Fig. 1*), sole moderate to severe COPD as well as in the control group (20.0±1.4 mm vs 17.5±1.5; 18.6±1.7 and 17.0±1.3 mm respectively). Meanwhile, SVRV in moderate to severe COPD patients with concomitant DM2 was smaller (by 3%; p<0.05) in contrast to sole moderate to severe COPD patients. Thus, SVRV in the moderate to severe COPD patients with DM2 made up 68.5±2.6 mL as compared with the control and mild COPD patients plus DM2 (72.8±1.5 mL and 71.6±1.3 mL respectively; p<0.05). CO in COPD patients of various degree of severity with or without DM did not significantly differ from the control.

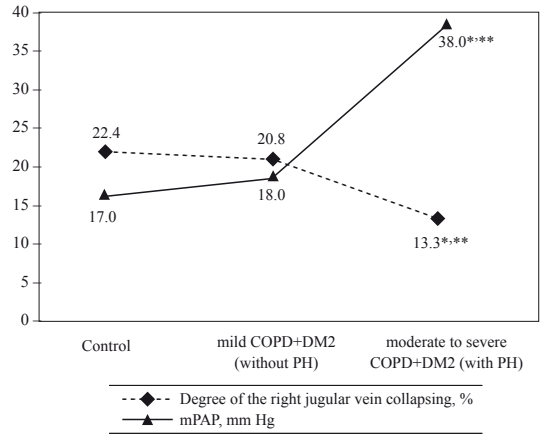
The antegrade biphasic flow by tDErjv was revealed in all patients. The ratio of Df/Sf did not differ among the control, DM1 and mild COPD plus DM1 groups (0.63±0.02; 0.64±0.01 and 0.67±0.01 respectively). Thus, mPAP values were made up 17.0±2.0; 17.0±1.0 and 18.0±1.0 mmHg respectively in these

***Figure 1*. Diameter of pulmonary trunk (mm)**



* p<0.05 vs control; ** p<0.05 vs mild COPD + DM2; ▼ p<0.05 vs mild COPD; ▼▼ p<0.05 vs moderate to severe COPD + DM2

***Figure 2*. Degree of the right jugular vein collapsing during inspiration in COPD plus DM2**



* p<0.05 vs control; ** p<0.05 vs mild COPD plus DM2

***Table 5*. The data of transcutaneous Doppler sonography of the right jugular vein in COPD and DM2 patients**

Parameter	Control (n=30)	COPD+DM2 (n=55)	COPD (n=40)	DM2 (n=30)
Systolic flow (Sf), m/s	0.19±0.02	0.07±0.03	0.10±0.02	0.17±0.02
Diastolic flow (Df), m/s	0.12±0.01	0.08±0.01	0.09±0.01	0.11±0.01
Ratio of Df/Sf	0.63±0.02	1.14±0.12	0.90±0.07	0.65±0.02
mPAP, mm Hg	17.0±2.0	34.2±4.0*, **,***	30.3±3.0*	17.0±2.0

* p<0.05 vs control; ** p<0.05 vs DM2; *** p<0.05 vs sole COPD

***Table 6*. The data of transcutaneous Doppler sonography of the right jugular vein according to COPD severity**

Parameter	Mild COPD +DM2	Moderate to severe COPD +DM2	Mild COPD	Moderate to severe COPD
	(n=20)	(n=35)	(n=12)	(n=28)
Sf, m/s	0.18±0.01	0.06±0.01	0.18±0.01	0.07±0.02
Df, m/s	0.12±0.01	0.08±0.01	0.12±0.01	0.09±0.01
Ratio of Df/Sf	0.67±0.02	1.33±0.08	0.67±0.02	1.28±0.04
mPAP, mm Hg	18.0±1.0	38.5±5.0*,**,***	18.0±1.0	34.0±4.0

* p<0.05 vs mild COPD+DM2; ** p<0.05 vs mild COPD; *** p<0.05 vs moderate to severe sole COPD

groups (p>0.05). In sole COPD patients as well as in COPD plus DM2 was detected the level of mPAP ~25 mm Hg at the ratio of Df=Sf, meanwhile at the ratio of Df:Sf>1.0, mPAP was >35 mm Hg. Sf was greater than Df in the control and DM2 groups (*Tab. 5*).

There was an increase of the ratio Df/Sf in the 1st and 2nd groups in a contrast to the control (by 2.0 times and 1.8 times respectively; p<0.05 vs the control). That is, while of Df/Sf ratio increased, mPAP increased too. The level of mPAP in the moderate to severe COPD patients was about the same that was obtained by SE according to tDErjv (*Tab. 6*).

The comparative analysis showed the high informing value of the collapsing degree of the right jugular vein at inspiration for PH diagnosis in COPD patients. Thus, decrease of degree collapsing of this vein at inspiration (at the standard point – isthmus of the thyroid) was the objective qualitative marker of PH. The collapsing degree of right jugular vein at inspiration was equal to 22.4±0.5%; 22.4±0.3% and 20.8±0.2% in the control, DM1 and mild COPD patients plus DM1 respectively (p>0.05). There were no significant differences of the collapsing degree of right jugular vein in mild COPD patients plus DM2 as compared with the control.

The collapsing level of this vein was higher (p<0.05) in the mild COPD plus DM2 patients without PH as compared with 13.3±0.2% in the moderate-severe COPD patients plus DM2 with PH (mPAP=38.0±5.0 mm Hg by SE). Thus, mPAP was increased in the process decreasing of the collapsing degree of the right jugular vein at inspiration (*Fig. 2*).

We have found the high and significant correlations among a collapsing degree of the right jugular vein at inspiration and mPAP level (r= -0.76; p<0.05 in COPD patients plus DM2 and r=-0.92; p<0.05 in the moderate to severe COPD plus DM2 patients burdened by PH). We revealed that tDErjv was more sensitive method for verification PH in COPD patients than SE (χ^2=13.5; p<0.05). Thus, tDErjv allowed determining the mPAP in all COPD patients, even with the severe lung emphysema.

Discussion

Hypoxic PH in COPD progresses over the time and its severity usually correlates with the degree of airflow obstruction and impairment of pulmonary gas exchange [4,6]. The rate of PH progression is slow (at an average rate 0.6 mm Hg per year) [4] and usually mPAP is only moderately elevated, even in the patients with advanced COPD [6]. The development of PH was more closely linked to the evolution of arterial gases than to initial PH value [4].

In COPD, changes in pulmonary circulation may start several years before PH is apparent at rest. Hypoxic pulmonary vasoconstriction and remodeling of pulmonary vessels are the most significant factors, which contribute to the development and maintenance of PH in COPD [36]. Hypoxic stimulus exerts opposite actions on systemic and pulmonary circulation – dilates systemic arteries and constricts pulmonary arteries. Probably, the impairment of endothelial function is associated with an altered response to hypoxic stimulus that further worsens of the gas exchange.

Pulmonary vascular abnormalities in COPD patients with middle-to-moderate severity mainly consist of thickening of the intima of pulmonary muscular arteries which reduces the lumen size and an increased proportion of muscularised arterioles [37-40]. The results of morphometric studies showed conspicuous changes in the structure of pulmonary muscular arteries in patients with mild COPD [37,38].

An endothelial dysfunction in pulmonary arteries has been shown at both ends of COPD spectrum – end-stage disease and early mild disease [38,41]. The impairment of endothelial function results from changes in the expression and release of vasoactive mediators (NO, prostacyclin, ET-1).

In COPD patients with a hypoxic PH, mPAP is not markedly elevated and the rate of progression PH is slow [36]. Therefore, RV has some time to adapt to such a modest increase in the pressure load. When mPAP is chronically elevated, RV dilates, with the increases in both end-systolic and end-diastolic volumes. In COPD, the SVRV is usually maintained, whereas the RV ejection fraction (RVEF) is reduced [36]. Systolic ventricular dysfunction is defined by a decrease of RVEF. In COPD, RVEF can be reduced and its value is inversely related to mPAP [42].

Disorders of RV function could be a cause by the action of hypoxemia on myocardium. It leads to the systolic dysfunction of RV. A verification of this fact is the significant decrease of SVRV in the moderate to severe COPD patients (against a background of the marked bronchial obstruction) with concomitant DM2 as compared with mild COPD patients.

We detected the strong significant correlation between SVRV and CO, which increased during COPD development (from r=0.65 in mild COPD plus DM2 up to r=0.82 in the moderate to severe COPD plus DM2). These data could be an evidence of the remodeling process in RV as well as development hypertrophic stage of heart adaptation to appearance of PH.

It has been shown [43,44] that in clinically stable COPD patients the contractility of RV lies within normal limits, irrespective of mPAP value. But, during acute exacerbation of COPD, when mPAP increases markedly, the contractility of RV is reduced in patients with clinical signs of right-heart failure [8,45]. In COPD the CO is usually preserved and it might even rise during exacerbation episodes [46,47], even when there are apparent signs of RV failure.

A combination of COPD plus DM1 had negative interdependence. Thus, an acute exacerbation of COPD caused decompensation of DM1 in all these patients and (we detected I grade of ketoacidosis in 87% of cases) it required an increasing of the usual insulin dose. COPD acute exacerbation with concomitant DM2 caused additionally: the increase of insulin dose in 18% of these patients; combined therapy in 15% and switch on base bolus regimen of insulin treatment in 31% of the patients. In part, decompensation of current DM2 or DM1 had negative consequences on the evolution of COPD, favored to a long persistence inflammation in pulmonary tissue and reduced the term of COPD remission. Meanwhile, in mild COPD patients with DM1 the parameters of pulmonary hemodynamics were comparable with the control. This fact could testify the compensation of systolic function of RV in these patients.

More evident expressiveness of PH in COPD plus DM2 patients was caused by an increase of the hypoxia against a background of DM2 decompensation as well as by more pronounced disorders of microcirculation and gases balance of venous blood in these patients as compared with sole COPD patients ($pvO2=28.9\pm3.2$ vs 32.0 ± 2.2 mm Hg respectively; $p<0.05$). In view of that hypoxia is the condition, which can be corrected in the process of therapy, probably; steady and irreversible character of microvascular disorders caused larger increase of mPAP in patients with such combined pathology.

We also performed the parallel comparison of diagnostic value of PH assessment by the collapsing degree of right jugular vein at inspiration as well as by SE (with quantitative evaluation of PH). This analysis showed the high informative value of qualitative detection of PH by the collapsing degree of right jugular vein in these patients (criterion of McNamara $\chi^2=6.21$; $p<0.05$) even in severe lung emphysema and obesity.

It has been recognized for many years that DM has a significantly greater incidence of angina pectoris, acute myocardial infarctions, congestive heart failure and other manifestation of atherosclerosis as compared to non-diabetic population [48,49]. More recently it has been shown that ventricle performance can be impaired (diabetic cardiomyopathy), even in absence of ischemic heart disease [50-59]. Both left ventricle systolic [55,60] and diastolic functional abnormalities [56-58] occur in DM2 patients. Diastolic dysfunction can even be seen in young diabetics [59]. It has been shown recently that early abnormalities in cardiac function can occur in DM2 patients with only minor abnormalities in glucose metabolism [61].

Conclusions

Decreasing of the collapsing degree of the right jugular vein during inspiration was the objective qualitative marker of hypoxic PH in COPD patients. Transcutaneous Doppler sonography of the right jugular vein revealed about the same mPAP as by SE, but allowed to determine mPAP in all COPD patients, even with the severe lung emphysema. DM2 had a negative influence on pulmonary hemodynamics. Thus, PH was more

severe in COPD plus DM2 patients as compared with COPD only.

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Solving the problem of antidepressant selection in Lithuania

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Abstract

Purpose: To ascertain the opinion of psychiatrists of the factors those determine antidepressants selection.

Material and methods: An original questionnaire of 30 questions, which deals with reliance of antidepressants selection according to the subtype of depression, was represented for a quarter of all Lithuanian psychiatrists.

Results: Respondents for depression with obsession – 36% chose paroxetine. It is interesting that despite the controversial opinion about the TCA prescribing according to their side effects profile and safety to use, our respondent chose amitriptyline for the melancholic depression with suicidal thoughts (50.2%) and for the anesthetic depression (28%). In some cases there is no unanimous opinion among the psychiatrists – data scattering was received in selection, the respondents chose different antidepressants from different groups in similar frequency. For the treatment of the adynamic depression – 7.6% – amitriptyline, 12.1% – citalopram, 10.6% – reboxetine, 10.6% – venlafaxine, for the anxious depression – 15.2% – amitriptyline, about 20% – citalopram, 15.2% – mirtazapin, for the anesthetic depression – 14.3% – escitalopram, 9% – sertraline, 8.3% – venlafaxine. There is no clear tendency or prevailing antidepressant.

Conclusions: Psychopathological peculiarity of depression can be one of the most important criteria in antidepressant selection. However, in many cases, the subtype of depression is ascertained empirically and based solely on the personal experience and clinical practice of the psychiatrist. There are no clear diagnostic criteria or practical guidelines for the reliable verification of the psychopathological subtype of depression, which would allow for the selection of a more adequate and prompt treatment for the patient.

Key words: depression, type of depression, selection of antidepressants.

Introduction

There are more than two tens of registered antidepressants in Lithuania and all of them are indicated as effective drugs for depression treatment. A new dual-action antidepressant has appeared recently as well as several novel antidepressants have been presented for the Drugs Control Agency that are at different research stages now. It is estimated that there will be more antidepressants generated during the next decade that will have different mechanism of action from those of the current ones [1,2].

There is a quite wide spectrum of antidepressants nowadays. However, a big assortment of pharmaceuticals results in various problems that the physicians must take into consideration while selecting an optimal treatment [3]. This appears to be a difficult task, though the identification of treatment failures is quite an easy one. Often do physicians and their patients ask themselves as to which one is the best choice, whether other pharmaceuticals bring better results, how to make a decision, if there are no clear guidelines for treatment? Unfortunately, practicing therapists have poor experience-based references for a proper choice of antidepressant. The majority of reviews of theoretical approach and practical guidelines relating to antidepressant consumption conclude that each sort of these pharmaceuticals is equally efficient, thus the recommendations for a certain antidepressant is based on the aspects of side effects, tolerance, patient opinion, and price. Patients react differently to antidepressants. Many of them go through trial after trial with little or no improvement at all. Eventually, some people

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find antidepressants that help them achieve remission; however, others do not. Some practical guidelines detect a quite diverse response to treatment depending on a clinical profile. For example, American Psychiatric Association (APA) set Practice Guideline for the Treatment of Major Depressive Disorder claim that the atypical symptoms and those of anxiety, melancholia, and border person disorder typical of non-psychosis, unipolar depression disorder might be related to a different response to the antidepressant. According to this guideline, it is recommended to give preference for the selective serotonin reuptake inhibitors (SSRI) and avoid of bupropion while treating depression with high anxiety, whereas, in case of obsessive-compulsive symptoms, SSRI with clomipramine are preferred, as well as tricyclic antidepressants (TCA) are recommended in case of a severe and melancholic depression; atypical depression is typically treated by SSRI or monoamine oxidase inhibitors (MAOI) while avoiding TCA [4].

Unfortunately, the data of these practical guidelines that guide the psychiatrists' selection of an antidepressant is quite limited in its scope and utility. The majority of the depressed patients are treated in outpatient settings. Melancholia and the episode of severe depression are comparatively rare in a current outpatient psychiatry practice [5]. Generally, anxiety disorders are comorbid conditions in depression [6,7]; the APA practical guide states that bupropion might act as an *anxiogenic* and therefore it should not be given to patients. Though MAOI and TCA can be useful for patients with anxious depression, other pharmaceuticals are given the preference. Practical guide does not cover possible impact of a specific symptom and type of depression on the selection of antidepressant.

It would be interesting to explore the criteria of antidepressant prescription that Lithuanian physicians use, as there is no exact information that would elucidate the selection of antidepressant. There have been only a few researches on the practice of the psychiatrists' prescription of antidepressants. The majority of articles focus on the tendencies of prescription rather than the argument for the prescription of certain pharmaceuticals [8-12]. The research of the factors that have influence on psychiatrists' choice of antidepressant can disclose the spheres for further scientific research in order to confirm or deny the tendencies of selection. Now, the information that would prove the significance of clinical criteria for the choice of antidepressant is lacking. Presumably, the non-clinical aspects, such as the economic factors of market, will have more impact on the choice of pharmaceuticals. Therefore, it is difficult to contradict the restrictions of the pharmaceutical guidelines.

This research is based on the opinion of Lithuanian psychiatrists of the antidepressants and the factors that determine their selection. It has been conducted by giving them a questionnaire. This article focuses on the discussion as to what factors affect the selection of antidepressant for treatment.

Methods

The survey had been conducted from January till March, 2005. The psychiatrists from different regions of Lithuania participated therein. The stratified sample was chosen; first, the biggest Lithuanian hospitals were chosen and 20 per cent of psychiatrists who worked therein were questioned pro rata in incidental order, independently of gender, age, occupation, work experience, etc. When the questionnaires were given to the respondents, they were informed about the objective of this survey. The respondents filled in the questionnaires anonymously.

In the questionnaire, there were 30 questions that included the respondents' demographic information, their opinion of 14 the most popular antidepressants in Lithuania and of the factors that have influence on the selection of antidepressant. They were asked to evaluate the efficiency of antidepressants, their tolerance in the scale from 1 to 5, where 1 means very low efficiency or tolerance and 5 means very good efficiency or tolerance; and, according to their significance, to rank 5 factors that have the most significant impact on the selection of depression treatment. The statements from the questionnaire are displayed in the tables of **Result** part of this article. These statements were formulated on the basis of the review articles, manuals and the clinical experience of the authors. Data analysis was performed with Statistical Package for the Social Sciences (SPSS) 10 for Windows software.

Results

The sample group consists of 133 psychiatrists, which approximately covers a quarter of all the Lithuanian psychiatrists. The answers of 18 respondents were excluded; 9 questionnaires were not sent back to the researchers.

The working experience of more than a half of psychiatrists exceeded 20 years (50.4%). The number of patients treated from depression distributed quite evenly among the respondents, which means that approximately 29 per cent of the respondents treat from 20 to 50 patients annually, the same percentage of the respondents treat from 50 to 100 patients, as well as more than 100 patients yearly; only 13 per cent of the respondents treat less than 20 patients during the mentioned period.

Generally, Lithuanian psychiatrists chose the following groups of antidepressants: SSRI, TCA, and noradrenergic and specific serotonergic antidepressants (NaSSA).

The respondents ranked the following antidepressants as the best ones: mirtazapin, escitalopram, citalopram, amitriptyline according to the efficiency and escitalopram, citalopram, sertraline, mirtazapin according to the tolerance (*Tab. 1*). While estimating the antidepressants according to their efficiency, mirtazapin was acknowledged as very effective (5 points) by 58.5 per cent of the respondents; within the same group, escitalopram was acknowledged as very effective by 55.3 per cent and citalopram by 48.9 per cent of them. While estimating the antidepressants according to the tolerance 66.4 per cent of the respondents acknowledged escitalopram as very good (5 points); within the same group, 51.6 and 50 per cent of the respondents claimed citalopram and sertraline as fully tolerated respectively.

Research data confirm that, in general, the selection of antidepressant was influenced by the complexion of depression symptoms, the tolerance of the pharmaceutical and the comor-

***Table 1*. Evaluation of the antidepressants by the efficiency and tolerability. Mean efficiency points (from 1 – very low up to 5 – very good) and mean tolerability points (from 1 – very low up to 5 – very good)**

	Mean efficiency points	Mean tolerability points
Mirtazapin	4.45	4.24
Escitalopram	4.42	4.63
Citalopram	4.13	4.45
Amitriptyline	4.13	2.56
Paroxetine	4.04	4.13
Sertraline	3.94	4.39
Venlafaxine	3.9	3.95
Clomipramine	3.56	3.17
Nortriptyline	3.4	2.64
Tianeptine	3.21	4.08
Imipramine	3.19	2.67
Fluoxetine	3.17	3.68
Bupropion	3.06	3.64
Doxepine	3.03	3.19

bid disorders (*Tab. 2*). According to the frequency, the patient's previous response to the treatment was the second factor that influenced the choice of practitioners. Some other factors, such as sexual dysfunction, patient opinion and co-operation during the consumption of the pharmaceuticals, that are often discussed in literature rarely did make any influence on the selection of antidepressant.

There was a possibility to indicate new criteria in the questionnaire concerning the factors that influence the selection of antidepressant but there were no records in the answered forms.

The results of the selection of antidepressant depending on the subtype of depression are displayed in the charts.

Discussion

The depressions are not the homogenous group in regard to their psychopathological structure. Nowadays classifications notice some peculiarities (subtypes) of depression – psychotic depression, depression with catatonic features, with or without somatic symptoms, agitated depression, etc. (ICD-10, DSM-IV TR). We uphold the view of some European and American psychiatrists that some forms of disturbances (symptoms) can markedly prevail over the picture of depression [13-18].

On that ground we have chosen some psychopathological "subtypes" of depressions in our survey: adynamic, anaesthetic, agitated (anxious), depression with obsessions and depression with clear suicidal thoughts. In common with clear diagnostic criteria for depression in all cases we note some specific peculiarities of psychopathological structure of each "subtype".

In the structure of adynamic depression there are prevailing psychomotor suppression, thinking process and movements are going slow, loss of energy, inability to make everyday social activities, strong feeling of disability and asthenia, gone sensation, sometimes – even inability to get up from the bed or get about. In case of anaesthetic depression the patients complain of prevailing loss of feelings, anhedonia, blur vision and weak perception of surroundings, inability to understand what is going on, depersonalization and derealization. In the structure of anxious depression there are marked prevailing feelings of inner tension, anxiety, trouble, angst, psychomotor agitation. In case of obsessive depression there were low self-esteem and prevailing obsessive thoughts of worthlessness, shoddy, booming around, picayune, contemptible, pathetic, pitiful and pygmy. In case of depression with strong suicidal thoughts there were prevailing feelings of hopelessness, purposeless, disability, things looking black, being at a deadlock.

The objective of the study was to explore and comprehend the criteria that guide psychiatrists in the selection of antidepressants for the patient with depression. As the number of the researches that assess the influence of clinical symptoms on different responses to the new generation antidepressants is relatively small, the scientific exploration of the psychiatrists' practice of prescription would be an interesting and useful one.

Most often were the SSRI, TCA and NaSSA groups of antidepressants selected; this might be due to the fact that many antidepressants of these groups are effective and well tolerated. The aspects of efficiency and safety are analyzed in much of clinical research. In this study, the authors try to analyze other factors possibly significant for the selection of antidepressants.

The results of the study reflect few significant factors that are important for the treatment of depression. According to the

***Table 2*. The factors generally impacting antidepressant selection**

	Factors impacting antidepressant selection	N	Choice of practitioners (%)
1.	The character of symptoms, type of depression	128	96.2
2.	Tolerability and safety of the medication	102	76.7
3.	Comorbid physical disorders	92	69.2
4.	Preceding response to treatment	87	65.4
5.	Personal experience of treatment with specific antidepressant	69	51.8
6.	Patient's age	61	45.8
7.	Pharmacodynamic and pharmacokinetic characteristics of medicament	54	40.6
8.	Patient's opinion, motivation to use medication, compliance	20	15
9.	Price of the medication	19	14.3
10.	Sexual dysfunction	18	13.5
11.	Pharmacy concerns' influence	1	0.75

Figure 1. **Antidepressants selection for the treatment of adynamic depression**

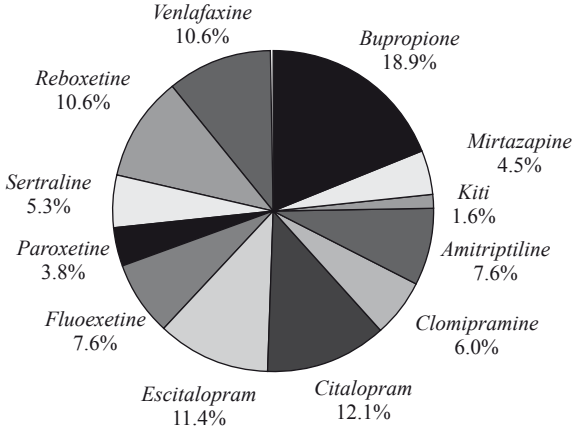
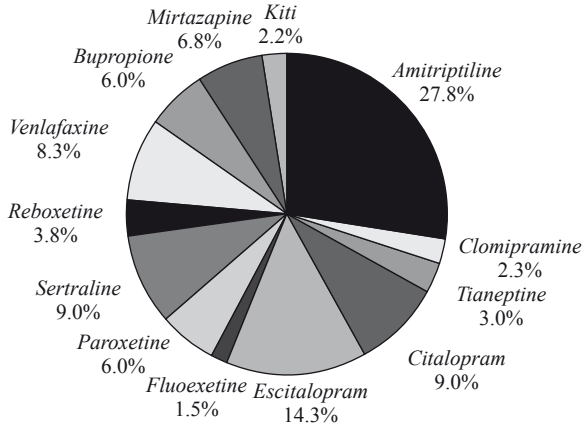


Figure 2. **Antidepressants selection for the treatment of anaesthetic depression**



respondents, one of the most important factors while selecting the antidepressant is the subtype of depression. In many cases (even 96.2%), the specialists noted that the character of depression symptoms has major influence on the selection of antidepressant. During the analysis of the selection of the antidepressant group by the subtype of depression, statistically significant results were retrieved (Monte Carlo χ^2=169, df=20, p<0.001). In authors' opinion, they are quite novel and important.

The factors of tolerance and safety were given the second place (76.7%). Given that the respondents treat a very wide contingent of patients in their practice, this is not strange. Comorbid physical disorders noted in the third place (69.2%) approved this assumption. The patient's opinion, the price of pharmaceuticals and sexual dysfunctions (as the side effect), in turn, were set for as the least significant factors for the selection of antidepressant. The relative costs of the antidepressants in Lithuania are pretty similar compared to one another with the exception of TCA group. These antidepressants are almost 3-4 times cheaper then other. For example, one month course of treating with SSRIs or NaSSA costs about 140-150 litas (40-44 EUR) and with TCA – about 20-60 litas (6-17 EUR). 80 per cent of prices are compensated by Lithuanian government.

Family histories of good medication response or bipolar affective disorder, comorbid psychiatric disorders (such as alcohol abuse, psychosis and others) were not included into the questionnaire. But there was possibility for psychiatrists to write it down in line "other" if the doctor seemed it important for the selection of the antidepressant. There were no notes about that.

In the authors' opinion, the most interesting results are retrieved during the analysis of the respondents' perspectives to treatment of a particular subtype of depression. In many cases, the respondents selected citalopram and escitalopram (23.5%) as well as bupropion (18.9%) for the treatment of adynamic depression (*Fig. 1*). In this particular case, the selection of bupropion is consequent, for its chemical structure is similar to that of amphetamine and stimulators. However, the selection of citalopram and escitalopram is clinically less grounded in the case of adynamic depression.

The anaesthetic subtype of depression was mostly treated with TCA (amitriptyline; 27.8%) or with citalopram and escitalopram (23.3%) (*Fig. 2*). The data about the adequate treatment strategies of this sort of depression is insufficient, which might be influenced by the complicated psychopathological structure of this subtype of depression. It has been proposed to use big or maximum doses of MAOI (this group of antidepressants is not reflected in the results of the study because it is not registered in Lithuania), SSRI, and TCA [19,20].

Many clinical studies discovered that anxiety is prevalent in half or sometimes even more than a half of depressed patients. Anxious depression is a frequent phenomenon and the anxiety as the symptom often influences the selection of antidepressant. Unfortunately, there is no research that demonstrates the advantage of any antidepressants for this large group of patients. On the contrary, several former studies were not successful in proving the difference of the response to various categories of antidepressants. For example, Rush et al. published the articles that disclosed no difference between bupropion and sertraline while treating the depressed patients and assessing the HAM-A scale [21]. Similar results were published by Akkaya et al. after they had conducted a comparative research of venlafaxine XR and reboxetine [22], Versiani et al. after the comparison of fluoxetine and amitriptyline [23]. The respondents' opinion of the selection of antidepressants is quite diverse (*Fig. 3*). One part of the psychiatrists preferred citalopram and escitalopram (34.3%), whereas others chose paroxetine (19.1%), mirtazapin (15.2%) or amitriptyline (15.2%). While in some sources, venlafaxine was chosen by only 3 per cent and sertraline by 8.4 per cent of the respondents. Thus there is no unanimous opinion among the respondents as to what antidepressant is the best one for treatment of anxious depression; however, there is a strong tendency to select SSRI, NaSSA and TCA.

The analysis of the respondents' opinion on the treatment of depression with suicidal tendencies has provided with interesting results. 50.2 per cent of the psychiatrists claimed that they would choose amitriptyline (*Fig. 4*). In such cases, 26.8 per cent of the respondents would choose citalopram and escitalopram. In the authors' opinion, the preference of TCA might be influenced by a strong and quick therapeutical effect, good

***Figure 3*. Antidepressants selection for the treatment of anxious depression**

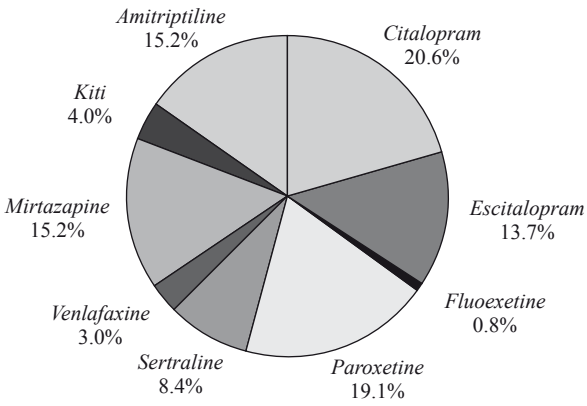
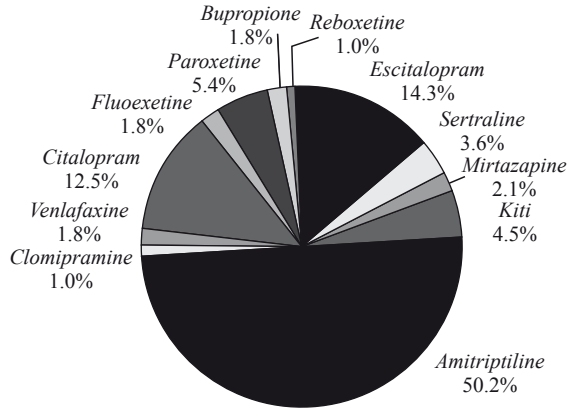


***Figure 4*. Antidepressants selection for the treatment of depression in prevalence of intense suicidal thoughts**



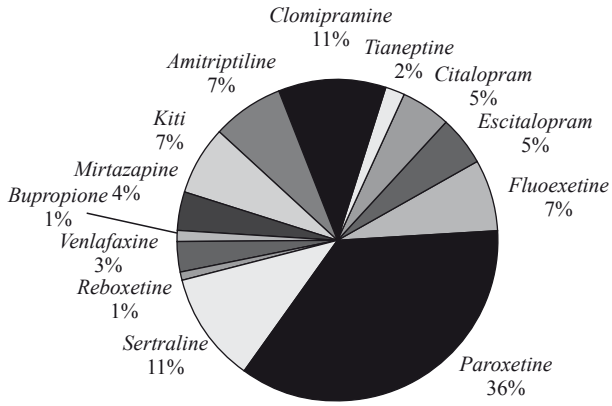
knowledge of pharmaceutical characteristics and a long clinical experience of treatment with TCA. In the case of a life-threatening situation (the danger of suicide), an acute side effect of TCA loses its significance. According to some researchers, the problem lies in that TCA themselves evoke the risk of suicide in case of overdose and are not recommended for the treatment of depression with suicide tendencies. It might be comment as patients with suicidal tendencies mostly are treated in inpatient settings in Lithuania. Suicidal tendency is the one of few cases when forced hospitalization can be considered according Lithuania's law. Treatment with TCA in inpatient department is not so dangerous for case of overdose on prescribed medication.

According to the references, TCA (clomipramine) and SSRI group are the best means for treatment of depression with clear obsessive-compulsive component. Most often did the respondents of the study choose paroxetine (36%), clomipramine (11%), which partly complies with the references of previous sources, and sertraline (11%) (*Fig. 5*). The frequency rate of the selection of other TCA, SSRI, and NaSSA was relatively low.

Thus there have been no clearly defined criteria and recommendations for the treatment of depression that would be coherent with psychopathological structure of depression until now. Although a huge number of specialists would choose the treatment (antidepressant) depending on the psychopathologic structure of depression. It complies with the results of research that have been conducted recently and the articles that turn to the psychopathologic structure of depression and a possibly diverse response to the antidepressants, subject to the subtype of depression more and more often [24].

In this study, the antidepressants that were ranked highly by the psychiatrists partly reflect the pharmacy market. The data of IMS Health Inc database on sales of antidepressants in Lithuania during the period of 2002-2004 shows that the consumption of SSRIs increased by 27.82 per cent and the one of TCA declined by 10.78 per cent; the consumption of other antidepressants increased by nearly three times. The cost of antidepressants increased up to 26 million litas (approximately 10 million US dollars) in the year of 2004; 68.15 per cent of them were intended for the SSRI group. Gladly, the results

***Figure 5*. Antidepressants selection for the treatment of depression with obsessions**



of this study do not reflect any influence from the part of the pharmacy companies on the prescriptions made by the psychiatrists in Lithuania. Only one respondent noted that he takes into consideration the name, authority and the advertisements of a pharmacy company while he selects the antidepressant.

A contemporary policy of health seeks to reduce the number of hospitalized patients is based on a holistic attitude towards the patient and his/her partnership with the physician, whereas the paternalistic model of intercourse is being criticized. Various researches display that partnership between the physician and the patient based on mutual understanding and trust has influence on the results of a therapeutical process. The success of consultation depends on a mutual agreement to the etiology, diagnosis and the way of treatment. The higher the equivalence rate of partnership between the therapist and the patient is, the more the latter will be liable to follow the plan of the treatment [25-27]. During the study, it had been noticed that rarely did the patient's opinion and the partnership while consuming pharmaceuticals have any influence on the psychiatrists' selection. Only 15 per cent of the respondents take into consideration the opinion of the patients. Obviously, in Lithuanian psychiatry

the paternalistic aspect of the therapist – patient model is still strong.

In summary, a glance at the opinion of a part of psychiatrists on the antidepressants and the patterns of their prescription reflects national tendencies of pharmaceutical prescriptions. Often were the SSRI group antidepressants chosen as effective and well tolerated pharmaceuticals. Interestingly, according to studies performed in other states, the priority is given to other antidepressants. For instance, in France, clomipramine, paroxetine and amitriptyline are estimated as the most effective antidepressants and tianeptine, paroxetine and citalopram as well tolerated (28). In the United States, citalopram, bupropion and sertraline are prescribed most often. In some countries, there is an opinion that TCA is already history and the prescription of this pharmaceutical reflects the malpractice and negligence of the psychiatrist [29].

Conclusions

The results of the research disclose that the psychopathological peculiarities of depression can be one of the most important criteria in antidepressant selection. However, in many cases, the subtype of depression is ascertained empirically and based solely on the personal experience and clinical practice of the psychiatrist. There are no clear diagnostic criteria or practical guidelines for the reliable verification of the psychopathological subtype of depression. It has been assumed that it is expedient to maintain scientific research on drawing these guidelines, which would allow for the selection of a more adequate and prompt treatment for the patient.

Limitations and weaknesses

Limitations and weaknesses of the survey have mainly to do with the investigated group was generally inpatient departments' psychiatrists. Also, there were not included all types of depression. We were especially interested in these subtypes of depression with psychopathological anaesthetic, adinamic and anxious structure and different clinical features but with no guidelines on how to treat them. In this survey we discus on Lithuanian psychiatrists' opinion on treatment approach to make it clear if it is need in such practical guidelines that could help or make specialists work easier. That is why we do not discus on ECT and psychotherapy. Moreover, there is only one hospital where ECT is available in Lithuania. Therefore we attach importance to adequate antidepressant selection. We want to admit that psychotherapy is mostly provided by psychologists – psychotherapists in Lithuania.

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Serum concentration of biochemical bone turnover markers in vegetarian children

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Abstract

Purpose: In general, most children on well-planed vegetarian diets can achieve normal growth and development. However, elimination of animal products from the diet decreases the intake of some essential nutrients, such as calcium and vitamin D, and may influence bone metabolism. This is especially important in childhood and adolescence, when growth and bone turnover are most intensive. The aim of this study was to investigate the serum concentrations of biochemical bone turnover markers in prepubertal vegetarian children.

Material and methods: We examined 50 children on vegetarian and 50 on omnivorous diets aged 2-10 years. Dietary constituents were analyzed using a local nutritional program. Serum bone formation (OC, BALP) and resorption (CTX) markers were determined by specific enzyme immunoassays (ELISA) and 25-hydroxyvitamin D by the chemiluminescence method (CLIA).

Results: The average daily energetic value and the percentage of energy from protein, fat and carbohydrates in the diets were similar in both groups of children and were within the recommended range. The vegetarian children showed about a two-fold lower daily intake of calcium and vitamin D than their omnivorous counterparts. The level of 25-hydroxyvitamin D in the serum of vegetarian children was also nearly 2-fold lower compared with omnivores. In vegetarians, as compared to non-vegetarians, mean serum concentrations of OC, BALP and CTX were lower by about 20%, 10% and 15%, respectively.

Conclusion: Our preliminary results suggest that an inadequate dietary intake of calcium and vitamin D may impair bone turnover rate in vegetarian children. The parameters of bone metabolism should be monitored in these children in order to prevent bone abnormalities.

Key words: vegetarian diets, children, bone turnover markers, vitamin D.

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Introduction

Vegetarian diets can be healthy when they are well balanced and if a variety of foods is consumed [1-3]. However, vegetarian diets with exclusion of nutrients from animal foods may influence bone metabolism, especially in childhood and adolescence when growth and bone turnover are most intensive [4-7]. Some authors investigating adult vegetarians have described that decreased calcium and vitamin D intake resulted in lower plasma vitamin D concentration and bone mineral density (BMD) [8-11]. Apart from measuring bone mineral content and density, biochemical bone turnover markers showing global skeletal activity have lately been developed and validated for the assessment of the dynamics of bone formation and resorption processes. Among them, products of the osteoblast activity (osteocalcin – OC, bone alkaline phosphatase – BALP), which are markers of bone formation, and products of osteoclast activity as markers of bone resorption (collagen type I terminal telopeptide – CTX) are considered to be clinically useful [12,13].

Osteocalcin, the major non-collagenous protein, synthetized by osteoblasts plays an important role in the regulation of bone growth and in the correct deposition of the minerals in the matrix. Its expression follows the proliferative phase of osteoblastic differentiation, so it can be considered a marker of mature osteoblasts. Bone alkaline phosphatase located in the plasma membrane of osteoblasts is one of the isoenzymes of alkaline phosphatase which plays an active role in bone formation and

skeletal mineralization. BALP appears in the matrix maturation phase and is a marker of the middle stage of bone formation. During bone resorption, a molecule of collagen is degraded and small fragments are liberated into the blood-stream. The 8-amino acid sequence (Glu-Lys-Ala-His-Asp-Gly-Gly-Arg) found in the C-terminal telopeptide of the α1 chain of type I collagen, which can undergo beta-isomerization, has proven to be a sensitive marker of the bone resorption process.

Serum levels of OC, BALP and CTX are not stable throughout life and are greater in infants and children than in adults. Peak values occur at puberty. Children have significantly elevated bone marker levels due to high skeletal growth velocity and rapid bone turnover during childhood growth [14,15]. Many physiological and pathological processes may influence bone metabolism resulting in changes in serum concentration of bone turnover markers. Measurements of these parameters offer many advantages for investigating skeletal diseases in children and adolescents as well as monitoring the response to treatment. Little is known regarding bone metabolism status in children on vegetarian diets.

The aim of this study was to assess serum concentrations of biochemical bone formation and resorption markers in prepubertal vegetarian children.

Material and methods

We examined 100 healthy prepubertal children with two different nutritional habits (vegetarian and omnivorous diet) who had been referred during wintertime (from November to February) to the Institute of Mother and Child (Warsaw). None of the subjects was receiving any medications or had a history of metabolic bone disease or any serious health problem. The whole group of investigated children was ethnically homogenous. Among them 50 children (23 girls, 27 boys) aged 2-10 years presented at the Department of Nutrition for dietary consultation were vegetarians. In this group there were 28 lacto-ovo-vegetarians (did not consume meat, poultry, fish, but ate eggs and dairy products), 4 lacto-vegetarians (excluded eggs), 5 ovo-vegetarians (excluded milk products, but ate eggs) and 13 vegans (excluded all foods of animal origin).

Healthy children (n=50; 25 girls, 25 boys), range age 2-10 years on an omnivorous diet sent to our laboratory for routine analytical control were the reference group. The study was approved by the Ethics Committee of our institution and informed consent was obtained from parents of the examined children. Dietary constituents (especially calcium, phosphate, vitamin D) and nutrient supplementation data were assessed by questionnaire and calculated using the local nutritional computer program (Dieta2®, National Food and Nutrition Institute, Warsaw).

Venous blood samples were obtained from fasting patients in the morning (8-10). Serum was prepared by centrifugation (1000 g for 15 min at 4°C) and concentrations of calcium and phosphate were determined by colorimetric methods with commercially available kits from Hoffman-La Roche (Switzerland) on Cobas Integra analyzer. Remaining serum samples were frozen at -20°C for the analysis of bone turnover markers and vitamin D within 2 months. Serum OC was analyzed immunoenzymatically using the N-Mid Osteocalcin ELISA kits (Nordic Bioscence Diagnostics, Denmark) which is based on the application of two highly specific monoclonal antibodies against human OC by recognising the midregion (20-29 aa) and the N-terminal region (10-16 aa) of osteocalcin. The sensitivity of this assay is 0.5 µg/L, the intraassay imprecision (CV) – 3.4% and the interassay CV – 6.4%. BALP activity was evaluated by a specific enzyme immunoassay utilising a monoclonal anti-BALP antibody coated on the strip to capture BALP in the sample (Alkphase-B kit, Metra Biosystems, San Diego, USA). The sensitivity of this assay is 0.7 U/L and the intra- and interassay CVs are below 2.7%. Serum CTX was determined using the Serum CrossLaps ELISA kits (Nordic Bioscence Diagnostics, Denmark). This assay is based on monoclonal antibodies that recognise the beta-aspartate isomerized form of the sequence (Glu-Lys-Ala-His-Asp-Gly-Gly-Arg) derived from the C-telopeptide region of the type I collagen alfa1-chain. According to the manufacturer, the intra- and interassay imprecision (CVs) are less than 8.1% and the lower detection limit is 0.9 ng/mL. Serum 25-hydroxyvitamin D was determined by chemiluminescence immunoassay (CLIA) using (DiaSorin kits, Stillwater, USA). During the incubation, 25-OH vitamin D is dissociated from its binding protein, and competes with labelled vitamin D for binding sites on the antibody. The detection limit of the assay is 7.0 ng/mL, the intra- and interassays CVs are 6.4% and 3.4%.

All data were presented as mean value ± standard deviation (SD). The Statistica (version 6.0) computer software was used for statistical analysis. The differences were regarded as statistically significant at $p<0.05$.

Results

Vegetarian children were in the same age and had similar average Body Mass Index (BMI) 15.6±1.4 kg/m^2 as their omnivorous counterparts 16.0±1.3 kg/m^2. Mean daily energy intake and the percentage of energy from protein, fat and from carbohydrates were within the reference range and similar in both groups (*Tab. 1*). The daily intakes of calcium and phosphate of omnivorous children were in the recommended range [16]. In vegetarian children the intake of phosphate was adequate and calcium was below the recommended range. Dietary intake of vitamin D in both groups of the tested children was very low in according to the recommendations. In vegetarian children vitamin D intake was about two-fold lower than in omnivores ($p<0.001$).

Concentrations of calcium and phosphate in serum were in the physiological range in all tested children, but the 25-OH vitamin D level in vegetarians was two-fold lower as compared to that in non-vegetarians ($p<0.0001$) (*Tab. 2*). The mean serum levels of all measured biochemical bone turnover markers were significantly lower (OC by about 20%, BALP – 10% and CTX – 15%) in vegetarian children in comparison with omnivores.

***Table 1*. Average daily energy and nutrients intake of examined children compared to recommended daily intake**

	Vegetarian children	Omnivorous children	Recommended daily intake*
Energy values (kcal)	1 468±409	1 591±305	1 400-1 700
Energy from protein (%)	13.1±2.5	14.2±3.1	12.0-14.0
Energy from fat (%)	30.1±5.9	30.5±6.5	32.0
Energy from carbohydrates (%)	56.2±5.9	55.3±7.1	56.0-58.0
Dietary Ca (mg)	559±345	821±335*	800-1 000
Dietary P (mg)	910±337	949±294	800-1 000
Dietary vitamin D (µg)	1.37±1.10	2.43±1.38*	10.0

Data are shown as mean values ± SD, * p<0.001

* Szotowa W, Socha J, Charzewska J, Dłużniewska K, Jabłoński E, Kunachowicz H, Rudzka-Kańtoch Z, Ryżko J, Stolarczyk A, Weker H. Dzienne zalecenia żywieniowe dla dzieci i młodzieży. Ped Pol, 1995; 1: 1-12.

***Table 2*. Serum calcium, phosphate, vitamin D and bone turnover marker concentrations in vegetarian and omnivorous children**

	Vegetarian children	Omnivorous children
Ca (mmol/L)	2.35±0.12	2.39±0.15
P (mmol/L)	1.60±0.19	1.73±0.17
25 OH vitamin D (ng/mL)	13.9±7.2	29.8±4.7***
OC (µg /L)	71.0±20.1	88.9±17.5**
BALP (U/L)	94.5±21.5	104.5±28.4*
CTX (ng/L)	1 697±653	1 993±300*

Data are shown as mean values ±SD; * p<0.01, ** p<0.001, *** p<0.0001

Discussion

Adequate and appropriate nutrition is important for all individuals, but not all follow a diet that is optimal for bone health. Calcium and vitamin D are the specific nutrients most important for attaining peak bone mass and for preventing osteoporosis [17]. Besides the amount of calcium in the diet, its absorption is also a critical factor in determining the availability of calcium for bone development and maintenance. Vegetarians who consume milk products have intakes of calcium as high as those of omnivores, but strict vegetarians are at risk of calcium and vitamin D deficiency [8]. In vegan diets care must be taken to choose plant foods that are high in calcium, another option is calcium-fortified food or supplementation.

In our vegetarian children the daily intakes of calcium and vitamin D were below the recommended values. Serum concentrations of calcium in both groups of children were within the physiological range but vitamin D was two-fold lower in vegetarians. Childhood and adolescence are very important periods for bone metabolism because most of the peak bone mass is accumulated during these years. Deficiencies in some of the nutrient components (calcium, vitamin D) together with reduced serum concentration of vitamin D may retard relevant bone growth and development. Research data available show that bone mineral density of the lacto-ovo-vegetarian children was comparable to the general omnivorous population [18]. However, adolescents who consumed a vegan diet in early life, demonstrated a lower relative bone mass than their omnivorous counterparts [19]. Data from the existing literature regarding adult individuals on vegetarian diets (especially vegans) suggest that low BMD at clinically important skeletal regions was present [20]. According to other authors normal bone mass in vegetarians, especially in lacto-ovo-vegetarians was also observed [21].

To our knowledge there are only two reports presenting values of biochemical bone turnover markers in vegetarian subjects. Fontana et al. [20] in their study observed similar levels of CTX and BALP between adult omnivores and 18 vegetarians (54.2±11.5 years) consuming their respective diet for 3.6 years on average. In adolescent vegetarians (aged 9-15 years) Parsons et al. [22] found also similar values of bone metabolism parameters as in omnivores. There are no studies regarding bone metabolism markers in prepubertal children on vegetarian diets. In our study we detected significantly reduced concentrations (by about 10-20%) of serum bone metabolism markers in vegetarians compared with omnivores. The examined vegetarian children were on different kinds of diet, but mean values of bone turnover markers for the vegans and lacto-ovo-vegetarians were not significantly different.

In conclusion, our results suggest that an inadequate dietary intake of calcium and vitamin D may impair bone turnover rate in vegetarian children. Further studies of bone metabolism in children following vegetarian diets with vitamin and mineral supplementation are needed; this data may assist in deciding whether supplementation is useful and help to prevent bone abnormalities in their later life.

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Intensive care unit environment contamination with fungi

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Abstract

Purpose: The purpose of the study was evaluation of the fungal presence in the environment of an intensive care unit.

Material and methods: The environment testing was carried out at a chest clinic intensive care unit in Cracow, in December 2004. The materials to mycological examinations were sampled simultaneously from indoor air and room walls in 15 rooms: air samples twice daily while samples from the walls once daily, for five days. The findings were processed statistically. The t-test (Student) and F-test (Snedecor) were used. The border value of significance was 0.05.

Results: No fungi were found in 6 air samples out of 150 taken in 15 rooms. The mean number of fungi in the particular rooms in the whole sampling period varied from 172 to12 c.f.u.×m^{-3}. Out of 75 samples from the walls, fungi were present only in 19 of them. The mean numbers varied from 0 to 0.37 c.f.u.×cm^{-2}. The moulds *Aspergillus* sp., *Penicillium* sp. and *Cladosporium* sp. as well as yeast-like fungi *Rhodotorula rubra*, *Candida* sp. were most frequently isolated from the indoor air and the walls.

Conclusion: Significant difference between the numbers of fungi sampled in the morning vs in the evening occurred on the first, third and fourth days of sampling ($p<0.001$). Yeast-like fungi *Rhodotorula rubra* and moulds *Aspergillus* sp., *Cladosporium* sp., *Penicillium* sp. were isolated from indoor air in all of the rooms tested.

Key words: fungi, indoor air, intensive care unit.

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Introduction

Nosocomial infections are a serious medical problem, particularly in intensive care units. Even though the percentage of patients there does not exceed 10% of all of the hospitalisations, nosocomial infections comprise 25 per cent of the total number of such infections [1]. A high correlation is reported between the prevalence of infection and the duration of hospitalisation. There is evidence that 80% of nosocomial infections are transmitted through the medical staff's hands [2-4].

A dramatic increase in the prevalence of fungal infections was observed in the recent years. Opportunistic fungal infections lead to considerable increase in mortality rate at invasive treatment wards. Particularly, systemic mycoses are considered a cause of death in 88% of the cases [5]. The aetiological profile of fungal infections is changing: fungal species, formerly considered as harmless, increase their virulence. This results from the resistance of fungal strains to numerous antifungals, prolonged antimicrobial treatment, but also from poor general condition of the patients, caused by the underlying disease [6-8].

An epidemiological study performed by Kao et al. [9] gives evidence that candidaemia occurred in eight patients out of 100 000 people population yearly. In 19% of the patients, candidaemia developed before or on the day of hospital admittance. *Candida species* other than *C. albicans* were detected in 47% of the cases: *C. parapsilosis* 21%, *C. glabrata* 12%, *C. tropicalis* 10%, and *C. krusei* 4%.

Unlike usually endogenous invasive candidiasis, invasive aspergillosis is exogenous. Abundant sporulation, tiny size of the spores ubiquitous in the environment and their ability to survive in a wide range of temperatures enable them to reach pulmonary alveoli [2,10-13]. Nosocomial *Aspergillus* infections may be serious problem at hospital wards with patients suffering from neutropenia if construction works are done near of them [14]. In such circumstances, there is a high density of mould spores in the indoor air, particularly *Aspergillus*.

The objective of the study was evaluation of the fungal presence in the environment of an intensive care unit.

Material and methods

The environment testing was carried out at a chest clinic intensive care unit in Cracow, Poland, in December 2004. The materials to mycological examinations were sampled simultaneously from indoor air and room walls in 15 rooms: four bays, a treatment room, three bathrooms, a nurse's station, a doctor's room, a rest room, a corridor, a dirty annex, a ward kitchen and a washing room. The air samples were taken twice daily and the samples from the walls once daily, for five days.

To evaluate the presence of fungi in the indoor air, 150 air samples were colleted using aspiration method by means of a MAS 100 (Merck) device in 15 rooms of the ward.

Two hundred litres of indoor air were sampled in each of the rooms at a time. For this purpose, the sampling device was positioned in the middle of the room, 0.5 m above the floor. The windows and the doors of the room were closed during the sampling period. The air was aspirated to a Petri dish with Sabouraud Glucose Selective Agar medium, with gentamicin an chloramphenicol (manufactured by Oxoid Company). The antibiotics were added to prevent bacterial growth on the medium. Petri dishec with the material aspirate were then incubated at 27°C. The cultures were inspected, the fungal colonies were counted, and their morphology was evaluated after three days of incubation. The period of incubation depended on the fungal genus detected but id did not exceed fourteen days. After the incubation period, the real number of the fungal colonies was obtained using a statistical calculation table for the MAS 100, and then the number of fungi in one cubic metre was calculated using a formula:

$$X = \frac{a \times 1000}{V}$$

where a – the number of fungal colonies grown from the indoor air sample; V – the volume of the air sample aspirated (litres); and X – the number of fungi in one cubic metre of the air expressed in terms of the number of colony forming units in one cubic metre (c.f.u./m^{-3}).

The presence of fungi was also evaluated on the walls of the rooms, in which the air was tested. A total of 75 samples was taken using a Count-Tact technique (bioMerieux). The imprints on the plates with Sabouraud glucose medium with chloramphenicol were taken using a bioMèrieux applicator from a dry wall surface 1.5 m above the floor. In the rooms inhabited by mothers with children, the samples were taken above the mother's bed. The plates with the material samples were incubated at 37°C for three days and then moved to a thermostat with 27°C. The number of colonies on the plate was then counted and the number of fungi on one square centimetre of the wall surface was calculated using a formula:

$$X = \frac{a}{\pi r^2}$$

where a – the number of fungi on the imprint plate; r – the diameter of the plate in cm; and X – the number of colony forming units per one square centimetre of the wall (c.f.u./cm^2).

The fungal colonies grown in the cultures were counted according to the accepted standards and identified using procedures accepted in mycology. Moulds were evaluated macroscopically an microscopically on the basis of their appearance in the culture as well as their morphological features in direct preparations stained with lactophenol and methylene blue (Merck). When the evaluation of a preparation was doubtful, a slide microculture was made for further identification. The yeast-like fungi were Gram-stained and cultured on starvation media. Photographs were taken of the macroscopical appearance of the fungi.

The findings were processed statistically. The t-test (Student) and F-test (Snedecor) were used. The border value of significance was 0.05.

Results

Out of 150 air samples taken in the rooms, only 6 did not contain fungi. During the morning sampling, no fungi were isolated in the bay № 4 on the first day of testing, in the bays № 1 and № 2 on the second day, and in the treatment room on the fourth day. During the evening sampling, no fungi were detected in the indoor air in the bay № 4 and in the nurses' room on the first day of sampling.

The highest number of fungi (720 c.f.u.×m^{-3}) was detected in bay № 2 on the third day of testing. The remaining numbers of fungi sampled in the bays did not exceed 70 c.f.u.×m^{-3}, i.e. did not exceed Polish standards for the bays.

In other rooms such as corridors, bathrooms, rest room and kitchen, higher number of fungi were sampled in evening. The results are shown in *Fig. 1*.

The average number of fungi in one cubic metre of air within the whole testing period varied between 172 c.f.u.×m^{-3} in the bay № 2 to 12 c.f.u.×m^{-3} in the bay № 4. The comparative analysis using the F test revealed significant differences ($p<0.001$) between the numbers of fungi sampled in the morning vs. in the evening on the first, third and fourth days of sampling which is shown in *Fig. 2*.

Out of 75 samples taken from the walls, fungi were present in 19 of them: their mean number varied between 0 and 0.37 c.f.u.×cm^{-2}. No fungi were found on the walls in the treatment room, bays № 2 and № 3, nurse's station and bathroom № 2. (*Fig. 3*).

The most abundant fungi in the rooms tested were moulds: *Aspergillus* sp., *Penicillium* sp. and *Cladosporium* sp. Those fungal genera were found in each of the rooms tested during the entire testing period. The dominating species of yeast-like fungi, present in all of the rooms was *Rhodotorula rubra*, while *Candida* sp. was also frequent. Fungi belonging to this genus were present in the indoor air in four rooms; three of them were inhabited by the patients (*Tab. 1*).

Discussion

According to Polish guidelines there are three classes of hospital wards cleanness. The intensive care units are classi-

Figure 1. **Numbers of fungi c.f.u.×m^{-3} isolated from the indoor air in the morning and evening in the rooms during entire assay period**

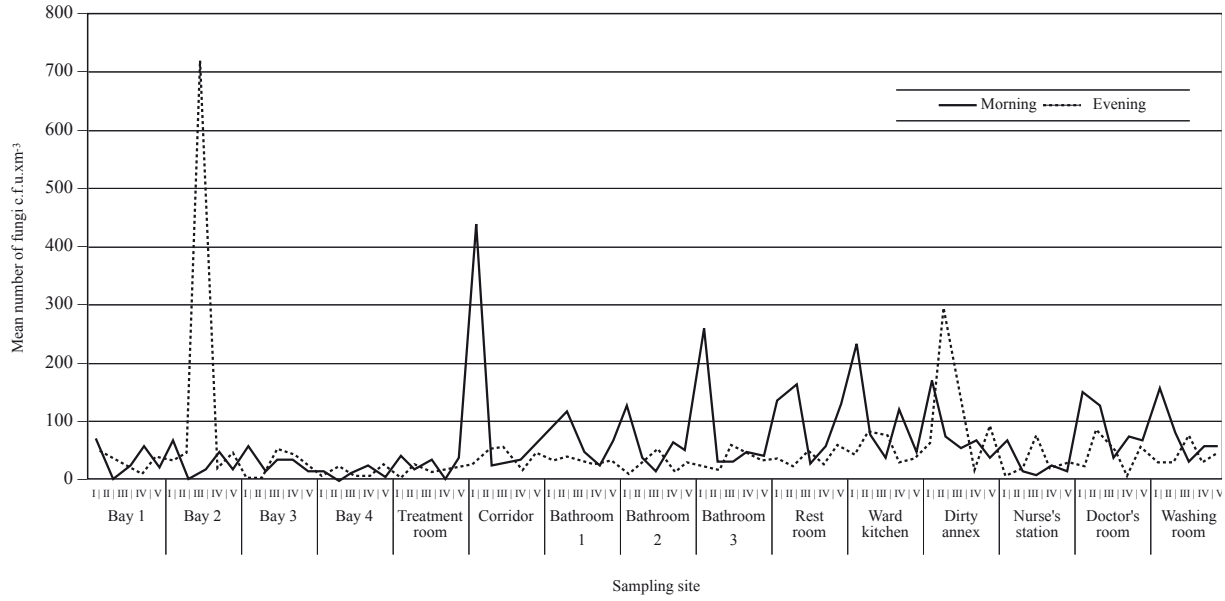

Figure 2. **Medians of the mean numbers of fungi isolated from the indoor air of the rooms tested**

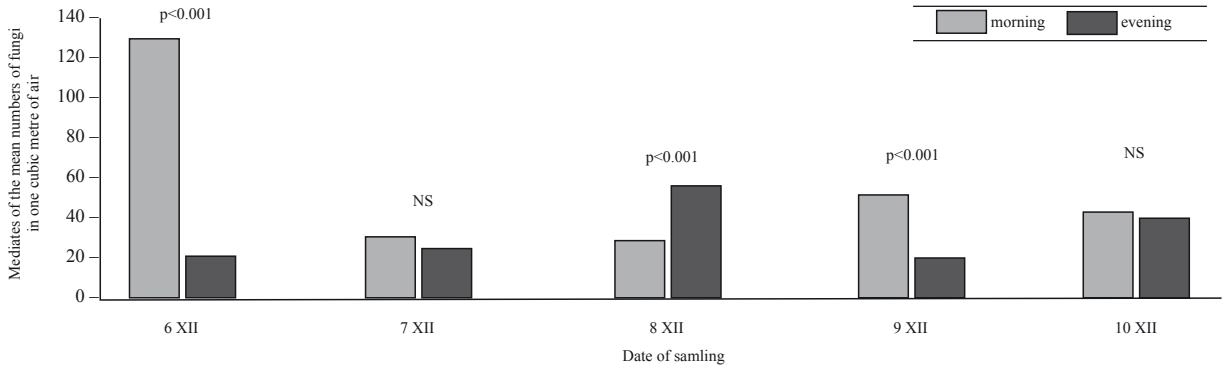

Figure 3. **Numbers of fungi isolated from the walls during the entire testing period**

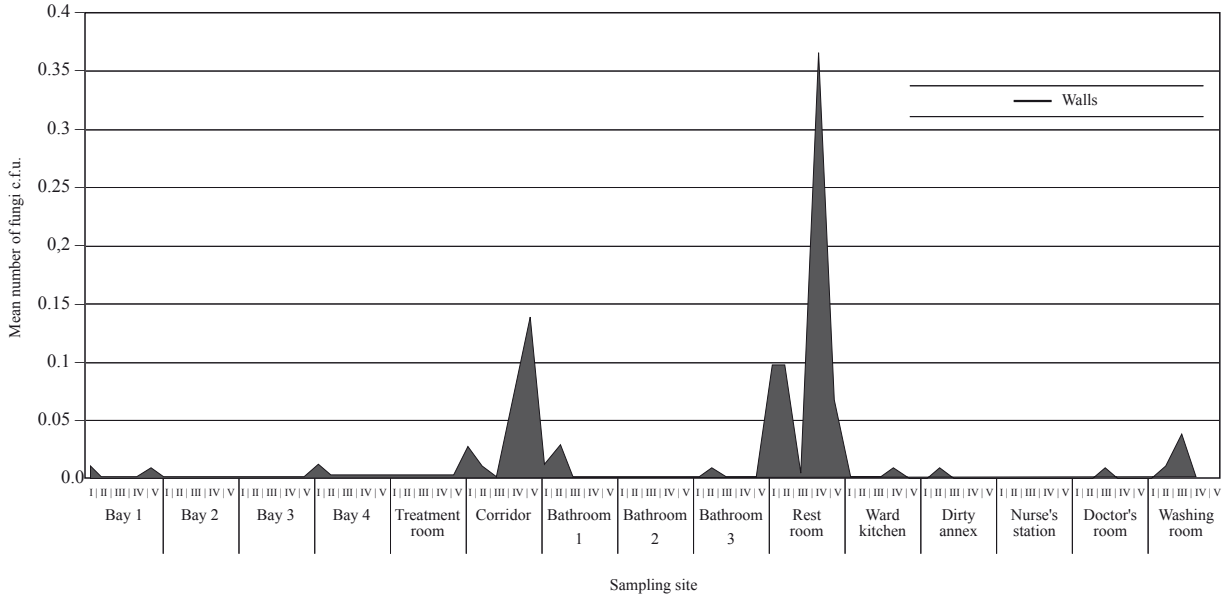

***Table 1*. Genera and species of the fungi isolated from the indoor air of the rooms during the entire testing period**

Genera and species of the fungi isolated from the indoor air	Room number														
	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Candida sp.	+	-	+	+	-	-	-	-	-	-	-	-	-	-	+
Rhodotorula rubra	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Penicillium sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Cladosporium sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Aspergillus sp.	+	+	+	+	+	+	+	+	+	+	+	+	+	+	+
Botritis sp.	-	-	-	-	+	+	-	+	-	-	+	-	-	+	+
Stachybotrys sp.	-	-	-	-	-	-	+	+	-	-	-	-	+	+	+
Acremonium sp.	-	-	-	-	-	-	-	-	-	-	-	-	-	+	+
Alternaria sp.	-	-	-	-	-	+	-	-	+	-	-	+	-	-	-
Rhizopus sp.	-	-	-	-	-	-	-	-	-	+	-	-	-	-	+
Mucor sp.	-	-	-	-	-	+	-	-	-	+	-	-	-	-	-
Other moulds	+	+	+	-	+	+	+	+	+	-	+	+	+	-	+

1, 2, 3, 4 – bays, 5 – treatment room, 6 – corridor, 7-9 – bathrooms, 10 – rest room, 11 – ward kitchen, 12 – dirty annex, 13 – nurse's station, 14 – doctors' room, 15 – equipment washing room

fied as class 2 which means presence of microorganisms not exceeding 300 colony forming units in one cubic metre (300 c.f.u.×m^{-3}) of indoor air [15]. The number of c.f.u.×m^{-3} allowed in operating theatres is 0 while in treatment rooms – 50. In other hospital rooms – 200 c.f.u.×m^{-3} [16]. In this study, the average number of fungi varied from 12 to 172 c.f.u.×m^{-3}, i.e. did not exceed the class 2 of cleanness.

In the present study, lower numbers of fungi were observed in the evening vs the morning sampling. Detailed comparative statistical analysis with the F-test for two samples showed ($p<0.001$) that the above phenomenon occurred on the first, third and fourth days of the measurements. The findings are consistent with those obtained by the investigators in Cracow and Białystok [14,17-19]. The decrease of the number of fungi in the evening might have been caused by ventilation of the rooms and/or lower number of people present in the rooms by day.

Even though *Candida albicans* is still the most frequent fungal pathogen at intensive care units, an increase of infections caused by other than *C. albicans Candida* species is observed. Particularly dangerous and hard to treat are the strains *Candida parapsilosis*, *Candida glabrata* and *Candida tropicalis*. Those fungal species are often resistant to azole antifungals [1,2,4,7].

As a rule, candidiasis is an endogenous infection, however, exogenous infections are also possible. Numerous studies give evidence that *Candida* fungi are detected in the indoor air at, e.g., surgical, haematological and obstetric wards; they are a potential source of infections, especially in risk group patients [5,9,11-13,20-22]. In the present study, *Candida* fungi were isolated from indoor air and from the walls in four rooms out of fifteen tested at the intensive care unit. Three of the rooms were bays.

The role of infections caused by the moulds *Mucor* and *Rhizopus* in severely ill patients at intensive care units is increasing. The fungi may invade patients with inhaled air as well as through the equipment used in diagnostics and care of the patients.

In a study carried out at an intensive care unit in Spain, a gastrointestinal tract zygomycosis was caused by *Rhizopus microsporus*. The infection was transferred through spatulas used by the medical staff. Zygomycosis was a complication of the underlying disease and contributed to an increase in mortality [22]. In our study, *Mucor* and *Rhizopus* were isolated only from indoor air in corridor and washing room, and from the walls in a rest room.

Moulds belonging to the *Botrytis* genus play a role in hypersensitivity reactions and may cause allergic alveolitis, the so-called vineyard worker's lung. The presence of those fungi in the indoor air may lead to allergy [3].

In our study, moulds belonging to the *Botrytis* genus were found in several rooms: in the treatment room, doctors' room, bathroom №1, corridor and kitchen. Probably, the moulds were brought to the ward with contaminated grapes. Similar contamination was detected in other our study at a invasive diagnostics ward in chest clinic, where this fungal species was isolated in six rooms [18].

The mortality in systemic aspergillosis is high as compared with other systemic mycoses. Most often, infection with *Aspergillus* occurs *via* inhalation. The tiny spores readily invade upper and lower airways and may produce lung aspergillosis in risk group patients [11-13,23].

In France, a systemic *Aspergillus fumigatus* infection occurred in a patient eleven days after liver transplantation. At the same time, lung aspergillosis caused by the same fungal species was detected in two patients at an intensive care unit [20]. Large amounts of *Aspergillus* were isolated in all of the rooms at the intensive care unit.

It is not surprising that large amounts of *Aspergillus* were found in the indoor air tested because that fungal genus is ubiquitous. Even though it is harmless for healthy people, it may be dangerous for the patients of risk groups, including those treated in surgical wards and intensive care units. Therefore, it appears, that indoor air monitoring focused on the presence of fungi is an important procedure in wards where risk group patients are treated. Such a procedure should be routine in hospitals, and particularly at intensive care units.

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Analysis of the upper gastrointestinal tract bleeding prevalence in patients treated due ischaemic heart disease

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Abstract

Purpose: The analysis concerning the frequency of bleedings from the upper part of the gastrointestinal tract in group of patients suffering from ischaemic heart disease (IHD) treated and not treated with coronaroplasty. The other aim of the study was to analyse the incidence of using particular groups of drugs.

Material and methods: 150 patients were included in the study, aged from 17 to 86. They were divided into three groups: I group – patients treated with coronaroplasty (n=50), II group – patients who were not treated with coronaroplasty (n=50), III control group (n=50). The patients filled in a questionnaire (among other things the questions concerned methods and period of treating heart ischaemia and stomach complaints. The documentation of the keyhole examinations of the upper part of the digestive tract was analysed in flashback).

Results: Bleeding from the upper part of the gastrointestinal tract (only single episode) was noticed in 5 patients (treated with IHD) (3.33%). All these patients belonged to the group II. Endoscopic examination of the upper part of gastrointestinal tract was carried out in 4 of these patients and haemorrhagic gastritis has been found.

The following drugs were more frequently used in patients treated with coronaroplasty: acetylsalicylic acid, clopidogrel, ticlopidine. Acenocoumarol was more frequently used in patients not treated with coronaroplasty. The differences were not significant and concerned the usage frequency of the following drugs: beta-blockers, calcium canal blockers, ACE, systemic nitrates and statins.

Conclusions: Bleeding from the upper part of gastrointestinal tract occurred more frequently among patients not treated with coronaroplasty. The following drugs were used more frequently in the group of patients treated with coronaroplasty: acetylsalicylic acid, ticlopidine and clopidogrel, but acenocoumarol was used more frequently in the group of patients treated only pharmacologically.

Key words: ischaemic heart disease, gastrointestinal bleeding, coronaroplasty.

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Introduction

Bleedings from the upper part of gastrointestinal tract are the most popular life-threatening emergencies in gastroenterology. It has been estimated that such complications occur in 1/1000 inhabitants a year [1,2]. The mortality rate due to bleedings from the gastric and duodenal ulcers was 10% [3]. Risk of bleeding concerning patients with chronic peptic ulcer disease, not treated with drug inhibiting hydrochloric acid secretion, was 2-3% [2]. According to other reports, 50% of bleedings from the gastrointestinal tract were caused by chronic peptic ulcer disease [4].

There are the following causes of bleeding from the upper part of gastrointestinal tract: gastric or duodenal ulcer – 30-45%, erosive or haemorrhagic gastritis – 10-20%, oesophageal varices 10-30%, Mallory-Weiss syndrome – 5-15%, non-malignant gastric tumours, blood vessels diseases, malignant gastric tumours, oesophagitis, haemorrhagic diathesis, others (mechanical injuries, aorta-jejunal fistula, stress ulcer) [5].

The majority of bleedings requires endoscopic treatment due to the fact that process of haemostasis is disturbed in the acid environment. After vessel injury, first of all the platelets start to adhere to collagen of the endothelium basement membrane, the platelets aggregate and form the platelet plugs that provide haemostasis for several hours till the fibrin formation. The platelets

***Table 1*. The analysis of sex, place of residence, age, weight of patients and period of treating the heart ischaemic disease**

Characteristic		Group I n/%	Group II n/%	Group III n/%	Statistical significance
sex	male	40/80	33/66	28/56	p=0.037 (IS)
	female	10/20	17/34	22/44	
place of residence	city	35/70	43/86	42/84	p=0.93 (NS)
	village	15/30	7/14	8/16	
age	medium	62.9	64.1	51.7	p=0.001 (WIS)
	median	61.5	61.5	55	
	minimum	39	47	17	
	maximum	80	82	86	
BMI	medium	26.6	28.3	25.8	p=0.02675 (IS)
	median	27.0	27.9	25.5	
	minimum	18.4	18.3	18.2	
	maximum	39.2	40.4	40.6	
period of treating (months)	medium	58.4	68.6		p=0.5061 (NS)
	median	36	36		
	minimum	1	1		
	maximum	396	312		

aggregation is reduced by half in the pH environment equal to 6.4, but it is abolished in the pH equal to 5.4. There are frequent recurrences in the case of spontaneous bleeding arrest. Pepsin is reactivated at the pH level <4 and as a proteolytic enzyme it initiates clot dissolving [2].

Treatment is divided into an endoscopic and pharmacological one. There are the following endoscopic methods for the treatment of bleeding from the upper part of gastrointestinal tract: the injection methods (using isotonic saline solution, adrenaline, sclerosing agents, agents increasing clotting capability, tissue glues), the thermal methods (electrocoagulation, thermal probe, microwave coagulation, argon laser), the mechanical methods (rubber bands, clips) and the combination of above mentioned methods. Pharmacological treatment includes drugs inhibiting hydrochloric acid secretion in the stomach (proton pump inhibitors) and prostaglandins analogues (i.e. misoprostol) [5].

The following cardiologic drugs are the most frequent cause of bleedings from the gastrointestinal tract:

a) heparin – bleeding is caused by the excessive blockage of fibrin formation and inhibition of proper haemostasis. This symptom depends on the dose, patient haemostatic response, administration method and other factors connected with a patient clinical state. It was stated that the bleeding frequency increases together with the increase of heparin dose and administration method [6]. Heparin can also cause thrombocytopenia and concomitant arterial thrombosis due to the aggregating platelets. This process is supposed to be evoked by immunological complexes IgG-heparin, but venous thrombosis can be a result of heparin neutralisation by the factor 4 that is released from the aggregating platelets during the treatment with this preparation [6];

b) derivative of coumarin – bleeding is caused by a decrease of vitamin K dependent clotting factors. Predisposing factors for bleeding occurrence are also diseases that affect decrease of above mentioned factors (intestinal malabsorption syndrome, hypermetabolic states, liver damage, alcoholism, renal diseases, haemorrhagic diathesis) and also gastric and duodenal ulcer disease and surgical procedures [6];

c) acetylsalicylic acid – bleeding, beside complications connected with chronic peptic ulcer disease, can be caused by an inhibition of the platelets aggregation and by diminished synthesis of clotting factors VII and IX [7]. Acetylsalicylic acid as an acid substance becomes the non-ionised form in the acid environment, more easily penetrating through the mucus, which passes through phospholipid cell membrane into the much more alcalic inside of the stomach mucosa cells. Acetylsalicylic acid is accumulated there, inhibiting cyclooxygenase 1 (COX1) and by this way inhibiting prostaglandins and prostacyclins production (gastric and duodenal mucosa protective factors) [4].

The aim of the study was to perform the analysis concerning the frequency of bleeding from the upper part of the gastrointestinal tract in the group of patients suffering from ischaemic heart disease (IHD) treated and not treated with coronaroplasty, in comparison with the control group. The other aim of the study was to analyse the incidence of using particular groups of drugs in investigated groups.

Material and methods

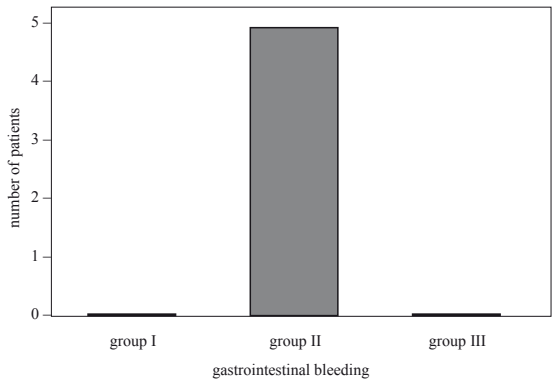
One hundred fifty patients were included into the study, aged from 17 to 86 years. Patients were treated in the Chair and the Department of Cardiology and Internal Diseases of the Ludwik Rydygier Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń and in the Clinical Ward of Cardiology and Cardiosurgery of the Military Clinical Hospital No10 in Bydgoszcz.

There were 100 patients with diagnosed IHD and 50 of them belonged to the comparative group with other cardiological problems (patients treated due to arterial hypertension, arrhythmias, patients diagnosed in a case of fainting or syn-

***Table 2*. Analysis of bleedings from the upper part of the gastrointestinal tract in particular groups**

	Group			Total	Statistical significance		
	I n/%	II n/%	III n/%	n/%	group I,II,III	group I and II and also control group III	group I and II
bleeding from the upper part of the gastrointestinal tract	0/0	5/10	0/0	5/3,33	p=0.0057 (WIS)	p=0.1078 (NS)	p=0.0218 (IS)
Number of treating	50	50	50	150			

***Figure 1*. Analysis of bleedings from the upper part of the gastrointestinal tract in particular groups**



cope). Patients were divided into three groups: I – patients with diagnosed IHD, treated with coronaroplasty and pharmacologically – 50 patients, II – patients with diagnosed IHD, not treated with coronaroplasty but they were treated pharmacologically – 50 patients, III – patients constituting comparative group – 50 patients.

Results were analysed statistically using SAS/Statistica. (The importance of differences between numbers in particular subgroups were verified by means of a test χ^2 on levels $p \leq 0.05$ (IS) and $p \leq 0.01$ (WIS). Due to the expected low numbers the Ystes correction was made and non-parametric variances of the analysis of Kruskal Wallis alternation (age, period of disease). The single-factor analysis of alternation and tests post hoc Sheffe (BMI) [8] were used for the analysis of three groups (I, II, III).

Results

There were 67% of males and 33% of females in the investigated group of 150 patients. Eighty percent of patients lived in the town and 20% lived in the country. The average age in the studied group was 59.6 years (from 17 to 86 years). The average BMI value was 26.9 (from 18.2 to 40.6), but the average duration of treatment on account of ischaemic heart disease was 42.3 months (from 1 to 396 months). Detailed analysis is shown in *Tab. 1*.

Bleeding from the upper part of the gastrointestinal tract, was found in 5/150 patients (3.33%). All bleeding patients belonged to the group II. Endoscopic examination of the upper part of the gastrointestinal tract was performed in 4 patients (one patient did not agree to this examination) and haemorrhagic gastritis was diagnosed.

The statistically higher incidence of bleeding from the upper part of the gastrointestinal tract was proved to occur among patients from group II, comparing all groups (p=0.0057), but also group I and II of patients who underwent or not underwent coronaroplasty (p=0.0218). Detailed analysis is shown in *Tab. 2* and in *Fig. 1*.

It was proved that the following drugs were statistically more frequently used in patients treated with coronaroplasty: acetylsalicylic acid, clopidogrel, ticlopidine.

Acetylsalicylic acid (ASA) was used in group I in 100% of patients, but in 84% of patients in group II (p=0.0099). The reason for renunciation of using ASA among patients from the group II was allergy or this drug as withdrawn in two patients due to chronic peptic ulcer disease.

Clopidogrel was used in 64% of the patients treated with coronaroplasty (in 8% of patients from the group II) (p=0.000), but ticlopidine in 56% of those patients (in 28% of patients from the group II) (p=0.0046). Acenocoumarol was statistically significantly more frequently used in patients not treated with coronaroplasty – in 20% (in 4% of patients from the group I) (p=0.0138). The fibrinolytic treatment was statistically significantly more frequently applied in patients not treated with coronaroplasty – 38%, but in patients treated using invasive methods – 16% (p=0.0132).

Some of investigated patients (group I i II) were treated due to ischaemic heart disease up to several years (from 1 to 396 months). During this time some of them suffered from myocardial infarctions, treated with fibrynolysis, independently of the subsequent treatment with coronaroplasty or without coronaroplasty.

The statistically significant differences were not proved, analysing the frequency of applying such drugs as beta-blockers, calcium canal blockers, angiotensin II convertase inhibitors (ACE), systemic nitrates and statins, but beta-blockers were observed to be more frequently used in group II and the remaining drugs were more frequently used in group I (p>0.05). However the difference was not statistically significantly. Detailed analysis is showed in *Tab. 2* and in *Fig. 1*.

Discussion

Ischaemic heart disease (IHD), but particularly myocardial infarction is connected with the necessity for drugs administration for many years. Our own studies revealed bleeding from the upper part of the gastrointestinal tract in 5 patients (treated due to IHD) (5%). All patients belonged to the group II. Endoscopic examination was performed in 4 patients (one patient did not agree to be examined) and haemorrhagic gastritis was

***Table 3*. Analysis concerning the incidence of particular drugs groups application in patients treated (group I) and not treated with coronaroplasty (group II)**

Drugs group	Group		Total	Statistical significance
	I n/%	II n/%	n/%	
acetylsalicylic acid	50/100	42/84	92/92	p=0.0099 (WIS)
clopidogrel	32/64	4/8	36/36	p=0.0000 (WIS)
ticlopidine	28/56	14/28	42/42	p=0.0046 (WIS)
acenocoumarol	2/4	10/20	12/12	p=0.0138 (IS)
beta-blocker	36/72	41/82	77/77	p=0.2346 (NS)
Ca-canal blocker	7/14	3/6	10/10	p=0.3173 (NS)
ACE inhibitor	45/90	39/78	84/84	p=0.1017 (NS)
nitroglycerin	12/24	11/22	23/23	p=0.8122 (NS)
statins	34/68	28/56	62/62	p=0.2164 (NS)
streptokinase and/or heparin	8/16	19/38	27/27	p=0.0132 (IS)
number of patients	50	50	100	

stated. Our own studies also proved that drugs as acetylsalicylic acid, clopidogrel, ticlopidine, were statistically significantly more frequently used in patients treated with coronaroplasty. Acetylsalicylic acid was used in group I in 100% of patients, but in 84% of patients in group II.

Our results are in accordance with other authors' reports. According to Jayaprakash et al. [9], 90% of patients suffering from ischaemic heart disease have taken aspirin.

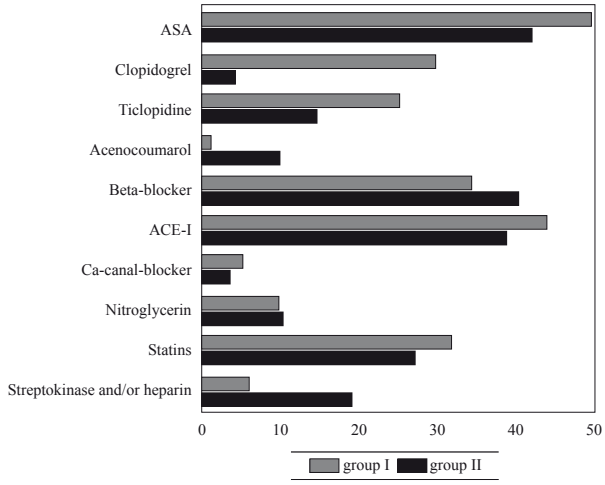
Six percent of patients from our studied group were forced to stop treatment with acetylsalicylic acid (allergy or chronic peptic ulcer disease).

According to Reguła et al. [10], the necessity for interruption of ASA treatment due to gastrointestinal complications was 14.5% and 12% according to McCarthy [11].

Bleedings from the upper part of the gastrointestinal tract are described in the literature to occur most frequently after non-steroidal anti-inflammatory drugs, including aspirin. Singh et al. [12] report that it occurs in 1-4%, according to Serrano et al. [13] – in 4.5%, according to Ibanez et al. [14] – in 4.0%. In accordance with Laine [15], gastrointestinal complications caused by non-steroidal anti-inflammatory drugs application, appear in 3-4.5% including severe complications like bleeding, perforations and death – in 1.5%. According to He et al. [16] aspirin increases twice the probability of bleeding from the gastrointestinal tract. Other reports concerning aspirin inform about gastrointestinal tract complications of patients suffering from ischaemic heart disease comparing with patients not suffering from this disease. In accordance with Bar-Dayan et al. [17], bleeding from the gastrointestinal tract is more frequent in patients who apply anticoagulative drugs. Serrano et al. [13] also more frequently observed bleeding from the upper part of the gastrointestinal tract in patients with ischaemic heart disease.

Other authors claim that the main reason for gastrointestinal bleedings are non-steroidal anti-inflammatory drugs that constitute 20-30% of gastrointestinal bleedings according to Muszyński et al. [18], 52% according to Loginov et al. [19] and 40% according to Langman [20]. In accordance with Krasowski [5], erosive or haemorrhagic gastritis after applying non-steroidal anti-inflammatory drugs, is the reason for

Figure 2. Analysis concerning incidence of particular drugs groups application in patients treated and not treated with coronaroplasty



10-20% of bleedings. Sapoznikov et al. [21] proved that among 318 patients suffering from gastrointestinal bleeding, 28% of them have been using aspirin for 30 days.

Our own studies revealed described bleedings as single incidents. There are a lot studies in the literature concerning gastrointestinal bleedings recurrence in patients treated with non-steroidal anti-inflammatory drugs. Laine et al. [22] stated probability of repeated bleeding in 4% of patients.

Other studies concern gastrointestinal bleedings caused by other remaining antiplatelet drugs. In accordance with Schamig et al. [10], ticlopidine significantly decreases the risk of haemorrhagic complications in comparison with other anticoagulants. Meissner et al. [23] observed 84 patients treated due to myocardial infarction and he found bleedings after streptokinase in 10 cases – 12%, including 5-6% of deaths (there were central nervous system bleedings in 3 cases, retroperitoneal haemorrhage in one case and one haemorrhage from the wound) in remaining 5 patients. The next 3 cases of bleeding included intramuscular bleedings, 1 case of bleeding from duodenal ulcer and 1 case of spontaneous rupture of the spleen.

According to Landefield et al. [24], patients who use oral anticoagulants for three years will develop gastrointestinal bleeding. In accordance with Solet et al. [25], gastrointestinal bleedings occur 2.5 times less frequently after ticlopidine and clopidogrel than after aspirin, but application of these two groups of drugs causes three times as large increase of bleedings amount. Ibanez et al. [14] observed gastrointestinal bleedings after clopidogrel in 2.3% of patients, but after ticlopidine in 3.1% of patients. According to Ng et al. [26], clopidogrel is connected with the increased risk of gastrointestinal bleeding.

During our study, bleedings in other systems and organs were not observed in the respondents. Haemorrhagic complications in other systems during the treatment with anti-aggregative drugs were described among others by McKevitt et al. [27], who noted bleedings into the central nervous system after clopidogrel in 9%, after heparin in 17%. Cay et al. [28] described bleedings into the central nervous system after clopidogrel. Bleedings into the lungs after clopidogrel and receptor IIb/IIIa inhibitors were described by Gill et al. [29]. According to Yusuf et al. [30], severe bleedings – it means bleedings that cause significant disability, loss of vision after blood effusions into the eye or bleedings requiring transfusion of 2 blood units – in patients using both clopidogrel and acetylsalicylic acid occur with the incidence of 3.7%. In accordance with Jones et al. [31], there were no differences concerning haemorrhagic complications in patients using acetylsalicylic acid or clopidogrel, but these complications occur more frequently in patients who apply these two drugs at the same time. Andryś et al. [6] described the internal programme concerning notification of side effects during applied therapy in the Department of Clinical Pharmacology of Medical University of Poznań. This programme was based on observations in the hospital departments of State Clinical Hospital No 1 of Medical University of Poznań during the years 1991-1994. The suspicion about side effects during the antithrombotic therapy was notified in 18 patients. Bleedings from the gastrointestinal tract after treatment with heparin were observed in 1 patient, bleedings from urinary tracts or genital tracts in 2 patients and 2 patients revealed duodenal bleeding proved in endoscopy.

It is plaucible to formulate the following conclusions on the basis of the research carried out:

1. Bleeding from the upper part of gastrointestinal tract more frequently occurred among patients not treated with coronaroplasty,
2. The following drugs were more frequently used in the group of patients treated with coronaroplasty: acetylsalicylic acid, ticlopidine and clopidogrel, but acenocoumarol was used more frequently in the group of patients treated only pharmacologically.

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Traumatic rupture of the gallbladder after blunt abdominal trauma

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Abstract

The extremely rare case of 83-year old woman with rupture of the gallbladder due to blunt abdominal trauma is presented. Patient's general condition was complicated because of coagulopathy caused by oral anticoagulant what has contributed to intra-abdominal haemorrhage. The rupture of the gallbladder and rupture of the liver were found during operation. The diagnosis of rupture of the gallbladder due to blunt abdominal trauma is difficult to establish before exploration and often coexists with injury to the liver. The treatment of choice in rupture of the gallbladder is cholecystectomy. In patients after abdominal trauma, beside damage of parenchymatous organs, the injury to other organs should be taken into consideration, even if they occur very rarely.

Key words: gallbladder rupture, gallbladder trauma, blunt abdominal trauma.

Introduction

There are relatively few cases of traumatic rupture of the gallbladder that has been published. The increasing amount of traffic accidents causes the great necessity of improvement diagnostic and therapeutic standards. Parenchymatosus organs such as liver and spleen are usually injured during blunt abdominal trauma. The gallbladder is sheltered from trauma by anatomic localisation. It is partly surrounded by the liver and ribs, omentum and intestine protect it. Reported prevalence of injury to the gallbladder in blunt or penetrating abdominal trauma range from 1 to 2% [1-5]. Injuries of the gallbladder are usually associated with damage to other abdominal organs. The aim of this study is to report extremely rare case with rupture of the gallbladder due to blunt abdominal trauma in 83-year old woman.

Case report

A 83-year old woman was admitted to our Department with acute abdominal pain after blunt abdominal trauma caused by fall on the floor. The patient sustained simultaneously superficial trauma of right eyebrow arch. She was chronically treated because of atrial fibrillation, arterial hypertension and coronary insufficiency and she has been taking oral anticoagulant.

The physical examination revealed the abdomen wall tense and painful in all four quadrants with positive Blumberg's sign and palpable mass in right hypochondrium. The patient was in haemorrhagic shock. The measured blood pressure was 90/50 mm Hg. The pulse was arrhythmic with rate 120 beats/minute.

Laboratory findings showed: leucocytosis – 17.7×103/µl, hemoglobin – 7.8 g/dl, hematocrit value – 22.8%. Blood clotting tests revealed coagulopathy: kaolin–kephalin time (APTT) – 60.1 seconds and International Normalised Ratio (INR) – 2.05. Abdominal ultrasonography demonstrated free liquid below margin of right liver flap, around spleen and between intestinal loops, moreover, there was a difficulty in identification of not homogeneous structure below margin of the liver. Abdominal roentgenogram showed some fluid – filled dilated small bowel loops.

Three units of fresh frozen plasma and two units phenotype – matched packed red blood cells have been transfused to the patient. Afterwards the patient was qualified for emergency laparotomy. During surgery, 2 000 ml of liquid blood and clots were recognized; furthermore free irregular sharp – edged gallstones were found intraperitoneally (*Fig. 1*). Then

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***Figure 1*. Gall stones**

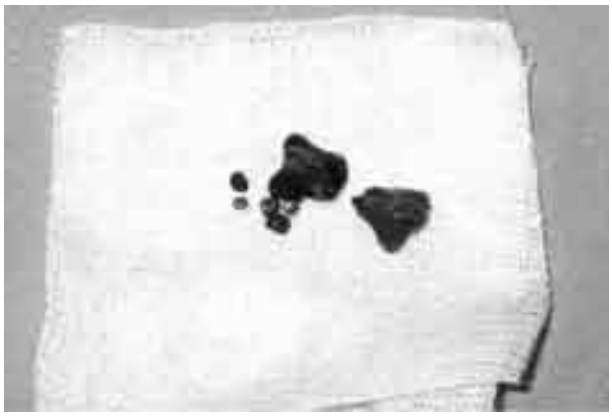

***Figure 2*. Rupture of the gallbladder and rupture of the liver**

approximately 4 cm in length rupture of the gallbladder with thicken wall in its fundus area and bleeding deep rupture of fifth liver segment 7 cm in length were found (*Fig. 2*). The liver was sutured and cholecystectomy was performed. The abdomen was cleaned, drained and closed in layers. The patient was transferred from operating theatre to the Intensive Care Unit because of signs of circulatory and respiratory insufficiency. Histopathological examination revealed gangrenous inflammation of the gallbladder.

Discussion

Most of the reported cases of injuries to the gallbladder up to the present are results of falls (as in our case), kicks and blows, and nowadays as well of sports and motor vehicle traumas [1,6]. The classification traumatic gallbladder injury includes lacerations, avulsion, contusion and the acute inflammatory condition of traumatic cholecystitis [1].

The diagnosis of rupture of the gallbladder due to blunt abdominal trauma is difficult to establish before exploration and often coexists with injury to the liver. Usually getting worse patient's general condition does not give much time for full diagnosis. Contrast enhanced computed tomography is a sensitive method for diagnosis of abdominal trauma and is very helpful for diagnosis gallbladder rupture [6], but the first line of diagnosis, especially when we have urgent situation should be ultrasonography, even if its sensitivity is not 100% [6-8]. Biliary isotope scintigraphy can reveal free intra-abdominal leakage of bile when the gallbladder is ruptured [6]. Some authors suggest that non-visualization of the gallbladder at ultrasonography or at computer tomography scans should raise the suspicion of traumatic gallbladder avulsion or rupture [9]. Another method which quickly helps to diagnose the character of intraperitoneal fluid after blunt abdominal trauma is peritoneal puncture and diagnostic peritoneal lavage. If there are diagnostic difficulties in stable patients laparoscopy can be performed. It is useful as diagnostic as well as therapeutic method [6]. The laparoscopy enables to perform hemostasis of small liver injuries with electrocoagulation and it gives possibility for cholecystectomy when gallbladder is ruptured. When laparoscopy does not give therapeutic success it should be conversed for laparotomy.

The treatment of choice in rupture of the gallbladder is cholecystectomy [9]. In patients after abdominal trauma, beside damage of parenchymatous organs, the injury to other organs should be taken into consideration, even if they occur very rarely.

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Diffuse nodular lymphoid hyperplasia of the gastrointestinal tract in patient with selective immunoglobulin A deficiency and sarcoid-like syndrome – case report

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Abstract

Nodular lymphoid hyperplasia is uncommon in adult patients. Associated diseases are common variable immunodeficiency (CVI) and lymphoid tissue malignancies. In this case report we focus on clinical presentation and differential diagnosis of diffuse nodular lymphoid hyperplasia of the gastrointestinal tract coexisting with selective immunoglobulin A deficiency and sarcoid – like syndrome.

Key words: nodular lymphoid hyperplasia, IgA deficiency, sarcoid-like syndrome.

A forty-year-old man with the history of sarcoide-like syndrome in 1998 was admitted to Department of Gastroenterology because of unspecific symptoms including upper abdominal pain, bloating, loose stools (1-3 per day). He had not taken any medication during preceding months. The physical examination was unremarkable, except for slight abdominal tenderness on palpation.

Laboratory tests were normal, except for insignificant increase of CRP (5.8 mg/l, norm: 0-5 mg/l), alpha-1 globulin (0.35 g/dl, 4.7%, norm: 2-4.5%) and urine alpha-amylase (469 IU/l, norm: 0-380 IU/l). Gastrointestinal (GI) tract bacterial and parasitic infections were excluded. Abdominal ultrasonography and chest X-ray showed no abnormalities. CT of the lungs revealed nodular peribronchovascular interstitial thickening, small subpleural nodules and mild lymph node enlargement (*Fig. 6*), observed morphological features suggest process with perilymphatic distribution, upper and mid lung zones predominance was typical for sarcoidosis although it did not exclude other diseases.

Endoscopic examinations including gastroduodenoscopy, colonoscopy and wireless capsule endoscopy revealed multiple pedunculated and sessile polyps, 2-10 mm in diameter located in the duodenum (*Fig. 1*), small bowel (*Fig. 2*) and on the ileocaecal valve; the mucosa was otherwise normal. The most involved segments of the GI tract were the proximal jejunum and distal ileum; no polyps were seen in the oesophagus, stomach and colon, however, the mucosa of the latter was granulated. The polyps were also seen on radiological examination as multiple round and oval filling defects, 1 to 5 mm in size (*Fig. 3*). Abdominal computed tomography demonstrated thickening of the small intestine wall particularly in the region of ileocaecal valve. In addition, multiple small (up to 11 mm) lymph nodes were detected in the small intestine and transverse colon mesentery and in the periaortal and pericaval region (*Fig. 4*).

Histopathological examination of the polypectomy specimen from the duodenum and terminal ileum showed stimulated reactive lymphatic follicles covered with normal mucosa, a picture corresponding to lymphoid polyp (*Fig. 5*). Immunohistochemical staining (CD 20, CD 3, CD 43, cyclin D1, MIB1) excluded a lymphoproliferative process.

Immunological tests revealed significantly low serum IgA level (10 mg/dl, norm: 80-310), whereas total IgG, IgG 1-4 subclasses, IgM and IgE levels were normal.

The final diagnosis was diffuse nodular lymphoid hyperplasia of the GI tract in a patient with sarcoid-like syndrome as a rare manifestation of selective IgA deficiency.

Discussion

Nodular lymphoid hyperplasia is a lymphoproliferative disease that cause still remains unknown [1]. The occurrence

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***Figure 1*. a-b) Endoscopic picture of polyps (3-5 mm in diameter) in the duodenum. Similar abnormalities were found in the terminal part of the small intestine**

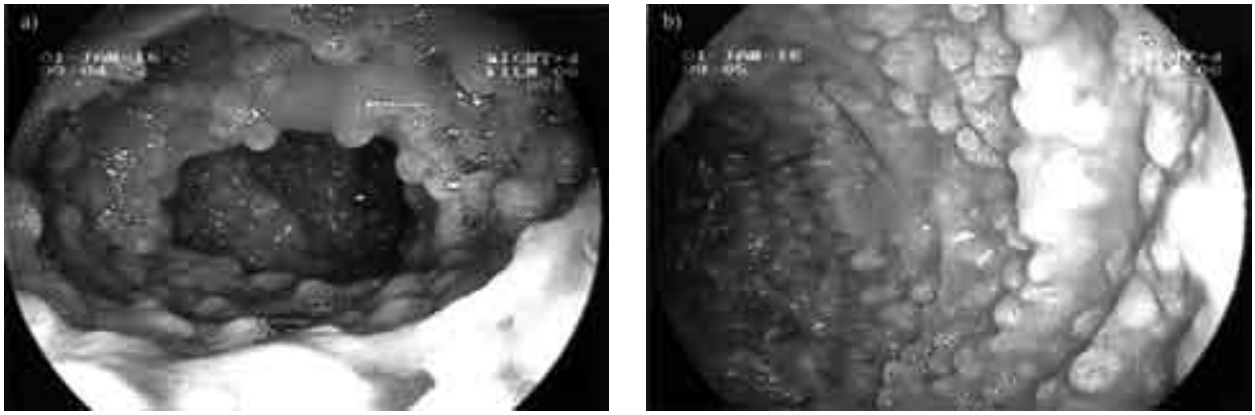
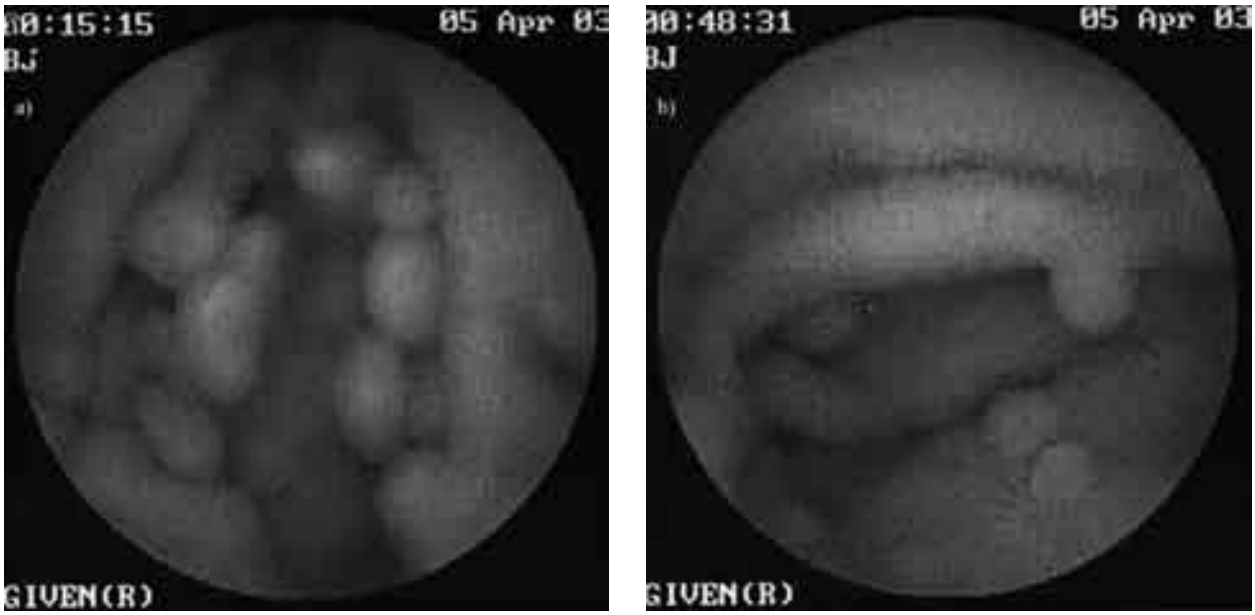


***Figure 2*. a-b) Wireless capsule endoscopy revealed multiple, small polyps in the small bowel, located mainly in the proximal jejunum and distal ileum**



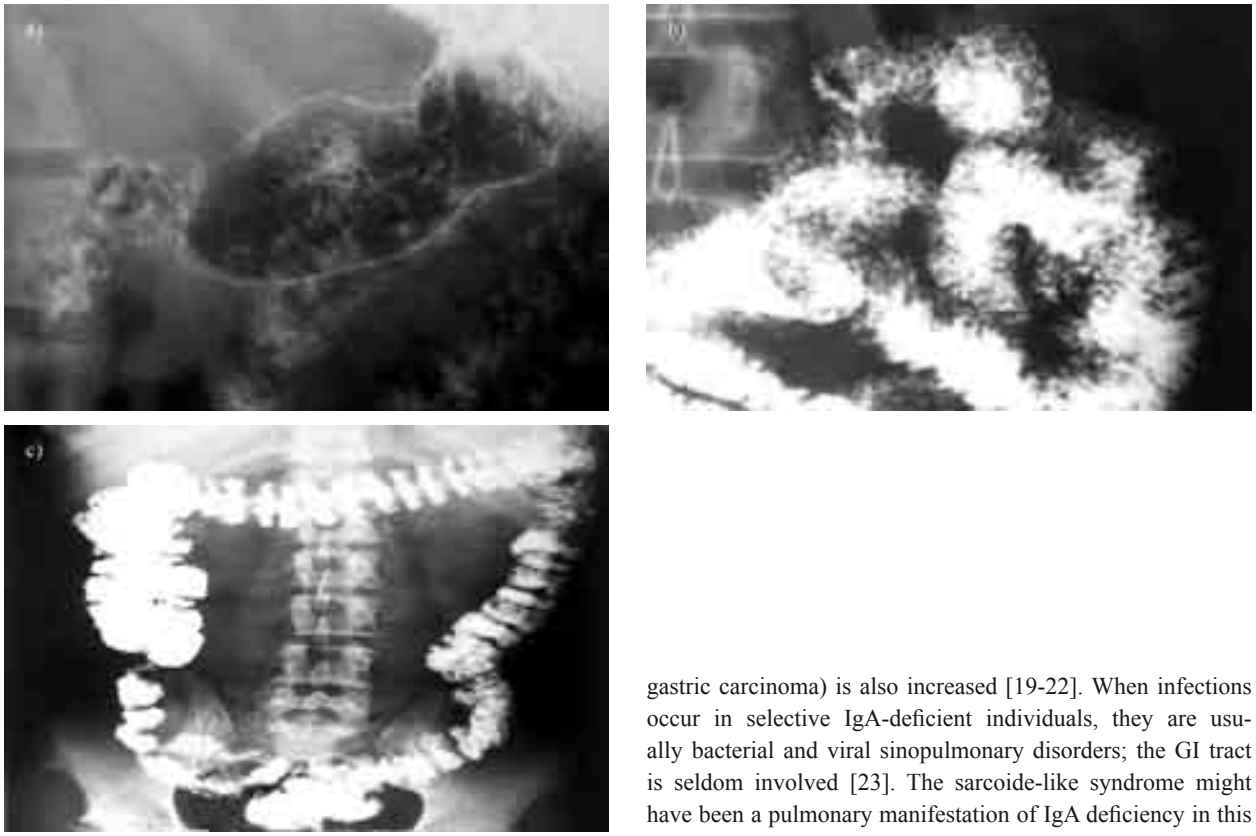
of nodular lymphoid hyperplasia is rather rare. It has been reported to appear in about 20% of patients with common variable immunodeficiency syndrome [1-7]. In some cases it was observed to associate with intestinal lymphoma [8-11]. Nodular lymphoid hyperplasia was also reported in adult patient without any kind of immunodeficiency [12-13]. In some cases the infection with Giardia lamblia was found [14]. There is a theory of the local immune response to the antigens as a stimulators in GI tract, but still no antigen is defined.

Nodular lymphoid hyperplasia always requires precise differential diagnosis from the other polyposis conditions, especially malignant lymphoma and familial adenomatous polyposis. The most often localisation of hyperplastic lymphoid nodules usually described as innumerable polypoid lesions, is the small bowel, especially the terminal ileum, but they can occur in the stomach and in the colon as well. In some rare cases the polypoid lesions themselves can cause bleeding or intestinal obstruction [15], but if these polyps are small they typically themselves do not cause any clinically significant symptoms. The frequent gastrointestinal symptoms described by patients usually result from underlying conditions like malabsorption syndrome or coexisting diseases like immunodeficiences and infections, so it is always important to define them and to undertake the appropriate therapy.

Nodular lymphoid hyperplasia in cases with no complications does not require any special treatment, however, the patients should undergo the prophylactic examinations. Nodular lymphoid hyperplasia is a benign disorder and usually the evolution of the disease is benign but in some cases lymphomatous association and transformation was documented [16]. The risk of malignancy in patient with coexisting hipogammaglobulinemia, especially the risk of lymphoma and gastric carcinoma is higher [17].

The cases of diffuse nodular lymphoid hyperplasia connected with hipogammaglobulinemia, usually coexist with common variable immunodeficiency syndrome, but in the case of our patient we found only selective immunoglobulin A deficiency and no other immunological defects.

***Figure 3*. Radiological examination shows innumerable nodules suggesting nodular lymphoid hyperplasia: a) in the stomach and duodenum; b) duodenum and small bowel; c) colon. The lesions are round or oval, 1-5 mm in diameter**



Selective IgA deficiency is defined as the total absence or severe deficiency of the IgA class of immunoglobulins in blood serum and secretions. Other immunoglobulins, such as IgM and IgG are present in normal or increased levels. This disorder is the most common primary immunodeficiency. The specific function of IgA is to protect the body's mucosal surfaces from infection. Although about 50% of the people with IgA selective deficiency are asymptomatic and free of complications [18], in some cases severe IgA deficiency can cause recurrent infections of mucosal tissues, allergies, celiac-like enteropathy or autoimmune disorders. The risk of malignant disorders (lymphoma, gastric carcinoma) is also increased [19-22]. When infections occur in selective IgA-deficient individuals, they are usually bacterial and viral sinopulmonary disorders; the GI tract is seldom involved [23]. The sarcoide-like syndrome might have been a pulmonary manifestation of IgA deficiency in this patient. The diseases and genetic disorders reported to be associated with selective IgA deficiency and GI tract are as follows: Crohn's disease, celiac disease, intestinal nodular hyperplasia and recurrent giardiasis. There is no treatment for selective IgA immunodeficiency syndrome.

In this article we would like to put an emphasis on the extremely extensive range of changes in the GI tract and their coexistence with only selective IgA deficiency and with sarcoid-like syndrome as a pulmonary manifestation of the disease. At present the patient receives symptomatic treatment (antibiotics and probiotics) for recurrent GI infections. He undergoes prophylactic examinations in order to exclude a malignant process every year.

***Figure 4*. a-b) Abdominal computed tomography demonstrates lymph nodes (up to 11 mm) enlargement (small arrows) and thickening within colonic wall (big arrows)**

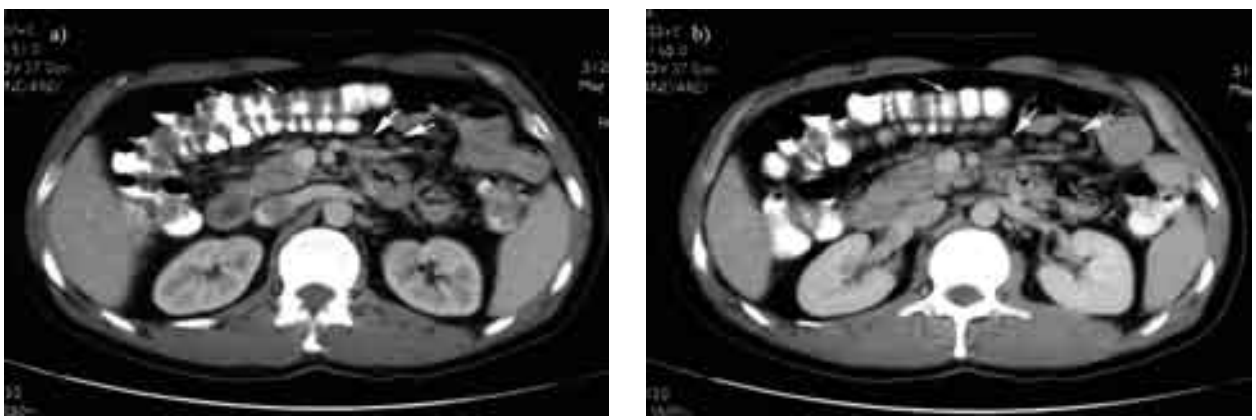


Figure 5. **A biopsy specimen from the terminal ileum polyp: a the lesion is covered by intact epithelium (hematoxylin-eosin, magification x 120); b lymphoid infiltrates (hematoxylin-eosin, magification x 280); c focal lymphoid hyperplasia and large lymphoid follicles with prominent germinal centres (hematoxylin-eosin, magification x 280)**

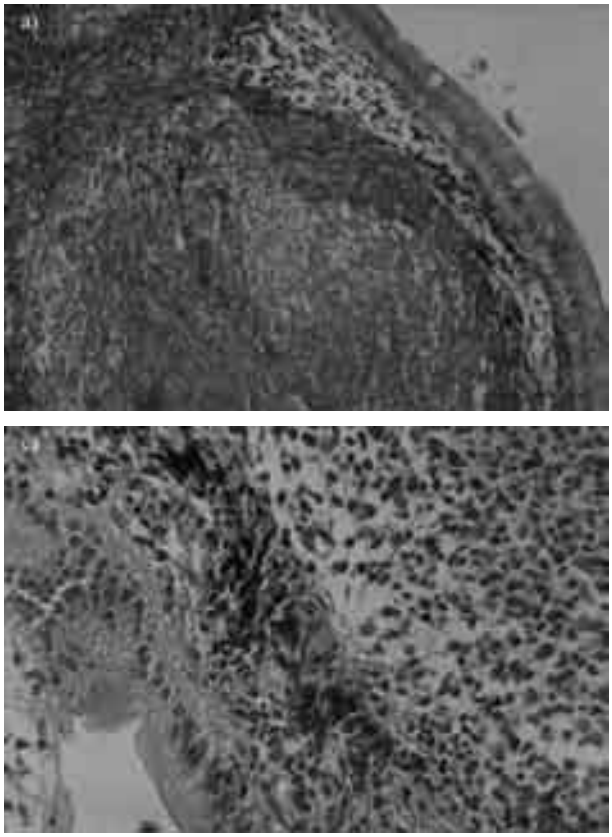
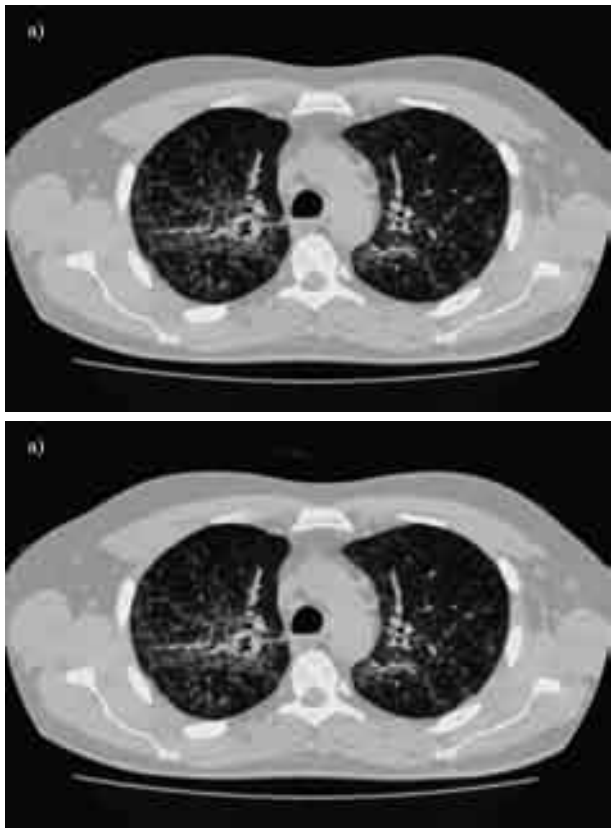


Figure 6. **a-b Computed tomography of the lungs revealed: nodular peribronchovascular interstitial thickening, small subpleural nodules and mild lymph node enlargement**



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