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The mechanical strength of orthodontic elastomeric memory chains and plastic chains: An in vitro study

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Abstract

Background. The loss of physical properties of orthodontic chains has been a topic of discussion among scientists and clinicians, motivating efforts to improve elastic materials and minimize the decrease of force. Orthodontic companies have introduced memory elastomers, which, according to the manufacturers, have improved mechanical properties.

Objectives. The aim of the study was to evaluate the effects of stretching elastomeric chains submerged in 37°C artificial saliva.

Material and methods. The study assessed 2 types of chains. The first phase of the study evaluated 1) the tensile strength of the chains; and 2) elongation at the time of tearing in an environment outside of the oral cavity (without exposition). The second phase of the study evaluated 1) permanent deformation after stretching; 2) elongation at the time of tearing; and 3) the tensile strength of the chain in conditions similar to those present in the oral cavity.

Results. In the experiments using artificial saliva, pronounced force decay was observed in the plastic chain, in which, after just 7 days, force decreased almost by half compared to the initial value, with a continuous downward trend. The memory chain, however, showed increased elasticity, and after the first week of exposition the force decay at the time of tearing was around 20% of the initial value. Force decay at the time of tearing remained at a steady level between 14.4 and 25.4% throughout the whole period of exposition. In the plastic chain this value oscillated between 50.3 and 55.9%. In the experiments assessing permanent deformation of the chains performed after each week of exposition, the specimens prepared from the memory chain stretched from 8 cm to approximately 9.5 cm after exposition, while the specimens prepared from the plastic chain stretched to approximately 13 cm.

Conclusions. Memory chains are more effective in orthodontic treatment due to diminished loss of mechanical and elastic capabilities, when compared to plastic chains.

Key words: force decay, initial strain, tearing, stretching, elastic chain

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The dynamic development of orthodontic techniques and products is allowing orthodontic therapy to reach new heights in positive treatment results, which in turn is making the use of permanent dental braces much more common.

The benefits of permanent dental braces include full control over dental alignment and precise use of forces, which makes them especially useful in treating dental anomalies and in interdisciplinary orthodontic, surgical and prosthetic treatment. Effective treatment is often not limited to the use of one type of brace; it frequently requires the implementation of precise and coordinated forces through the introduction of orthodontic archwires and supplementary elements.

Among those supplementary elements, elastic orthodontic elements called elastomers have been used for years. In addition to properties such as pliability and elasticity, elastomers are easy to use, reduce the risk of intra-oral trauma, do not require the cooperation of patients and are cheap. They are produced in a wide variety of colors, and patients adapt to them easily during orthodontic treatment. Disadvantages include a decrease of force that is noticeable over time, difficulties in oral hygiene, increased dental plaque retention compared to metal elements and a loss of color through the absorption of fluid and dye from food.¹ There are many types of elastomers, including, most commonly, separators, ligatures, rotation wedges, elastic threads and chains. They are an indispensable type of orthodontic accessory because they supply the force needed to move teeth, e.g. canine retraction and closing interdental spaces. Elastomers are most commonly made of 2 types of materials: natural or synthetic latex, the latter being increasingly more popular due to better mechanical and elastic properties, as well as the fact that more and more people are allergic to natural latex. Chains are produced as colorless or colored polyurethane elastomers with varying force (closed, short, long chains). Closed chains produce the biggest initial force. Less force is exerted by short chains in which the chain links are separated by short connectors. Even smaller forces are at play in long chains where the spaces between the chain links are longer. Loss of the initially generated force constitutes the biggest clinical problem. Studies have shown a 28–50% loss of initial force as early as 8 h after chain insertion into the oral cavity. After 24 h, the rate of initial force loss decreases significantly but is still observed in the following 2 to 3 weeks.^{2–4} For practitioners, the correlation between the loss of force of the elastic elements and the time lapsed signifies that the chains need to be regularly changed every 5 to 6 weeks.

For years, the loss of physical properties of orthodontic chains has been a topic of discussion among scientists and clinicians, motivating efforts to improve elastic materials and minimize the decrease of force. Orthodontic companies have introduced memory elastomers which, according to the manufacturers, have improved mechani-

cal properties. Elastomers used during orthodontic treatment are exposed to harsh conditions in the oral cavity, such as high temperature, significant humidity and spicy, highly seasoned food. These factors, apart from influencing the esthetics of the elastomers, significantly decrease their efficacy.

The aim of the study was to evaluate and compare the mechanical strength of elastomeric memory chains and plastic chains in *in vitro* conditions.

Material and methods

Two types of orthodontic chains – memory chain and plastic chain (American Orthodontics, Sheboygan, USA) – were chosen for the study. The type of factory in which the elastic materials are produced has a significant influence on their force decay. The composition and properties of the chains given by the manufacturers are often similar or nearly identical, but scientific studies show that the differences are very significant. For that reason, the materials chosen for this study were all manufactured by one company.

The study was carried out in 2 phases. The first phase of the study evaluated 1) the tensile strength of the chain and 2) elongation at the moment of tearing, both in an environment outside of the oral cavity (without exposition). The second phase of the study evaluated 1) the permanent deformation after stretching, 2) elongation at the moment of tearing and 3) the tensile strength of the chain, all in conditions similar to those present in the oral cavity. The specimens were constantly submerged in artificial saliva at 37°C over periods of up to 4 weeks; the procedure was repeated at weekly intervals ($t = 1$ week, 2 weeks, 3 weeks and 4 weeks).

The orthodontic elastic chains were subjected to the study within 2 weeks of delivery from the manufacturer's warehouse and were stored in a cupboard, at room temperature, in order to avoid UV radiation and excessive humidity prior to the experiments. Colorless chains were chosen for the study to eliminate the pigmentation effect in the elastic materials.^{5–7}

A total of 35 specimens, 8 cm each, were taken from each type of chain. In the first phase of the study of the mechanical properties, both types of chain were placed, without exposition, in a Zwick 1445 Universal Testing System machine (Zwick GmbH, Ulm, Germany) and a tear test was performed at a cross-head speed of 50 cm/min. Additionally, an elongation-at-tear test was performed. All the tests were carried out by the same person, at room temperature.

In the second phase of study, 8 cm specimens of both chain types were stretched 100%, i.e. to a length of 16 cm. The stretched chains were then attached to mandrels and submerged in artificial saliva at 37°C. The artificial

saliva was prepared according to EU regulation PN-EN 12868:2001, paragraph 5.8.

After the exposition time lapsed – i.e. 1, 2, 3 or 4 weeks – the stress on the specimens was lifted. After being taken out of the artificial saliva, the chains were dried, and, after a 30-min rest, each specimen was measured in order to study the permanent deformation. An 8 cm section was then cut from the middle of each of the stretched, submerged and dried specimens and placed in the Universal Testing System machine, where a tear test was performed at a cross-head speed of 50 cm/min. Additionally, an elongation-at-tear test was performed. All the tests were carried out by the same person, at room temperature.

Data analyzed came from an interval scale and were shown as the mean value and standard deviation (mean \pm SD). In order to compare the two independent groups (memory chains and plastic chains) Student's t-test was used. Compliance with normal distribution of data was verified using the Shapiro-Wilk test. Pairwise comparisons of strength and elongation when exposed to saliva and temperature were carried out using an analysis of variance (ANOVA). The post hoc Tukey's test was used when the results showed significant differences to determine homogeneous groups (Table 1). The statistical analysis was carried out using STATISTICA v. 10.0 software

(StatSoft Inc., Tulsa, USA). All the tests were analyzed at a significance level of $\alpha = 0.05$

Results

The test results are presented in Tables 2–6. Table 2 shows the results of the tensile strength test for both memory chains and plastic chains, before exposition (time 0) and after submersion in artificial saliva after 1, 2, 3 and 4 weeks. The results show the mean force in newtons (N) registered at the moment of tearing. The plastic chains exhibited more tensile strength than the memory chains did, and the difference was statistically significant. The force required to tear the plastic chains equaled 30.4 N, which was 37.6% higher than the force needed to tear the memory chains. Time spent submerged in artificial saliva decreased the mechanical strength of both chain types. The biggest decrease in the force value necessary to break the plastic chains was registered after the 4th week, while in case of the memory chains the biggest decrease was after the 1st week. Furthermore, the difference between the memory chain's initial tensile strength and its tensile strength after 4 weeks of exposition decreased by almost 50%. The changes in tensile strength are expressed as percentage values in Table 3.

Table 1. Pairwise comparison

Comparison parameter	Tukey's pairwise comparison, $\alpha = 0.05$				
	memory chain			plastic chain	
Tensile strenght 0		b			b
Tensile strenght 1 week	a			a	b
Tensile strenght 2 weeks	a	b		a	b
Tensile strenght 3 weeks	a			a	
Tensile strenght 4 weeks	a	b		a	
Elongation 0			c		b
Elongation 1 week	a	b		a	
Elongation 2 weeks		b		a	
Elongation 3 weeks	a	b		a	
Elongation 4 weeks	a			a	

Table 2. Tensile strenght

Tensile strenght Mean (SD)	0	1 week	2 weeks	3 weeks	4 weeks
Memory chain	22.1 (1.2)	19.4 (1.2)	21.2 (1.1)	20.2 (1.4)	20.6 (1.0)
Plastic chain	30.4 (1.1)	27.6 (2.4)	28.7 (1.1)	27.2 (1.8)	26.2 (3.0)
Student's t test – p value	p < 0.0001	p < 0.0001	p < 0.0001	p < 0.0001	0.0006

Table 3. Tensile strenght change

Tensile strenght change %	1 week	2 weeks	3 weeks	4 weeks
Memory chain	19.4 (1.2)	21.2 (1.1)	20.2 (1.4)	20.6 (1.0)
Plastic chain	27.6 (2.4)	28.7 (1.1)	27.2 (1.8)	26.2 (3.0)

Table 4. Elongation

Elongation % (SD)	0	1 week	2 weeks	3 weeks	4 weeks
Memory chain	414 (9.1)	337 (21.2)	354.4 (25.5)	333.3 (27.5)	308.9 (37.9)
Plastic chain	400.7 (54.3)	199 (25)	190.9 (25.6)	193 (29.6)	176.7 (34.1)
Student's t test – p-value	0.535002	p < 0.0001	p < 0.0001	p < 0.0001	p < 0.0001

Table 5. Elongation change

Elongation change %	1 week	2 weeks	3 weeks	4 weeks
Memory chain	-18.6	-14.4	-19.5	-25.4
Plastic chain	-50.3	-52.4	-51.6	-55.9

Table 6. Permanent deformation

Permanent deformation	1 week	2 weeks	3 weeks	4 weeks	Avg
Memory chain	9.3	9.7	9.5	9.4	9.475
Plastic chain	12.7	13.2	13.5	12.9	13.075

Table 4 presents the results of the elongation-at-tear test for both types of chain. In the first phase of the study, without exposition, the memory chains showed better elastic properties than the plastic chains; however, the difference was not statistically significant.

In the tests conducted with artificial saliva a significant decrease of elongation was observed in the plastic

chains. After just 7 days of exposition, the elongation-at-tear value was 50.3% of the initial value, with a tendency for further decrease. The memory chains, on the other hand, exhibited more elasticity and after the first week of exposition showed only a 20% decrease in the elongation-at-tear value when compared to the initial value. For the remainder of the exposition time, the decrease in the

elongation-at-tear value of the memory chains stayed between 14.4 and 25.5%. In case of the plastic chains, this value oscillated between 50.3 and 55.9% (Table 5). The statistically significant difference between those values points to the higher elasticity of the memory chains.

In the test for permanent deformation carried out after each week of exposition, the length of the specimens was measured 30 min after being removed from the mandrels. The memory chain specimens stretched from 8 cm to approximately 9.5 cm after 7 days of exposure and the plastic chain specimens stretched to approximately 13 cm. Exposition time did not significantly influence the deformation values of either type of chain. The results are presented in Table 6.

Discussion

The hypothesis that elastomers are materials that remain stable throughout the entire time they are in the oral cavity and that they do not lose strength during treatment can be categorically rejected. The first studies and published results about decreases in the mechanical properties of elastomers appeared in the 1970s. The authors' own research points a gradually decreasing force in both plastic chains and in modified elastomers such as memory chains. It is worth mentioning that the latter exhibit greater elasticity and the decrease in elongation-at-tear value was almost 50% of the decrease noted for the plastic chains. The problem of force decay in elastic orthodontic elements constitutes a significant clinical issue and is of interest to researchers. Different studies have reported force decay ranging from 24 to 85% after 28 days.^{8–14} Masoud et al. compared the mechanical properties of thermoplastic and thermoset elastic chains and found significant force decay in thermoplastic chains, on average 20% higher than in thermoset chains.¹⁵ Kersey et al. evaluated the strength of elastics, and showed that the force decay after 24 h equaled 17% and the value of the forces generated was lower than that given by the manufacturer.¹⁶ A study by Kanchana et al. found that the initial force in elastics decreases by 30% in the first hour.¹⁷ Mikulewicz et al. demonstrated that the rate of force relaxation in elastics after 24 h varies between 5.7 and 17.7%, depending on type of elastic.¹⁸

Environmental factors such as water, fluids, saliva, food and temperature impact changes in the mechanical properties of elastic elements. Fraunhofer et al. demonstrated that the exposition of elastic chains to saliva and superficial fluoride necessitates an increase in length of elongation to generate 150 g and 300 g force.¹⁹

Josell et al. studied chains from several different manufacturers, and demonstrated that all the chains lose their physical qualities most rapidly on the first day or even the first hour of use. This trend then continues over 2–4 days.²⁰

During orthodontic treatment the elastic elements are stretched to generate force. Stretching a chain stresses the molecular polymer within it. The procedure initially strengthens the durability of the elastic chain and causes it to generate the force necessary for orthodontic treatment.²¹ This is possible because the material “wants” to return to its initial form and size. It is crucial for the material not to get stretched to its limits, as that will cause permanent deformation and excessive broadening. Quantifying the loss of physical properties depends on the speed and strength with which the material is stretched. With enough force applied it is possible to tear the material. Rock et al. showed that stretching the elastic elements 300% or more produces an unfavorable response that causes the material to reach its limit of elasticity. Stretching the material 200% is frequent in orthodontic treatment.²² Huget et al. demonstrated that stretching chains 50% causes less force decay than stretching it 100 or 200%.²³

The results obtained in the present study are consistent with the published work of authors worldwide. In both chain types, the biggest force decay was observed after the first 7 days. Subsequently it decreased slightly, remaining at a fairly constant level. A significantly lower loss of physical properties was observed in the memory chain. The authors of the study have confirmed, in contradiction to studies conducted by Ash et al. and Andreassen et al., that the loss of elastic properties also occurs when artificial saliva is used.^{24,25}

The results of the present study confirm the need to exchange the elastic elements used in orthodontic treatment every 4 weeks in order to maintain a moderately constant force value. Memory chains are more effective in orthodontic treatment due to a lower rate of loss of mechanical and elastic properties when compared to plastic chains.

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Cytotoxicity of anticancer drugs and PJ-34 (poly(ADP-ribose)polymerase-1 (PARP-1) inhibitor) on HL-60 and Jurkat cells

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Conflict of interest

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Abstract

Background. The majority of the clinical trials with poly(ADP-ribose)polymerase-1 (PARP-1) inhibitors were conducted or are ongoing in patients with solid tumors, while trials with leukemia patients are less frequent. Surprisingly scarce data is available on the combinatory effects of PARP inhibitors with DNA damaging antitumor drugs in leukemic cells (primary cells or established lines).

Objectives. The aim of the present study was to assess the effect of PJ-34 (PARP-1 inhibitor) on the cytotoxicity of different antileukemic drugs with different DNA damaging mechanisms and potency (doxorubicin, etoposide, cytarabine and chlorambucil) in human leukemic Jurkat and HL-60 cells.

Material and methods. Different exposure scenarios were applied: 1) 72 h simultaneous incubation with PJ-34 (2.5 or 5 μ M for Jurkat and HL-60 cells, respectively) and a drug used at a wide concentration range; 2) preincubation of the cells with PJ-34 for 24 h and then with a combination of PJ-34 + drug for an additional 48 h; 3) preincubation of the cells with the drug for 24 h with a subsequent incubation with a combination of PJ-34 + drug for an additional 48 h. Cytotoxicity was assessed using a WST-1 reduction test.

Results. It was determined that PJ-34, when used in all 3 scenarios, did not induce any significant enhancement of cytotoxicity of the drugs either in Jurkat or in HL-60 cells.

Conclusions. Although the results do not confirm the beneficial effects of PARP inhibition in combination treatment of the leukemic cells, we propose that future studies including an additional step with the inhibition of DNA repair by homologous recombination should provide promising results.

Key words: PJ-34, doxorubicin, cytarabine, chlorambucil, etoposide

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Poly(ADP-ribose) polymerases, also termed ADP-ribosyltransferases with diphtheria toxin homology (ARTDs) according to a new nomenclature, catalyze the polymerization of ADP-ribose units from NAD⁺ on acceptor proteins, leading to the formation of linear or branched polymers of ADP-ribose. The PARP superfamily encompasses at least 18 enzymes involved in several biological processes, including transcriptional regulation, DNA repair, cell cycle regulation, hypoxic response, inflammation, spindle pole function, oncogene-related signaling and cell death.¹ Poly(ADP-ribose)polymerase-1 (PARP-1), an abundant and ubiquitous enzyme, is the best characterized member of the family. It accounts for 80–90% of detectable poly(ADP-ribose) synthesis following DNA damage.

Recently, PARP inhibitors were shown to selectively target DNA double-strand break (DSB) repair-deficient breast cancer type 1/2 susceptibility protein (BRCA1/2) null cells for killing.^{2,3} The general understanding of this phenomenon, called synthetic lethality (i.e. when inactivation of either of 2 genes alone allows cell viability but simultaneous inactivation of both genes causes cell death), is that after inhibition of PARP-1 (a component of the DNA single-strand break (SSB) repair machinery), unrepaired SSB lesions are converted into DNA DSB during DNA replication and require activation of homologous recombination (HR) repair proteins (e.g. BRCA1/2) for their resolution. Hence, BRCA1/2 functionally null tumor cells treated with a PARP inhibitor accumulate DNA DSB and undergo cellular death. Besides its major role in detecting SSB, PARP-1 was shown to bind to and assist the repair of other damaged DNA structures, including stalled replication forks and DSB, having an effect on both HR and non-homologous end-joining repair processes (NHEJ). Assuming a high propensity of tumor cells towards genome instability and the complexity of PARP-related DNA repair routes, it is not surprising that PARP-1 inhibitors have raised many expectations as potential clinical anti-tumor drugs. These expectations have led to many trials with different generation PARP inhibitors used in monotherapy or in combination with other drugs (<http://clinicaltrials.gov>). Interestingly, so far the majority of the trials have been conducted (or are ongoing) in patients with solid tumors, while leukemia, myeloproliferative disorders or other hematological malignancies were less common. This situation is reflected by relatively scarce data in scientific literature on the combinatory effects of PARP inhibitors with other DNA damaging antitumor drugs in leukemic cells (primary cells or established lines).

In our study, we decided to assess the influence of a well known PARP-1 inhibitor, PJ-34, on the cytotoxic effects of different antileukemic drugs showing different DNA damaging mechanisms and potency. To this end, we selected doxorubicin (DNA intercalation and inhibition of topoisomerase II, DNA and RNA polymerases, DNA

alkylation, disruption of calcium homeostasis and generation of free radicals), etoposide (pure topoisomerase II inhibitor), cytarabine (antimetabolite incorporating into DNA and interfering with DNA and RNA synthesis) and chlorambucil (alkylating agent of the nitrogen mustard type). PJ-34 is a very potent PARP inhibitor with half maximal effective concentration (EC₅₀) of 20 nM, which is 10,000 times lower than the EC₅₀ of 3-AB⁴. For screening purposes, we selected Jurkat and HL-60 cells, which are well established human leukemic in vitro models. Although using a simultaneous coincubation of cells with different agents is currently the most common practice, in the present study, different exposure scenarios were applied. To this end, two general assumptions were made: 1) to preincubate the cells with PJ-34 for a longer time (24 h) to develop not only PARP inhibition, but also other recently-postulated potential PARP-independent effects (e.g. changes of cell cycle distribution^{5,6} or activation of the cytoprotective phosphatidylinositol-3 kinase (PI3K)-Akt pathway), and 2) to superimpose PARP inhibition on a fully-developed DNA damage and DNA damage response/repair, i.e. commencing the co-exposure to PJ-34 after 24 h of preincubation of the cells with a drug.^{5,6}

Material and methods

Chemicals and reagents

The doxorubicin was from Sequoia Research Products (#SRP04660d), chlorambucil was purchased from Enzo Life Science (#ALX-400-049-G001), Cell Proliferation Reagent WST-1 (4-[3-(4-iodophenyl)-2-(4-nitrophenyl)-2H-5-tetrazolio]-1,3-benzene disulfonate, #11644807001) was obtained from Roche, RPMI 1640 + GlutaMAX culture medium (#61870) and Fetal Bovine Serum (#10270-106) were purchased from Life Technologies and the Mycoplasma Detection Kit - MycoProbe (#CUL001B) was from R&D Systems. All other chemicals including etoposide (#E1383), cytarabine (#C1768), PARP inhibitor VIII - PJ34 (#P4365), penicillin-streptomycin (#P0781) and trypsin-EDTA (#T4049) were from Sigma Aldrich.

Cell lines

The human T cell leukemia cell line (Jurkat - DSMZ #ACC 282) and the human acute myeloid leukemia cell line (HL-60 - DSMZ #ACC 3) were obtained from Deutsche Sammlung von Mikroorganismen und Zellkulturen GmbH (Braunschweig, Germany).

The cells were cultured in suspension in RPMI 1640, supplemented with 10% heat-inactivated fetal bovine serum and antibiotics (penicillin 100 U/mL and 100 µg/mL streptomycin). The cells were incubated in a 5% CO₂ humidified atmosphere. They were screened for *Mycoplasma* spp. infection using a Mycoplasma Detection Kit.

Cytotoxicity assessment – WST-1 reduction test

The cytotoxicity of doxorubicin, etoposide, cytarabine, chlorambucil and PJ-34 on Jurkat and HL-60 cells was measured using a colorimetric WST-1 reduction test. The assay is based on the conversion by viable cells of light red tetrazolium salt WST-1 to the yellow formazan derivative, whose optical density is measured spectrophotometrically.

In brief, Jurkat cells (4×10^3 cells per well) or HL-60 cells (1.5×10^3 cells per well) were seeded in 50 μ L into a 96-well plate (NUNC #167008) and exposed to test substances added as a $\times 2$ concentrated solution in 50 μ L of RPMI, for 24, 48 or 72 h. Then 10 μ L of the WST-1 reagent was added to each well and the microplate was placed at 37°C for 1.5–2 h. After 1 min shaking, the optical density of the formazan product was determined using a Multiscan RC spectrophotometer (Labsystems Helsinki, Finland) with a 450 nm filter and 620 nm filter as a reference. The results were expressed as the percent of cell survival (OD of exposed vs OD of non-exposed cells (control)).

The effect of PARP-1 inhibitor – PJ-34 on the cytotoxicity of doxorubicin, etoposide, cytarabine and chlorambucil on Jurkat and HL-60 cells was also studied. In these experiments the cells were exposed to a combination of a drug with PJ-34 (used at maximum non-cytotoxic concentration determined in preliminary experiments) for 72 h, or they were preincubated with PJ-34 or with the drugs for 24 h. After the preincubation, the cells were treated with a combination of drugs with the inhibitor for an additional 48 h. At the end of the exposure, the viability of the cells was assessed in a WST-1 reduction test.

Statistical analysis

The data was expressed as the mean \pm SD from the indicated number of separate experiments. The Inhibitory Concentrations inducing 50% decrease in viability (IC₅₀) with Confidence Intervals (CI) were calculated using GraphPad Prism v. 6.01 for Windows (GraphPad Prism Software, Inc., USA). After log transformation, the model of nonlinear regression (log(inhibitor) vs normalized response – variable slope) was applied.

Results

The cytotoxicity of PJ-34 (PARP-1 inhibitor) and selected drugs in Jurkat and HL-60 cells after 24-, 48- and 72-h exposure.

During 72-h incubation, the PJ-34 inhibitor decreased cell survival in a dose-dependent manner (Fig. 1). A higher sensitivity of Jurkat cells was observed, i.e. calculated IC₅₀ values for all time-points were 2-fold less in comparison to HL-60 cells. Based on the results of further

studies on the 72-h exposures, the PJ-34 concentration of 2.5 μ M and 5 μ M were selected for Jurkat and HL-60 cells, respectively.

The cell viability at these concentrations exceeded 70%. Prior to any studies on the inhibitory effects of PJ-34, a thorough dose-response cytotoxicity analysis of selected antitumor drugs on both cell lines was conducted. The data (Fig. 2) suggests a rather diverse potency of the drugs, with DOX and CYT showing the highest cytotoxic activity. In most cases (except CYT), after 72 h of incubation, the HL-60 cells were 2- (CHL) to 5- (ETO) fold more sensitive to the drugs in comparison to Jurkat cells.

Cytotoxicity of selected anti-cancer drugs in combination with PJ-34

Assuming different effects of PJ-34 on drug cytotoxicity depending on the extent of developing cellular damage and DNA damage response, 3 models of PJ-34-drug co-incubations were applied, i.e. 1) simultaneous co-incubation (scheme in Fig. 3), 2) 24-h preincubation with a drug or 3) 24-h preincubation with PJ-34 (scheme in Fig. 4). In each case, the cytotoxicity was determined after a total of 72 h of exposure.

Fig. 1. Viability of Jurkat and HL-60 cells after 24, 48 or 72-h exposure to PJ-34. WST-1 reduction test (n = 3–4). For each exposure, PJ-34 IC₅₀ value has been calculated

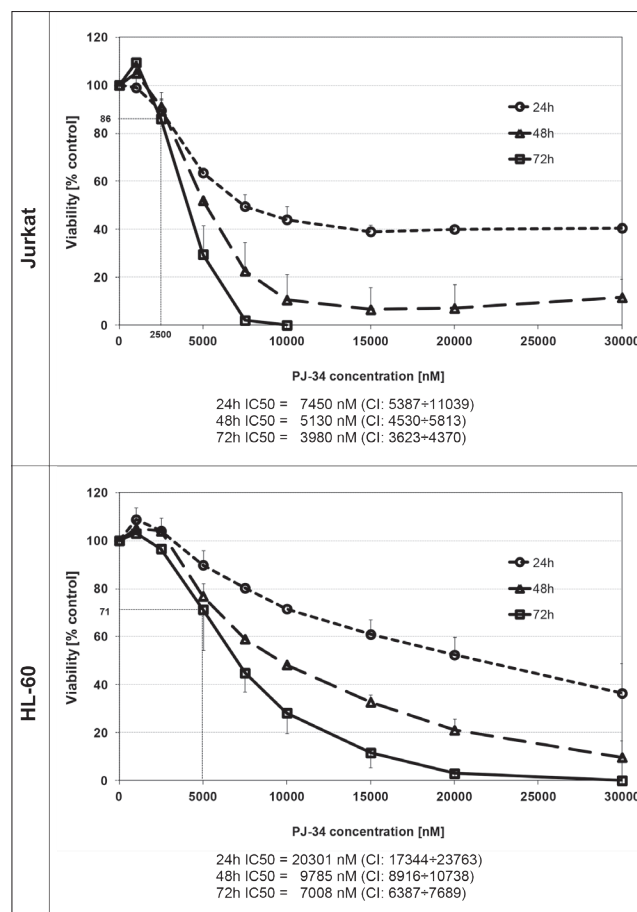
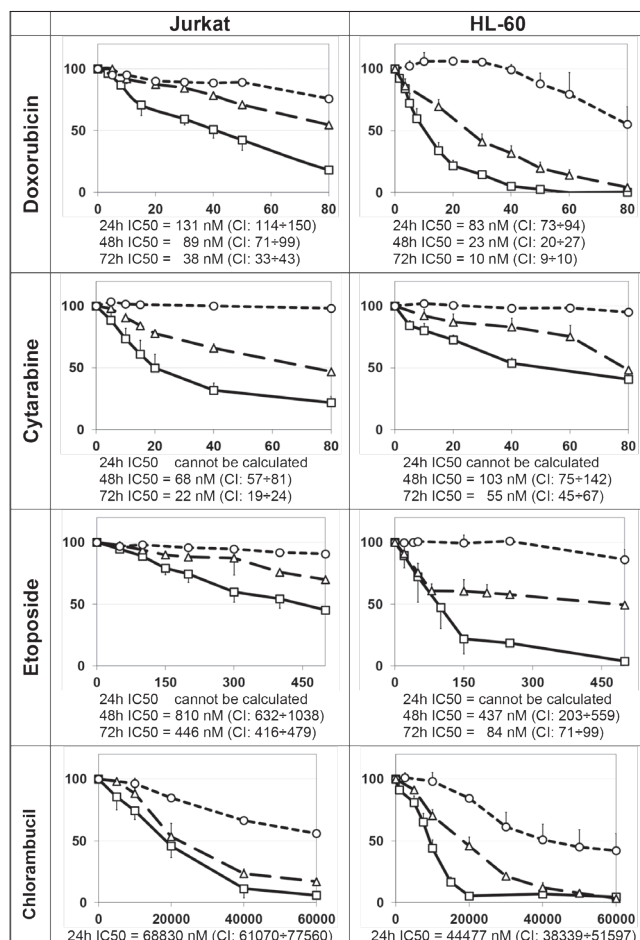


Fig. 2. Viability of Jurkat and HL-60 cells (% control; the Y axis) after the exposure to selected anti-tumor drugs at the concentrations indicated (nM; the X axis): doxorubicin, cytarabine, etoposide or chlorambucil for 24 h (circles), 48 h (triangles) or 72 h (squares). WST-1 reduction test (n = 3–4). For each exposure IC50 value has been calculated



It was determined that PJ-34 when used simultaneously with the drugs did not induce any significant enhancement of cytotoxicity of the drugs either in Jurkat or in HL-60 cells (Fig. 5).

Similarly, PJ-34 did not significantly influence the cytotoxic potential of the drugs either when the cells were preincubated for 24 h with PJ-34 and then co-exposed with PJ-34 + drug for an additional 48 h (Jurkat cells: Fig. 6B; HL-60 cells: Fig. 7B) or when they were preincubated with a drug for 24 h and then co-exposed for an additional 48 h with drug-PJ-34 combination (Jurkat cells: Fig. 6A; HL-60 cells: Fig. 7A).

Fig. 3. Scheme of 72-h experiments – simultaneous incubation of Jurkat or HL-60 cells with a single drug (green line) or combination of an anti-cancer drug with PJ-34 (red line)

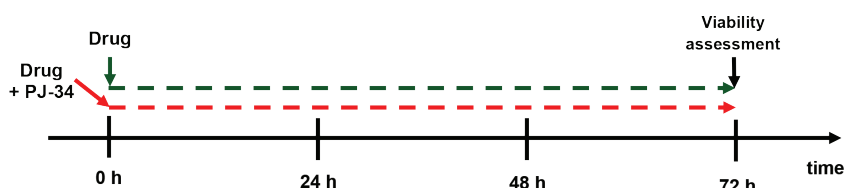
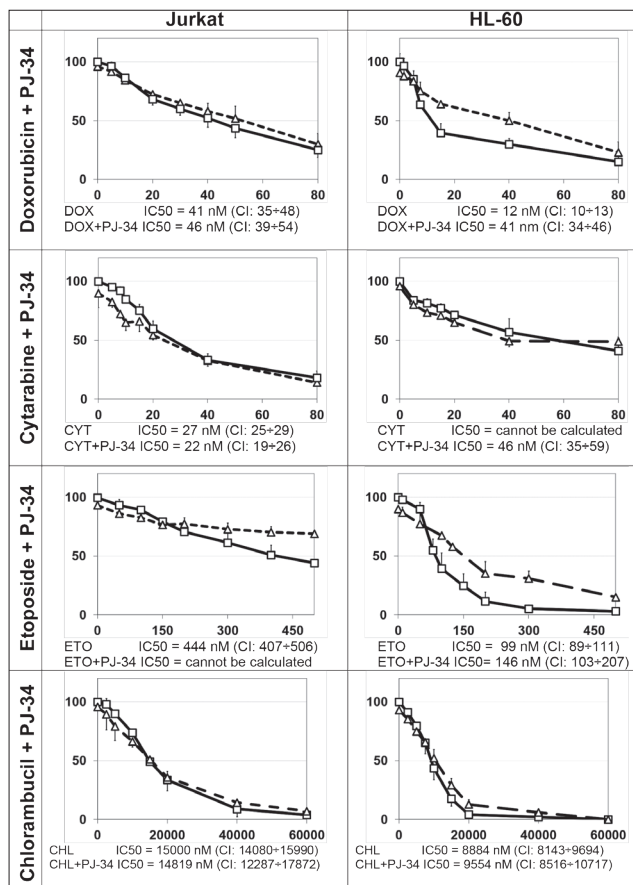


Fig. 5. Viability of Jurkat and HL-60 cells (% control; the Y axis) after the exposure to a combination of an anti-cancer drug (nM; the X axis): doxorubicin, cytarabine, etoposide or chlorambucil with PJ-34 (at 2.5 μ M for Jurkat cells and 5 μ M for HL-60 cells) for 72 h. WST-1 reduction test (n = 3–4). Squares – the drug, triangles – the combination of the drug with PJ-34. For each exposure IC50 value has been calculated



Discussion

In our study we hypothesized that increased DNA damage caused by the addition of PJ-34 to selected DNA-damaging drugs could produce a measurable increase in cytotoxicity in leukemic Jurkat and HL-60 cells. This concept is in line with current ideas of designing combined therapies where, by sensitizing tumor cells to cytotoxic agents, a lower dose could be given while maintaining the same relative efficacy and reducing the toxic side effects.

Although many reports indicate the usefulness of PARP-1 inhibitors in enhancing the cytotoxicity of DNA-damaging drugs in solid tumors, especially with BRCA1/2 deficiency, the experience with leukemic cells is relatively scarce. For many years, PARP-1 and PARP-2 have been recognized as central components of the Base Excision Repair/Single-strand break repair process (BER). However, recently PARP-1 has been found to be activated by other types

of lesions including DNA crosslinks, stalled replication forks and double-strand breaks.⁷ PARP-1 can bind to and be activated by DSB both in vitro and in vivo.⁸ It is predominantly involved in the HR-dependent repair of DSB at disrupted replication forks. While PARP-1 appears not to be involved in executing HR as such, some data indicates that it actively operates in the HR-dependent restart of stalled replication forks.⁹ The current model for PARP1-mediated replication fork stability assumes that if SSB results in fork collapse, resulting in a one-ended DSB and SSB in the sister chromatid, then PARP-1 binds the SSB and/or DSB and recruits XRCC1, thereby promoting the SSB repair process to repair the sister chromatid.¹⁰ PARP-1 may also repress Ku70/Ku80 binding at the one-ended DSB and thereby enabling HR-mediated template switching to promote fork

Fig. 4. Scheme of 24-h preincubation experiments. A) HL-60 or Jurkat cells were preincubated for 24 h with the drug, then for subsequent 48 h with a combination of the drug and PJ-34; B) HL-60 or Jurkat cells were preincubated for 24 h with PJ-34, then for subsequent 48 h with a combination of the drug and PJ-34

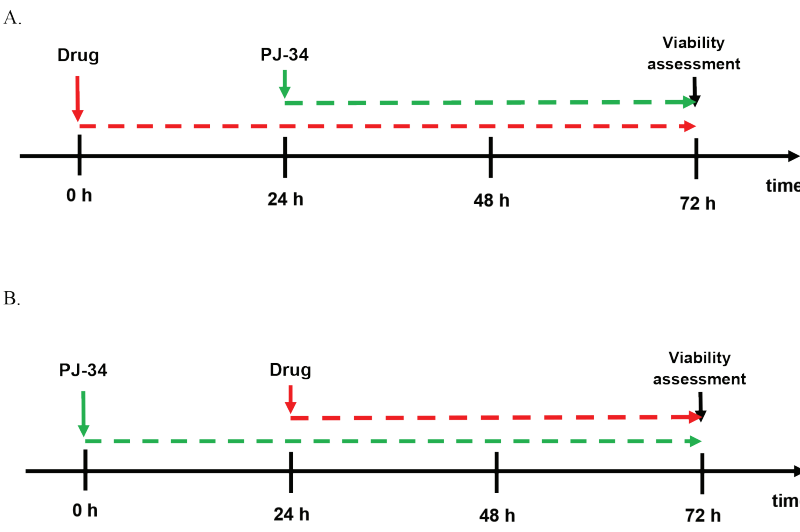
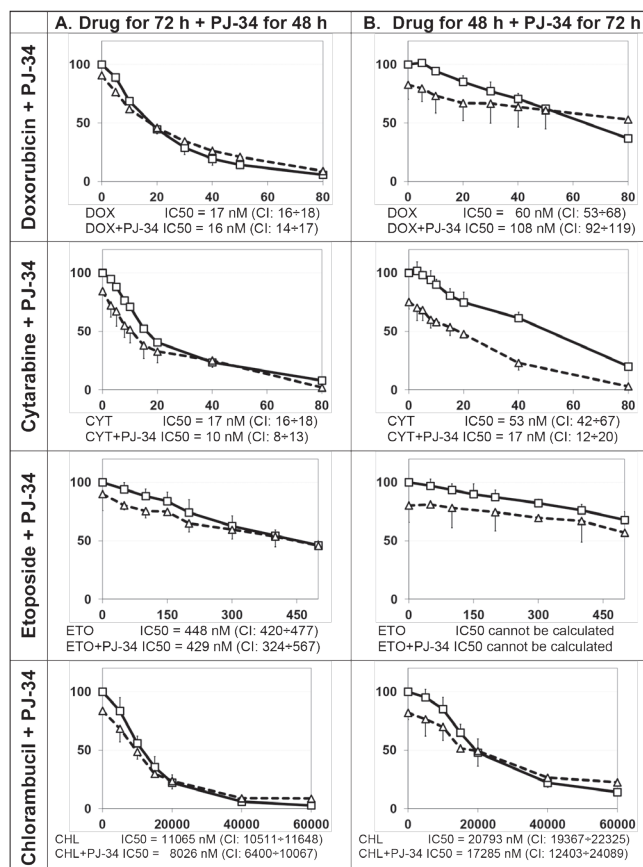


Table 1. Mechanisms of action and types of DNA repair induced by the selected drugs

Drug selected	Mechanisms of action	Type of repair of the DNA damage induced
Doxorubicin (DOX)	Topoisomerase II (Top2) inhibitor; “poisons” the enzyme by stabilizing the DNA cleavage complexes, resulting in DNA strand breaks ²¹ DNA intercalation, inhibition of DNA and RNA polymerases, DNA alkylation, disruption of calcium homeostasis, generation of free radicals; DOX-induced ROS generate mutagenic base modifications and drive the formation of additional bulky lesions in the form of DNA adducts and crosslinks, lesions which cause replication fork stall and collapse; the higher cytotoxicity of DOX compared to ETO may be due to a higher frequency of DNA double strand breaks and/or by the formation of more persistent cleavage complexes	Two major pathways are active in the repair of DNA DSB: NHEJ and HR repair; the phase of the cell cycle in which DNA damage is induced is critical in determining which of the response processes predominate: because HR requires a homologous DNA sequence it is prevalent in mitotic cells when a copy of the target DNA is available for exchange; DSB generated in the G1 phase of the cell cycle are repaired by NHEJ
Etoposide (ETO)	Potent and the most selective Top2 cleavage complexes-targeted drug currently in the clinic; does not intercalate DNA; Top2 cleavage complexes produced form in a monotonic manner without decrease at high drug concentration; the complexes are readily reversible upon drug wash out, in contrast to anthracyclines; despite the similar mechanisms of action of ETO and DOX, the kinetics of cleavage complex formation and recovery varies, the same as the ratio of single strand to double strand Top2 mediated DNA breaks ²¹	
Cytarabine (CYT)	Primarily involves inhibiting DNA synthesis; after activation by phosphorylation, the triphosphates of CYT are incorporated into DNA opposite dG and inhibit DNA synthesis by stalling replication forks; when incorporated into DNA, CYT is also a potent inhibitor of topoisomerase I and II	– repair mechanism not clear; nuclear co-localization of Mre11, Rad50 and Nbs1 with phosphorylated ATM and H2AX, increases in response to CYT; function of ATM and MRN complex at sites of stalled replication forks is unknown, but they may prevent fork collapse, which otherwise could lead to DSB and chromosomal aberrations ²⁴ – suggested DSB formed as a result of unresolved stalled replication forks may be repaired by HR or NHEJ
Chlorambucil (CHL)	Most probably alkylates the nitrogenous bases of DNA (e.g. formation of O6-chloroethylG, O4-chloroethylT adducts) and forms inter- and intrastrand crosslinks; formation of crosslinks results in uncoiling and twisting of the DNA helix ^{22,23} these structural changes in the DNA duplex result in the inhibition of DNA synthesis and DNA replication, DSB and finally cell death	– primary chloroethyl adducts at O6-G are repaired by direct base repair by O6-alkylG-DNA alkyltransferase – highly cytotoxic interstrand crosslinks require nucleotide excision repair (NER) factors (e.g. XPF-ERCC1) for incision and HR or NHEJ to complete repair – intrastrand crosslinks repaired by NER

Fig. 6. Viability of Jurkat cells (% control; the Y axis) preincubated with an anti-cancer drug at the concentrations indicated (nM; the X axis) for 24 h (A) or with PJ-34 at 2.5 μ M (B) and then with the combination of both agents for subsequent 48 h. WST-1 reduction test (n = 3). Squares – the drug, triangles – the combination of the drug with PJ-34. For each exposure IC₅₀ value has been calculated

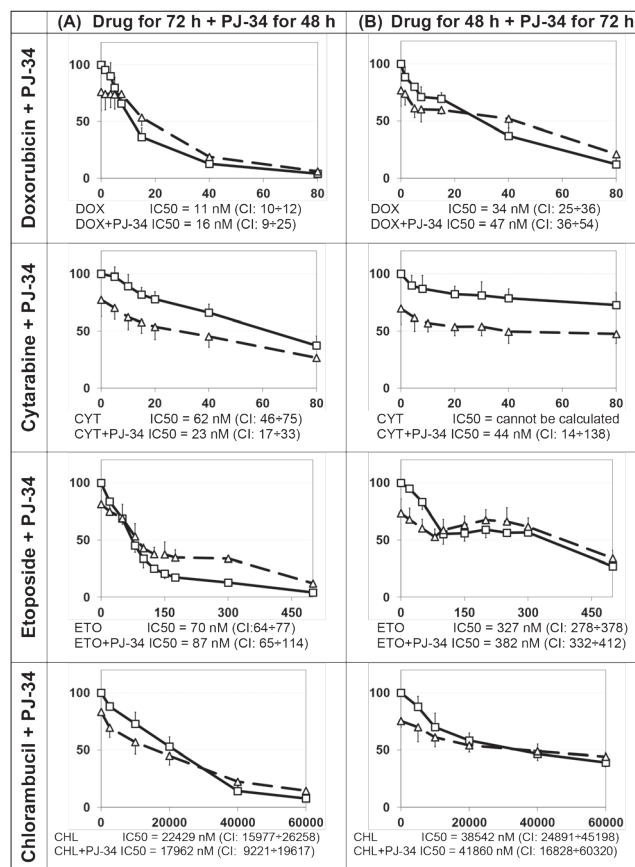


restart, preventing aberrant NHEJ repair of the DSB. PARP-1 may also promote HR directly, e.g. by regulating Mre11 nuclease activity at the DSB.¹¹

The data quoted strongly suggests that PARP-1 inhibitors may be potentially useful in neoplasms defective in a vast array of DNA damage repair pathways, not only in BER (SSB repair) or HR (DSB repair). For this reason, in the present study, drugs with different putative modes of DNA-damaging activity were used (Table 1). Moreover, assuming an as-yet unidentified effect of PARP-1 inhibition depending on the timing of DNA damage induction (hence the extent of the damage and development of signaling of the damage), we applied 3 different exposure scenarios. Such approach is fully supported by many clinical trials with PARP inhibitors in combination with chemotherapy, where it is often difficult to define an optimum combination dose and schedule that would improve the therapeutic ratio.

In spite of the extended investigations, we did not observe any satisfactory effect of PJ-34 on the drugs cytotoxicity in either treatment schedules. Available literature data indicates rather divergent effects of PARP inhibition in leukemic cells, ranging from increased sensitivity to

Fig. 7. Viability of HL-60 cells (% control; the Y axis) preincubated with an anti-cancer drug at the concentrations indicated (nM; the X axis) for 24 h (A) or with PJ-34 at 5 μ M (B) and then with the combination of both agents for subsequent 48 h. WST-1 reduction test (n = 4). Squares – the drug, triangles – the combination of the drug with PJ-34. For each exposure IC₅₀ value has been calculated



the drugs, through no effects, to even increased resistance of the cells. For example, 5'-aza-2'-deoxycytidine (a DNA methyltransferase inhibitor) failed to increase the cytotoxicity of PARP inhibitors (KU-0058948 and PJ-34); in contrast, MS275 (a histone deacetylase inhibitor) potentiated the cytotoxic effect of KU-0058948 and PJ-34 in all PARP inhibitor-sensitive leukemic cells.¹² In human leukemia K562 cells, AG14361 (a PARP inhibitor) caused a 2-fold sensitization to camptothecin-induced cytotoxicity.¹³ CEP-8983 (a novel PARP inhibitor) synergized with bendamustine (a nitrogen mustard derivative) in killing primary chronic lymphocytic leukemia cells in vitro.¹⁴ Olaparib sensitized ATM null lymphoid tumor cells in vitro and in vivo to DNA-damaging agents.¹⁵ On the other hand, the pre-treatment of HL-60 cells with 3-aminobenzamide (3-AB) or 6(5H)-phenanthridinone (PARP inhibitors), resulted in resistance to, rather than potentiation of, apoptotic death induced by DNA-damaging agents, idarubicin, etoposide and fludarabine.¹⁶ As can be seen, in spite of the very attractive hypothesis of the synthetic lethality and theoretical usefulness of PARP inhibitors in enhancing the DNA-damaging effects of antitumor drugs, the above-mentioned divergent results in

leukemic cells are rather unexpected, with the underlying mechanisms probably being very complex.

The reasons for the lack of PJ-34 effects observed in the present study are unclear, however some potential explanations can be provided:

a) as we did not measure PARP activity, it cannot be excluded that the cells may have constitutively reduced protein expression, which may exert different effects compared to catalytic inactivation by PJ-34. Indeed, there are reports indicating that low PARP levels (and activity) attenuate responsiveness to PARP inhibitors.¹⁷ In this case, decreased PARP protein might be selectively advantageous to withstand the “poisoning” activity of drug-induced DNA-PARP aggregates.¹⁸ Quite similarly, the cytotoxic effect of topoisomerase inhibitors requires and is positively correlated with the levels and activity of topoisomerases;¹⁹

b) in our studies, we used the highest allowable concentration of PJ-34 which, after 72 h, did not induce a significant cytotoxicity in the cells. However, again, it cannot be excluded that a potential residual activity of PARP-1 in PJ-34-treated cells might suffice for rescuing the drug’s cytotoxic effects. Even if the PARP activity in the cells was not fully inhibited, we believe that such a condition much better reflects the clinical situation, where full inhibition of PARP-1 with currently-used inhibitors is impossible to achieve because of over-toxicity;²⁰

c) the cells may have an efficient DNA repair capacity via different paths, which was sufficient to repair DNA damage after exposure to the drugs. For example, it is well recognized that cells deficient in DNA DSB repair are highly sensitive to the chemical inhibitors of PARP, however, cells with intact DNA DSB-response pathways repair damage with high fidelity and accordingly show very little sensitivity to PARP inhibitors.

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A laboratory study evaluating the pH of various modern root canal filling materials

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Abstract

Background. Alkaline pH is responsible for antibacterial activity and the stimulation of periapical tissue healing. It neutralizes the acidic environment of inflammatory tissues in the periapical region of the teeth and favors bone repair by activating tissue enzymes.

Objectives. The aim of this study was to evaluate and compare in vitro the pH of 8 root canal filling materials (sealers and points) –AH Plus Jet (AH), Apexit Plus (AP), Endomethasone N (END), Epiphany (EP), GuttaFlow (GF), gutta-percha (G), Resilon (R), Tubliseal (T).

Material and methods. 0.1 g of each material ($n = 6$) was placed in dialysis tubes and immersed in 20 mL of deionized water. The control contained deionized water (pH 6.6) with an empty tube. The pH values were recorded immediately after immersion (baseline) and after 1, 2, 24, 48, 120, and 192 h with a pH-meter. Data were statistically analyzed using the Student's t -test and 1-way analysis of variance ($p < 0.05$).

Results. Nearly all the materials had pH significantly higher than the control ($p < 0.05$). There were significant differences in the pH between the materials tested at each time point ($p < 0.001$). The highest pH was exhibited by EP, followed by AP and AH. The lowest pH was shown by GF, G and R.

Conclusions. Among the materials studied, only EP, AP and AH Plus were able to elevate the pH level that would allow inactivation of microorganisms in the root canals and promote healing of inflamed periapical tissues. However, the low alkalizing potential of G and R can be modified by the concomitant application of sealers producing alkaline pH.

Key words: pH, endodontics, hydroxyl ion release, root canal obturation

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Increased health awareness among the population and the wish to save their natural teeth, accompanied by up-to-date knowledge and more effective working techniques of dentists, currently make it possible for people to preserve the teeth that in the past would have been extracted. Endodontics is the branch of dentistry concerned with the treatment of diseases of the pulp and periapical tissues. Root canal treatment is a safe and effective means of saving the teeth that otherwise would be lost. Endodontic therapy involves the removal of diseased pulp tissue and the subsequent shaping, cleaning, and hermetic obturation of the root canals to prevent their recontamination. Although this procedure results in removing blood vessels and nerves from the pulp cavity, it can preserve the tooth function successfully for many years provided the treatment is performed properly.¹ In some cases, multiple visits are required to complete endodontic therapy, during which inter-appointment dressings, such as calcium hydroxide are applied into the root canal.^{2,3}

Calcium hydroxide releases hydroxyl ion, thus favoring alkaline pH, which is responsible for its antibacterial effect and stimulation of the periapical tissue healing. When used as a temporary dressing, it kills microorganisms actively by damaging the plasma membrane, DNA and proteins of microorganisms.⁴ It has been shown that strongly alkaline pH inhibits growth, or even kills *Enterococcus faecalis* – facultative anaerobic Gram-positive cocci responsible for root canal treatment failures.⁵ The alkaline pH does not only impede infection development, but also neutralises the acidic environment of inflammatory tissues in the periapical region and favours bone repair by activating tissue enzymes (alkaline phosphatase).⁶ The effect of calcium hydroxide seems to be directly proportional to their alkaline potential.⁷

Currently, there is a tendency to limit the number of appointments necessary to complete root canal therapy. It is commonly believed that there is no need to apply temporary dressings into the root canal several times, as a similar effect can be achieved with their single application. Reducing the number of sessions in endodontic treatment eliminates the risk of complications, including the loss of temporary filling or tooth fracture, which can result in treatment failures. Thus, in endodontic therapy a one-visit model is proposed as a standard, with the shaping, cleaning and hermetic obturation of the root canal being performed during one appointment.⁸ In light of this fact, it seems important that the functions of inter-appointment dressings could be replaced by the final root canal filling materials.

The primary functions of the root canal filling are obturation and sealing of the root canal space.⁹ To fulfil these requirements, the simultaneous use of 2 materials is generally recommended: basic, in the form of central core material (gutta-percha or Resilon), and accessory, in the form of paste sealing spaces between core material and the root canal wall.

Since the studies involving the alkalisating abilities of root canal filling materials are relatively scarce, the aim of the current study was to evaluate and compare in vitro the pH of commercially available sealers and points most commonly used in the dental practice.

Material and methods

Table 1 shows the composition of the materials used in the study. All sealers were prepared according to the manufacturer's instructions. Shortly after manipula-

Table 1. Materials used in the study, their compositions and manufacturers

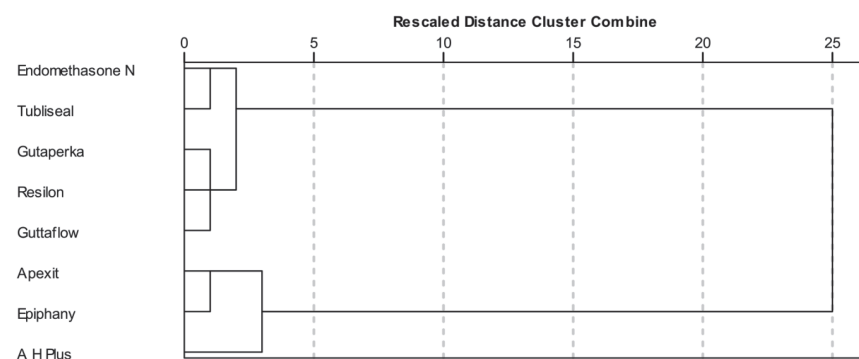
Name	Source	Active ingredients
AH Plus™	Dentsply DeTrey GmbH, Konstanz, Germany	bisphenol-a epoxy resin, bisphenol-f epoxy resin, calcium tungstate, zirconium oxide, silica, iron oxide pigments, dibenzyl diamine, amino adamantane, tricyclodecane-diamine, silicone oil
Apexit® Plus	Ivoclar Vivadent AG, Schaan, Lichtenstein	calcium salts (hydroxide, oxide, phosphate), hydrogenised colophony, disalicylate, bismuth salts (oxide, carbonate), highly dispersed silicon dioxide, alkyl ester of phosphoric acid
Endomethasone N Eugenol	Septodont, Cedex, France Chema – Elektromet, Rzeszów, Poland	zinc oxide, hydrocortisone acetate, thymol iodide, barium sulfate, magnesium stearate, eugenol
Epiphany	Pentron® Clinical Technologies, LLC Wallingford CT, USA	• organic part: bisgma, ethoxylated bisgma, udma, hydrophilic difunctional methacrylates • inorganic part: calcium hydroxide, barium sulphate, barium glass, bismuth oxychloride, silica
Gutta-Flow®	Coltene/Whaledent GmbH+Co. KG, Langenau, Germany	gutta-percha powder, polydimethylsiloxane, silicone oil, platin catalyst, zirconium dioxide, nano-silver, coloring
Gutta-percha points	VDW® GmbH Munchen, Germany	gutta-percha, zinc oxide, barium sulfate, pigment agent
Resilon points	Pentron® Clinical Technologies, LLC Wallingford CT, USA	• organic part: thermoplastic synthetic polymer – polycaprolactone, • inorganic part: biactive glass, bismuth oxychloride, barium sulphate
Tubli-Seal	Kerr Italia S.p.A., Salerno, Italy	zinc oxide, barium sulfate, oleo resin, oils/modifiers, thymol iodide, eugenol

Table 2. pH of 8 endodontic materials tested at different times

Material		Time (h)						
		0	1	2	24	48	120	192
AH Plus (AH)	mean	10.04a	10.06a	10.09a	9.99	9.78	9.53	9.11
	SD	0.18	0.17	0.17	0.28	0.43	0.57	0.78
	minimum	9.87	9.90	9.95	9.72	9.16	8.67	8.01
	median	9.97	9.98	10.02	9.86	9.67	9.40	8.98
	maximum	10.31	10.31	10.34	10.40	10.34	10.19	10.02
Apexit (AP)	mean	9.92a	10.11a	10.20a	10.98a	11.09a	11.20a	11.26a
	SD	0.11	0.11	0.13	0.05	0.06	0.06	0.09
	minimum	9.73	9.93	9.98	10.89	11.00	11.12	11.10
	median	9.91	10.11	10.22	10.99	11.10	11.22	11.29
	maximum	10.07	10.23	10.34	11.06	11.17	11.26	11.35
Endomethasone N (END)	mean	7.09b	7.39b	7.41b	7.47*b	7.49b	7.32b	7.20\$ ^b
	SD	0.14	0.29	0.18	0.18	0.15	0.11	0.13
	minimum	6.89	7.01	7.20	7.21	7.27	7.12	6.98
	median	7.10	7.41	7.42	7.48	7.47	7.33	7.23
	maximum	7.28	7.75	7.70	7.71	7.69	7.45	7.32
Epiphany (EP)	mean	9.99a	10.71	11.04	11.28a	11.29a	11.21a	11.23a
	SD	0.22	0.14	0.26	0.09	0.07	0.04	0.05
	minimum	9.70	10.54	10.78	11.16	11.20	11.15	11.16
	median	10.07	10.74	11.05	11.29	11.28	11.21	11.25
	maximum	10.18	10.89	11.30	11.38	11.37	11.28	11.28
GuttaFlow (GF)	mean	6.51*d	6.53cd	6.56cd	6.39cd	6.07# ^{\$}	5.63#	5.02
	SD	0.05	0.04	0.07	0.17	0.18	0.38	0.31
	minimum	6.46	6.49	6.41	6.17	5.86	5.15	4.57
	median	6.49	6.52	6.59	6.45	6.10	5.80	5.11
	maximum	6.58	6.58	6.60	6.54	6.27	5.98	5.36
Gutta-percha (G)	mean	6.16*c	6.41ce	6.33*c	6.42ce	6.55#cd	6.15#cd	6.27*#d
	SD	0.13	0.10	0.07	0.14	0.11	0.04	0.05
	minimum	6.05	6.30	6.24	6.29	6.40	6.11	6.20
	median	6.11	6.37	6.32	6.37	6.55	6.13	6.26
	maximum	6.36	6.57	6.42	6.65	6.70	6.21	6.35
Resilon (R)	mean	5.89c	6.47cf	6.53ce	7.04#* ^{\$}	6.83*ce	6.61*ce	6.86#bce
	SD	0.17	0.09	0.09	0.24	0.23	0.09	0.06
	minimum	5.70	6.35	6.40	6.85	6.68	6.52	6.80
	median	5.84	6.47	6.54	6.92	6.70	6.60	6.86
	maximum	6.10	6.58	6.65	7.45	7.20	6.73	6.95
Tubliseal (T)	mean	7.04b	7.39b	7.41b	7.45\$ ^b	7.31b*	7.23\$* ^b	7.11* ^{bc}
	SD	0.16	0.36	0.14	0.20	0.17	0.20	0.21
	minimum	6.85	6.94	7.17	7.12	7.09	7.01	6.89
	median	7.06	7.47	7.46	7.53	7.30	7.24	7.06
	maximum	7.20	7.83	7.57	7.63	7.60	7.54	7.44
control		6.6 d	6.6 def	6.6 *de	6.6 #de	6.6 \$de	6.6 \$de	6.6 de
p-values		*p = 0.006 a-d p > 0.05	a-f p > 0.05	*p = 0.043 a-e p > 0.05	#p = 0.002 *p = 0.006 \$p = 0.011 a-e p > 0.05	\$p = 0.001 *p = 0.006 #p = 0.007 a-e p > 0.05	\$p = 0.002 *p = 0.004 #p = 0.027 a-e p > 0.05	*p = 0.001 \$p = 0.023 #p = 0.038 a-e p > 0.05

The values which have not been tagged with identical letters and symbols in the columns indicate statistically significant differences at a level of $p < 0.001$; The values which have been tagged with identical letters a,b,c,d,e,f, in the columns are not statistically significant ($p > 0.05$); SD - standard deviation.

Fig. 1. Dendrogram illustrating the similarities in pH value of examined materials



The tested materials which are closest to each other in pH level are connected by vertical lines and form a cluster. The position of the lines on the scale (at the top of the figure) indicates the distances between clusters: the closer to the scale center, the greater similarity in pH level (details in the text).

tion, 0.1 g of each material was placed into dialysis tubes (Sigma Aldrich Chemie, Steinheim, Germany) and transferred into separate plastic vials, containing 20 mL of deionized water. A total of 6 samples were used for each material. The vials were hermetically sealed and kept in an incubator at 37°C.

Before each measurement, the vials were shaken for 5 s to ensure uniform hydroxyl ion distribution. The pH values were recorded immediately after immersion (baseline) and after 1, 2, 24, 48, 120, and 192 h with a pH-meter (ISE 710A, Orion Research Inc., Boston, USA), previously calibrated with solutions of known pH (4, 7, 10). Each sample was measured twice, and the mean value was recorded. The experiment was performed in static conditions (without changing the deionized water).¹⁰ The pH of the deionized water in which an empty tube was immersed was measured in all study periods (control).¹¹

Statistical analysis was performed using the software package STATISTICA 8.0 (StatSoft). One-way analysis of variance, ANOVA, for independent samples was applied to compare pH of the materials at each time point. If the difference was significant, individual comparisons were performed by Tukey's multiple comparisons test. The level of significance was set at $p < 0.05$. Hierarchical cluster analysis with a dendrogram, using average linkage between groups, was used as the classification method. Pearson's correlation coefficient was applied to measure the strength and direction of the linear relationship between the pH of the materials and the time of the experiment.

Results

The obtained results are listed in Tables 2 and 3, and presented in Fig. 1. The dendrogram (Fig. 1) presents 3 separate clusters of materials which are most similar to each other in terms of pH. The greatest similarity in pH was found in the following groups: the first cluster consisted of alkaline materials such as AP, EP and AH, the

second one was composed of neutral materials -G, R, GF. The third cluster contained acidic materials -END, T.

The mean pH values and SD measured for the study materials at different time points are presented in Table 2. The controls showed no noticeable change over the experimental period.

The majority of the materials demonstrated significantly higher pH as compared to the control, except for GF at baseline and after 1, 2, 24 h, G after 1, 48, 120, 192 h and R after 1, 2, 48, 120, 192 h.

Generally, the pH of the materials differed between individual clusters and these differences were statistically significant ($p < 0.001$).

The highest pH was exhibited by EP, followed by AP and AH. All 3 materials had a very similar pH at baseline (no statistically significant differences, $p > 0.05$). EP had significantly higher pH than AH at all other time points ($p < 0.001$), and compared to AP after 24, 48, 120, 192 h. AH showed statistically lower but still alkaline pH than AP after 24, 48, 120 and 192 h ($p < 0.001$) (Table 2).

The pH of GF, G, and R did not differ significantly after 1 and 2 h ($p > 0.05$). The baseline R showed a lower pH than those of GF and G, but in the last period of the experiment the pH of R increased ($p < 0.001$) (Table 2). In the first 2 h, G had a lower pH than GF, but over time the pH of G rose and was statistically significant after 48, 120, and 192 h (Table 2).

The pH of GF, G, and R did not differ significantly after 1 and 2 h ($p > 0.05$). The baseline R showed a lower pH than those of GF and G, but in the last period of the experiment the pH of R increased ($p < 0.001$) (Table 2). In the first 2 h, G had a lower pH than GF, but over time the pH of G rose and was statistically significant after 48, 120, and 192 h (Table 2).

Table 3. Correlations between mean values of materials pH and the duration of the experiment

Material	Pearson's correlation coefficient	Level of significance	Correlation
	r	p	
AH	-0.649	0.000	strong negative
AP	0.769	0.000	strong positive
END	-0.177	0.262	poor negative
EP	0.468	0.002	moderate positive
GF	-0.940	0.000	strong negative
G	-0.256	0.102	poor negative
R	0.395	0.010	moderate positive
T	-0.272	0.082	poor negative

r – Pearson's correlation coefficient; strong correlation $r > 0.6$; moderate correlation $0.3 < r < 0.6$; poor correlation $r < 0.3$; p – level of significance.

END and T were characterised by very similar and statistically insignificant pH values during all experimental periods ($p > 0.05$) (Table 2).

The analysis of the pH values of the materials as a function of time showed that only 2 sealers (EP and AP) were characterised by a gradual increase in pH until the final hours of the experiment. The pH value of other materials, after a slight increase, was either continuously decreasing (AH, GF) or stabilised (G, R, END, T).

A correlation was demonstrated between pH of the materials and time of the experiment. AP, EP and R showed positive and statistically significant correlations ($p < 0.05$) (Table 3). The other materials exhibited negative correlations, which were statistically significant for AH, GF, G and R ($p < 0.05$).

Discussion

The experimental method consisting in placing root canal filling materials in plastic tubes and immersing them in vials with an aqueous medium for a varying period of time in order to evaluate the pH of sealers is well established in literature. The dialysis tubes simulate the single-rooted teeth and, therefore, eliminate the anatomic variables found within the root canals of the teeth. According to Beltes and al., this method offers simplicity, time economy, and guarantees the reproducibility of measurements and easy comparisons of results.¹²

Among the materials tested, Epiphany (9.99–11.29) and Apexit Plus (9.92–11.26) had the highest pH. This may be due to the presence of calcium hydroxide in their composition. When the materials were placed in an aqueous solution, calcium hydroxide dissociated into hydroxyl and calcium ions increasing the pH in the surrounding medium.¹³ AH Plus presented a slightly lower but still alkaline pH (10.09–9.11). The pH values observed in the present study were higher than those obtained by other authors. Tanomaru-Filho et al. demonstrated that Epiphany produced the pH of 7.11–9.04 throughout a 28-day observation period.¹⁴ Faria-Junior et al. evaluated a new version of the Epiphany sealer – Epiphany SE (with acidic resin monomers added) and obtained pH values in a range of 5.25–5.72.^{15–20} Apexit Plus caused alkalinisation at the level of 7.5–10.79 and AH Plus in a range of 6.04–7.81. These discrepancies may be explained by various experimental conditions (different sample mass, evaluation of the release of hydroxyl ions after material setting, replacement of the surrounding medium after each measurement).

Zinc oxide-eugenol sealers, gutta-percha, Resilon and GuttaFlow exhibited neutral or slightly acidic pH. These observations are in agreement with earlier reports.^{14,20,21}

Maintaining the alkaline environment during the root canal treatment and after its completion seems to be desirable from the clinical point of view.⁷ It has been proven that the growth and development of osteoblasts, i.e. cells

crucial for the healing of periapical tissues, depends on the pH in the extracellular fluid. In the acidic environment, osteoblast activity decreases, and even a slight drop in pH can inhibit their function. Precipitation of calcium and phosphate salts in tissues and mineralisation processes, on the other hand, are supported by the alkaline pH.²²

One of the most frequently used biochemical markers of osteoblast activity and mineralisation processes in bones is alkaline phosphatase (ALP). It liberates free phosphate ions, which in turn react with calcium ions to form calcium phosphate precipitates in the organic bone matrix. Optimal pH for this enzyme activity can be varied in different biological systems, ranging from 8.0 to 10.8.²³

The alkaline pH of root canal filling materials, dependant on hydroxide ion release, appears to be responsible for their antibacterial effect. Estrela et al. have proved that at a pH greater than 9, bacterial enzymes can be irreversibly inactivated, resulting in loss of their biological activity.¹⁵ As the experiment shows, only materials in the alkaline group could produce the pH level favouring an alkaline phosphatase activity and promoting an antimicrobial action.^{2,24}

Therefore, attempts are made to incorporate alkalinising substances, such as calcium hydroxide into root canal filling materials. Tanomaru-Filho et al. observed a beneficial effect of adding 20% of calcium hydroxide to Epiphany sealer.²⁵ This resulted in a significant increase in the release of hydroxyl ions and thereby an elevation of pH values during the 28-day experimental period. Moreover, the addition of calcium hydroxide to Epiphany promoted better consistency for its use as a retrograde filling material, following root-end resection.¹⁴ Duarte et al. also have shown that the $\text{Ca}(\text{OH})_2$ addition to AH Plus favored a more alkaline pH. The authors emphasise, however, that when the material is used as a sealer, 10% addition of calcium hydroxide thickens the material too much, and they recommend a 5% incorporation.²⁰ Da Silva and Leonardo point out that merely the presence of calcium hydroxide in the composition of a sealer does not assure the release of an adequate amount of hydroxyl ions in the final product. The ions may not be released due to the interaction with other material components or after material setting.²⁶

Still, it should be remembered that in clinical conditions the alkalinising abilities of endodontic materials can be modified by dentine tissues. However, dentine seems to be a stronger buffer for acids than for alkalis. Main buffer properties depend on dentine hydroxyapatites together with water and a layer of adsorbed ions. The released layer adhering to apatite crystals reacts with various chemical compounds used in endodontic therapy and can modify their pH. The whole dentine tissue has been shown to be a more effective buffer than hydroxyapatite alone, indicating a contribution of dentine organic components to its buffer capacity.²⁷

Conclusions

Among the materials studied, only Epiphany, Apexit Plus and AH Plus were able to elevate the pH level that would allow the inactivation of microorganisms in the root canals and stimulate healing of inflamed periapical tissues. Gutta-percha, Resilon and GuttaFlow did not increase the pH sufficiently to stimulate biologically beneficial processes. The low alkalinising potential of gutta-percha and Resilon can, however, be modified by the concomitant application of sealers producing alkaline pH.

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Relationship between dietary antioxidant index (DAI) and antioxidants level in plasma of Kraków inhabitants

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Abstract

Background. Some literature data indicate that antioxidant-rich food may significantly increase antioxidants in serum and decrease the oxidative stress but results are ambiguous.

Objectives. The aim of this study was to estimate the total antioxidant capacity of food intake among the inhabitants of Kraków, Poland on the basis of dietary antioxidant index (DAI) and evaluation the relation between DAI and the level of antioxidants in plasma.

Material and methods. Examination included 70 (37 women and 33 men) non-smoking inhabitants of Kraków aged 46.4 ± 13.7 years. DAI was investigated on the basis of Food Frequency Questionnaire including 145 food items. DAI was measured using the method by Benzi and expressed as FRAP (mMol/L). In plasma samples total antioxidant status (TAS) expressed as FRAP and malondialdehyde (MDA) concentration as a marker of lipids peroxidation were measured.

Results. The mean value of DAI of all examined persons was 46.74 ± 25.5 mMol/L (in female group: 54.13 ± 27.7 mMol/L; in male group: 37.83 ± 19.5 mMol/L; $p < 0.05$). The highest contribution in total DWA value had fruits (48.7%) opposite to vegetables (9.3%). Statistically significant positive correlations between DAI and FRAP in plasma was found in all: $r = 0.42$ and in female: $r = 0.54$ groups (not significant in men group: $r = 0.20$). Statistically significant negative correlation of DAI with MDA (malonylaldehyde) in female (-0.49) and male (-0.51) groups.

Conclusions. The obtained results confirmed the hypothesis that the intake of antioxidants in daily diet (measured as DAI) might increase antioxidants defense (measured by TAC as FRAP) and decrease oxidative stress (measured by MDA concentration in plasma). The dietary modification towards higher consumption of antioxidants (especially in men) should be highlighted in prevention of diseases in which oxidative stress play considerable role.

Key words: FRAP, MDA, healthy people, dietary antioxidant index, Food Frequency Questionnaire

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The integrated antioxidant defense system plays the main role in the body's defense against reactive oxygen species (ROS) and oxidative stress.¹ Antioxidants scavenge free radicals, ROS and reactive nitrogen species from cells, preventing or reducing the damage caused by the oxidation of body tissues.² Free radicals can adversely alter lipids, proteins and DNA, and can trigger a number of human diseases.³ Oxidation leading to free radical formation can be accelerated by stress, smoking, alcohol intake, sunlight, air pollution and other factors.¹ The antioxidant defense system includes the enzymatic system (e.g. superoxide dismutases [Cu/Zn, Fe and Mn types], catalase and glutathione peroxidase) and the non-enzymatic system (e.g. ascorbic acid, tocopherols, tocotrienols [vitamin E], copper, zinc and selenium).^{4–10} The main source of non-enzymatic antioxidants seems to be a well-balanced diet. Antioxidant activity and synergistic interactions between molecules in food and blood plasma are reflected by the total antioxidant capacity. In nutritional epidemiology, the dietary antioxidant index (DAI) is usually used to assess the intake of antioxidants from food. Evaluating the DAI is important because current literature data indicate that a diet enriched with multicomponent antioxidant foods like fruit, vegetables and vegetable oils, grains and cereal-based products, fresh herbs and certain beverages may significantly increase the concentration of antioxidants in plasma and therefore decrease oxidative stress.¹¹

Many dietary antioxidants may contribute to cellular protection against radicals and other ROS. Many food products are rich sources of antioxidants. The main source of antioxidants in food are fruit and vegetables containing vitamins C and E, selenium and carotenoids such as beta-carotene, lycopene, lutein and zeaxanthin. Vitamin E (8 structural isomers of tocopherols/tocotrienols) is one of the most widely distributed antioxidants in nature; α -tocopherol is the best known and shows the most antioxidant activity.¹² Vitamin E can to some degree prevent the consequences of oxidized LDL, and vitamin C provides nitric oxide synthase (NOS) activity.¹³ Good sources of specific antioxidants include anthocyanins in eggplant, grapes and berries; beta-carotene in pumpkin, mangoes, apricots, carrots, spinach and parsley; flavonoids in tea, green tea, citrus fruits, red wine, onions and apples; lycopene in tomatoes, pink grapefruit and watermelon; vitamin A in sweet potatoes, carrots, milk and egg yolks; vitamin C in black currants, kiwi fruit, mangoes, broccoli, spinach, peppers and strawberries; vitamin E in vegetable oils (such as wheat germ oil), avocados, nuts, seeds and whole grains; zinc in seafood, lean meat, milk and nuts. Any of these may contribute significantly to the total DAI value.¹⁴

The aim of the study was to assess the daily intake of antioxidants among inhabitants of the Kraków metropolitan area on the basis of the DAI. Additionally, links between the DAI and the FRAP and MDA in plasma were examined.

Material and methods

Seventy healthy volunteers (37 women and 33 men) were enrolled in the study, which lasted for a period of 4 months (February to May, 2015). The participants were 21 to 73 years old (mean age: 46.4 ± 13.7 years). They were nonsmokers. They all provided written consent, and the study protocol was approved by the Bioethics Committee of the Jagiellonian University Medical College.

Each participants' dietary antioxidant index (DAI) was investigated on the basis of a food frequency questionnaire (FFQ). The questionnaire was prepared on the basis of American Dietetic Association recommendations.¹⁵ In the FFQ 145 food items were classified into groups such as fruit and dried fruit, vegetables and vegetable oils, sweets, grains and cereal-based products, mixed dishes, fresh herbs and spices and beverages. It was stated in the questionnaire that every nutritional position represents all forms of a particular food (fresh, frozen, dried, cooked or packed in cans) – e.g. "apples" means fresh apples, apple juice, compote, apple pie, etc.

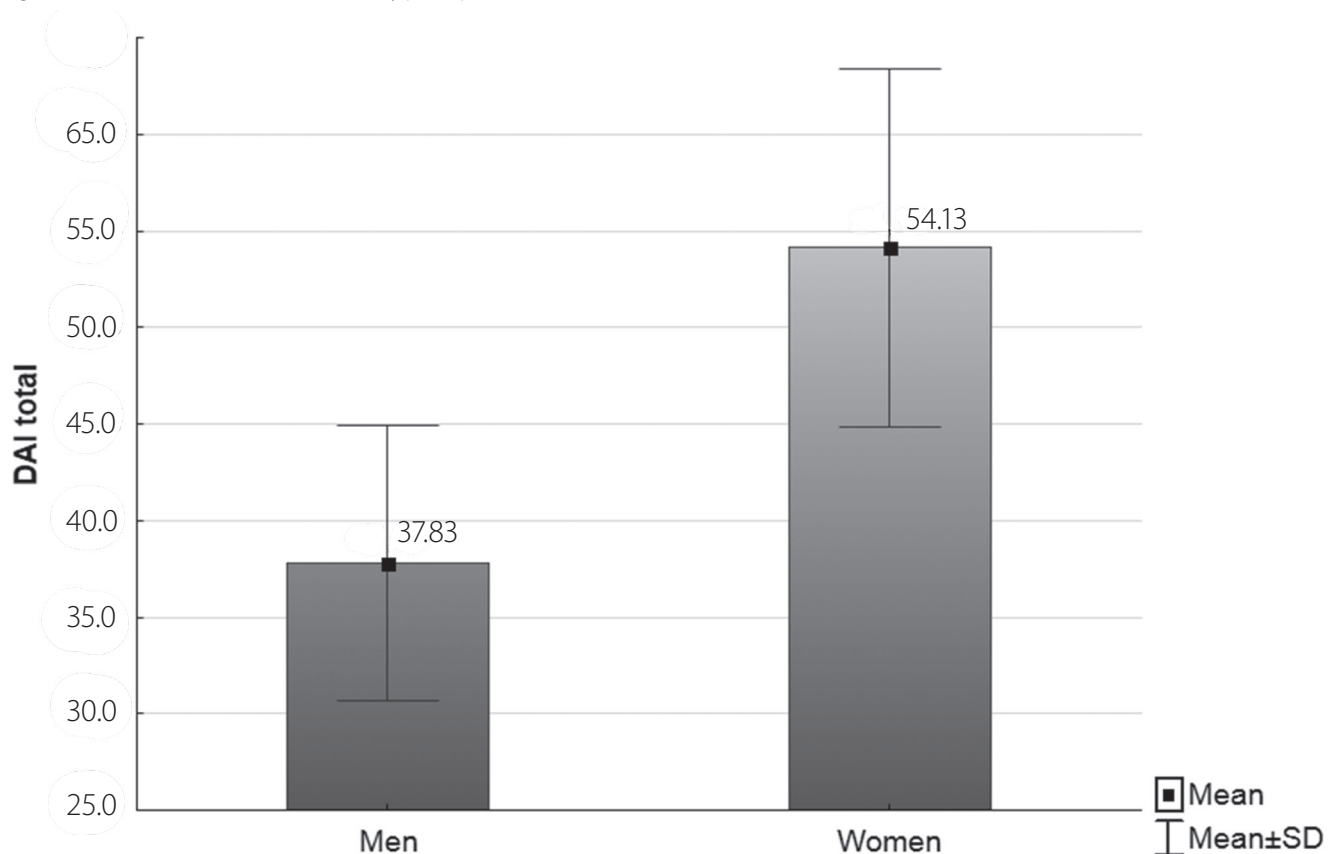
The participants reported how often they had eaten each food in the previous month. In order to calculate the DAI for each participant, previously published databases from the USA and Italy, containing the most commonly consumed foods, were used. The size of a medium portion (100 g of a product, the volume of a glass, the content of a soup spoon or the amount of product necessary to spread over two pieces of bread, etc.) was included for every nutritional product in the questionnaire. Medium serving sizes were given as a reference; half of a medium portion was treated as a small portion, and one and a half or more of a medium portion was considered a large portion.

The total antioxidant capacity of the participants' food intake (their dietary antioxidant index) was measured using the method described by Benzie.¹⁶ This method exploits the ability of a given sample to reduce ferric ions to ferrous ions (FRAP), which are bound in a colored complex with tripyridyl-S-triazine. In the questionnaire the FRAP value of a medium portion of each product was included. The survey participants could declare small, medium or large portions. A small portion was counted as half of the FRAP value for a medium portion, and a large portion was counted as 1.5 times the FRAP value of a medium portion. The FRAP values specific for the size of the portion were multiplied by the number of portions and the total was divided by 30 days. This result was treated as the FRAP value of the daily nutritional intake (DAI).

DAI values for the male and female subgroups were compared using Student's *t* test.

Fasting blood samples were collected from the 70 participants once after a 12 h overnight fast. The blood samples were drawn into tubes containing K3 EDTA and were centrifuged within 4 h of collection from the participant. Plasma samples were collected and stored at -30°C until the analyses of FRAP and MDA were done.

Fig. 1. DAI values in the male and female study participants



The total antioxidant status, expressed as FRAP, and malondialdehyde (MDA) concentration, as a marker of lipid peroxidation, were measured in the plasma samples. MDA was assayed by determining the coupling of MDA with thiobarbituric acid (TBA) under acid pH conditions at 95°C. The TBA-MDA complex was determined fluorometrically at 532 nm.^{17,18} MDA levels were expressed in mMol/L.

All the statistical analyses were carried out using STATISTICA for Windows software (v. 10.0, StatSoft Polska, Kraków, Poland). Differences were considered statistically significant if $p < 0.05$.

Results

The mean value of the DAI of the entire study group was 46.74 ± 25.5 mMol/L. The mean DAI value for the female participants (54.13 ± 27.7 mMol/L) was statistically significantly higher ($p < 0.05$) than the mean for the male participants (37.83 ± 19.5 mMol/L).

The contribution of particular groups of food products, expressed as mMol/L and as proportional contributions, in the entire study group and for the male and female subgroups are presented in Tables 1 and 2.

In the entire study group as well as in the male and female subgroups, fruit contributed the most to the total

Table 1. The contribution of particular groups of food products in the total dietary antioxidant index (DAI) in the study group

Groups of food products	Men n = 33	Women n = 37	Total n = 70
	mMol/L		
Fruit and dried fruit	20.52	24.69	22.70
Vegetables	3.56*	4.92*	4.34
Fresh herbs and spices	0.74	3.08	2.01
Grains and cereal-based products (pastas, rice)	4.32*	7.96*	6.31
Mixed dishes (traditional Polish soups)	0.78	0.89	0.84
Fish and seafood	0.24	0.33	0.30
Fats and oils	0.12	0.11	0.12
Sweets (chocolate, honey, jam)	0.29	0.90	0.62
Beverages (coffee, tea, wine)	7.26*	11.23*	9.43
mMol/L	37.83	54.13	46.74

* statistically significant results.

Table 2. The contribution percentages of particular groups of food products in the total dietary antioxidant index (DAI) in the study group

Groups of food products	Men n = 33	Women n = 37	Total n = 70
	mMol/L		
Fruit and dried fruit	54.22	45.62	48.72
Vegetables	9.41	9.09	9.28
Fresh herbs and spices	1.95	5.69	4.30
Grains and cereal-based products (pastas, rice)	11.42	14.70	13.50
Mixed dishes (traditional Polish soups)	2.06	1.65	1.80
Fish and seafood	0.63	0.62	0.63
Fats and oils	0.33	0.21	0.27
Sweets (chocolate, honey, jam)	0.77	1.66	1.33
Beverages (coffee, tea, wine)	19.19	20.76	20.17
%	100	100	100

Table 3. The concentration of FRAP (mMol/L) and MDA (nMol/mL) in the study group

	Men n = 33		Women n = 37		All n = 70	
	X	SD	X	SD	X	SD
FRAP mMol/L	1.07	0.42	1.19	0.44	1.14	0.43
MDA nMol/mL	9.12	7.75	10.14	7.60	9.66	7.16

Table 4. The values of correlation coefficients among the male and female study participants

	Men n = 33	Women n = 37
plasma FRAP mMol/L versus DAI	0.20	0.54*
MDA nMol/mL versus DAI	-0.51*	-0.49*

*statistically significant results.

dietary antioxidant index (48.7% in the full group). Beverages, especially coffee, were in the second position (20.2% in the whole study group), and next were grains and cereal-based products (13.5% in the full group). Vegetables were eaten very rarely (only 9.3% of the total DAI value in the entire group). The contributions of the remaining food products (fresh herbs and spices, fish and seafood, fats and oils, mixed dishes and sweets) were low: 8.3% in the entire study group).

It was found that there were statistically significant differences between men and women in the DAI values for vegetables, grains and cereal-based products and beverages (Fig. 2).

The plasma total antioxidant ability, expressed as FRAP, and plasma MDA levels are presented in Table 3.

In the entire study group as well as in the male and female subgroups, a statistically significant correlation was found between the mean DAI value and FRAP in plasma ($r = 0.42$). A negative correlation was found in all 3 groups between the mean DAI value and plasma MDA level ($r = -0.45$) (Fig. 3).

The correlation coefficient between FRAP in plasma and the DAI was statistically significant only among the female participants ($r = 0.54$). In male subgroup this coefficient did not reach statistical significance ($r = 0.20$). The correlation coefficients between the DAI and MDA in plasma were statistically significant in both the male and female subgroups (Table 4).

Discussion

According to the Food-Based Dietary Guidelines created by the National Nutrition Institute in Warszawa and the Polish Ministry of Health, a healthy diet should include fruit and vegetables in amounts equal at least half of the daily food intake, with a 3 : 1 proportion of vegetables to fruit. The amount of fats should be low, and where possible substituted with vegetable oils or soft margarines.¹⁹ The results of this study showed that vegetables were eaten too rarely and their contribution to the total DAI value was too low. Fruit contributed the most to the total DAI values. As in the present study, other authors have found that among both healthy and ill subjects the main source of natural antioxidants in the daily diet is fruit.²⁰ For many people in Poland fruit is bought and consumed mainly during the summer. Plasma antioxidant capacity was lower in autumn and in winter, compared to relatively the high level during the summer; the lowest was noted in spring.²¹ This discrepancy is caused by higher consumption of fruit (e.g. strawberries, raspberries, cherries, gooseberries, currants) and seasonal vegetables (young beet leaves, kohlrabi, asparagus, sorrel, green beans, zucchini). Moreover, it should be noted that the antioxidant potential of fruits and vegetables during winter is lowered because of transportation and long periods of storage. Therefore the recommendation for Polish people should be to increase consumption of fruit and vegetables from local producers (e.g. cultivated in greenhouses) during the spring, autumn and winter. In addition, consumption of citrus fruit, fruit/vegetable drinks and spices can be suggested to fill shortages in antioxidative potential when fresh seasonal products are not available.

Currently, specialists in nutrition consider dietary enrichment with functional foods and supplements a very

Fig. 2. The statistically significant differences in DAI (Student's t-test) of consumed vegetables, grain and cereal based products and beverages between female and male groups

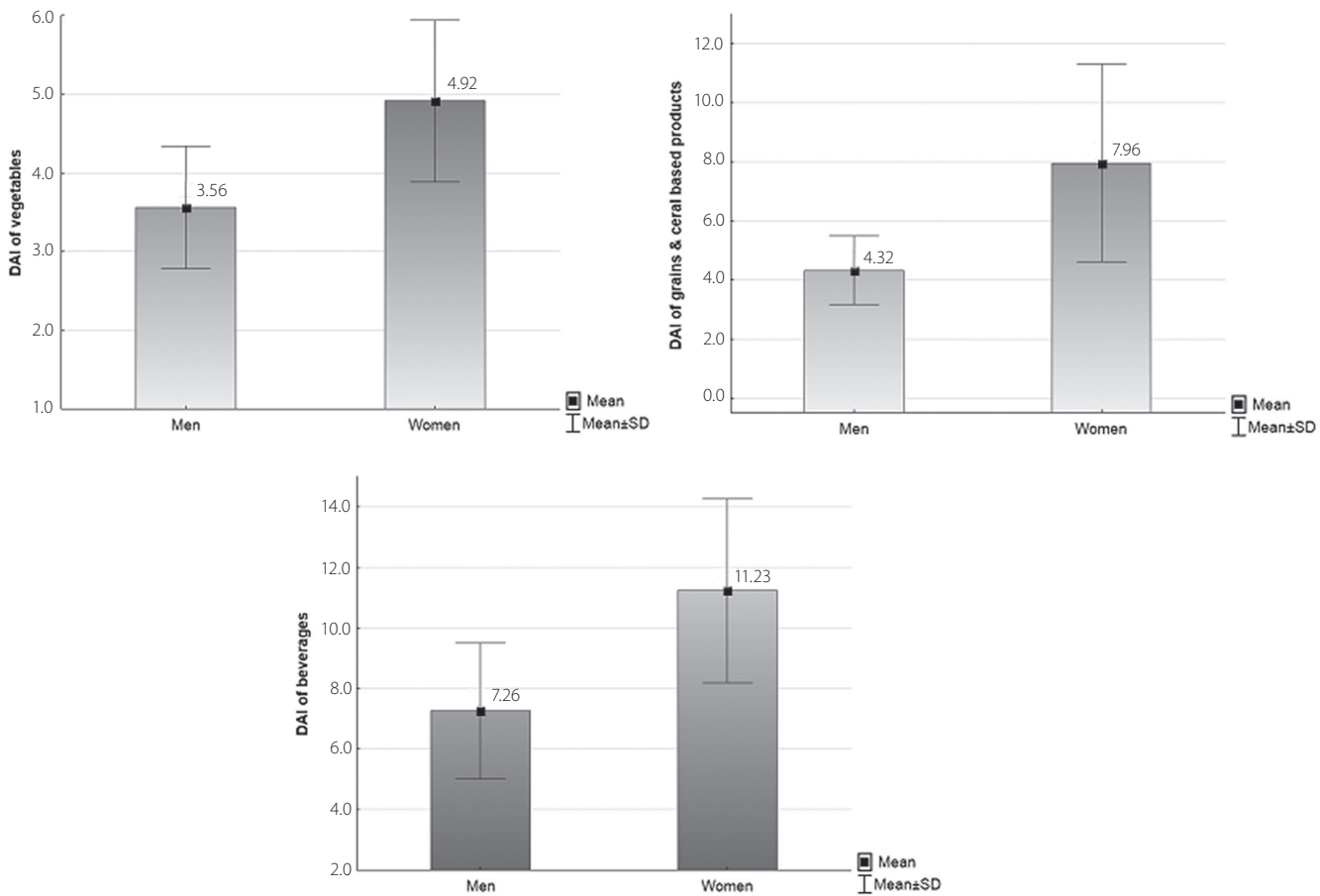
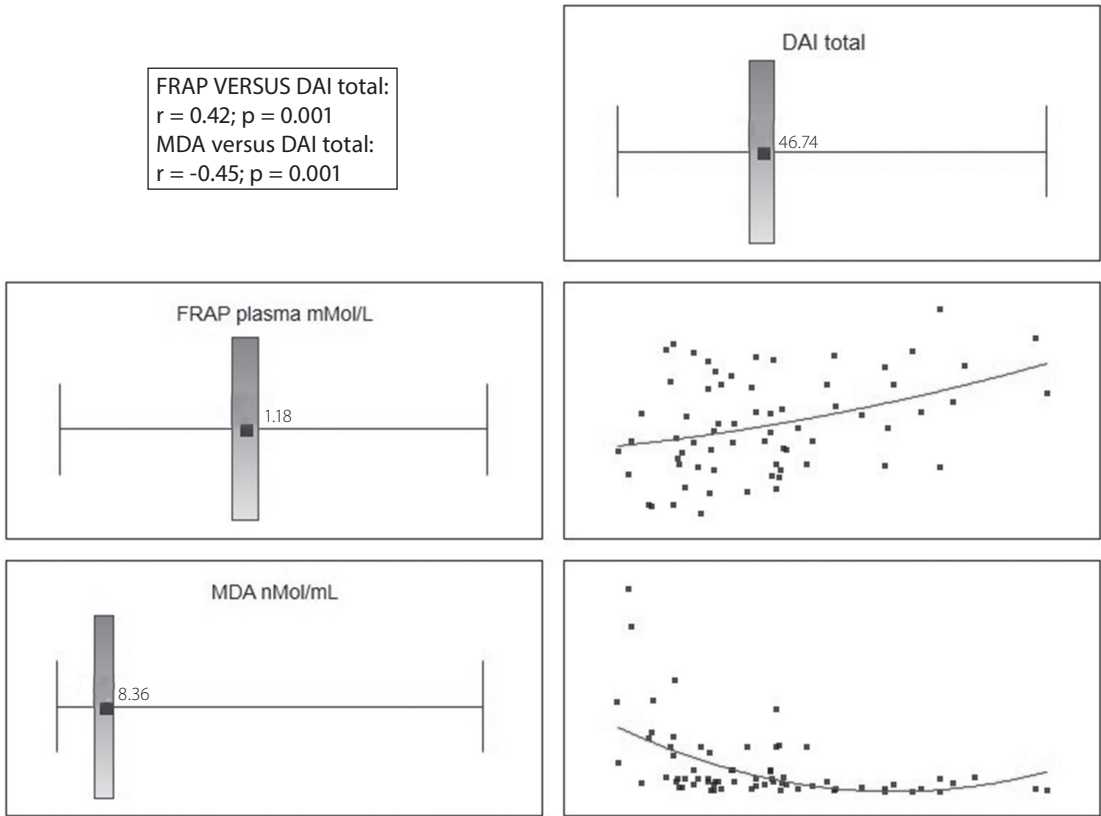


Fig. 3. The values of correlation coefficients in all examined groups



important factor in the prevention of degenerative disorders caused by oxidative stress.²² Unfortunately, the results of large studies are not optimistic.²³ Some recent studies – the Women's Antioxidant Cardiovascular Study (8,000 female health professionals, aged 40 years or older), the Women's Health Study (40,000 healthy women at least 45 years old) and the selenium and vitamin E Cancer Prevention Trial (SELECT; 35,000 men aged 50 or older) – showed that antioxidant supplements did not help prevent diseases caused by oxidative stress.^{24–26} The evidence suggests that antioxidant supplements do not work as well as the naturally occurring antioxidants in food. Moreover, taking supplements in high doses can be harmful. Dietary supplements may cause side effects, trigger allergic reactions or interact with prescription and over-the-counter medicines.^{23,27} Therefore, a healthy diet remains the best way to provide sufficient antioxidant intake. Many researchers have shown that sufficient consumption of fruit and vegetables is associated with a reduced rate of some diseases (especially coronary heart disease) and with a lower risk of all-cause mortality – particularly cardiovascular mortality, but also cancer mortality.^{28,29}

It is worth pointing out that it is not clear whether these results are related only to the amount of antioxidants in vegetables and fruit, or to other components of these products, or to lifestyle factors such as stress, air pollution, etc. People who eat more antioxidant-rich food might also be more likely to be physically active and less likely to smoke, and it is clear that physical exercise and not smoking are crucial in the prevention of cardiovascular diseases, stroke, intestinal cancer, etc. All these factors may decrease the risk of disease, but the results of the present study confirmed a direct link between the DAI and the level of TAS and lipid peroxidation markers in plasma, indicating that a diet rich in antioxidant foods should also decrease the risk of oxidative stress disorders. It is also important to emphasize that the positive correlation between the DAI and plasma MDA in both the male and female subgroups suggests that a diet rich in antioxidants may reduce the risk of developing various pathological conditions, such as MDA-induced cell injury via, as Ayala et al. wrote, “intramolecular or intermolecular protein/DNA crosslinking that may induce profound alteration in the biochemical properties of biomolecules”.³⁰ Unfortunately the present study found no correlation between DAI and the total antioxidant status of plasma expressed as FRAP in men, which may be partially explained by the lower intake of antioxidants in their diet (in comparison to women).

Conclusions

The results of the study confirmed the hypothesis that the intake of antioxidants in the daily diet might increase

antioxidant defense (measured by the total antioxidant status of plasma expressed as FRAP) and decrease oxidative stress (measured by plasma MDA concentration). Introducing an anti-oxidant diet should therefore become an interesting approach to the prevention of disorders caused by increased oxidative stress.

Dietary modification towards higher consumption of natural antioxidants (especially by men) should be implemented as a public health strategy.

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An evaluation of selected oral health indicators and cariogenic bacteria titer in patients with *Helicobacter pylori*

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Abstract

Background. Studies based on polymerase chain reaction (PCR) techniques indicate that *Helicobacter pylori* can be constantly or temporarily present in the oral cavity in virulent or non-virulent form. *Streptococcus mutans* exerts a strong inhibitory effect on *H. pylori*.

Objectives. The aim of the present study was to investigate the prevalence and virulence of *H. pylori* in the oral cavity and the correlation of these factors with oral health and cariogenic bacteria titer.

Material and methods. The study involved 108 adults who were positive in urease tests for *H. pylori* presence in the gastric mucosa. Group I consisted of 50 patients with positive saliva tests using PCR for the presence of *H. pylori* DNA, while group II comprised 58 patients with negative tests. The research material consisted of saliva and dental plaque. To determine the density of *S. mutans* and *Lactobacillus*, commercially available *S. mutans* and LB sets were used.

Results. *H. pylori* DNA was found in the oral cavities of 46% of the patients who had tested positive in urease tests for the presence of these bacteria in the stomach. Among those who tested positive for the presence of *H. pylori* in the oral cavity, virulent strains were identified in 16% of the patients. Approximal plaque index (API) and bleeding on probing (BOP) were found to be significantly higher in patients with confirmed *H. pylori* in the oral cavity. This group also had a smaller number of *S. mutans* colonies.

Conclusions. *H. pylori* is found more often in patients with poor oral hygiene. Oral sanitation and hygiene instructions should be considered relevant as a complement to eradication therapy.

Key words: PCR, saliva, *Lactobacillus*, dental plaque, *Streptococcus mutans*

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Helicobacter pylori is a Gram-negative spiral bacterium from the ϵ -proteobacteria family. The optimal temperature for the bacterium's existence ranges from 36–42°C, at a pH of between 5 and 7 in microaerophilic conditions. *H. pylori* plays a significant role in the pathogenesis of gastric carcinoma and mucosa-associated lymphoid tissue (MALT) lymphomas.²⁴

The bacterium is highly sensitive to acids, and protects itself from their influence with help of its mucus layer and the production of urease in large amounts. This enzyme, encoded by 7 ure genes, constitutes one of the major factors of pathogenic activity, hence the division into pathogenic urease-positive strains and nonpathogenic urease-negative strains, which only occur in laboratory culture conditions.²⁵

Helicobacter pylori occurs in 3 forms: living spiral culture forms, which are virulent and infectious in character and cause inflammation in experimental animals; living spherical non-culture forms, which have less ability to colonize and cause infections in experimental animals; and residual degenerative forms of dying *H. pylori*.¹

The incidence and prevalence of *H. pylori* infection in the human population is estimated at 50%. In developing countries with low socio-economic conditions the rate is around 80–100%, and in developed countries it is in the region of 20–40%. Humans are considered the main carrier of this bacteria. Routes of transmission have been described based on oral-oral, fecal-oral and gastro-oral theories.²

Laboratory tests have shown that some strains of *H. pylori* have the ability to form biofilm in vitro. The formation of biofilm is associated with the binding of individual bacteria, which form microcolonies that grow in 3 dimensions.² Biofilm is prevalent in the oral cavity, and spiral and spherical forms of *H. pylori* can be detected in dental plaque.¹

The presence of *H. pylori* in the oral cavity has been confirmed by polymerase (PCR).³ In other studies *H. pylori* has been isolated from saliva, dental plaque and various pathological lesions in the oral cavity.^{4–6} The amounts of *H. pylori* genetic material found vary widely from study to study. For example, Song et al. isolated bacteria from 97% of dental plaque samples and 55% of saliva samples, whereas Cammarota et al. confirmed its presence in only 3.2% of dental plaque samples.^{7,8} There are some difficulties in comparing these results due to the use of different primers, PCR protocols and sampling techniques. Chamanrokh et al. suggested the potential occurrence of non-culture spherical forms in the oral cavity.⁹

Some studies indicate that the *H. pylori* strains isolated from the oral cavity differ from gastric strains, whereas other researchers have stated that identical strains are present in both the oral cavity and the stomach.^{7,10} In most cases, patients who tested positive for *H. pylori* in the oral cavity also had positive gastric biopsy results, but a large number of patients with gastric infections do not present co-infection in the oral cavity.¹¹

H. pylori in the oral cavity may also interact with the components of its ecosystem. A study by Okuda et al. indicates that *Porphyromonas gingivalis* and *Fusobacterium nucleatus* interact with *H. pylori* strains.¹²

The bacterial component of the cariogenic process plays a critical role in the pathogenesis of changes. *Streptococcus mutans* and *Lactobacillus acidophilus* are currently considered to be the most cariogenic species of bacteria. *S. mutans* bacteria are the primary cariogenic colonizers. Their development and high titer may help to drive *H. pylori* out of its niches in the oral cavity, and when *H. pylori* numbers are low, ideal conditions for *S. mutans* colonization are present. In turn, *Lactobacillus* is a prominent member of the late cariogenic flora family with an unconfirmed impact on the *H. pylori* population. Investigating the tendency for *S. mutans* and *Lactobacillus* to interact with *H. pylori* may help to clarify the conditions that the bacteria need to exist in the oral cavity. This would help to ensure effective diagnoses and eradication therapy for *H. pylori*.

The aims of the study were to assess the presence and degree of virulence of *H. pylori* bacteria in the saliva and dental plaque of patients with positive urease results from a gastric biopsy; to evaluate selected indicators of oral health in patients with confirmed and unconfirmed *H. pylori* present in their saliva and dental plaque; and to determine the *Streptococcus mutans* and *Lactobacillus* bacteria index in the oral cavities of patients with confirmed and unconfirmed *H. pylori* in their saliva and dental plaque.^{1–3}

Material and methods

The study was conducted on adult patients (both sexes) at the Endoscopy Unit of the Central Clinical Hospital in Katowice, Poland, which is part of the Department of Gastroenterology and Hepatology of the Medical University of Silesia in Katowice. As part of the gastroenterological diagnostic process, patients underwent a gastroscopy that included an urease test. A total of 108 patients with positive *H. pylori* urease tests in the gastric mucosa qualified for the next stage of the study. The participants provided written consent for the study and had to meet strict inclusion criteria for the study groups, as follows:

- a positive urease test result during a gastroscopy;
- aged over 18;
- no severe general diseases affecting their oral health (e.g., neoplastic diseases, systemic diseases, untreated diabetes);
- no inflammatory conditions in their oral mucosa that significantly affected the environment (erosive lesions, ulcerations, RAS syndrome, fungal infections, etc.);
- a minimum of 24 teeth in the oral cavity.

Patients who did not meet the above criteria, as well as pregnant women, people wearing removable partial

dentures, patients treated with bismuth compounds and/or antibiotics in the previous 2 months and patients who had undergone previous eradication therapy or immuno-suppressive therapy were excluded from the study. The age of the 108 subjects accepted ranged from 18 to 68 years, with an average age of 42 years. Women made up 60.2% of the study group.

Patient interviews and physical examinations were conducted in separate rooms. The prepared questionnaires were filled in with data obtained from the patients. Selected dental indicators – namely the Simplified Oral Hygiene Index (OHI-S, devised by Greene and Vermillion), approximal plaque index (API) and bleeding on probing (BOP) – were determined. The API is used to evaluate oral hygiene by assessing the presence of dental plaque in interdental spaces. BOP is used to evaluate the degree of bleeding from periodontal pockets and rapidly estimate the severity of a patient's gingivitis. In a simplified procedure assessments are made of the palatal surfaces and linguistic quadrants 1 and 3 and 2 and 4 of the vestibular dentition quadrant.

Procedures for sampling and preserving the genetic material

A total of 3 mL of stimulated saliva was collected from each patient along with a small quantity of dental plaque from the lingual side or palatal surface of a molar. Each patient, in a fasting state, received a paraffin cube and a sterile plastic vial. By holding the paraffin cube in the oral cavity and chewing it gently the patient was able to deposit stimulated saliva in the opened vial. Once 3 mL of saliva (the level marked on the previously prepared vial) was obtained, plaque was collected using a disposable probe and deposited in the vial with the saliva. The vial with the study material was secured with a factory default plastic cap and mounted in a rack in a portable refrigerator at about 5°C. Then a non-invasive bacteriological examination was performed to determine the bacteriological index of *S. mutans* and *Lactobacillus*, using ready-to-use Dentocult SM and LB kits (Orion Diagnostica, Espoo, Finland).

The kits were placed in an incubator set at 37°C. The result for *S. mutans* was read after 3 days of incubation, whereas the *Lactobacillus* result was recorded after 4 days. The quantitative results for the bacterial colonies were evaluated with the Dentocult SM and LB kits. They were determined in colony-forming units/mL (CFU/mL). In the case of *S. mutans* these values were additionally ranked by the manufacturer according to classes. *Lactobacillus* bacteria were evaluated quantitatively using the ready-to-use interpretative keys accompanying the Dentocult LB kits, which assessed them as < 10³, 10³, 10⁴, 10⁵ or 10⁶ CFU/mL.

The study material in the form of collected saliva and dental plaque was properly secured and immediately transported to the Department of General Medical Biology at the Medical University of Silesia in Katowice to determine the presence of *H. pylori* genetic material using

PCR. On the basis of the results, the study participants were divided into 2 groups: group I, which consisted of 50 subjects (including 29 [26.9%] women) with positive PCR saliva test results for the presence of *H. pylori* DNA, and group II, consisting of 58 subjects (including 36 [33.3%] women) with negative results.

The gastric biopsies were collected in 1.5 mL tubes containing 200 µL of RNAlater nucleic acid stabilizer (Sigma-Aldrich, Hamburg, Germany). They were properly secured and then transported to the laboratory, where they were stored at -20°C until the beginning of the analysis. They served as positive control standards in the PCR process.

The vials containing 3 mL of saliva with a small amount of dental plaque suspended in it were sent to a laboratory, where they were stored at -20°C until the beginning of the analysis.

Genomic DNA extraction

The dental plaque was scraped from the patient's teeth and put in 3 mL sterilized tubes. The samples were frozen at -20°C before being transported to the laboratory. The DNA was extracted using a QIAamp® DNA Mini Kit (Qiagen, Germany) in accordance with the protocol provided in the manual kit (QIAamp DNA Mini and Blood Mini Handbook 04/2010).

PCR

The PCR amplification procedure was carried out with a Mastercycler Personal PCR thermal cycler (Eppendorf, Hamburg, Germany). The PCR mix included the following: 12.5 µL of PCR Master Mix, including a ready-to-use solution containing "Taq" DNA Polymerase, dNTPs, MgCl₂ and reaction buffers (Promega, Madison, USA); 10 pM of each primer (forward and reverse); and 2.5 µM of each dNTP. Approximately 400 ng of the isolated DNA was used as a template. Nuclease free water was added to make up the reaction volume (25 µL). The DNA extracted from *H. pylori* obtained from the gastric biopsies with positive urease test results served as a positive control. The primer sets used in the PCR process for this study are presented in Table 1.

The PCR amplification results were viewed using 1.5% agarose gel with GelStar Stain (Lonza, Visp, Switzerland) under ultra-violet light and photographed.

The full text of the laboratory procedures that were used in this study is available on request via e-mail.

Statistical analysis

The statistical analysis of the results was performed using STATISTICA software, v. 10 (StatSoft, Tulsa, USA). Median values, mean values and standard deviation

Table 1. Primer sequences with PCR product

Primers	Sequence of primers 5'–3'	Gene	Annealing temperatures (°C)	Product size (bp)	Source
HPU1/HPU2	CGTGCATACCCCTATTGAGG CACGCTCTTTAGCTCTGTG	Urease A	62-60-58-56-54	380	Song et al. 1999 with modifications
HP1/HP2	CGTTAGCTGCATTACTAGAGAG CATTACTGACGCTGATTGCGC	16sr RNA	66-64-62-60-58	110	Song et al. 1999 with modifications
HPOS/HPOAS	GTGTGGGAGAGGTAGGTGGA TGCCTTAGCTGCATTACTGG	16sr RNA	62-60-58-56-54	216	Chaudhry et al. 2001
EHG-U/EHC-L	CCCTCAGCCCATCAGTCCCAAAAA AAGAAGTCAAAAACGCCCAAAAC	860 bp DNA region	58	417	Song et al. 1999
ET-5U/ET-5L*	GCCAAATCATAAGTCCGAAGAA' TGAGACTTTCTAGAAGCGGTGTT	860 bp DNA region	58	230	Song et al. 1999
CagA	GATAACAGGCAAGCTTTTGAGG CTGCAAAAGATTGTTGGCAGA'	CagA	62-60-58-56-54	349	Yamaoka et al. 1999

*ET-5U/ET-5L – primers for nested PCR, which are directed to the 417-bp product of primers EHG-U/EHC-L.

tions were calculated. The statistical hypotheses based on these results were verified using the non-parametric Mann-Whitney U test. In addition, the test χ^2 of independence was used to analyze differences between the data measured on a weak measurement scale. The decision to use the Mann-Whitney test was dictated by several cases of deviations in the distribution of the characteristics compared to normal distribution, which was checked using the Shapiro-Wilk test. To verify the statistical hypotheses, the following levels of statistical significance were adopted: $p > 0.05$ – no significance; $p < 0.05$ – statistical significance; $p < 0.01$ – high statistical significance.

Approval for the study was obtained from the Ethics Committee of the Silesian Medical University (No. KNW/0022/KB1/161/10/I/11, dated 20.09.2011). The laboratory tests were subsidized by the Department for Science and International Cooperation of the Medical University of Silesia (Contract No. KNW-1-074/D/1/0).

Results

Of the 108 subjects known to have *H. pylori* present in the stomach, molecular testing showed that 50 had *H. pylori* DNA present in their saliva samples and in the plaque suspended in the saliva (group I). In 58 patients genetic tests revealed no *H. pylori* DNA present in the oral cavity despite confirmed infection of the gastric mucosa (group II). Therefore, the presence of *H. pylori* bacteria in the oral cavity was detected in 46% of the patients with urease-positive bacteria detected in the stomach.

Data tables for oral health indicators

The mean OHI-S value, API and BOP percentages were higher in group I than in group II, and the Mann-Whitney U test showed that these differences were statistically significant in the cases of the API and BOP (API: $p = 0.0028$, BOP: $p = 0.0013$). The difference between the two groups' OHI-S values was not statistically significant ($p = 0.4324$).

Test results of *Streptococcus mutans* and *Lactobacillus* cultures

The statistical analysis showed significant differences in the number of *S. mutans* bacterial colonies in the 2 study groups. A higher number of bacterial colonies was observed in study group II compared to group I ($p = 0.0065$). The reverse trend was observed in the case of *Lactobacillus* cultures: The number of colonies of this type was higher in group I than in group II; however, the difference was not statistically significant ($p = 0.3363$).

Of the 50 patients who tested positive for the presence of *H. pylori* in their oral cavities (group I), virulent strains (cagA+) were confirmed in 8 cases, which represents 16% of the group. Due to the small size of the subgroup known to have the virulent strain, it was difficult to reliably assess the differences in average oral health and cariogenic bacteria values, both in the case of the 2 subgroups, as well as with respect to group I as a whole. However, no statistically significant correlations were present, either in the subgroup with known virulence or in the subgroup with non-virulent strains.

One fact observed was that patients with virulent strains of *H. pylori* detected in the oral cavity did not use antibacterial mouthwashes (0/8 patients). The percentage of patients with nonvirulent strains of *H. pylori* in the oral cavity who used mouthwashes was 31% (13/42 patients). Among patients in group II, who tested negative in the PCR examination for *H. pylori* in their oral cavities, 41% used mouthwashes (24/58 patients).

In patients with virulent strains of the bacteria more frequently suffered from active gastrointestinal diseases: 87% of the patients from the virulent subgroup had at least one such disorder. In the subgroup with non-virulent strains, the percentage of patients with diseases of this type was 62%, as compared with 68% of people with no *H. pylori* present in the oral cavity. Due to the small size of the subgroup with virulent strains (cagA+) these results were not statistically significant.

Discussion

The research conducted by the authors of the present study consisted in isolating *H. pylori* DNA from the saliva of patients who had tested positive for the presence of this bacteria in their gastric mucosa during a gastroscopy. The presence of *H. pylori* in the oral cavity has been the subject of numerous studies and has aroused controversy. Its presence in this environment in residual or transient form is a fact. On the other hand, the frequency and amount of bacteria in the oral cavity has not been fully established. To date, only a few mechanisms of interaction have been shown to exist between different bacteria, and this can help to improve our understanding of the occurrence and role of *H. pylori* in pathologies of the oral cavity. Its presence in plaque is highest in the area around the molars and lowest in the region of the premolars and incisors.¹³ This fact may be due to oxygen exposure, which increases in the mesial direction, and this in turn can lead to the formation of distal niches.

Many different methods have been developed to detect *H. pylori* in the oral cavity. The PCR method, however, is the most accurate and most suitable for research purposes. The most sensitive method for testing saliva involves the use of EHC-U/EHC-L primers. The only disadvantages of molecular methods are that they are expensive and are technically more difficult compared to breeding, histological methods and rapid urease tests. The 16S rRNA gene marker has been shown to be the most accurate method.¹⁴

In the present study, the detection rate of *H. pylori* in the oral cavity of patients with urease-positive bacteria in their stomach was 46%. This is comparable to the findings of a study by Gebara et al., in which, out of the 100 patients who tested positive for *H. pylori* in gastric mucosa, positive results for the presence of the bacteria in the oral cavity were noted in 43.3% of cases.¹⁵ Accord-

ing to other researchers, PCR DNA analysis revealed *H. pylori* in the oral cavity in 3–89% of cases.^{4,7,8,14,16} Such disparate results may be due to different procedures employed for sampling, or a consequence of the primers used. This is because microorganisms similar in shape as well as other urease-producing bacteria may in fact be present in complex biofilm. Dental plaque has been called “a reservoir for genotypes”, where horizontal gene transfer can take place between the bacteria that reside in the biofilm.^{17,18} The genotypic variability of this bacteria manifests itself in the presence of point mutations and changes in the gene sequence. This may lead to both false positive and false negative results if the PCR test is limited to determining a single gene. The present study therefore identified 4 different genes, namely: 16S rRNA (two kinds of primers), *UreA*, 860bp DNA region (2 types of primers) and *cagA*. Recognition of a combination of at least 2 different, simultaneously occurring genes was regarded as a positive result.¹⁸ Individually marked genes were analyzed for the sensitivity and specificity of the primer. A 16S rRNA region that amplifies the 106bp fragment and the region from human leukocytes presents low specificity. This may consequently lead to false positive results. The urease gene may not be present in all strains of *H. pylori*, which means there is a chance of obtaining false negative results. Other species of bacteria that secrete urease in the plaque, such as *Streptococci*, *Actinomyces* or *Haemophilus spp*, can cause false positives.^{18,19}

The research team of Silva et al. demonstrated a correlation between the colonization of supragingival plaque and oral health parameters – specifically, between the percentage of the surface covered with dental plaque and the bleeding rates of periodontal pockets.¹⁶ Patients with higher values of these 2 indicators were likely to report the presence of *H. pylori* in their oral cavities. The authors also demonstrated a 98% DNA sequence identity in gastric, saliva and plaque bacteria. The present study shows a statistically significant difference between the 2 study groups in terms of the amount of interdental plaque (API) and bleeding from periodontal pockets (BOP): Higher values of both indices were observed among the patients with *H. pylori* present in the oral cavity.

The present study, based on a simplified OHI-S index, showed that *H. pylori* was more likely to be detected in patients with higher levels of this index, despite the lack of any statistical significance. When analyzing mean values and extremes of the index, the simplified model should be abandoned so that the total index for all teeth can be measured to ensure greater research accuracy. Most of the tooth surfaces marked in a simplified indicator test are easily accessible for hygiene procedures, while other areas may remain uncleared. This fact may have an impact on the total index.

Considering all 3 of the oral hygiene indices used in this study – the OHI-S, API and BOP – it can be concluded that a patient's oral hygiene status affects the diagnosis

and treatment of *H. pylori* infections. Jia et al. emphasize the importance of dental plaque as a temporary reservoir of bacteria and show that long-term professional management reduces re-infection of the stomach following eradication treatment.^{19,20} The failure of triple eradication therapy for treating *H. pylori* in the oral cavity is a consequence of a failure to achieve an effective concentration of antibiotics in the saliva and dental plaque.^{19,20} Okuda et al. and Andersen et al. demonstrated that strains of *P. gingivalis* and *F. nucleatum* strongly co-aggregate with *H. pylori* strains.^{1,12} Okuda et al. also observed that *Streptococcus oralis*, *Streptococcus mitis*, *Streptococcus mutans*, *Streptococcus*, *Streptococcus sorbinus*, *Actinomyces naeslundii*, *Prevotella intermedia* and *Prevotella nigrescens* produce bacteriocin-like, anti-*H. pylori* inhibitor proteins.¹² In patients with appropriate oral hygiene, low or even no trace amounts of *H. pylori* bacteria may be present in the oral cavity. This is explained by the fact that the bacterium is driven out by early colonizers of the oral cavity. Gebara et al. suggest that a high percentage of positive *H. pylori* results in the oral cavity is associated with poor oral hygiene: As noted earlier, among their patients who tested positive for the presence of *H. pylori* in the gastric mucosa, the same bacteria was present in 43.3% of patients whose oral hygiene was clearly worse than the rest of the study group.¹⁵ Analyzing the data, it must be assumed that in the *H. pylori* positive group of patients in the above studies there was a predominance of late colonizers in the oral cavity, or cariogenic bacteria, i.e., microorganisms of the *Lactobacillus* group.^{12,15} The present study indicates a trend towards a higher incidence of bacterial colonies of *Lactobacillus* in patients with *H. pylori* in the oral cavity than in those in whom this bacteria was not present in the oral cavity. However, the difference was not statistically significant.

The authors observed that none of the people in the subgroup with virulent strains of *H. pylori* in the oral cavity used antibacterial mouthwashes, while 31% of the people diagnosed with non-virulent strains used mouthwashes. The percentage of mouthwash users in group II, where no *H. pylori* was detected in the oral cavity, was 41%. The absence of *H. pylori* in patients using mouthwashes most probably does not indicate the direct impact of mouthwashes on this bacterium, but rather on the biofilm as a whole. This causes subsequent quantitative and qualitative changes in plaque mass in relation to the proportion of *Lactobacillus* and *Streptococcus* strains.

Various studies have reported a negative correlation between the presence of *H. pylori* and the number of *S. mutans* colonies.¹² Likewise, the present study showed a statistically significant increase in the density of *S. mutans* bacterial colonies in patients without *H. pylori*.

Two research teams, Assumpção et al. and Momtaz, were able to show the presence of *H. pylori* strains with virulent factors in the oral environment by determining factors of bacterial pathogenicity in molecular stud-

ies.^{21,22} Positive genotype *babA2*, *cagA* and *vacA* bacterial strains involve a higher risk of metaplastic changes developing when compared to gene-free strains. Hacker and Kaper introduced the concept of a pathogenicity Island (PAI). This theory defines a DNA segment with a specific nucleotide composition, at the end of which the *cagA* gene is located, which is responsible for encoding a highly immunogenic *cagA* protein.^{22,23} It is found in 60–80% of *H. pylori* strains, and its presence is associated with the production of the vacuolating cytotoxin (*vacA*). The *H. pylori* *cagA* genotype and *vacAs1m1* are associated with an increased risk of adenocarcinoma of the stomach. Assumpção et al. found a genotypic similarity (*cagA* and *vacA*) between *Hp* bacterial isolates in the stomach and the oral cavity.²¹ According to those researchers, the *H. pylori* in plaque is associated with acute gastrointestinal disorders, such as gastroesophageal reflux, which can lead to the virulent strains *vacAs1m1+* and *cagA+* colonizing the mouth. The present study indicates that the vast majority of people (87.5%) with virulent *H. pylori* strains in the oral cavity suffer either from active stomach ulcers (5/8 patients) of varying degrees of severity, or from gastroesophageal reflux disease (4/8 patients). Half of the respondents had undergone eradication therapies between 2 and 5 years previously and were currently diagnosed with a reinfection.

Both the research conducted by the authors of this study and an analysis of the literature on the subject indicate that every individual undergoing eradication therapy should be provided with dental care so that they can receive dental treatment and instructions in oral hygiene.

Summary and conclusions

H. pylori was observed in the oral cavity of 46% of the patients that had a positive urease test in a biopsy of the gastric mucosa. The presence of virulent strains was observed in 16% of the patients with confirmed *H. pylori* in the oral cavity.

Higher BOP, OHI-S and API values, and thus poor hygiene and gum inflammation, were noted in patients that had positive urease tests in biopsies of the gastric mucosa as well as *H. pylori* confirmed in the oral cavity.

Fewer colonies of *Streptococcus mutans* were found in patients with confirmed *H. pylori* in the gastric mucosa and the oral cavity than in patients without *H. pylori* confirmed in the oral cavity.

A higher number of *Lactobacillus* colonies were observed in patients with *H. pylori* present in the oral cavity than in patients with no *H. pylori* in the oral cavity. However, the correlation was not statistically significant.

Every individual who undergoes eradication therapy should receive dental care in order to check the state of their oral hygiene and eliminate the potential risk of reinfection from oral niches.

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The effects of recombinant human granulocyte colony-stimulating factor mouthwash on radiotherapy-induced oral mucositis in locally advanced nasopharyngeal carcinoma patients

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Abstract

Background. Acute oral mucositis is a common complication of radiotherapy for nasopharyngeal carcinoma (NPC) patients.

Objectives. The aim of the study was to observe the effects of recombinant human granulocyte colony-stimulating factor (rhG-CSF) on radiotherapy-induced oral mucositis in locally advanced NPC patients.

Material and methods. The study involved 64 locally advanced NPC patients that were randomly allocated to receive either rhG-CSF mouthwash (2 µg/mL rhG-CSF; group A, n = 34) or a compounded mouth rinse (10 µg/mL vitamin B12, 0.48 mg/mL gentamicin and 0.04 mg/mL dexamethasone in saline; group B, n = 30) during radiotherapy. Both mouthwashes were used 6 times daily at the onset of oral mucositis, and the treatments continued until the end of all intensity-modulated radiotherapy sessions. Oral mucositis was graded according to the Radiation Therapy Oncology Group acute radiation morbidity scoring criteria. A visual analog scale was used to assess peak mouth pain once a week, and the duration of oral mucositis was recorded.

Results. In comparison with group B, the patients in group A had a significantly lower incidence of oral mucositis of grade 3 or above (38.2% vs 66.7%, $p < 0.05$) and less peak mucosal pain in the 5th, 6th and 7th weeks of radiotherapy ($p < 0.05$). group A patients also had shorter durations of oral mucositis (35.1 days vs 39.4 days, $p < 0.05$) and lower peak swallowing function scores ($p < 0.05$).

Conclusions. The rhG-CSF mouthwash may be more effective than the compounded mouth rinse in preventing and treating radiotherapy-induced mucositis and mucositis-related pain, and thus improving the quality of life for locally advanced NPC patients. These effects should be further investigated in a prospective controlled study.

Key words: recombinant human granulocyte colony-stimulating factor, oral mucositis, radiotherapy, locally advanced nasopharyngeal carcinoma

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Nasopharyngeal carcinoma (NPC) is the most common malignant tumor of the head and neck, and radiotherapy is the main radical treatment.¹ However, radiotherapy may lead to complications, such as acute oral mucositis, especially in locally advanced NPC patients. Oral mucositis results from injury to the epithelial cells that line the oral cavity. This damage causes changes that range from mild atrophy to severe ulceration, and these symptoms are aggravated with the accumulation of the radiation dose. Serious consequences include pain requiring opioid analgesia, potentially life-threatening infections, inadequate nutrition requiring parenteral feeding, and prolonged hospitalization.^{2,3} Currently, no standard therapy prevents or treats severe oral mucositis.⁴ Most patients are treated with a topical mouth rinse containing vitamin B12.⁵

Recombinant human granulocyte colony-stimulating factor (rhG-CSF) is a hematopoietic growth factor that promotes the proliferation and differentiation of neutrophils.⁶ It has been widely used to increase the neutrophil count in patients with advanced neoplasms and to reduce the magnitude of chemotherapy-induced neutropenia.^{7,8} Another hematopoietic growth factor, human granulocyte-macrophage colony-stimulating factor (GM-CSF), has been shown to prevent and treat chemotherapy- and radiation therapy-induced oral mucositis in patients with head and neck cancer.^{9,10} However, information is lacking about the effect of rhG-CSF on radiotherapy-induced oral mucositis in locally advanced NPC patients. The present randomized controlled study was designed to observe the effects of rhG-CSF mouthwash on oral mucositis in locally advanced NPC patients.

Material and methods

Patient selection and characteristics

Patients who met the following criteria were eligible for the study: 1) age 18 or over; 2) histologically proven nasopharyngeal squamous cell carcinoma (clinical stage III or IV); 3) Karnofsky performance status > 70; 4) normal complete blood counts, liver function tests, renal function tests and blood sugar tests; 5) no history of prior radiation therapy or cytotoxic chemotherapy; and 6) no autoimmune disease.

A total of 64 patients were enrolled from January 2012 to December 2013 at the Department of Oncology at the First Affiliated Hospital of Yangtze University (Jingzhou, China). Informed consent was obtained from all the patients. There were 22 female patients and 42 male patients, and the median age was 48 years (range: 32–70 years). According to the American Joint Committee on Cancer (AJCC) 7th edition staging system, 35 patients had stage III malignancies and 29 patients had stage IV malignancies. Twenty-six of the patients were tobacco users. The patients' characteristics are shown in Table 1.

All the patients were treated with definitive radiotherapy. Cisplatin (30 mg/m²) was administered weekly for 6 weeks as a radiosensitizer. Intensity-modulated radiotherapy was adopted using 6 MV photons generated from a linear accelerator. The prescribed median doses were 73.92 Gy in 33 fractions for gross tumor volume, 69.96 Gy in 33 fractions for the positive lymph node, 60.06 Gy in 33 fractions for the high-risk clinical target volume, and 50.96 Gy in 28 fractions for the low-risk clinical target volume. The dose constraints of the organs at risk were determined in accordance with the Radiation Therapy Oncology Group (RTOG) 0615 protocol.

In addition, all the patients underwent dental prophylaxis in the form of scaling and fluoride application, and dental cavity filling and extraction were performed prior to radiotherapy. Patients with artificial dentures were advised not to wear them during the procedures. Dentists verified that none of the patients had developed oral mucositis prior to radiotherapy. The patients were counseled against using phenol- or alcohol-containing mouthwashes, tobacco, alcoholic beverages, very hot food, very cold food or spicy food. No intraoral tumor ablative procedures that altered the mucosal surfaces were carried out.¹¹ All the procedures were performed in accordance with the hospital's ethical guidelines.

Administration of rhG-CSF

The patients were randomly assigned to group A or group B. Group A patients were treated with the rhG-CSF mouthwash (saline containing 2 µg/mL rhG-CSF; QILU Pharmaceuticals, Jinan, China) at the onset of oral mucositis. Group B patients were treated with a compounded mouth rinse (saline containing 10 µg/mL vitamin B12, 0.48 mg/mL gentamicin and 0.04 mg/mL dexamethasone). Both of the oral rinses were used 6 times daily until the end of all the radiotherapy sessions. During each use, the rhG-CSF mouthwash or compounded mouth rinse was kept in the oral cavity for 3 min.

Oral mucositis was graded according to RTOG acute radiation morbidity scoring criteria. The nurses in charge of the study patients examined their oral cavities daily as part of each morning's routine care from the start of radiotherapy until all the radiotherapy sessions were over. Mouth pain was assessed using a visual analog scale (VAS) once a week during radiotherapy, and the peak mouth pain score was recorded. The duration of oral mucositis was calculated from the onset of oral mucositis to recovery. Peak swallowing problems were self-reported by patients and determined using a VAS of 0–5.¹²

Statistics

The χ^2 test and Student's t-test were used to analyze the data. SPSS Statistics 17.0 software (SPSS Inc., Chicago, USA) was used. A p-value < 0.05 was defined as statistically significant.

Table 1. Characteristics of patients with locally advanced nasopharyngeal carcinoma

Characteristics	Group A	Group B
Number of patients	34	30
Gender: number (%)		
male	22 (64.7%)	20 (66.7%)
female	12 (35.3%)	10 (33.3%)
age: mean (range)	48.6 (35–70)	47.3 (32–68)
Karnofsky performance status: number (%)		
> 80	30 (88.2%)	27 (90%)
= 80	4 (11.8%)	3 (10%)
NPC stage: number (%)		
III	19 (55.9%)	16 (53.3%)
IV	15 (44.1%)	14 (46.7%)

Table 2. The effect of rhG-CSF mouthwash on radiotherapy-induced oral mucositis in locally advanced nasopharyngeal carcinoma patients

Group	Number of patients	Grade of oral mucositis	
		1–2	3–4
A	34	21 (61.8%)	13 (38.2%)*
B	30	10 (33.3%)	20 (66.7%)

* $p = 0.02$ vs Group B; χ^2 test.

Results

The patients' characteristics are shown in Table 1. All the patients completed the radiotherapy procedure. Group A had 34 patients and group B had 30 patients. The majority of patients in both groups had Karnofsky performance status scores that were greater than 80. There was no obvious heterogeneity between characteristics of the 2 groups.

The rhG-CSF mouthwash decreased the incidence of severe radiotherapy-induced oral mucositis. More than 80% of the patients suffered from oral mucositis when the radiation dose was increased to 20 Gy. Administration of the rhG-CSF mouthwash improved oral mucositis. In comparison with group B, the incidence of oral mucositis of grade 3–4 significantly decreased in group A ($p < 0.05$, Table 2). Oral mucositis of grade 3–4 was observed in 38.2% and 66.7% of patients in group A and group B, respectively. One patient in group A and 4 patients in group B had severe oral mucositis of grade 4, and these patients received antibiotics for local infections. Good tolerance to radiotherapy was observed in 61.8% of patients with oral mucositis of grade 1–2 in group A.

The rhG-CSF mouthwash decreased the severity of radiotherapy-induced mouth pain. Mouth pain was gradually aggravated with the accumulation of the radiation dose. The most severe pain occurred in the 5th and 6th weeks of radiotherapy. Eighty-six percent of the patients used painkillers in accordance with the World Health

Organization three-step analgesic ladder principle. At each time point, the peak pain score was recorded prior to painkiller use. Compared with group B, the pain scores of the patients in group A were decreased in the 5th, 6th and 7th weeks of radiotherapy (Fig. 1, $p < 0.05$). Therefore, treatment with the rhG-CSF mouthwash decreased the incidence and severity of mucositis and improved pain relief in the mouth.

The rhG-CSF mouthwash shortened the duration of radiotherapy-induced oral mucositis. As shown in Fig. 2, the mean durations of oral mucositis

were 35.1 days and 39.4 days in group A and group B, respectively ($p < 0.05$). This suggests that the rhG-CSF mouthwash can shorten the duration of radiotherapy-induced oral mucositis and promote recovery from oral mucositis.

The rhG-CSF mouthwash improved radiotherapy-induced swallowing function scores. All the patients had different degrees of decline in swallow function. One patient in group B (but none in group A) required total parenteral nutrition. Compared with group B, the peak mean swallowing function scores in group A were significantly improved (Fig. 3, $p < 0.05$).

Side effects of the rhG-CSF mouthwash

No side effects such as nausea and vomiting were observed with the rhG-CSF mouthwash. All the patients completed the treatment.

Fig. 1. The effect of rhG-CSF mouthwash on mouth pain scores of locally advanced nasopharyngeal carcinoma patients during radiotherapy, as assessed with a visual analog scale. Group A patients received the rhG-CSF mouthwash treatment, and group B patients received a compounded mouth rinse treatment. Results are mean \pm standard deviation (SD)

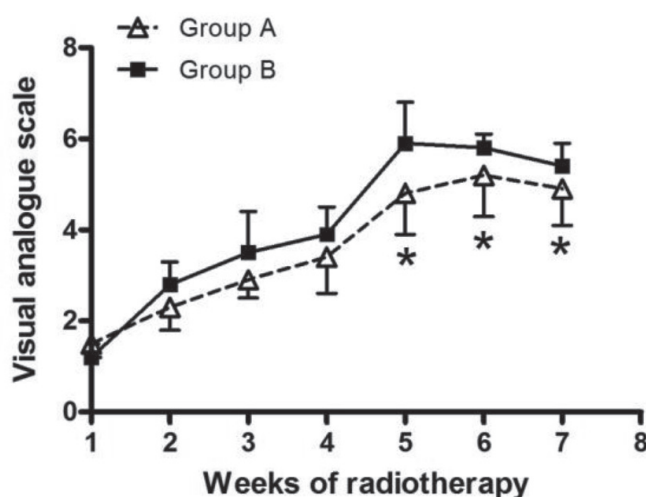


Fig. 2. The effect of rhG-CSF mouthwash on the duration of radiotherapy-induced oral mucositis in locally advanced nasopharyngeal carcinoma patients. Group A patients received the rhG-CSF mouthwash treatment, and group B patients received a compounded mouth rinse treatment. Results are mean \pm SD

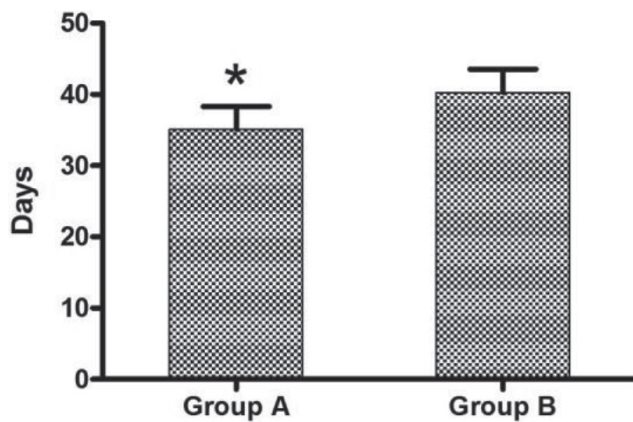
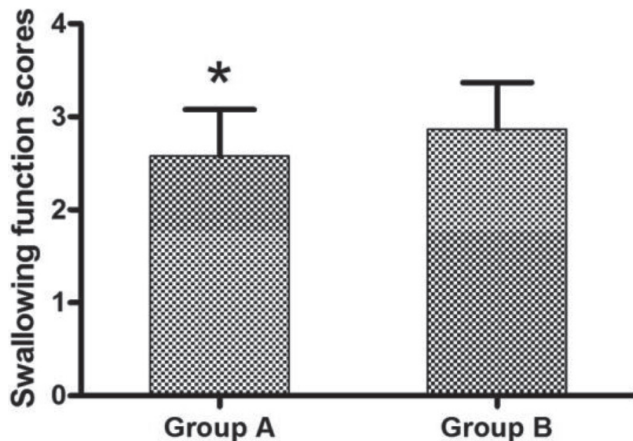


Fig. 3. The effect of the rhG-CSF mouthwash on peak mean swallowing function scores that were induced by radiotherapy in locally advanced nasopharyngeal carcinoma patients. Group A patients received the rhG-CSF mouthwash treatment, and group B patients received a compounded mouth rinse treatment. Results are mean \pm SD



Discussion

Radiation inevitably injures normal tissues around the tumor in NPC patients. In particular, oral mucosal epithelial cells are extremely sensitive to radiation, and acute oral mucositis is a common complication of radiotherapy for NPC.¹³ The incidence of acute oral mucositis of grade 3–4 is 85% in radiotherapy patients with advanced NPC.² This complication seriously affects the patients' quality of life and nutrition status. Oral mucositis can increase the potential risk of oral infection, which could result in suspension of radiotherapy and a poor curative effect.¹⁴

In this study, topical application of rhG-CSF mouthwash was demonstrated to be effective for the prevention and treatment of radiotherapy-induced oral mucositis in locally advanced NPC patients. Significant decreases in the duration and severity of oral mucositis, reductions in the degree of pain and improvements in swallowing function

were attributed to the effects of the rhG-CSF mouthwash. The control group received a standard compounded mouth rinse that has been used in the authors' department with good results. The compounded mouth rinse was also effective against oral mucositis.

Importantly, rhG-CSF is a 19.6-kDa glycoprotein that is characterized as a growth factor for hematopoietic progenitor cells.¹⁵ It has been approved by the US Food and Drug Administration and the European Medicines Agency (EMA), and is commonly used to treat neutropenia and to mobilize bone marrow hematopoietic stem cells for transplantation.¹⁶ A previous study showed that GM-CSF influences the proliferation and differentiation of stem cells and regulates several functions in mature leukocytes, macrophages and dendritic cells in the mucosa and dermis.^{17,18} Therefore, the present authors speculated that the potential mechanism of the effects of rhG-CSF on oral mucositis was associated with the proliferation and differentiation of mucosal stem cells.

In summary, the topical application of rhG-CSF effectively prevented and treated radiotherapy-induced oral mucositis in locally advanced NPC patients. Furthermore, treatment with rhG-CSF improved the quality of life for these patients without inducing any side effects. Therefore, these effects should be further investigated in a prospective, controlled study.

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An outbreak of leptospirosis imported from Germany to Poland

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation;

D – writing the article; E – critical revision of the article; F – final approval of article

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Conflict of interest

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Abstract

Background. Leptospirosis is a zoonotic disease caused by spirochetes of the *Leptospiraceae* family. In both humans and animals the main route of infection is indirect contact – through water or other products contaminated with urine containing spirochetes. Infection most commonly occurs through ingestion of water or food contaminated with *Leptospira* spp.

Objectives. The aim of the study was to characterize cases of leptospirosis imported to Poland from Germany in 2014 and to analyze methods that are helpful for making a diagnosis.

Material and methods. The 10 patients examined were reported as suspected leptospirosis cases on the basis of clinical symptoms and epidemiological investigations. They originated from different regions of Poland and had been working together at a strawberry plantation in the Cloppenburg district of Lower Saxony in Germany. Blood and urine samples were tested by PCR and serum samples by serology. All ELISA positive and negative cases were examined using a reference microscopic agglutination test (MAT).

Results. In the tested group, 6 individuals (60%) were seropositive according to the ELISA, and 2 of them were confirmed by the MAT. The PCR results for the blood and urine samples were negative.

Conclusions. Using the ELISA in the diagnosis of leptospirosis allowed the disease to be identified much faster, differentiating classes of antibodies and recognizing levels of them that are too low to be detectable by the MAT.

Key words: ELISA, outbreak, leptospirosis, diagnosis

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In August 2014, a leptospirosis outbreak was recognized in Poland. All the infected people originated from different regions of Poland, and all had worked during the summer on a strawberry plantation in Germany.

Leptospirosis is a zoonotic disease caused by spirochetes of the *Leptospiraceae* family. In animals, the course of the disease is asymptomatic. Spirochetes colonize the renal tubules of the hosts and a large number of them are excreted into the environment with the urine.^{6,10} In both humans and animals the main route of infection is indirect contact –through water or other products contaminated with urine containing spirochetes. Infection most commonly occurs through ingestion of water or food contaminated with *Leptospira* spp.

In humans the disease is usually biphasic. The first stage – bacteremia (leptospiremia) – persists for one week, followed by the second stage, with immune response and leptospiruria. The second stage can lead to tissue damage directly by leptospira or by inflammatory processes developed as a result of the infection. In humans, leptospirosis has various clinical pictures, from fever of unknown origin or flu-like illness to the classic form of Weil's disease or meningitis. Many patients show spontaneous recovery within 10–14 days.^{9–11} *Leptospira* spp. can survive several months in the intraocular fluid, occasionally causing chronic, recurrent uveitis.¹¹ These bacteria may be also excreted with the urine for several months after infection.⁶

Certain professions are associated with increased risk of infection, including farmers, miners, veterinarians, sewage systems workers and garbage collectors. Leptospirosis outbreaks have been observed in Poland in various regions, mainly in Southern Poland.^{3,8,15} Most cases were identified by the clinical symptoms and physical examination. In the period from 2009 to 2012 sporadic cases requiring hospitalization were reported. According to national epidemiological data, from 2011 to 2013, only 6 cases were reported. However, the number of recognized cases probably underestimated the actual number, due to limited laboratory capacity. In Poland, only 3 medical laboratories are equipped to diagnose leptospirosis and this is probably the reason for such a low number of recognized cases.

The host's humoral response plays a major role in *Leptospira* spp. infection, so serological tests are useful in identifying the infection. IgM antibodies specific for *Leptospira* spp. are detectable as early as 5–7 days after the onset of the first symptoms of the disease, and they persist for at least 5 months. Detecting these antibodies is evidence of acute and/or active infection. IgG antibodies are detected 7 days after the onset of symptoms and persist for years.^{1,7}

Laboratory methods for identifying leptospirosis include urine culture, a reference microscopic agglutination test (MAT), an enzyme-linked immunosorbent assay (ELISA), and an immunofluorescence assay (IFA). Bacteria are isolated from human blood, urine and cere-

brospinal fluid, depending on the stage of the disease.^{9,12} Molecular diagnostic methods are also used, mainly the polymerase chain reaction (PCR). DNA gene fragments of *Leptospira* spp. have been detected in various clinical specimens including serum, urine, aqueous humor of the eye and tissue sections.¹³

Based on the results of amplification reactions and gene fragment sequencing it is possible to determine pathogenic and non-pathogenic human and animal leptospira strains.^{4,13} It is especially useful to apply molecular methods in the incubation stage of the disease, when the specific antibodies are produced. DNA is detected in the blood during the first 5–15 after the onset of symptoms. The number of bacteria in the serum at that time ranges from 10^5 to 10^9 leptospira/L. The results of an ELISA (class of IgM antibodies) and PCR in clinical materials taken at the same time are positive in about 60% of infected individuals.¹³

The aim of the present study was to characterize all the cases of leptospirosis imported to Poland from Germany in 2014, and to analyze laboratory methods helpful for identifying the infection.

Material and methods

Material was collected from 10 individuals returning from Germany (4 women and 6 men) with suspected leptospirosis. Two of the women were 45 years old, and the others 22 and 23 years old; the men were 18, 20, 21, 23, 26 and 56 years old. They presented with high fever (39–40°C), chills, myalgia, and flu-like symptoms. All but patient 5 were hospitalized 1 to 7 days after the onset of symptoms, and they were treated with antibiotics immediately. Patient 5 was treated at a community health center. Detailed characteristics of the patients are presented in Table 1. In differential diagnosis, sciatica was initially diagnosed in patient 5. Patient 9 was tested for HAV, HBV, HCV, *Mycobacterium tuberculosis*, *Chlamydomphila pneumoniae* and *Bordetella pertusis* infections. Urine and blood cultures were performed in all the patients; in patients 6 and 9 the cerebro-spinal fluid was cultured as well.

The blood and urine samples for leptospirosis tests were obtained from the patients between August 18 and September 3 of 2014.

Serological tests

ELISA

IgM and IgG antibodies to *Leptospira* spp. spirochetes were tested in sera with the ELISA method (Serion Classic, Institut Virion-Serion GmbH, Wurzburg, Germany). Seropositive sera were examined using a reference MAT.

MAT (microscopic agglutination test)

The 16 serovars used were *Leptospira interrogans* species: Icterohaemorrhagiae, Canicola, Zanoni, Autumnalis, Bataviae, Hebdomadis, Australis and Pomona; *Leptospira borgpeterseni* species: Poi, Ballum, Tarassovi, Sejroe and Mini; *Leptospira weilii* species: Celledoni; and *Leptospira kirschneri* species: Cynopteri and Grippotyphosa.

Titers of 1 : 400 and higher were considered a confirmation of leptospirosis.

DNA preparation

Bacterial DNA was extracted from serum and urine samples with the QIAamp Tissue kit (QIAGEN GmbH, Hilden, Germany) according to the manufacturer's instructions. Concentrations of the extracted DNA, expressed as optical density, were measured with a Bio-Photometer (Eppendorf, Hamburg, Germany) at 260 nm. The DNA samples were stored at -20°C until tests were performed.

PCR

The extracted DNA samples were amplified to detect fragments of *Leptospira* spp. gene using primer pairs:

a) Amu1 (5'-CGC GCT GCA GTT ACT TAG TCG CGT CAG AAG-3') and Amu2 (5'-CGC GGT CGA CGC TTT CGG TGG TCT GCC AAGC-3'), which are complementary to the LipL32 gene fragment (occurring only in pathogenic species of spirochetes). These primers amplify a 756 bp fragment of the LipL32 gene.

b) G1(5'-CTG AAT CGC TGT ATA AAA GT-3') and G2 (5'-GGA AAA CAA ATG GTC GGA AG-3'), which amplify a 285 bp fragment (plasmid DNA).

The 50 µL reaction mixtures contained PCR buffer with 200 µM dNTPs, 50 pmol of each primer and 2.5 U Hot-Star Taq Plus DNA polymerase (QIAGEN GmbH, Hilden, Germany). An aliquot of 5 µL of DNA template was added to each reaction mixture. The cycling conditions were as follows: 3 min at 95°C, followed by 40 cycles of 30 s of denaturation at 95°C, 30 s of annealing at 60°C and 61°C, 1 min of elongation at 72°C and finally 7 min of elongation at 72°C. PCRs were performed in a Mastercycler gradient apparatus (Eppendorf, Hamburg, Germany). Each run of PCR testing included positive and negative controls (DNA from the strains collection and water, respectively). All the amplicons were analyzed in electrophoresis with 1.5% agarose gel stained with Midori Green Advance DNA Stain (Nippon Genetics Europe GmbH, Dueren, Germany).

Results

The patients examined had been reported as suspected leptospirosis cases on the basis of clinical symptoms and

epidemiological findings. Epidemiological investigations showed that the examined individuals originated from different regions of Poland and that they had been working together at a strawberry plantation in the Cloppenburg district of Lower Saxony in Germany. All 10 of the examined individuals worked on the strawberry plantation along with 4 other Polish individuals hospitalized in Germany due to leptospirosis presenting as Weil's disease. The precise dates when they worked in this region during the summer were not available.

All but 1 of the 10 patients were hospitalized in Poland; the exception (Patient 5) was treated at a community health center. IgM specific *Leptospira* spp. antibodies were detected in 6 of the patients and IgG in 2 of them. The main symptoms of the infected individuals were fever (approx. 40°C), chills, muscle and joint pain and headaches. Two patients presented meningitis, and 1 patient developed renal impairment (Table 1).

Four seronegative individuals had similar symptoms to those presented by seropositive ones. The diagnosis of leptospirosis in those patients was based on epidemiological data from anamnesis, negative blood and urine cultures and other negative test results. Those 4 patients did not agree to, or did not report for, the control tests.

All ELISA positive and negative cases were examined using a MAT. Positive results were confirmed by MAT for 2 individuals. Based on the results of the MAT, *Leptospira kirschneri* (Grippotyphosa titer 1 : 800 and Cynopteri titer 1 : 400) was the species that caused the infection.

All of the PCR results for the blood and urine samples were negative: Bacterial DNA was undetectable in the blood and urine.

Ceftriaxone was the medication most frequently used to treat the patients examined; other antibiotics used were ampicillin, amoxicillin and ciprofloxacin. Because of the serious condition of patients 8, 9 and 10, empirical treatment with ciprofloxacin and amoxicillin or ciprofloxacin and tarcefoksym was recommended. All the seropositive patients were cured and discharged in good general condition.

Discussion

In August 2014, a leptospirosis outbreak was recognized in Poland. The patients had been infected outside Poland, on a strawberry plantation in Germany, and the disease spread across the border. International travel as well as regional environmental and occupational exposure now constitute a major independent risk factor for disease acquisition.³

Saito et al. reported that in 2014 soil and environmental water were collected and cultured using "a novel combination of five antimicrobial agents for selective isolation of *Leptospira* from contaminated samples".¹⁶ They found that both saprophytic *Leptospira* spp. and pathogenic

Table 1. Characteristics of patients suspected of leptospirosis

No.	Age	Sex	Clinical symptoms on admission to the hospital	Onset of symptoms / date of hospitalization	Date of blood sampling (no. of days after the onset of symptoms)	ELISA		MAT	PCR	Treatment duration
						IgM U/mL	IgG U/mL			
1	45	female	fever up to 40°C, chills, myalgia, pain in the calves	5.08.2014 / 6.08.2014	18.08.2014 (13)	19	4	negative	negative	ceftriaxone i.v. 3 x 1 g (12 days)
2	23	female	fever up to 40°C, chills, myalgia, arthralgia, headache	2.08.2014 / 4.08.2014	18.08.2014 (14)	29	6	negative	negative	ceftriaxone i.v. 3 x 1.5 g (14 days)
3	20	male	fever up to 40°C, abdominal pain, myalgia	3.08.2014 / 8.08.2014	18.08.2014 (15)	78	30	negative	negative	ceftriaxone i.v. 3 x 1 g (10 days)
4	23	male	meningitis, fever, headache, arthralgia, stiff neck	24.08.2014 / 28.08.2014	29.08.2014 (5)	10.5	11	negative	negative	ampicillin i.v. 4 x 1.5 g (10 days)
5	22	female	fever up to 39°C, myalgia, arthralgia, headache, nausea, abdominal pain, renal dysfunction (pollakiuria)	30.07.2014 (not hospitalized, treated from 16.08.2014)	02.09.2014 (35)	31	1.8	negative	negative	amoxicillin p.o. 2 x 1 g (21 days)
6	18	male	meningitis, headache	29.08.2014 / 30.08.2014	01.09.2014 (4)	58	4.6	positive	n. d.	ceftriaxone i.v. 3 x 1 g (6 days)
7	21	male	fever up to 40°C, nausea, pain of muscles, bones, joints	10.08.2014 / 13.08.2014	18.08.2014 (8)	11	9	negative	negative	Ceftriaxone i.v. 3 x 1 g (8 days)
8	45	female	fever up to 39°C, abdominal pain, headache, vomiting, urinary tract infection of unknown etiology	7.08.2014 / 12.08.2014	19.08.2014 (5)	4.7	0	negative	negative	ciprofloxacinum 2 x 0.2 g (3 days) amoxicillin p.o. 2 x 1 g (14 days)
9	26	male	fever up to 40°C, chills, headache, sore throat, hepatitis (SIRS-systemic inflammatory response syndrome)	11.08.2014 / 18.08.2014	03.09.2014 (23)	3.8	1.5	negative	negative	ciprofloxacinum 2 x 0.2 g (17 days) tarcefoksym i.v. 3 x 2 g (17 days)
10	56	male	fever up to 39°C, hacking cough, RCC susp. (renal cell carcinoma)	20.08.2014 / 27.08.2014	03.09.2014 (14)	0	3	negative	negative	ciprofloxacinum 2 x 0.2 g (9 days) tarcefoksym i.v. 3 x 2 g (9 days)

The level of specific antibodies to *Leptospira* spp. by ELISA: IgM class: negative < 15 U/mL, borderline range from 15 U/mL to 20 U/mL, positive > 20 U/mL. IgG class: negative < 10 U/mL, borderline range from 10 U/mL to 15 U/mL, positive > 15 U/mL, n.d. – not done.

Leptospira spp. are widely distributed in the environment, and “hypothesized that soil serves as a reservoir for *Leptospira* spp. and infectious source for leptospirosis”.¹⁶

Similar outbreaks of leptospirosis among seasonal strawberry harvesters that have been reported recently pointed out the difficulties with early recognition of the disease.⁵ Leptospirosis has frequently been overlooked or misdiagnosed due to its varied clinical presentations, which range from relatively mild symptoms, such as headache or nausea, to more severe forms, associated with meningitis or renal impairment.

This highlights the need for rapid diagnostic tests for early screening of leptospirosis to replace the less sensitive MAT in outbreak investigations. In the group examined in the present study only 2 patients with seropositive ELISAs were confirmed by MAT (Table 1).

Cultures and the serological MAT method are both laborious and time-consuming. At the same time the results obtained are not clear-cut. Identification of *Leptospira* species using MAT requires constant maintenance of live cultures of multiple strains representing different serotypes of each spirochete species (recognized species of these bacteria have been classified into 25 serogroups and 300 serotypes, therefore it is very difficult to collect and maintain cultures of all of them). Interpretation of the MAT result of a single serum sample is also difficult. Therefore, examinations of paired sera or several repetitions for every patient are needed to monitor dynamic of antibody levels.¹⁴ ELISA kits, on the other hand, detect antibodies specific to all *Leptospira* serogroups. Moreover, these tests allow the class of antibodies to be determined.

For the purposes of the current study, ELISA IgM class antibodies were the best parameter, regardless of the stage of disease, because not all patients produce IgG antibodies. A diagnostic ELISA test was used to detect the presence of specific antibodies in both IgM and IgG classes to obtain information about the complete immune condition of the patient. In 1995 Silva et al. studied 2 groups of leptospirosis patients, 57 of them in the acute phase and 10 during convalescence. IgM class antibodies were detected on the 2nd day of symptomatic disease and were observed in 100% of patients up to the 5th month. Further, 66.7% of the patients had IgM class antibodies after 7 months, and 50% had them after the 12th month of the onset of symptoms. IgG class antibodies were detected on the 7th day of symptomatic disease in 9.1% of the patients, with maximum reactivity (87.5%) between the 2nd and 3rd months; they were not detected at all in 1 patient.¹⁷ Early detection of specific IgG antibodies and IgM antibodies may indicate reinfection.¹⁶

Moderately increased IgM titers accompanied by low IgG titers have also been found. As early as 1985, Terpstra et al. observed that the combination of moderately increased IgM titers and low IgG titers was most often observed in the first 10 day of the disease. This characteristic pattern was found in approximately 2/3 of the sera

obtained from leptospirosis patients during the acute phase of the disease. Terpstra et al. also confirmed observations by Adler et al. that it was not always possible to demonstrate specific IgG in the first few weeks after the onset of disease.^{2,18}

Conclusions

Using the ELISA in diagnosing leptospirosis allowed the disease to be identified much faster, differentiating the class of antibodies and recognizing them at levels too low to be detectable by the MAT. In the near future, however, it is necessary to develop an appropriate, sensitive PCR method and to establish appropriate materials and testing times using the ELISA and MAT, to complete the determination and recognition *Leptospira* species.

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IL-6, IL-8 and TNF- α levels correlate with disease stage in breast cancer patients

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Conflict of interest

None declared

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Abstract

Background. Breast cancer is the most common cancer in Chinese women. Inflammation contributes to tumor progression and can be induced by excessive production of pro-inflammatory cytokines such as interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor- α (TNF- α). However, how their levels relate to the expression of estrogen receptors (ER), progesterone receptors (PR) and human epidermal growth factor receptor 2 (HER2) by the tumor has not been investigated.

Objectives. The aim of the study is to more fully understand the significance of serum IL-6, IL-8 and TNF- α in breast cancers with different ER, PR and HER2 status.

Material and methods. Preoperative serum samples were collected from 110 patients diagnosed with ductal carcinoma and 30 healthy control subjects. IL-6, IL-8 and TNF- α levels were determined by enzyme-linked immunosorbent assay (ELISA). Associations of cytokine levels with clinical tumor stage were evaluated, and correlations of serum cytokine levels with ER, PR and HER2 expression were determined using the Pearson correlation coefficient.

Results. Serum levels of IL-6 and IL-8 were significantly higher in the subjects with ductal carcinoma than in the controls, and strongly correlated with clinical tumor stage, lymph node metastasis, and ER and HER2 antigen expression ($p < 0.05$). TNF- α levels in stage III carcinoma patients were significantly higher than in the controls ($p < 0.01$) and were associated with lymph node metastasis ($p < 0.01$). A strong positive correlation was found between IL-8 and TNF- α levels in the cancer patients ($p < 0.001$).

Conclusions. The study showed that IL-6, IL-8 and TNF- α levels correlated with clinical disease stage and lymph node metastasis as well as with ER and HER2 antigen expression. Specifically, IL-6 and IL-8 seem to have significant potential as prognostic cancer biomarkers. Analyzing serum cytokine levels might help identify patients with a poor prognosis who may benefit from more aggressive disease management.

Key words: breast cancer, biomarkers, interleukin-6, TNF- α , interleukin-8

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Breast cancer is the most common form of cancer among Chinese women and constitutes a large public health burden. The most common type of breast cancer is ductal carcinoma in situ, which is often associated with inflammation. Cells involved in the inflammatory response are attracted by cytokines and chemokines and may contribute to the development and progression of breast cancer.^{1,2} Most cytokines are overexpressed in cancer tissues compared to normal tissues, and overexpression correlates with a poor prognosis.^{2–4} Numerous cytokines, such as interleukin-6 (IL-6), interleukin-8 (IL-8) and tumor necrosis factor- α (TNF- α), have been implicated in the initiation and progression of ductal carcinoma.^{5–10}

IL-6 is a pro-inflammatory cytokine that has multiple functions.¹¹ It is involved in the regulation of immune reactions, hematopoiesis and the inflammatory state.^{2,11,12} IL-6 has an important role in tumor progression, as it can inhibit the apoptosis of cancer cells and stimulate tumor angiogenesis.^{13,14} Clinical studies have shown that serum IL-6 levels were increased in patients with breast cancer, and this increase correlated with tumor stage and poor patient survival.^{15,16} IL-8 is a chemokine that has an autocrine and/or paracrine tumor-promoting role, and significant potential as a prognostic and/or predictive cancer biomarker.⁷ IL-8 plays a specific role in breast cancer, and increased serum IL-8 levels are associated with positive lymph node status and higher-stage tumors.¹⁵ Finally, TNF- α is a necrotic factor in the tumor microenvironment that promotes tumor growth and migration.^{4,8} Elevated circulating TNF- α levels correlate with higher tumor stages and lymph node metastasis.^{4,6,17} Furthermore, in ductal carcinomas, the steroid hormone receptors progesterone receptor (PR) and estrogen receptor (ER) may also play important roles in cancer progression; it has been shown that the level of the expression of ER, PR, and human epidermal growth factor receptor 2 (HER2) by tumors is associated with tumor prognosis.^{12,17–19} Although the presence of IL-6, IL-8 and TNF- α

in breast cancer has been noted by many researchers, whether the levels of these cytokines in the serum are correlated with the PR, ER, and HER2 status of the tumor is not clear.^{6,15,16} Furthermore, whether there is a correlation among the cytokine levels themselves has not been addressed previously.

In the present study, to more fully understand the significance of serum IL-6, IL-8 and TNF- α in breast cancers with different ER, PR and HER2 status, cytokine levels were measured in samples isolated from patients with ductal carcinoma and from control subjects. Correlations between the cytokine levels and the clinical stage were then analyzed, as well as ER, PR and HER2 antigen expression.

Material and methods

Sample collection

Samples were collected from 110 female patients (Table 1) diagnosed at Huai'an First People's Hospital (Huai'an, China). The age of the patients ranged from 35 to 68 years. Individuals were eligible if they were primary breast cancer patients who had not received any prior treatment. Patients who presented with additional conditions, such as other malignancies, advanced organ failure or active infection, were also excluded. The patients examined were in clinical stage I, II, or III according to the TNM classification. The clinical diagnosis was routinely confirmed by histopathological examination of the tumor tissue samples. ER, PR and HER2 status was determined at the protein level by immunohistochemistry. The control group was comprised of 30 healthy women. Preoperative serum samples were collected before the initiation of treatment. The samples were stored at -80°C until the analysis. The present study conformed to the ethical standards of the World Medical Association Helsinki Declaration and was approved by the Ethics Committee of Huai'an First People's Hospital Faculty of Medicine. All the patients in the study had signed informed consent forms at Huai'an First People's Hospital.

Measurement of serum cytokine levels

Levels of IL-6, IL-8 and TNF- α in the patients' sera were determined by enzyme-linked immunosorbent assays (ELISAs) according to the manufacturer's instructions (R&D Systems, Inc., Shanghai, China).

Statistical analyses

Means and standard deviations were calculated. The data were evaluated using Student's *t*-test for the patient group relative to the control group, and Tukey's multiple comparison test for more than 2 study groups using

Table 1. Demographic data of the study participants

Group	Age (years)	Number
Healthy subjects	28–57	30
Ductal carcinoma	35–65	110
HER2 positive	35–63	45
PR positive	37–65	60
ER positive	37–65	55
Stage I	35–47	25
Stage II	30–59	50
Stage III	34–65	35

GraphPrism 6.0 software (GraphPad, La Jolla, USA). The values $p < 0.05$ (*), $p < 0.01$ (**) and $p < 0.001$ (***) were considered statistically significant. The Pearson correlation coefficient was used to assess correlations between the levels of IL-6, IL-8 and TNF- α in the cancer patients.

Results

Serum IL-6 levels in ductal carcinoma

Serum IL-6 levels in the subjects with ductal carcinoma were significantly higher than those in the healthy women ($p < 0.001$; Fig. 1A). Serum IL-6 levels correlated with the clinical tumor stage and lymph node metastasis. IL-6 concentrations were significantly increased in the patients with stage II and III carcinomas and lymph node metastases ($p < 0.01$; Fig. 1B, 1C).

Next, the relationship between serum IL-6 levels and the ER, PR and HER2 status of ductal carcinomas was analyzed. Levels of IL-6 were significantly higher in the sera collected from patients with ER⁺ tumors than in the sera from those with ER⁻ tumors ($p < 0.001$; Fig. 1D). Interestingly, serum IL-6 levels were significantly higher in the patients with HER2⁻ ductal carcinomas than in those

with HER2⁺ carcinomas ($p < 0.001$; Fig. 1F). Serum IL-6 levels did not correlate with the PR status of the tumors (Fig. 1E).

Serum IL-8 levels in ductal carcinoma

The serum IL-8 levels in the ductal carcinoma patients were significantly higher than in the control group ($p < 0.001$; Fig. 2A). Serum IL-8 levels also correlated with the clinical tumor stage and lymph node metastasis. IL-8 levels were significantly elevated in the patients with stage II and III carcinomas and lymph node metastases ($p < 0.01$; Fig. 2B, C). It was further found that the IL-8 levels correlated with the ER and HER expression of the tumor tissue in the carcinoma patients ($p < 0.001$; Fig. 2D, F). The levels of IL-8 were significantly higher in ER⁻ and HER2⁺ tumor patients than in ER⁺ or HER2⁻ patients. However, the IL-8 levels did not significantly differ between patients with PR⁺ and PR⁻ tumors (Fig. 2E).

Serum TNF- α levels in ductal carcinoma

Although a slight increase in TNF- α levels was observed in the ductal carcinoma patients compared to the control group, this difference was not significant (Fig. 3A). How-

Fig. 1. Serum IL-6 levels: A) in healthy women and in patients with ductal carcinoma; B) in different clinical tumor stages; C) in patients with lymph node metastasis; D) ER positive vs ER negative; E) PR positive vs PR negative; F) HER2 positive vs HER2 negative. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$

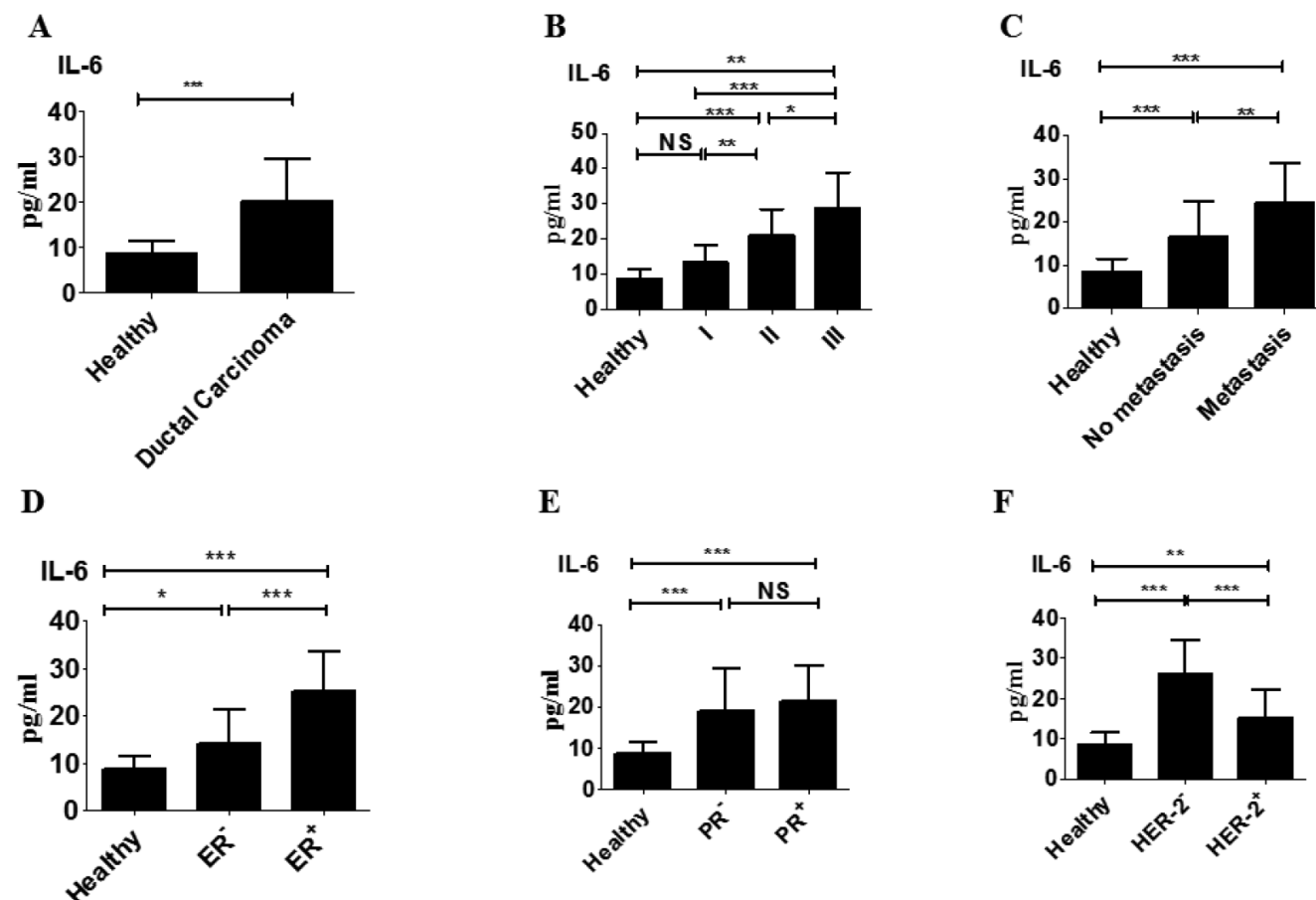


Fig. 2. Serum IL-8 levels: A) in healthy women and in patients with ductal carcinoma; B) in different clinical tumor stages; C) in patients with lymph node metastasis; D) ER positive vs ER negative; E) PR positive vs PR negative; F) HER2 positive vs HER2 negative. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$

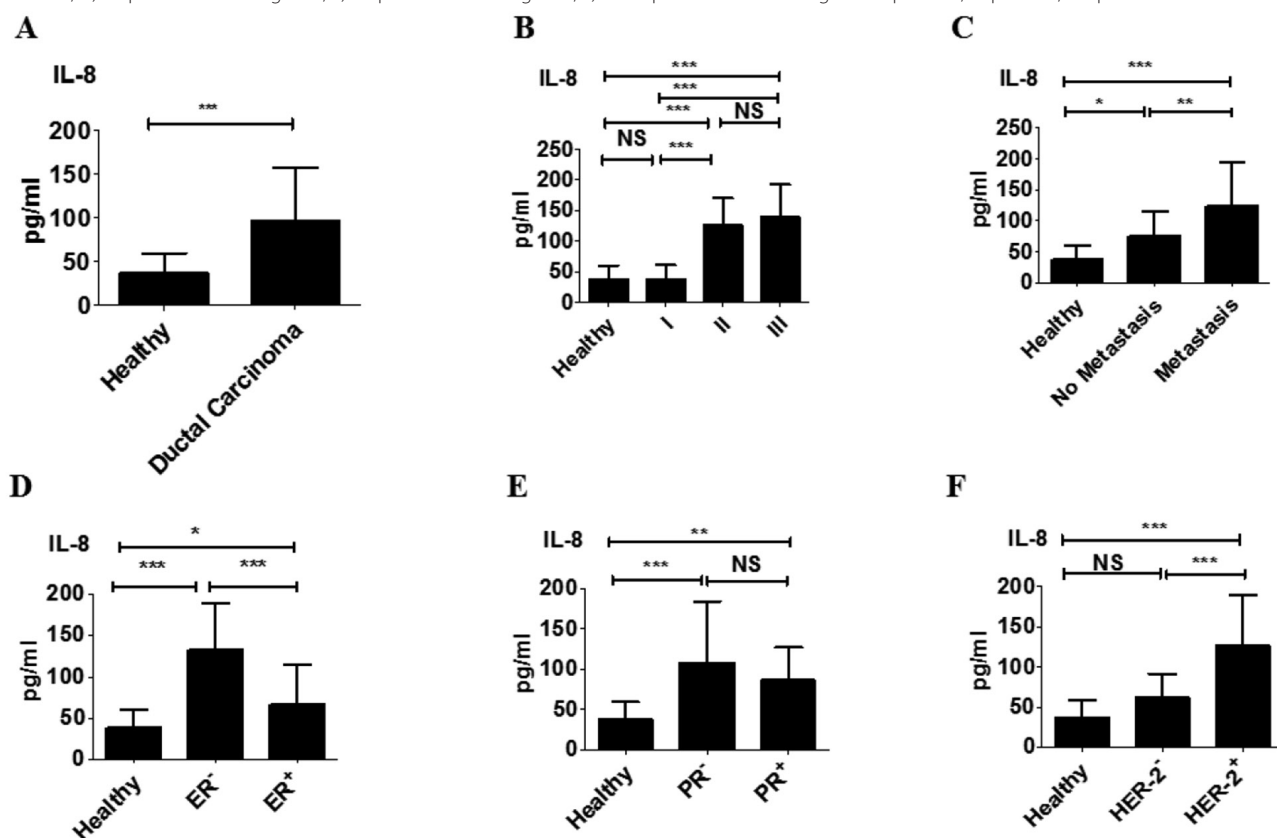


Fig. 3. Serum TNF- α levels A) in healthy women and in patients with ductal carcinoma; B) in different clinical tumor stages; C) in patients with lymph node metastasis; D) ER positive vs ER negative; E) PR positive vs PR negative; F) HER2 positive vs HER2 negative. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$

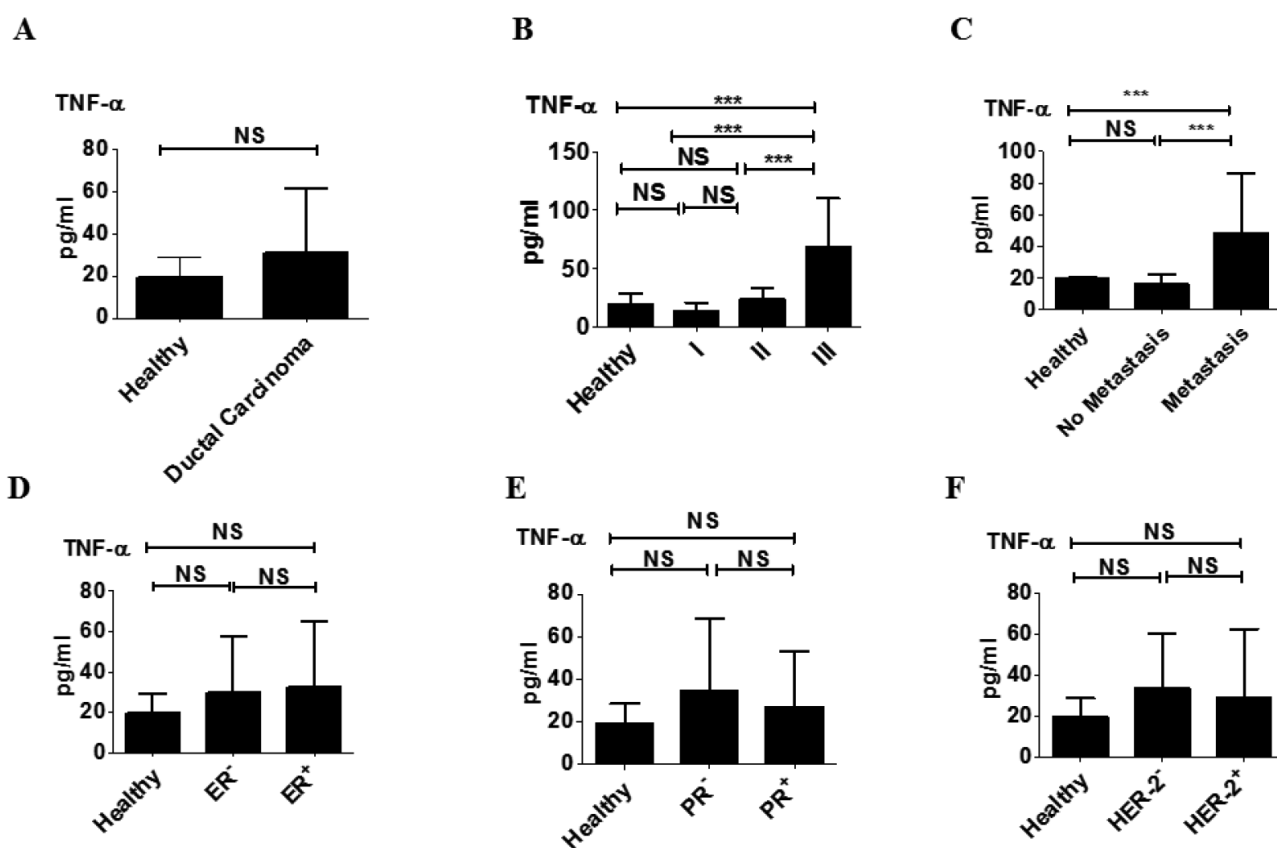
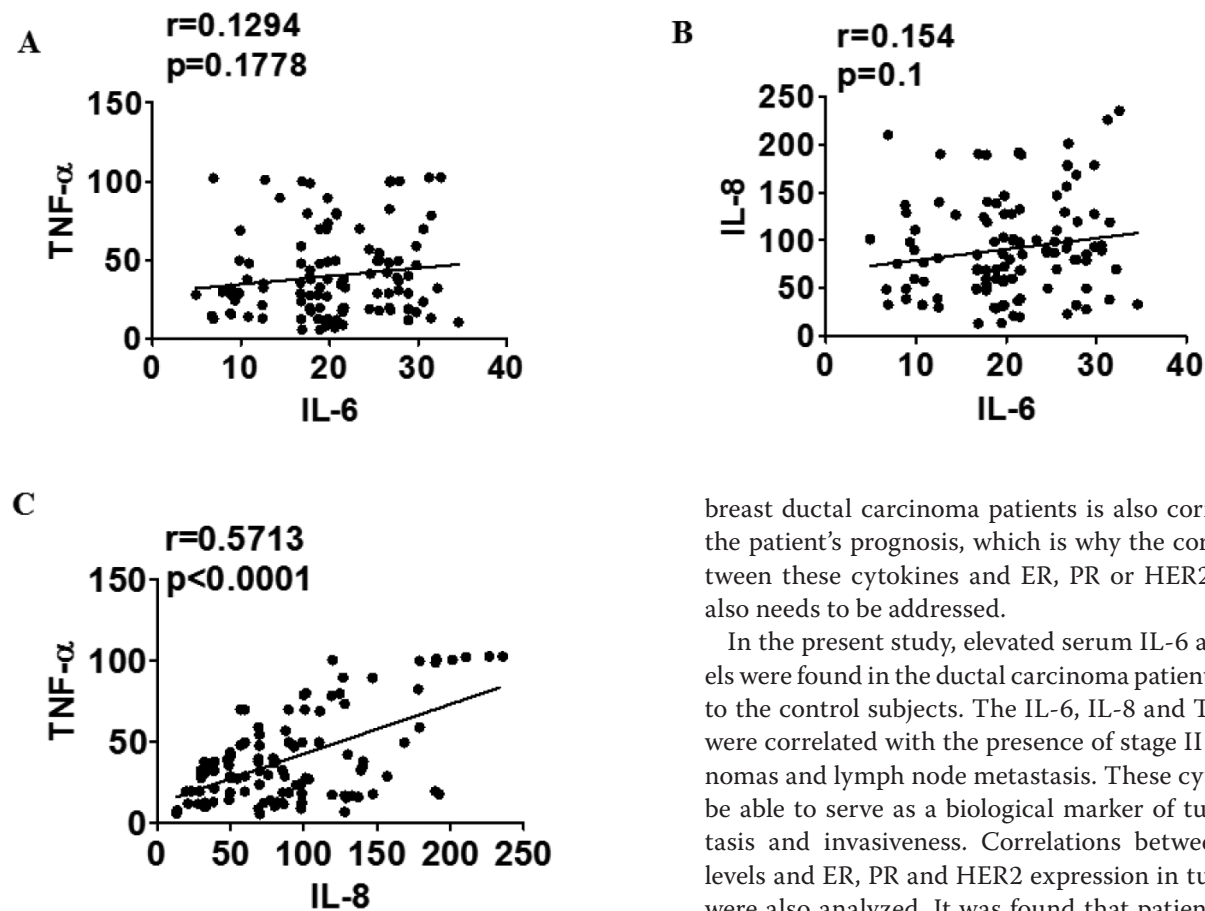


Fig. 4. Correlations between cytokines IL-6, IL-8 and TNF- α : A) IL-6 and TNF- α ; B) IL-8 and IL-6; C) IL-8 and TNF- α 

ever, the serum TNF- α levels of stage III carcinoma patients were significantly higher than those in the healthy controls ($p < 0.001$; Fig. 3B). Serum TNF- α levels also correlated with clinical tumor stage and lymph node metastasis ($p < 0.001$; Fig. 3B, 3C). TNF- α levels did not correlate with ER, PR or HER2 expression in the tumor tissues (Fig. 3D, 3F).

Correlations between the levels of IL-6, IL-8 and TNF- α in cancer patients

In the samples isolated from the ductal carcinoma patients, a significant positive correlation was found between the levels of TNF- α and IL-8 ($r = 0.8571$, $p < 0.001$; Fig. 4C), but not between TNF- α and IL-6 levels (Fig. 4A). Similarly, no correlation was found between the levels of IL-6 and IL-8 (Fig. 4B).

Discussion

It has been reported that high serum levels of IL-6, IL-8 and TNF- α are correlated with strong tumor invasiveness and poor prognosis.^{2,6,7,11,20} However, whether there is a correlation between these cytokines and the tumor stage and lymph node metastasis is still not very clear. Furthermore, the expression of ER, PR and HER2 in

breast ductal carcinoma patients is also correlated with the patient's prognosis, which is why the correlation between these cytokines and ER, PR or HER2 expression also needs to be addressed.

In the present study, elevated serum IL-6 and IL-8 levels were found in the ductal carcinoma patients compared to the control subjects. The IL-6, IL-8 and TNF- α levels were correlated with the presence of stage II or III carcinomas and lymph node metastasis. These cytokines may be able to serve as a biological marker of tumor metastasis and invasiveness. Correlations between cytokine levels and ER, PR and HER2 expression in tumor tissues were also analyzed. It was found that patients with ER+ or HER2- tumors have increased serum IL-6 levels versus those with ER- or HER2+ tumors; however, IL-8 levels were higher in ER- and HER2+ tumor patients compared with those with ER+ or HER2- tumors. While ER+ breast cell lines secrete lower IL-6 levels than ER- cells, it is possible to speculate that IL-6 production from breast tumor cells may constitute only a small fraction of total serum IL-6 content. In addition to tumor cells, T cells, macrophages, and B cells are able to secrete IL-6.^{13,14,21,22} In the late stage of ductal carcinoma, tumor tissues are infiltrated by numerous T cells and M2 macrophages; these cells produce large amounts of IL-6 and promote the metastasis of breast tumor cells.^{23–25} Thus, IL-6 production by immune cells likely accounts for the increased IL-6 serum levels in patients with ER+ ductal carcinomas. For IL-8 production, it has been suggested that ER expression can downregulate IL-8 production in tumor cells, and breast cancer patients who lack ER expression have worse prognoses.^{7,26} HER2 is a tumor antigen that is expressed by different tumors (breast cancer, lung cancer, gastric cancer) and can elicit a host immune response; therefore, it can be hypothesized that HER2 expression in breast cancer may directly promote the production of IL-8.²⁷

The relationship among cytokine levels was also analyzed, and a strong positive correlation was found between IL-8 and TNF- α . Therefore, IL-8 and TNF- α might act synergistically in the initiation and development of

tumors. However, there was no correlation between IL-6 levels and IL-8 or TNF- α levels. Although tumor cells are able to produce all 3 cytokines, the authors speculate that the main origin of these cytokines in tumor patients is different. Surprisingly, the data from the present study revealed that serum TNF- α levels did not significantly differ between the healthy controls and the ductal carcinoma patients. However, the serum TNF- α levels were elevated in stage III breast cancer patients as compared with those with stage I cancers and the healthy control subjects, meaning that only late stages tumor patients secrete high levels of TNF- α (Fig. 3B). It is interesting to note that TNF- α levels were not correlated with ER, PR or HER2 expression, although there was a strong positive correlation between TNF- α levels and IL-8 levels. The authors speculate that the main TNF- α producing cells (such as macrophages) may not be affected by ER, PR or HER2 expression, which could explain this result.^{28,29} Another possibility is that the production of TNF- α is not only correlated with IL-8 levels, but also associated with other factors, such as IL-1 β .^{4,30}

In this study, a correlation was found between cytokine levels and tumor stages, lymph node metastasis, and the ER, PR and HER2 status of tumors. However, it is still unclear whether the elevated cytokine levels are merely a result of the presence of late stage and/or metastatic tumors or if their elevated levels contribute to the progression of tumors to an advanced clinical stage. More research is needed to investigate how the ER, PR and HER2 expression affects the production of IL-6 and IL-8.

The present study suggests that increased serum levels of IL-6, IL-8 and TNF- α are associated with breast ductal carcinoma. Specifically, the levels of these cytokines correlate with the clinical stage of the disease and with ER and HER2 antigen expression by the tumors. Thus, these cytokines seem to have significant potential as prognostic cancer biomarkers.

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Binder syndrome: Clinical findings and surgical treatment of 18 patients at the Department of Plastic Surgery in Polanica Zdrój

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Abstract

Background. Binder syndrome (BS) is an uncommon congenital underdevelopment of the maxilla and nasal skeleton. Other clinical features include a hypoplastic or absent anterior nasal spine; a short, flat nose with short columella; an acute nasolabial angle; a convex upper lip and class III malocclusion.

Objectives. The aim of the study was to outline the major characteristics of BS and to present a variety of surgical treatment methods.

Material and methods. The study included 18 patients treated in the authors' department from 1989 to 2013.

Results. The patients were predominantly women, aged 6 months to 34 years. Nine patients did not present any co-morbidities, but in the other 9 the most common co-morbidities were a unilateral cleft lip and palate, followed by a cleft palate, a bilateral cleft lip and palate, a cleft lip, GERD, gluten intolerance, oligophrenia, goiter and foot malformation. Most of the patients had not been operated on previously. The most common procedure carried out was an iliac crest bone graft. In 4 patients, no procedures other than cleft lip and palate repair were undertaken. In 3 cases a Le Fort I osteotomy was performed to correct the patients' orthognathic status. No major or life-threatening complications were noted. In 2 cases, due to a high degree of resorption of bone grafts, multiple secondary grafting of bone, cartilage and deepithelialized skin was necessary to obtain satisfactory results.

Conclusions. In BS surgical treatment is the treatment of choice. It results in adequate correction of facial retrusion. However, due to various degrees of bone resorption, the results are not lifelong. No unequivocally superior surgical strategy in BS has been presented so far. Most disagreement among authors is related to the need for and timing of maxillary osteotomy, the choice between bone and cartilage grafting in nose reconstruction and columella lengthening. Although alloplastic materials offer the tempting advantage of fast and simultaneous augmentation of deficient tissues, their use may risk prolonged infections and extrusion, resulting in exacerbations of deformities.

Key words: Binder syndrome, maxillary underdevelopment, bone graft, cartilage graft, resorption

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The first patient who presented Binder syndrome (BS) might have lived in Quarai, New Mexico, in the 14th century (ca. 1375–1450 AD). The skeleton of a female approximately 16–17 years old was described during a repatriation by the Smithsonian Institution National Museum of Natural History. A thorough examination of the skeleton revealed several characteristics indicating that the individual could have been affected by Binder syndrome.¹

Binder syndrome, also referred to as maxillonasal dysplasia or frontonasal dysplasia, is an uncommon congenital entity characterized by a series of disorders mainly affecting the skeleton of the midface. The features of Binder syndrome were first described by Noyes in 1939, but it was Binder who, 20 years later, first named it as a distinct clinical entity characterized by arhinoid face, distorted nasal bone positions, a hypoplastic or absent anterior nasal spine, a short, flat nose with a short columella, an acute nasolabial angle, a convex upper lip and predominant class III malocclusion resulting from intermaxillary hypoplasia, and whom the syndrome has been named after ever since.^{2,3} A characteristic inclination of the upper incisors is often marked. Other clinical features that are sometimes seen include absent or atrophic frontal sinuses, cervical vertebral disorders and other skeletal malformations, as well as (rarely) mental retardation.^{4–6}

Antoneli et al. investigated audiological and brainstem electrophysiological profiles of patients diagnosed with frontonasal dysplasia and they found no abnormalities in the auditory system from the cochlea to the brainstem, while the mild conductive hearing loss found in the patients with BS and a cleft palate was related to the cleft.⁷ Most authors agree that a flat nose and midfacial retrusion are the two most common findings in Binder syndrome. The syndrome is therefore connected with such terms as “dish face”, “fossae prenasalis” and “facies scaphoidea”. However, the spectrum of frontonasal dysplasia severity is very wide and in some populations, such as the Japanese or Chinese, the frequency of BS occurrence may be underestimated due to features similar to maxillary hypoplasia among healthy individuals in these populations.⁵

The etiology of BS remains uncertain and evidence regarding the mode of inheritance in Binder syndrome is ambiguous. Based on a study of 60 families, Olow-Nordenram and Valentin proposed that BS is inherited in an autosomal recessive manner with incomplete penetrance or that it is multifactorial.⁴ In contrast, Gross-Kieselstein et al. suggested a dominant mode of inheritance, based on an examination of an affected mother and daughter.⁸ Many authors suggest that exposure to exogenous factors during pregnancy constitutes the main etiologic factor, naming vitamin K deficiency and vitamin K antagonist drugs (warfarin, acenocoumarin), anticonvulsive drugs, retinoic acid or birth trauma, leading to a disturbance of the prosencephalic induction center during embryonic growth.^{5,9} Some authors highlight a connection between

warfarin- and acenocoumarin-induced coagulation disorders in pregnancy and hemorrhage-induced nasal septum cartilage damage, leading to maxillary growth retardation that continues in the post-natal period. Howe et al. established that prenatal nasal septum growth is maximal in the 6th through 9th weeks of pregnancy.¹⁰ If this period overlaps with warfarin exposure, it results in warfarin embryopathy as a consequence of the hemorrhagic mechanism described above. Binder syndrome is therefore often regarded as a variant of chondrodysplasia punctata (CDP), characterized by maxillary growth retardation combined with calcified foci in the nasal septum, evidenced by radiograms performed early in life.^{11,12} Holmstrom et al. stated that inhibition of the ossification center at the lateral and inferior border of the pyriform fossa is responsible for the growth retardation that leads to the formation of characteristic features of Binder syndrome.¹³

In an animal model study Noguchi et al. investigated the correlation between transthyretin gene mutation and facial growth retardation.¹⁴ They concluded that the mutation leads to delayed transthyretin production, which in turn causes massive cell death in the nasal placode. This phenomenon leads to hypoplasia of the frontonasal region. It is significant that transthyretin plays an important role in retinoic acid delivery to the tissues. Both excess and deprivation of vitamin A, which is the precursor of retinoic acid, may lead to abnormal fetal development.¹⁵

In an extensive study performed on 24 patients diagnosed with Binder syndrome, Keppler-Noreuil et al. presented etiologic findings and associated disorders.⁶ They diagnosed 13 patients with trisomy 21, 2 with trisomy 18 and 1 with mosaic trisomy 21. In 1 case, vitamin K epoxide reductase deficiency was found; in another, Stickler syndrome; in another, fetal warfarin syndrome; and one case of Robinow syndrome was detected.

Other etiologic factors associated with BS are maternal autoimmune disorders such as systemic lupus erythematosus or mixed connective tissue disease.¹⁶

Olow-Nordenram et al. stated that 44% of a group of 43 patients presenting BS had malformations of cervical vertebrae, the atlas and axis being the most frequently affected.¹⁷ Another study revealed that 15 of 28 patients (53.5%) suffered from cervicospinal and craniospinal anomalies.¹⁸

Most diagnostic imaging in BS is based on classic lateral cephalograms, which show reduced anteroposterior dimensions of the anterior cranial base and maxilla and class III malocclusion. In recent years, supplementary diagnostic imaging methods such as 3D laser surface scanning have been introduced.¹⁹

Due to tremendous progress in prenatal diagnostic imaging, it is now possible to detect facial underdevelopment as early as the 21st week of gestation, or even at 14.5 weeks.^{20,21} This also permits prenatal differential diagnosis of BS and other syndromes characterized by

Table 1. Treatment course of Binder Syndrome patients treated in authors' department

No.	Sex	Age	Comorbidities	Operations	Complications
1	f	7	none	Iliac crest bone graft – nasal dorsum, maxilla	none
2	m	2	CL	CP reconstruction – Kilner – Wardill procedure	none
3	f	17	previous allogenic cartilage graft to dorsum	1) Iliac crest bone graft – nasal dorsum, maxilla 2) Iliac crest bone graft repositioning	bone graft displacement
4	f	18	oligophrenia, CP, goitre, feet malformation	bone graft – nasal dorsum, maxilla	none
5	f	8	none	1) Iliac crest bone graft – nasal dorsum, columella 2) Iliac crest bone graft – nasal dorsum	none
6	f	13	none	1) Nose osteotomy 2) Iliac crest bone graft – nasal dorsum, columella 3) Rib bone graft – nasal dorsum	none
7	f	0.5	CLP, gluten intolerance	1) CL reconstruction – Randall procedure 2) CP reconstruction – Veau procedure 3) Iliac crest bone graft – maxillary onlay, alveolar fissure	none
8	f	10	none	1) Iliac crest bone graft – nasal dorsum, columella 2) Iliac crest bone graft – nasal dorsum	none
9	m	2	BCLP, CL operated elsewhere	CP reconstruction – Veau procedure	none
10	m	29	none	Iliac crest bone graft – nasal dorsum	none
11	m	22	septoplasty – 6 years old Iliac crest bone graft – 19 years old	1) Le Fort „1,5” osteotomy 2) Iliac crest bone graft – nasal dorsum, columella 3) auricular cartilage graft – nasal septum support 4) rib bone graft – nasal dorsum 5) rib cartilage graft – nasal dorsum 6) dermal deepithelialised graft – nasal dorsum 7) columella reconstruction – iliac crest bone graft	bone graft resorption
12	f	23	none	1) Iliac crest bone graft – nasal dorsum, columella, maxilla 2) Le Fort I osteotomy 3) Iliac crest bone graft – nasal dorsum, septum	none
13	m	17	none	1) Iliac crest bone graft – piriform aperture 2) Iliac crest bone graft – nasal dorsum, columella 3) Iliac crest bone graft – nasal dorsum 4) columella plasty – Cronin procedure 5) Iliac crest bone graft – nasal dorsum, maxilla	bone graft resorption
14	f	23	GERD	1) Le Fort I osteotomy 2) Iliac crest bone graft – nasal dorsum 3) auricular compound graft - columella	none
15	f	34	CP	Iliac crest bone graft – nasal dorsum, columella	none
16	f	9	none	Iliac crest bone graft – nasal dorsum, maxilla	none
17	f	0.5	CLP	CL reconstruction	none
18	f	2	CLP	1) osmotic expanders CP reconstruction 2) palate secondary procedures	palate fissure

mid-facial underdevelopment, such as chondrodysplasia punctata, Robinow syndrome, Aarskog syndrome, Crouzon syndrome, Apert syndrome, achondroplasia, Stickler syndrome and Rudiger syndrome.

Most of the articles presenting Binder syndrome patients treatment include 20 cases or fewer. To date, no more than 250 cases of BS patients have been described in world medical literature.^{5,3,21}

Material and methods

The aim of the study was to outline the major characteristics of Binder syndrome and the variety of surgical treatment methods used with patients operated on in the authors' department and worldwide. Eighteen patients diagnosed with BS and treated at the Plastic Surgery Clinic of Wrocław Medical University in Polanica Zdrój (Poland) between 1989 and 2013 are presented.

Results

The patients were predominantly women (13 : 5), aged 6 months to 34 years. Nine patients did not present any co-morbidities; the most common co-morbidities noted were unilateral cleft lip and palate (3 patients), followed by cleft palate (2 patients), bilateral cleft lip and palate (1), cleft lip (1), GERD (1), gluten intolerance (1), oligophrenia (1), goitre (1) and malformation of the feet (1). The great majority of the patients (15/18) had not been operated on previously. One patient had had an allogenic cartilage graft implanted in the nasal dorsum; one had undergone a septoplasty and iliac crest bone graft; another had had primary reconstruction of a cleft lip.

Iliac crest bone grafts were the most common procedure implemented at the authors' department, to correct collapsed nasal dorsa (12 patients). The bone grafts were used either as solid compact bone grafts into the nasal dorsum or as spongy bone grafts to augment underdeveloped maxilla. In 6 cases the procedure was repeated due to graft resorption of varying degrees. In 1 case the need for re-grafting was caused by bone graft displacement in the postoperative period. In 2 cases the re-grafting procedure was performed using rib bone grafts. In 2 cases the degree of graft resorption was high enough to necessitate 3 re-grafting procedures or combined bone and cartilage grafting. In 1 of these cases, considering the high degree of both bone and cartilage graft resorption, a deepithelialized skin graft was used. In 4 patients, due to a low degree of maxillary underdevelopment and the patients' or their parents' preferences, no procedures other than cleft lip and palate repair were undertaken. In 3 cases Le Fort I or "high" Le Fort I osteotomies (so-called "Le Fort 1.5") were performed to correct the patients' orthognathic status. Two patients' columellas were

reconstructed, in 1 case by a compound auricular graft, and in the other by the Cronin procedure. The courses of treatment for all 18 patients are summarized in Table 1.

No major or life-threatening complications were noted. In 1 case, a palate fissure occurred, which necessitated secondary closure procedures. As mentioned above, in 2 cases, due to a high degree of resorption of bone grafts, repeated grafting of both bone and cartilage, as well as deepithelialized skin, was required to obtain satisfactory results.

Case presentations

The treatment histories of selected patients operated on in the authors' department are presented in detail below.

Patient 1

A 17-year-old male presenting mid-facial underdevelopment and nasal bridge lowering was admitted to the department for nasal reconstruction and maxillary augmentation (Fig. 1–3). He had been diagnosed with Binder syndrome at the age of 5 years in another plastic surgery center, and the treatment was prescheduled for when the patient reached postpubertal age. He was first operated on at the age of 18 years, when a calcaneus bone graft was performed in the area of the pyriform aperture and below the nasal wings in order to augment the underdeveloped region and gain support for further stages of surgical reconstruction. After 6 months nasal reconstruction was performed. Two blocks of compact bone were harvested from the iliac crest and sutured together to form an L-shaped bone graft, which was inserted into the nasal dorsum and columella, reconstructing both elements (Fig. 4–6). Due to resorption of the bone graft placed in the nasal dorsum, another dorsal bone graft was performed after 5 years. The next stage consisted in columella soft tissue correction. The final refinement procedure consisted in an iliac crest bone graft into the maxilla and nasal dorsum (Fig. 7–8).

Patient 2

A female patient treated at the department for maxillofacial underdevelopment and a soft palate cleft underwent primary soft palate reconstruction using the Wardill-Killner technique at the age of 4 years and further nasal reconstruction with the use of bone grafts. In the first stage, at the age of 24 years, the nasal dorsum and columella were reconstructed using an L-shaped bone graft inserted into a subperiosteal pocket. In the postoperative period, abnormal mobility of the graft was noted, followed by its intense resorption. As a result, another bone graft was performed 2 years later.

Fig. 1–3. Patient 1 before nasal reconstruction and maxillary augmentation

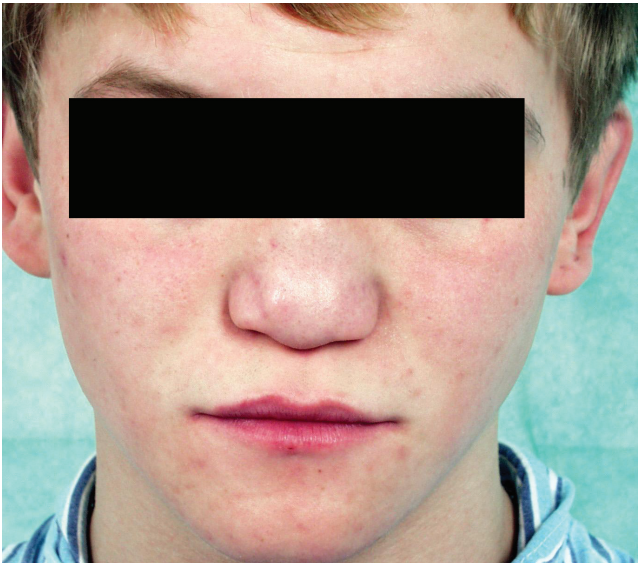


Fig. 1.



Fig. 2.



Fig. 3.

Fig. 4–6. Patient 1 after nasal reconstruction and maxillary augmentation (early result)

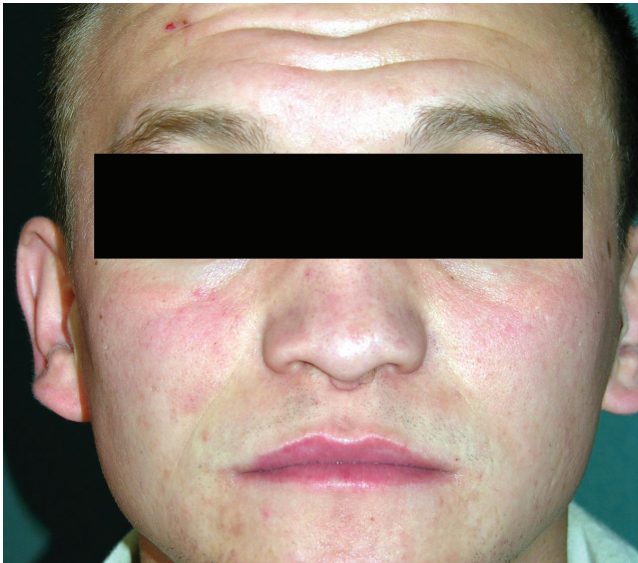


Fig. 4.



Fig. 5.



Fig. 6.

Fig. 7, 8. Distant result after columella soft tissues plasty and refinement procedures

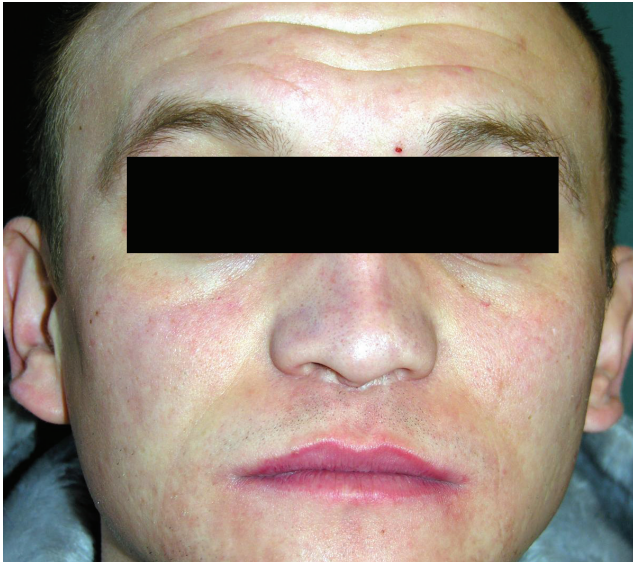


Fig. 7.

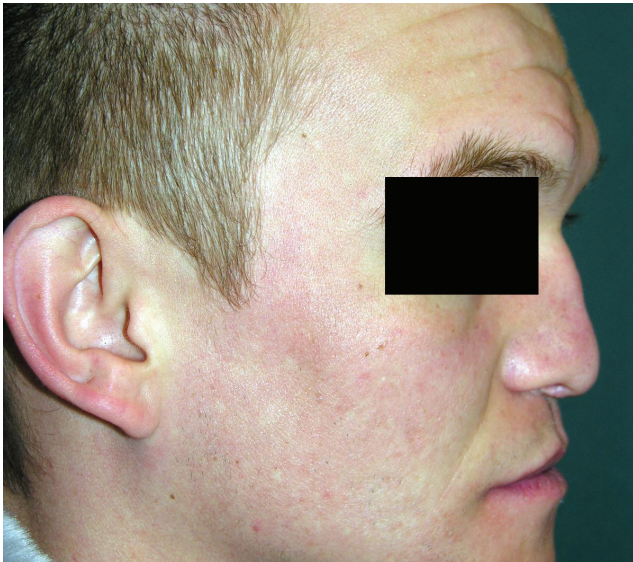


Fig. 8.

Patient 3

A 21-year-old male patient was admitted to the department for surgical treatment of the underdeveloped middle part of his face. His dominant concern was maxillary retrusion. In order to correct this deformity, as the first stage of surgical treatment a “high” Le Fort I osteotomy was performed. During the next 7 years, he underwent an L-shaped iliac crest bone graft onto the dorsum, columella reinforcement with an auricular cartilage graft, a rib bone graft onto the dorsum and maxilla. In the postoperative period, marked graft resorption occurred, which necessitated grafting cartilage and deepithelialized dermal grafts in order to achieve long-lasting volume.

Patient 4

A 23-year-old female patient was admitted to the department presenting the characteristic features of Binder syndrome. Her main concern was the underdevelopment of the nasal dorsum and maxilla. She was scheduled for an iliac crest bone graft to the deficient areas, followed by a Le Fort I osteotomy. Due to bone graft resorption, another bone graft was performed.

Patient 5

A 17-year-old patient was admitted to the department due to a high degree of nasal dorsum and maxilla underdevelopment (Fig. 9–13). Her surgical treatment consisted in a Le Fort I osteotomy (Fig. 14–19) followed by an iliac crest bone graft onto the dorsum and a composite auricular graft for columella reconstruction (Fig. 20–22).

Discussion

Although Binder syndrome is not a life-threatening entity nor it does compromise any important organs or systems, it contributes to major face dysmorphia resulting in an impaired social life, leading to a significant decrease in the quality of life. Therefore, patients presenting all or some characteristics of the syndrome require sophisticated surgical procedures to mask the syndrome's effects.

Surgical treatment is the treatment of choice in Binder syndrome. However, no unequivocally superior surgical strategy has been presented so far. In some instances, adequately planned and staged orthodontic treatment may produce good results, which can be further improved by performing a surgical procedure of limited extent, Cosseu et al. describe a Binder syndrome patient treated mainly by staged orthodontic procedures (transverse palate expansion, the use of a Delaire mask, as well as transverse and sagittal maxillary expansion), which produced excellent results in terms of angle class and maxillary underdevelopment correction.²² When the patient was 17, the outcome was further improved with a costal chondral graft to reconstruct the nasal dorsum. As a result, the patient hardly shows any signs of Binder syndrome at all.

Among the clinical features of Binder syndrome, maxillary hypoplasia and nasal skeleton underdevelopment are the most frequently addressed and corrected, enabling the best results to be achieved, and thus leading to improvement in the patients' appearance and the quality of their social life. Most authors perform maxillary osteotomies, bone or cartilage grafting, as well as local skin grafts to obtain maxillary advancement, nasal reconstruction and expansion of deficient soft tissues, respectively.

A certain degree of controversy exists about the need for and timing of a maxillary osteotomy in Binder syndrome.

Fig. 9–13. Patient 5 presenting high degree of nasal and maxillary underdevelopment



Fig. 9.



Fig. 11.



Fig. 10.

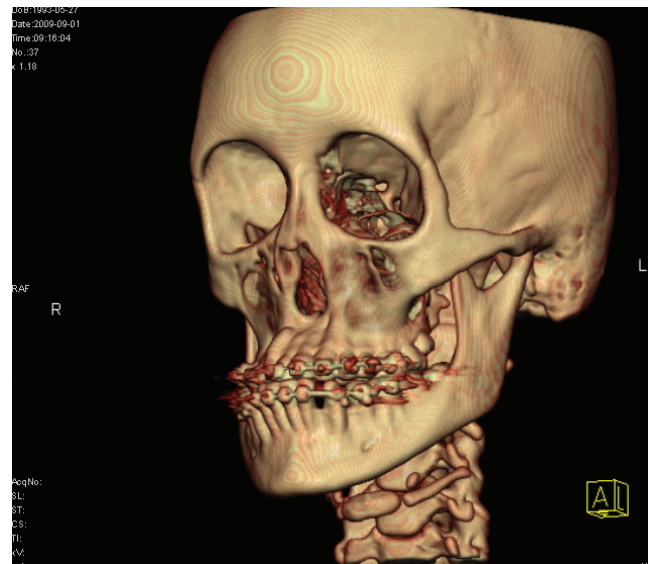


Fig. 12.



Fig. 13.

Based on 17 years of experience treating 24 cases of Binder syndrome at Chang Gung Memorial Hospital, Goh et al. stated that maxillary osteotomies were rarely required and were therefore reserved only for the patients with severe class III malocclusion that cannot be adequately treated using of orthodontic measures, whereas in other patients augmentation of the maxillary area by means of bone or cartilage grafts is sufficient.²³ The need for early osteotomies and other surgical procedures, such as bone or cartilage grafting, may be advocated for psychological reasons and the patient's desire for a normal appearance. Although some authors state that early, repeated bone or cartilage grafting may result in progressive midfacial expansion, others postpone aggressive surgical procedures until midfacial growth is completed, which usually oc-

curs in the mid-teenage years.^{23,24} Reconstructive procedures mostly involve augmentation of the pyriform and nasal base region with autologous costal cartilage grafts and, in cases when treatment was necessary before mid-facial growth was fully completed, with silastic implants.

The need for columellar skin lengthening has also been a subject of discussion. Tessier stated that the nasal skin may be expanded in short or small noses without any need for grafts or flaps.²⁵ Holstrom presented the opinion that in many cases of columella shortening there is no real lack of skin, and that adequate undermining of the skin in that area may result in sufficient stretching of the skin.¹³ On the other hand, many authors advocate local skin plasties in order to achieve columellar skin lengthening. Such procedures can lead to visible scar formation and in some cases serious scar retraction, resulting in further shortening of the columella.²⁶

Kruk-Jeromin et al. describe 5 patients presenting characteristics of Binder syndrome, 2 of whom were treated surgically.²⁷ A V-Y advancement of the columella was performed and an L-shaped bone graft harvested from the iliac crest was inserted into a subdermal pocket in the dorsum and columella. The other patients were treated only orthodontically. The authors stated that in some cases it is sufficient to apply only the aforementioned bone grafts, whereas in other cases maxillary osteotomy is crucial to achieve good results.

Gewalli et al. compared the results of Binder syndrome patients treated using cartilage and bone grafts; both techniques resulted in "an increased and normalized angle of convexity of the face and nasal tip projection".²⁸ Goh et al. stated that bone grafts showed a higher rate of resorption, and other authors prefer cartilage over bone for nasal augmentation due to its stability over time.²⁴ Bhatt et al. proposed L-shaped costal cartilage grafts for nasal columella and nasal dorsum enhancement, and recommended immersing the graft in a saline solution of gentamicin for 30 min prior to implantation in order to prevent it from warping.²⁹ Chummun et al. described the use of costochondral grafts for reconstructing craniofacial disorders, including 46 cases of Binder syndrome.¹¹ They concluded that this kind of procedure resulted in long-lasting correction of underdeveloped noses. Chiang et al. described a 3-dimensional nasal tip, columella, dorsum and alae reconstruction method using a single osseous and cartilaginous rib carved in a special manner to augment all the components of the retruded nose in BS patients.³⁰ Monasterio et al. state that the results of bone grafting are unpredictable, especially if the tissue cover remains tight.²⁴ They advocate cartilage grafts as the treatment of choice in Binder syndrome nasal reconstruction.

Another method of nasal reconstruction in Binder syndrome was presented by Kansu et al.: A case of a patient treated by placing a titanium screw into the floor of the pyriform fossa, which was supposed to act as the absent

nasal spine.³¹ Because the short-term result was regarded as satisfactory by the authors, no long-term follow-up results were reported. This meant that complications such as screw extrusion or injury-related perforation of the nasal tip, which is a risk when using such a method, were not excluded.

In the present study, most of the patients were affected with a cleft lip or palate, which is consistent with other authors' findings.^{10,32,33} In 2002, Mulliken et al. described an entity that they named "binderoid complete cleft lip or palate".³² Their patients (15 cases) presented cleft lip and/or palate accompanied by underdevelopment of the nasolabiomaxillary elements. The authors noted that a binderoid phenotype may occur not only in non-syndromic patients, but also in patients with chondrodysplasia punctata, cervical spine anomalies, fetal exposure to warfarin and (as noted above) a cleft lip and palate. The patients presented by Mulliken et al.³² were predominantly women (2 : 1), which was consistent with the observations of the present authors. This ratio is the reverse of what is observed in cases of isolated cleft lip and palate.

As in other authors' reports, the patients in the present study showed a wide spectrum of intensity of dysmorphic features, ranging from mild nasal hypoplasia to severe nasomaxillary underdevelopment accompanied by malocclusion.¹³

The surgical protocol used at the present authors' institution is based on early closure of the cleft lip/palate in patients presenting this co-morbidity. Nasolabiomaxillary underdevelopment was addressed in adolescence. In the most severe cases, maxillary advancement was performed by means of a Le Fort I or "high" Le Fort I osteotomy. In most patients, nasal dorsum reconstruction was undertaken by iliac crest bone graft. The dorsum and columella were reconstructed by an L-shaped bone graft, whereas the maxilla was augmented by using fragments of cancellous bone. The great majority of the patients presented intense resorption of the grafted tissues, requiring another bone graft. In most of the reoperations, auricular cartilage or deepithelialized skin grafts were used. The average number of surgical procedures per patient was 2.2, which is slightly lower than the 2.4 reported by Chummun et al.¹¹ This is a highly satisfactory number, especially considering the high rate of other congenital deformities that were also included in the surgical treatment plan (such as cleft lip and palate). In the authors' opinion, cartilage and dermal grafts show a lower incidence of resorption; this coincides with other authors' observations.^{11,28-30}

In the present study, no alloplastic materials were used to augment the nasolabiomaxillary region. Other authors report the use of silicone or high-density porous polyethylene implants in the nasal dorsum and columella.^{22,34} However, a high rate of complications with alloplastic materials, including extrusion of the implanted material, makes the use of autologous grafts preferable.

Fig. 14–19. Result after Le Fort I osteotomy

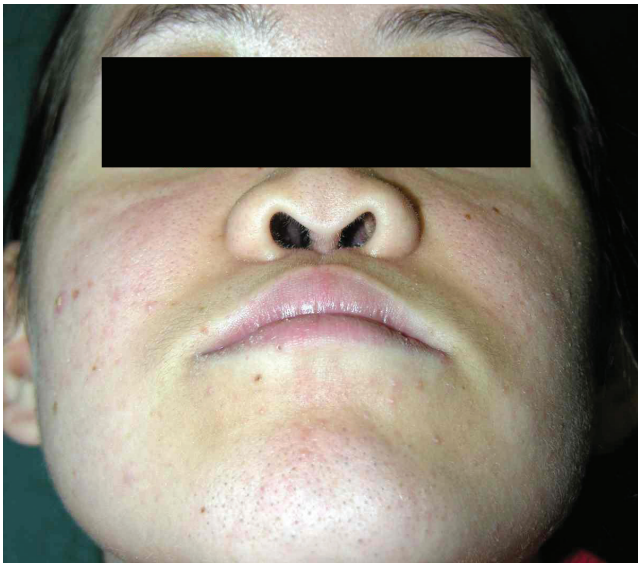


Fig. 14.



Fig. 17.



Fig. 15.



Fig. 18.



Fig. 16.



Fig. 19.

Fig. 20–22. Final result after composite auricular graft for columella reconstruction



Fig. 20.



Fig. 21.



Fig. 22.

The patients in the present study were also treated orthodontically, and logopedic rehabilitation was introduced in the early stages.

Conclusions

In BS, surgical treatment is the treatment of choice, resulting in adequate correction of facial retrusion. However, due to various degrees of bone resorption, the results do not last a lifetime. In some cases they need refinement procedures or even bone re-grafting.

No unequivocally superior surgical strategy in Binder syndrome has been presented so far. Most of the disagreement among authors is related to the need for and timing of maxillary osteotomy, the choice between bone and cartilage grafting in nose reconstruction, and columella lengthening.

Although alloplastic materials offer the tempting advantage of fast and simultaneous augmentation of deficient tissues, their use may risk prolonged infections and extrusion, resulting in exacerbations of deformities.

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Combined screening for early and late pre-eclampsia and intrauterine growth restriction by maternal history, uterine artery Doppler, mean arterial pressure and biochemical markers

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Abstract

Background. Pre-eclampsia is a systemic disease connected with high maternal and fetal morbidity and mortality. Despite significant progress achieved in perinatal medicine, pre-eclampsia is still one of the most significant current problems in obstetrics.

Objectives. The aim of the study was to establish diagnostic algorithms for early and late pre-eclampsia (PE) and intrauterine growth restriction (IUGR).

Material and methods. A total of 320 pregnant women between 11 + 0 and 13 + 6 weeks of gestation were recruited for a case-control study. The study group consisted of 22 patients with early PE, 29 patients with late PE and 269 unaffected controls. The following parameters were recorded: maternal history, mean arterial pressure (MAP), mean uterine artery pulsatility index (UtA-PI), and the concentrations of placental growth factor (PlGF), pregnancy-associated plasma protein A (PAPP-A) and free beta-human chorionic gonadotropin (free β -hCG).

Results. A multivariable stepwise logistic regression analysis indicated that the best screening model for the prediction of early PE is based on a combined analysis of maternal risk factors, UtA-PI and PlGF levels (sensitivity: 91%; specificity: 84%). The best screening model for the prediction of late PE is based on a combined analysis of maternal risk factors, UtA-PI and MAP (sensitivity: 85%; specificity: 83%). The most effective screening model for the prediction of IUGR is based on a combined analysis of maternal risk factors, UtA-PI and PlGF concentrations (sensitivity: 91%; specificity: 83%).

Conclusions. The integrated model of screening established in this study can be a valuable method to identify patients at increased risk of developing pre-eclampsia and related complications. The ability to predict the occurrence of pre-eclampsia in early pregnancy would enable maternal and fetal morbidity to be reduced through the introduction of strict obstetric surveillance as well as planned delivery in a reference center.

Key words: pre-eclampsia, placental growth factor, diagnostic algorithms

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Pre-eclampsia (PE) is a disease characterized by high maternal and fetal morbidity and mortality. As many as 3% of all pregnant women in Europe experience pre-eclampsia.^{1,2} The incidence of pre-eclampsia in healthy primiparous women ranges from 2 to 7%. Despite the significant progress achieved in perinatal medicine, pre-eclampsia is still one of the most urgent current problems in obstetrics. Nowadays patients at high risk of developing pre-eclampsia are distinguished on the basis of clinical features, the most important ones being pregestational diabetes, increased body mass index (BMI), a history of pre-eclampsia and chronic hypertension. However, these risk factors lead to the detection of only 30% of the patients who will suffer from pre-eclampsia, and are not effective in nulliparous women without risk factors in their medical history.^{3,4}

Pre-eclampsia prediction can be made more accurate by combining biochemical and biophysical markers, 3 of which – i.e. free beta-human chorionic gonadotropin (free β -hCG), pregnancy-associated plasma protein A (PAPP-A) and placental growth factor (PlGF) – can be used in first-trimester screening studies.^{5–9} To increase the sensitivity of screening studies for PE using multivariate analysis, a standardized technique for measuring blood pressure in the first trimester has been included. In the mid-1990s, uterine artery flow in the second trimester was shown to be an effective method characterized by high sensitivity but low specificity.¹⁰ Some reports indicate the value of uterine artery flow assessment in the first trimester.¹¹ Integrated screening studies performed sufficiently early should allow the detection of patients at increased risk of pre-eclampsia.

The early identification of patients at increased risk of developing pre-eclampsia is important for several reasons. Firstly, the effectiveness of preventive interventions is dependent on the early introduction of treatment modifying the placentation process.³ Secondly, early risk stratification allows patients to be correctly qualified into groups, and for survival rates to be increased through interventions in the high-risk group. Finally, accurate predictions will improve the quality of studies evaluating the potential role of preventive actions and the pathogenesis of pre-eclampsia.

The aim of the study was to establish diagnostic algorithms for early and late pre-eclampsia and intrauterine growth restriction.

Material and methods

The study population consisted of 320 pregnant women between 11 + 0 and 13 + 6 weeks of gestation between 2011 and 2015. The study was performed at the Perinatology and Gynecology Department of Polish Mother's Memorial Hospital Research Institute, a tertiary care center, and in two private practices: NZOZ Medyk W. Litwiński

(Włocławek, Poland) and NZOZ Sonomedica (Łódź, Poland). The group consisted of 240 participants without risk factors in their medical history and 80 with at least one risk factor for developing pre-eclampsia (pre-eclampsia in a previous pregnancy, chronic hypertension, elevated BMI, kidney diseases, diabetes, systemic diseases). Women with multiple pregnancies, those with the presence of significant fetal abnormalities and those who had experienced an abortion or termination of pregnancy were excluded from the present study. All the participants in the study signed a consent form, and the approval of the Hospital Ethics Committee to conduct the study was obtained.

The presence of the risk factors listed above did not lead to pre-eclampsia in every case. Based on the actual occurrence of pre-eclampsia, the study group was subdivided into 4 groups: 1) patients who did not suffer from pre-eclampsia (the control group; $n = 269$); 2) patients who suffered from the early form of pre-eclampsia ($n = 22$); 3) patients who developed late-onset pre-eclampsia ($n = 29$); 4) patients diagnosed with intrauterine growth restriction ($n = 43$). An analysis of potential risk markers in these 3 groups was carried out.

An accurate interview with each patient was conducted during the first antenatal visit. The following data were recorded: age, height, parity, smoking during pregnancy, type of conception, family history (including pre-eclampsia affecting mothers), elevated blood pressure before pregnancy, diabetes, pre-eclampsia in a previous pregnancy, systemic diseases, kidney disorders or antiphospholipid syndrome. In addition, the mean blood pressure was measured, a 5 mL blood sample was collected and an ultrasound scan was performed. During the ultrasound examination, the following measurements were recorded, in accordance with the Fetal Medicine Foundation guidelines: the crown-rump length, nuchal translucency, and the flow through the ductus venosus and uterine artery. An initial evaluation of the fetal anatomy was also performed.

The following visit, which took place between 20 + 0 and 23 + 6 weeks of gestation, included blood pressure measurement and an ultrasound scan, with an evaluation of fetal anatomy and growth to detect congenital fetal anomalies. Additionally, uterine artery flow was measured.

During the last visit, scheduled between 30 + 0 and 34 + 6 weeks of gestation, blood pressure was measured and an ultrasound scan was performed in order to evaluate the anatomy, Doppler flows and growth of the fetus.

The 5 mL blood samples taken during the first visit were collected in plastic tubes with ethylenediaminetetraacetic acid (EDTA). Following centrifugation, the serum was stored at -80°C . PlGF, PAPP-A and free β -hCG concentrations were evaluated using equipment that provides reproducible results (Delfia Xpress System, PerkinElmer, Waltham, USA).

Blood pressure was measured using equipment certified by the Fetal Medicine Foundation (3BT0-A2, Micro-

life, Taipei, Taiwan). The devices were calibrated before and at regular intervals during the study. The measurements were performed by appropriately trained doctors. The subjects were in a seated position with their arms at the level of the heart. An appropriate adult cuff size was selected for the participant's arm circumference (small: < 22 cm, average: 22–32 or large: 33–42 cm). After 5 min rest, the blood pressure was measured in both arms simultaneously at 1-min intervals until the differences between successive measurements fell within 10 mm Hg in systolic blood pressure and 6 mmHg in diastolic blood pressure in both arms. When stability was reached, the mean arterial pressure (MAP) of each arm was calculated as the average of the previous 2 stable measurements. The final result was taken from the arm with the highest MAP.

All the scans were performed transabdominally with a Voluson E6 and E8 ultrasound machine scanner (GE Healthcare, Chicago, USA). All the ultrasound and Doppler examinations were performed by doctors certified by the Fetal Medicine Foundation. The measurements were taken in accordance with Fetal Medicine Foundation guidelines. Pulsed wave Doppler was used to assess the uterine artery pulsatility index (UtA-PI). First, a sagittal section of the uterus was obtained and the cervical canal was identified. The transducer was then carefully tilted from side to side and color flow mapping was used to visualize each uterine artery. Pulsed wave Doppler was used with the gate set at 2 mm and an angle of insonation less than 30 degrees. After obtaining 3 similar consecutive waveforms, the PI was measured and the mean PI of the left and right arteries was calculated. The uterine artery pulsatility index measurements were taken in a similar way in the second trimester. The findings were collected and the risk of pre-eclampsia was assessed.

Statistical analysis

The mean PI, MAP, PlGF, PAPP-A and free β -hCG of the uterine artery were converted into multiples of median (MoM) and corrected for the crown-rump length of the fetus (CRL), the patient's age, BMI, smoking, parity, type of conception and racial origin. The distributions of the examined parameters were transformed into a Gaussian distribution after logarithmic transformation.

The measurements taken from the pre-eclampsia, IUGR and unaffected groups were compared. The comparison between the early pre-eclampsia, late pre-eclampsia and IUGR groups and the unaffected control group was made using the Mann-Whitney U test with post-hoc Bonferroni correction was used (the critical value for statistical significance was $p < 0.0167$). A p -value < 0.05 was considered significant.

A multivariable stepwise logistic regression analysis was carried out to calculate the risks of IUGR, early and late-onset pre-eclampsia based on a combination of maternal

risk factors, biochemical markers (PlGF, PAPP-A, free β -hCG concentrations) and biophysical markers (MAP, UtA-PI). The performance of the screening was described by receiver-operating characteristics (ROC) curves, areas under the curve (AUC), confidence intervals (CI 95%), sensitivity, specificity and detection rates, with a fixed false-positive rate of 10%. Prediction models were developed using multivariable logistic regression analysis consisting of the following components: univariate analysis, linear examination of predictors, an examination of the correlation between predictors, an evaluation of significant interaction between potential predictors, residual analysis and model evaluation. The p -value needed to be included in the logistic regression analysis was fixed at < 0.05 . The analysis results were presented using odds, Nagelkerke's R^2 and p -values.

The statistical analysis was conducted using R 3.1.1 software.

Results

The participants' characteristics are presented in Table 1. The early PE group was characterized by a higher BMI, a higher incidence of chronic HA, diabetes and kidney disease than the controls. The late PE group was characterized by a higher prevalence of smokers, women with a history of pre-eclampsia and chronic hypertension than the controls. In the IUGR group, the women were found to have a higher BMI, and more often suffered from diabetes, chronic hypertension and kidney disease than the controls.

The impact of the following factors on the prediction of the early, late pre-eclampsia and IUGR was analyzed: UtA-PI, UtA-PI MoM, MAP, MPA MoM, PlGF concentration, PlGF MoM, PAPP-A concentration, PAPP-A MoM, free β -hCG and free β -hCG MoM. Data for each parameter are presented in Table 2.

Multivariable stepwise logistic regression analysis was used to determine the effect of the following log-transformed factors: MPA MoM, mean UtA-PI MoM, PlGF MoM, PAPP-A MoM and free β -hCG MoM on the occurrence of early pre-eclampsia, late pre-eclampsia and intrauterine growth restriction. The results of the ROC analysis for early pre-eclampsia, late pre-eclampsia and IUGR are presented in Table 3.

The detection rates of the early pre-eclampsia, late pre-eclampsia and IUGR groups were also compared, with a fixed false-positive rate of 10% (Table 4).

The performance of the screening was described by the areas under the curve. The AUC values are described for all the parameters in Table 5.

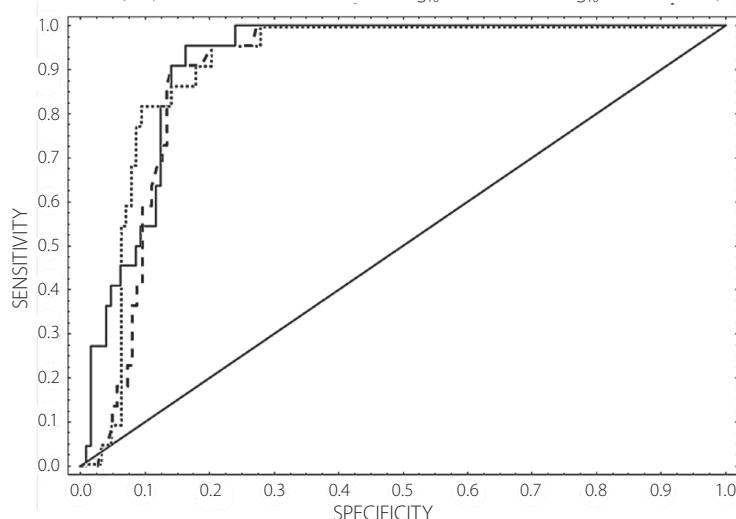
On the basis of the data, the best screening models for the prediction of early pre-eclampsia, late pre-eclampsia and intrauterine growth restriction were established and presented in ROC curves (Fig. 1–3).

Table 1. Participants' characteristics

	Control (n = 269)	Early pre-eclampsia (n = 22)	Late pre-eclampsia (n = 29)	IUGR (n = 43)
Age (years)	28 (25–31)	29.5 (26–34) ^{NS}	30 (27–33) ^{NS}	29 (26–34) ^{NS}
BMI (kg/m ²)	23.9 (22–27.6)	30.6 (23.1–32.3) *	26.5 (23.6–29.7) ^{NS}	28.7 (23.6–32.4) ‡
Smoking	21 (8%)	2 (9%) ^{NS}	6 (21%) ^{NS}	6 (14%) ^{NS}
Parity				
multiparous	115 (43%)	8 (36%) ^{NS}	23 (79%) ‡	21 (49%) ^{NS}
primiparous	154 (41%)	14 (64%) ^{NS}	6 (21%) †	22 (51%) ^{NS}
Preterm delivery	13 (5%)	5 (23%) *	14 (48%) ‡	11 (26%) ^{NS}
Miscarriage in medical history	32 (12%)	6 (27%) ^{NS}	6 (21%) ^{NS}	17 (40%) ‡
History of pre-eclampsia	27 (10%)	7 (31%) *	18 (62%) ‡	13 (30%) ^{NS}
Diabetes	13 (5%)	5 (23%) *	1 (3.4%) ^{NS}	8 (18%) *
Chronic hypertension	30 (11%)	12 (55%) ‡	9 (31%) *	25 (58%) ‡
Kidney disease	5 (2%)	6 (27%) ‡	2 (7%) ^{NS}	9 (21%) ‡
Pre-eclampsia in mother	19 (7%)	2 (9%) ^{NS}	3 (10%) ^{NS}	5 (12%) ^{NS}
AFS	0	0	0	0

Data are presented as n (percent) or median (first quartile-third quartile). Comparison between groups (early pre-eclampsia, late pre-eclampsia, IUGR) and the group of patients who didn't suffer from pre-eclampsia was made for dichotomous variables using the χ^2 test with appropriate corrections and for continuous variables using the Mann-Whitney U test. For both tests post-hoc Bonferroni correction was used (the critical value for statistical significance was $p < 0.0167$). ^{NS} $p > 0.0167$; * $p < 0.0167$; † $p < 0.001$; ‡ $p < 0.0001$.

Fig. 1. Receiver-operating characteristics (ROC) curves of maternal risk factors + Log₁₀ PIGF MoM (•••), maternal risk factors + Log₁₀ Uta-PI MoM (---) and maternal risk factors + Log₁₀ PIGF MoM + Log₁₀ Uta-PI MoM (—) in the prediction of early-preeclampsia



Maternal risk factors + Log₁₀ mean Uta PI MoM

$Y = -3.15$ (SE 0.63) + 6.8 (SE 2.88) \times Log₁₀ mean Uta PI MoM + 1.8 (SE 0.52) \times maternal risk factors.
Odds = e^Y ; $R^2 = 0.33$; $p < 0.0001$.

Maternal risk factors + Log₁₀ PIGF MoM

$Y = -2.6$ (SE 0.55) - 2.25 (SE 1.1) \times Log₁₀ PIGF MoM + 1.9 (SE 0.52) \times maternal risk factors.
Odds = e^Y ; $R^2 = 0.40$; $p < 0.0001$.

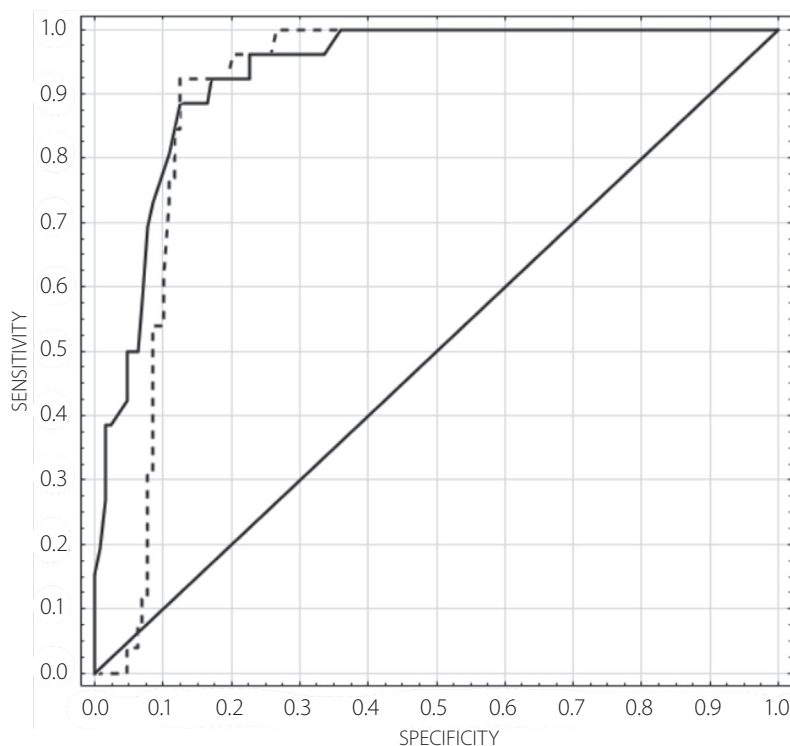
Maternal risk factors + + Log₁₀ PIGF MoM + Log₁₀ Uta PI MoM

$Y = -3.41$ (SE 0.69) - 2.6 (SE 1.2) \times Log₁₀ PIGF MoM + 7.09 (SE 2.88) \times Log₁₀ mean Uta PI MoM + 1.7 (SE 0.53) \times maternal risk factors.
Odds = e^Y ; $R^2 = 0.43$; $p < 0.0001$.

Table 2. Data for each marker in the four outcome groups

	Control (n = 269)	Early pre-eclampsia (n = 22)	Late pre-eclampsia (n = 29)	IUGR (n = 43)
Gestational age according to CRL (hbd)	12.5 (12.2–13)	12.3 (12–13) ^{NS}	12.6 (12.4–13) ^{NS}	12.4 (12.1–13) ^{NS}
CRL (mm)	63.6 (59.1–68.3)	60 (54.7–66.7) ^{NS}	64.7 (61.2–67.1) ^{NS}	61.5 (56.2–67.1) ^{NS}
DV PI	1.01 (0.91–1.11)	0.95 (0.86–1.02) ^{NS}	1.01 (0.92–1.05) ^{NS}	0.96 (0.91–1.04) ^{NS}
Mean UtA-PI	1.68 (1.32–2.09)	2.28 (2.1–2.56) ‡	2.18 (2.07–2.33) ‡	2.33 (2.12–2.62) ‡
Mean UtA-PI MoM	1.07 (0.86–1.29)	1.42 (1.26–1.62) ‡	1.4 (1.3–1.47) ‡	1.44 (1.3–1.73) ‡
PlGF concentrations (pg/mL)	27.4 (19.6–34)	15.55 (11.1–19) ‡	21 (16.7–25.6) *	20.5 (14.6–26.2) *
PlGF MoM	1.21 (0.93–1.57)	0.62 (0.51–0.96) ‡	0.92 (0.63–1.09) ‡	0.97 (0.59–1.12) ‡
PAPP-A concentrations (IU/L)	1.6 (1–2.6)	0.84 (0.67–1.13) ‡	1.03 (0.6–1.8) *	0.90 (0.6–1.2) ‡
PAPP-A MoM	1.01 (0.65–1.55)	0.67 (0.382–0.82) ‡	0.74 (0.33–1.09) *	0.49 (0.37–1.06) ‡
Free beta-hCG concentrations (IU/L)	42.1 (31.2–58)	41.65 (25.7–47) ^{NS}	42 (34.1–55.8) ^{NS}	40.3 (32.4–44.4) ^{NS}
Free beta-hCG MoM	1.14 (0.75–1.49)	1.08 (0.74–1.23) ^{NS}	1.25 (1.05–1.49) ^{NS}	1.12 (0.91–1.25) ^{NS}
Mean MAP	84.6 (78.4–92.6)	100.35 (93.1–103.7) ‡	96.4 (92.1–103.6) ‡	95.6 (90.2–102.2) ‡
Mean MAP MoM	0.99 (0.93–1.06)	1.15 (1.06–1.17) ‡	1.09 (1.06–1.16) ‡	1.12 (1.03–1.16) ‡
Age at delivery (hbd)	39 (37–40)	30 (29–32) ‡	37 (35–37) ‡	32 (29–34) ‡
Birth weight (g)	3450 (3050–3780)	1015 (850–1200) ‡	2920 (2450–3120) ‡	1297 (890–1760) ‡
Mean UtA-PI (secondtrimester)	1 (0.82–1.32)	1.63 (1.45–1.9) ‡	1.74 (1.42–1.88) ‡	1.62 (1.43–1.78) ‡

Data is shown as a median (interquartile range). Comparison between groups (early pre-eclampsia, late pre-eclampsia, IUGR) and group of patients who didn't suffer from pre-eclampsia was made using U Mann-Whitney test. The post-hoc Bonferroni correction was used (critical value for statistical significance was $p < 0.0167$). ^{NS} $p > 0.0167$; * $p < 0.0167$; ‡ $p < 0.001$; ‡ $p < 0.0001$.

Fig. 2. Receiver-operating characteristics (ROC) curves of maternal risk factors + Log₁₀ MAP MoM (—) and maternal risk factors + Log₁₀ UtA-PI MoM (- - -) in the prediction of late pre-eclampsia

Maternal risk factors + Log₁₀ mean UtA PI MoM
 $Y = -3.046 \text{ (SE 0.64)} + 7.058 \text{ (SE 3.02)} \times \text{Log}_{10} \text{ Sr UtA PI MoM} + 1.94 \text{ (SE 0.52)} \times \text{maternal risk factors}$
 Odds = e^Y
 $R^2 = 0.26$
 $p < 0.05$

Maternal risk factors + Log₁₀ mean MAP MoM
 $Y = -2.204 \text{ (SE 0.368)} + 22.75 \text{ (SE 7.463)} \times \text{Log}_{10} \text{ mean MPA MoM} + 1.205 \text{ (SE 0.297)} \times \text{maternal risk factors}$
 Odds = e^Y
 $R^2 = 0.29$
 $p < 0.001$

Table 3. The results of ROC analysis for early and late pre-eclampsia and IUGR

	Early pre-eclampsia				Late pre-eclampsia				IUGR			
	sensitivity	specificity	R ² Nagelkerke	p-value	sensitivity	specificity	R ² Nagelkerke	p-value	sensitivity	specificity	R ² Nagelkerke	p-value
PAPP-A MoM	0.63	0.47	0.04	*	0.79	0.61	0.18	†	0.65	0.54	0.12	*
PIGF MoM	0.86	0.62	0.19	‡	0.75	0.55	0.08	*	0.70	0.57	0.11	*
Free beta-hCG MoM	0.72	0.46	0.02	NS	0.93	0.13	0.00	NS	0.79	0.39	0.01	NS
Mean MAP MoM	0.81	0.50	0.06	*	0.90	0.56	0.14	†	0.76	0.54	0.08	*
Mean Uta-PI MoM	0.77	0.58	0.13	†	0.93	0.61	0.12	†	0.83	0.64	0.32	‡
DV PI	0.59	0.58	0.03	*	0.65	0.43	0.00	NS	0.60	0.58	0.02	NS
maternal risk factor +												
PAPP-A MoM	0.68	0.75	0.29	‡	0.86	0.66	0.26	†	0.86	0.77	0.42	‡
PIGF MoM	0.86	0.80	0.40	‡	0.72	0.70	0.20	†	0.86	0.78	0.41	‡
Free beta-hCG MoM	0.81	0.80	0.29	NS	0.86	0.42	0.15	NS	0.81	0.77	0.38	NS
Mean MAP MoM	0.77	0.77	0.30	NS	0.93	0.50	0.29	†	0.88	0.66	0.32	‡
mean Uta-PI MoM	0.90	0.78	0.32	‡	0.93	0.66	0.25	*	0.90	0.83	0.52	‡
DV PI	0.86	0.75	0.30	NS	0.62	0.61	0.15	NS	0.86	0.79	0.39	NS
Śr Uta-PI MoM + PIGF MoM	0.86	0.77	0.42	*	0.90	0.65	0.23	NS	0.93	0.76	0.53	*

NS p > 0.05; * p < 0.05; † p < 0.001; ‡ p < 0.0001.

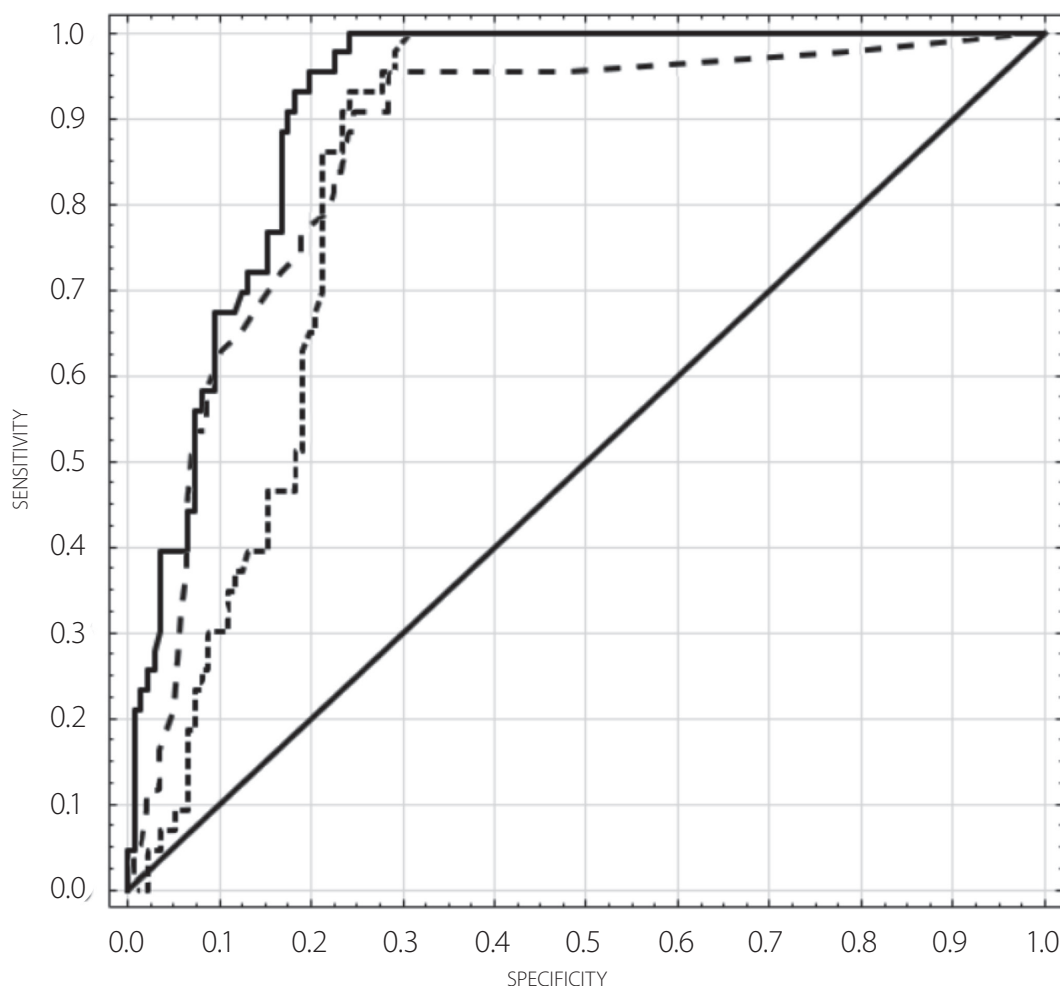
Discussion

Pre-eclampsia occurs in 2-3% of all pregnancies and is the main cause of maternal and fetal morbidity and mortality.¹ The diagnosis is based on clinical features such as high blood pressure and proteinuria, which are the final steps in the pathogenesis of pre-eclampsia, beginning with impaired implantation and growth of trophoblasts in the first trimester of pregnancy. Despite the lack of acknowledged methods of prevention, 2 important benefits can be gained from defining a group of patients who may be found to be at increased risk of pre-eclampsia during routine examinations performed in the first trimester of pregnancy. Firstly, greater obstetric supervision can be provided for

high risk patients, and secondly, an adequate response can be prepared in case of complications. The present analysis was conducted on a group of patients undergoing a routine scan in the first trimester of pregnancy in order to calculate the risk of chromosomal abnormalities and pre-eclampsia and its complications. The results of the statistical analysis indicate that multivariable screening based on the parameters investigated in this study allow the majority of pregnancies complicated by pre-eclampsia to be identified. This screening is more valuable in detecting early-onset pre-eclampsia than late-onset pre-eclampsia, with the respective sensitivities being 91 and 85%, while the respective specificities are 84 and 83%.

The combination of a detailed medical history with a blood pressure examination is the cheapest and the

Fig. 3. Receiver-operating characteristics (ROC) curves of maternal risk factors + Log₁₀ PIGF MoM (---), maternal risk factors + Log₁₀ MAP MoM (---) and maternal risk factors + Log₁₀ PIGF MoM + Log₁₀ UtA-PI MoM (—) in the prediction of intrauterine growth restriction



Maternal risk factors + Log₁₀ PIGF MoM + mean UtA PI MoM

$Y = -7.516 \text{ (SE 1.80)} - 0.123 \text{ (SE 0.573)} \times \text{Log}_{10} \text{ PIGF MoM} + 4.213 \text{ (SE 1.04)} \times \text{Log}_{10} \text{ UtA PI MoM} + 1.962 \text{ (SE 0.43)} \times \text{maternal risk factors}$
 Odds = e^Y ; $R^2 = 0.54$; $p < 0.05$

Maternal risk factors + Log₁₀ PIGF MoM

$Y = -1.283 \text{ (SE 0.68)} - 0.592 \text{ (SE 0.56)} \times \text{Log}_{10} \text{ PIGF MoM} + 1.87 \text{ (SE 0.38)} \times \text{maternal risk factors}$
 Odds = e^Y ; $R^2 = 0.42$; $p < 0.0001$

Table 4. The performance of the screening for pre-eclampsia and intrauterine growth restriction for each parameter separately and for a combination of maternal factors with biochemistry, mean UtA-PI and MAP, shown as the detection rate for a fixed false-positive rate (FPR)

	Detection rate (%) for a fixed false-positive rate (FPR)		
	early pre-eclampsia	late pre-eclampsia	IUGR
	FPR 10%	FPR 10%	FPR 10%
PAPP-A MoM	48.3	25.9	33.6
PIGF MoM	57	34.4	46.7
Free β -hCG MoM	20.3	6.9	21.9
Mean MPA MoM	45.5	24.1	34.9
Mean UtA-PI MoM	33.2	19.3	41.5
Mean UtA-PI (second trimester)	39.1	41.4	23.3
Maternal risk factors +			
PAPP-A MoM	36.4	27.6	55.8
PIGF MoM	81.8	58.6	30.2
Free β -hCG MoM	---	---	---
Mean MPA MoM	77.3	80.8	62.8
Mean UtA-PI MoM	59.1	48.3	67.4
DV PI	---	---	---

most readily available screening method. In the present analysis, screening tests based only on medical history were found to have a sensitivity of 41% and specificity of 95% (RR 3.42), which is consistent with previous analyses. According to Poon et al., 37% of patients who will develop early-onset pre-eclampsia and 29% of patients who will develop late-onset pre-eclampsia can be detected on the basis of medical history alone, albeit with a 5% false positive rate.³ If blood pressure is also measured during the examination, the area of possible pregnancy complications is expanded. The calculated detection rates for early pre-eclampsia, late pre-eclampsia and intrauterine growth restriction based only on blood pressure measurement are 45, 24 and 35% respectively, with a 10% false positive rate. These results are consistent with those of Manten et al., who observed higher blood pressure in patients with a history of pre-eclampsia, even when no pre-eclampsia occurred. These patients are at increased risk of developing chronic hypertension.¹²

Indirect evidence of impaired placenta perfusion in pre-eclampsia complicated pregnancies has been revealed by Doppler studies. An increase in the uterine artery pulsatility index in the first and second trimesters is observed in patients with pre-eclampsia, particularly in early onset type and IUGR. According to Plasencia et al., detection rates in early and late-onset pre-eclampsia based on the uterine artery pulsatility index were higher in the second trimester than in the first.^{13,14}

An understanding of the pathophysiology of pre-eclampsia would allow the factors participating in early angiogenesis and placentation to be evaluated as potential biochemical markers. The PAPP-A and free β -hCG examined in the present study are components of screening tests for chromosomal abnormalities. Placental growth factor – one of the factors playing a role in placentation – was found to be present in similar amounts to those identified in previous studies.^{15–17} The same studies also reported a decrease in PAPP-A levels between 11 + 0 and 13 + 6 weeks of gestation in patients who developed pre-eclampsia, after excluding pregnancies complicated by chromosomal abnormalities.^{15–17} Additionally, the present study found the level of PAPP-A to be significantly lower in early-onset pre-eclampsia, and a significant relationship was noted between the level of PAPP-A and the uterine artery pulsatility index. These findings are consistent with those of Poon et al. and Spencer et al., who showed the value of PAPP-A measurement in the prediction of early pre-eclampsia.^{18–20} Pregnancies

complicated by pre-eclampsia revealed lower levels of the markers and mediators of endothelial cell dysfunction as well as placental growth factor. A relationship was also observed between PIGF levels and the chance of developing early-onset pre-eclampsia, as previously noted by Akolekar et al. and Erez et al.^{21,22} In the present study, no relationship was observed between the level of free β -hCG and the occurrence of pre-eclampsia or its complications. These results are consistent with those of numerous studies conducted on large cohorts of patients.^{16,23}

However, detection rates for early pre-eclampsia, late pre-eclampsia and IUGR calculated separately for each parameter were disappointing. The respective detection rates for early pre-eclampsia, late pre-eclampsia and IUGR, calculated on the basis of multiples of the median, were 45, 24 and 35% for mean arterial pressure; 33, 19 and 41% for mean uterine artery pulsatility index; 57, 34 and 47% for PIGF concentration; and 48, 26 and 34% for PAPP-A concentration. The false positive rate was 10%. As a significant relationship was found between individual, biochemical and biophysical parameters, the next step was to create an integrated model of screening tests for pre-eclampsia – an approach analogous to the concept of early multifactorial screening for chromosomal abnormalities. The application of an integrated model of screening resulted in a spectacular improvement in the sensitivity of the test, especially regarding the early form of pre-eclampsia. Finally, after taking all

Table 5. Comparative evaluation of screening examinations for the prediction of early and late pre-eclampsia for maternal risk factors and maternal risk factors + additional factors

	Area under curve (AUC)		
	early pre-eclampsia	late pre-eclampsia	IUGR
PAPP-A MoM	0.62 (0.57 – 0.67) *	0.74 (0.70 – 0.78) †	0.69 (0.64 – 0.74) *
PIGF MoM	0.79 (0.75 – 0.83) ‡	0.66 (0.61 – 0.71) *	0.68 (0.64 – 0.72) *
Free beta-hCG MoM	0.59 (0.53 – 0.65) ^{NS}	0.61 (0.56 – 0.66) ^{NS}	0.55 (0.50 – 0.60) ^{NS}
Mean MAP MoM	0.77 (0.71 – 0.83) *	0.74 (0.69 – 0.79) †	0.73 (0.68 – 0.78) *
Mean UtA-PI MoM	0.75 (0.71 – 0.79) †	0.75 (0.71 – 0.79) †	0.82 (0.79 – 0.85) ‡
DV PI	0.64 (0.58 – 0.70) *	0.51 (0.46 – 0.56) ^{NS}	0.61 (0.56 – 0.66) ^{NS}
Maternal risk factors	0.85 (0.81 – 0.89) ‡	0.80 (0.76 – 0.84) ‡	0.83 (0.8 – 0.86) ‡
Maternal risk factors +			
PAPP-A MoM	0.85 (0.82 – 0.88) ‡	0.84 (0.81 – 0.87) †	0.85 (0.82 – 0.88) ‡
PIGF MoM	0.91 (0.89 – 0.93) ‡	0.86 (0.83 – 0.89) †	0.84 (0.81 – 0.87) ‡
beta-hCG MoM	0.81 (0.76 – 0.86) ^{NS}	0.55 (0.49 – 0.61) ^{NS}	0.81 (0.77 – 0.85) ^{NS}
Mean MAP MoM	0.92 (0.89 – 0.95) ^{NS}	0.93 (0.91 – 0.95) †	0.86 (0.83 – 0.89) ‡
Mean UtA-PI MoM	0.90 (0.87 – 0.93) ‡	0.87 (0.84 – 0.90) *	0.91 (0.89 – 0.93) ‡
DV PI	0.70 (0.66 – 0.76) ^{NS}	0.67 (0.63 – 0.71) ^{NS}	0.73 (0.69 – 0.77) ^{NS}
Sr UtA-PI MoM + PIGF MoM	0.92 (0.90 – 0.94) ‡	0.80 (0.77 – 0.83) ^{NS}	0.91 (0.89 – 0.93) *

CI 95% for AUC is presented in brackets; ^{NS} $p > 0.05$; * $p < 0.05$; † $p < 0.001$; ‡ $p < 0.0001$.

the parameters into account (i.e. personal history, mean arterial pressure, mean uterine artery pulsatility index, PIGF and PAPP-A concentration), the best screening model for early pre-eclampsia prediction was found to be based on a combination of maternal characteristics, the mean uterine artery pulsatility index and the placental growth factor concentration. The sensitivity and specificity of this model are 91 and 84% respectively. The best model for screening for IUGR is based on the same 3 factors, which may be related to the frequent coincidence of IUGR and early pre-eclampsia. The sensitivity and specificity of this model are 91 and 83% respectively. The combination of maternal risk factors and mean arterial pressure is the best screening tool for the prediction of late-onset pre-eclampsia, with sensitivity and specificity of 85 and 83% respectively. Early pre-eclampsia screening is particularly important because of the rapid course of the condition, the high risk of maternal and neonatal mortality and morbidity, as well as its short- and long-term maternal complications.

The main advantages of the present study are the inclusion of a large number of patients with risk factors at accurately specified gestational ages and clear criteria of pre-eclampsia. In addition, a higher detection rate was achieved by considering a variety of biochemical and biophysical markers (blood pressure measurement and Doppler studies of uterine arteries) along with a medical history taken at an accurately specified gestational age. However, a potential limitation of this study was

the large inter-patient variation among the risk factors, which made it difficult to obtain statistical significance for some parameters.

The ability to predict the occurrence of pre-eclampsia in early pregnancy enables maternal and fetal morbidity to be reduced through strict obstetric surveillance as well as planned delivery in a reference center. The integrated model of screening presented in this study can be valuable in defining the group of patients at increased risk of developing pre-eclampsia. Future studies are necessary on the potential role of pharmacological interventions starting in the first trimester of pregnancy with the aim of improving the quality of placentation and reducing the prevalence of pre-eclampsia.

Conclusions

Prediction of early pre-eclampsia is most effective when based on a combination of maternal risk factors, the mean uterine artery pulsatility index and placental growth factor concentration.

Prediction of late pre-eclampsia is most effective when based on a combination of maternal risk factors and mean arterial blood pressure.

Prediction of IUGR is most effective when based on a combination of maternal risk factors, the mean uterine artery pulsatility index and placental growth factor concentration.

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Decreased sL-selectin serum levels in sleep apnea syndrome patients with cardiovascular diseases

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Abstract

Background. Obstructive sleep apnea syndrome (OSA) is a common disorder associated with an increased risk of cardiovascular diseases.

Objectives. sL-selectin is an adhesion molecule released from the surface of leukocytes as they are activated and may inhibit leukocyte attachment to the endothelium. The aim of this study was to evaluate sL-selectin serum levels in OSA patients with cardiovascular diseases.

Material and methods. A total of 163 OSA patients were enrolled in the study. The mean age was 55.41 ± 8.63 years and the mean AHI (apnea hypopnea index) was $35.02 \pm 22.28/h$. A control group was composed of 59 healthy subjects. All subjects underwent a nocturnal respiratory polygraphy. sL-selectin serum levels were measured using the enzyme-linked immunosorbent assay (ELISA) method.

Results. sL-selectin serum levels were significantly lower in OSA patients than in the control group (1080.02 ± 175.29 vs 1350.73 ± 569.75 ng/mL, $p < 0.05$). In addition, there was a negative correlation between sL-selectin levels and AHI and DI and a positive correlation between sL-selectin levels and mean and minimum saturation. sL-selectin levels were lower in OSA patients with cardiovascular diseases than in those without co-morbidities. We also found that sL-selectin correlated positively with HDL-cholesterol (high density lipoprotein) and negatively with uric acid and CRP (C-reactive protein).

Conclusions. Our work, together with observations relating to other diseases and experimental studies, suggests that lower sL-selectin levels could play a role in an increased risk of cardiovascular complications in sleep apnea syndrome. However future studies are needed to understand the role of sL-selectin in sleep apnea syndrome.

Key words: cardiovascular risk, sleep apnea, P-selectin

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Introduction

The relationship between obstructive sleep apnea syndrome (OSA) and cardiovascular disease is an interesting one. OSA increases the risk of hypertension, myocardial infarction and stroke, and has an influence on insulin resistance, obesity and metabolic changes.^{1,2} Many mechanisms may be helpful in explaining this association with the most important being sympathetic activity, oxidative stress, inflammation and endothelial dysfunction.^{2–4} It has also been suggested that OSA-induced hypoxic stress may contribute to cardiovascular diseases through the activation of adhesion molecules.⁵

L-selectin is a member of the selectin family.⁶ This family of adhesion molecules includes 3 receptors expressed from various cells including endothelial cells (E-selectin), leukocytes (L-selectin) to platelets and endothelium cells (P-selectin).⁷ L-selectin is expressed by granulocytes, lymphocytes and monocytes. It plays an important role in the launch of the adhesion cascade and in the capture and rolling of leukocytes along the endothelial cells.^{6,8} L-selectin is susceptible to proteolytic cleavage and may exist in the blood stream in soluble form (sL-selectin). It is released from the surface of neutrophils as they are activated and is a marker of neutrophil activation. L-selectin inhibits leukocyte attachment to the endothelium by occupying receptors and in high concentration it may block adhesion completely.⁹

The aim of this study was to evaluate sL-selectin serum levels in OSA patients with cardiovascular diseases.

Material and methods

Patients

A total of 163 patients with newly diagnosed OSA were enrolled in the study. None of the patients examined had previously received OSA treatment. The group examined comprised 124 males and 39 females. The mean age was 55.41 ± 8.63 years. The mean AHI (apnea hypopnea index) was 35.02 ± 22.28 /h. The majority of subjects were overweight and obese with mean BMI (body mass index) 34.98 ± 7.55 . Many cardiovascular comorbidities were observed in the OSA patients. 113 patients had hypertension, 44 diabetes, 36 ischemic heart disease and 10 had suffered a stroke. The majority of patients with OSA had cardiovascular disease (75%, $n = 121$). All patients with comorbidities received standard treatment according to international recommendations. In the majority of cases, antihypertensive treatment included an ACEI (angiotensin converting enzyme inhibitor), usually in combination with drugs from different groups. Five patients with ischemic heart disease had medical history of CABG (coronary artery bypass graft). All of the diabetes patients were treated with oral hypoglycemic agents. We divided the OSA patients into 2 groups: those with cardiovascular

diseases ($n = 121$) and those without ($n = 42$). The first group included patients with one or more cardiovascular diseases. Only 42 of the patients presented OSA without any other cardiovascular disease.

A control group constituted 59 healthy subjects, including 28 females. All subjects from the control group underwent respiratory polygraphy tests. In addition, daytime sleepiness was not observed in any subjects in the control group. The mean age in the control group was 51.27 ± 12.97 years and the mean BMI was 29.47 ± 5.42 .

Polygraphy

All patients and all subjects from the control group underwent a nocturnal respiratory polygraphy using a Grass Aura Lite PSG (Warwick, USA). The following parameters were evaluated during 8 h of nocturnal sleep: AHI, desaturation index – DI, mean and minimum SaO_2 at the end of sleep apnea/hypopnea episodes. Apnea was defined as the cessation of airflow for more than 10 s and hypopnea as a reduction in airflow by at least 30% compared with its value during wakefulness for at least 10 s followed by a 4% or greater decrease in oxyhemoglobin saturation. An oxygen desaturation event was detected when oxygen saturation fell by at least 4%. In all cases, manual scoring was carried out after automatic scoring.

Selectin

Blood samples were collected from fasting subjects in the morning. After centrifugation for 10 min at 1467 RCF, the serum was extracted and stored at -80°C . sL-selectin serum levels were measured using the enzyme-linked immunosorbent assay (ELISA) method and the following kit: Human L- Selectin/CD62L (R&D Systems, Minneapolis, USA). The tests were performed according to the manufacturer's specifications. The ELISA microplate reader from MRXe Dynex Technologies (Chantilly, USA) was used. Additionally, the following biochemical parameters were measured in the blood serum sample: uric acid, CRP (C-reactive protein), total cholesterol, LDL cholesterol (low density lipoprotein), HDL cholesterol (high density lipoprotein), and triglycerides.

Statistical analysis

Statistical analysis was performed using CSS STATISTICA software for Windows (v. 5.0). Spearman's r correlation coefficient was used to assess the relationship between 2 variables and the Mann-Whitney U test was used to compare values between the 2 groups. Differences between samples were considered significant at $p < 0.05$.

This work was approved by the institution's relevant ethics committee: the Commission of Bioethics at Wrocław Medical University. We obtained written informed consent from all participants involved in our study.

Table 1. Selected parameters in examined group

Factor	Control group n = 59	OSA patients n = 163	Mann-Whitney U test p-value
Age (years)	51.27 ± 12.97	55.41 ± 8.63	ns
AHI/h	2.21 ± 1.90	35.02 ± 22.28	< 0.05
DI/h	2.88 ± 2.76	33.64 ± 24.22	< 0.05
sL-selectin (ng/mL)	1350.73 ± 569.75	1080.02 ± 175.29	< 0.05
BMI	29.47 ± 5.42	34.98 ± 7.55	< 0.05
CRP (mg/L)	3.7 ± 2.89	6.55 ± 6.24	< 0.05
Total cholesterol (mmol/L)	5.52 ± 1.12	5.38 ± 1.17	ns
LDL cholesterol (mmol/L)	3.39 ± 0.93	3.47 ± 1.18	ns
HDL cholesterol (mmol/L)	1.25 ± 0.34	1.18 ± 0.44	ns
Triglycerids (mmol/L)	2.04 ± 1.38	2.10 ± 1.22	ns

AHI - apnea hypopnea index; DI - desaturation index; BMI - body mass index; CRP - C-reactive protein; LDL - low density lipoprotein cholesterol; HDL - high density lipoprotein cholesterol.

Results

sL-selectin serum levels were significantly lower in OSA patients than in the control group (1080.02 ± 175.29 vs 1350.73 ± 569.75 ng/mL, $p < 0.05$) (Table 1). In addition, sL-selectin levels correlated negatively with AHI ($r_s = -0.162$; $p < 0.05$) and DI ($r_s = -0.134$; $p < 0.05$) while there was a positive relationship between sL-selectin levels and mean ($r_s = 0.201$; $p < 0.05$) and minimum ($r_s = 0.157$; $p < 0.05$) saturation.

sL-selectin levels were also lower in OSA patients with cardiovascular diseases compared with OSA patients without co-morbidities (1065.91 ± 179.30 vs 1120.66 ± 158.22 ng/mL, $p < 0.05$).

Spearman's r correlation coefficient showed that sL-selectin correlated positively with HDL-cholesterol ($r_s = 0.144$; $p < 0.05$) and negatively with uric acid ($r_s = -0.205$; $p < 0.05$) and CRP ($r_s = -0.188$; $p < 0.05$) (Table 2).

Discussion

The role of sL-selectin in sleep apnea syndrome is disputable and still not properly understood. We were only able to find a few studies on sL-selectin in OSA. Ohga et al. showed, in a relatively small group (6 OSA patients and 7 from the control group), that sL-selectin levels were higher in OSA patients compared to the levels in normal subjects. These authors also observed that sleep did not have an influence on sL-selectin levels in either the control group or OSA patients.¹⁰ Cofta et al. demonstrated similar results in a larger group (80 patients). These au-

thors also observed a gradual increase in the plasma concentration of L-selectin as the severity of the OSA worsened.¹¹

However other studies didn't show any differences in sL-selectin levels in sleep apnea syndrome. El-Solh et al. examined 4 adhesion molecules: ICAM-1 (intercellular adhesion molecule), VCAM-1 (vascular cell adhesion molecule), L-selectin and E-selectin in 15 patients with coronary artery disease (CAD) and moderate-to-severe OSA. All but the L-selectin molecules were significantly higher in OSA patients compared to the control group.⁵ Chinese authors demonstrated similar results evaluating the serum levels of adhesion molecules in OSA patients with and without hypertension. Serum levels of ICAM-1 and VCAM-1 were significantly higher in OSA patients with or without hypertension than in the control group. However serum levels of L-selectin did not differ in the groups examined.¹²

Our study showed that sL-selectin serum levels were significantly lower in OSA patients compared to the control group and that sL-selectin correlated negatively with OSA parameters such as AHI and DI. We found these results surprising as, to our knowledge, this

Table 2. The relationships between sL-selectin serum levels and selected parameters

Factor	sL-selectin serum levels	
	r_s	p-value
Age	-0.161	< 0.05
AHI	-0.162	< 0.05
DI	-0.134	< 0.05
BMI	-0.066	ns
CRP	-0.188	< 0.05
Total cholesterol	0.036	ns
LDL cholesterol	0.014	ns
HDL cholesterol	0.144	< 0.05
Triglycerids	-0.099	ns
Mean saturation	0.201	< 0.05
Minimum saturation	0.157	< 0.05
Uric acid	-0.205	< 0.05

AHI - apnea hypopnea index; DI - desaturation index; BMI - body mass index; CRP - C-reactive protein; LDL - low density lipoprotein cholesterol; HDL - high density lipoprotein cholesterol.

is the first study suggesting that sL-selectin may be lower in OSA patients and that this could correlate negatively with OSA parameters. These results could be linked to the high incidence of cardiovascular disorders in the group examined. However they may also change our point of view on the role of sL-selectin in OSA, especially given that we also demonstrated that sL-selectin levels were lower in OSA patients with cardiovascular diseases than in those without co-morbidities.

Decreased sL-selectin levels have been described in many cardiovascular disorders as well as after trauma. In type 2 diabetes, sL-selectin is not only decreased, but vascular complications are accompanied by a decrease in the leukocyte surface expression of L-selectin.¹³ Serum levels of sL-selectin are lower in acute ischemic stroke compared with higher levels of other adhesion molecules such as ICAM (intercellular adhesion molecule), VCAM (vascular cell adhesion molecule) and E-selectin.¹⁴ After severe trauma, a decrease in sL-selectin indicates an increased likelihood of lung failure and multiorgan dysfunction syndrome.¹⁵ Different studies have demonstrated that sL-selectin levels were lower in patients with stable angina, unstable angina and acute myocardial infarction and in patients with coronary artery disease and type II diabetes.^{16,17} Some authors even suggested that a marked fall in sL-selectin might constitute a marker for silent CAD (coronary artery disease) in patients with type 2 diabetes.¹⁷ Other studies indicate that the stimulation of leukocytes in patients with ischemic heart disease leads to down regulation of surface L-selectin expression, which results in a decrease in levels of circulating sL-selectin.¹⁶ In addition, the findings by Rozenberg et al. in an animal model suggest that L-selectin could have a protective role against atherosclerosis.¹⁸ In addition, we showed that sL-selectin correlated positively with HDL-cholesterol and negatively with uric acid and CRP. These correlations may also suggest the anti-atherosclerotic effect of sL-selectin. We also demonstrated that CRP was significantly higher in OSA patients ($p < 0.05$). Our findings are in agreement with many previous studies, having demonstrated that the activation of an inflammatory process plays an important role in sleep apnea syndrome and in atheromatosis development.^{19,20} In several studies, CRP was examined along with other inflammatory markers such as TNF- α (tumor necrosis factor), fibrinogen, interleukins IL-6 and IL-8, ICAM and VCAM.²¹

More recent studies also indicate that hyperuricemia could increase the risk of cardiovascular diseases and their complications, probably through the influence on endothelial dysfunction, oxidative stress and inflammation.²²

We realize that our study has a few limitations. First of all, there are differences in BMI between the OSA patients and control group and sL-selectin correlated negatively with age. The relationship between age and L-selectin is not clear. Some authors have suggested, that

L-selectin levels could decrease until 55 years old.²³ In addition, the age-related differences in other inflammatory markers have been described. This could be connected with changes in visceral adipose tissue accumulation.²⁴ Moreover, such a high percentage of OSA patients with cardiovascular diseases (75%) could have an influence on the proper interpretations of the results. It is difficult to assess whether the low serum level of sL-selectin was associated only with OSA or cardiovascular disease (or both entities), especially since the correlations demonstrated were weak (even though statistically significant). An additional analysis in patients with cardiovascular diseases but without OSA could be helpful in the better understanding of the sL-selectin changes observed. However, we did not include this group of patients. We also have to remember that the patients examined received drugs from different groups as statins, ACEIs or beta-blockers. We did not find in the literature any studies describing drug influences on sL-selectin serum levels, but experimental studies suggest that cholesterol lowering therapy in mononuclear cells from hypercholesterolemic patients could reduce mRNA and protein expression of L-selectin and other adhesion molecules.²⁵

Conclusions

Our work, together with observations relating to other diseases and experimental studies, suggests that decreased sL-selectin levels could play a role in an increased risk of cardiovascular complications in sleep apnea syndrome. However future studies are needed to understand the role of sL-selectin in sleep apnea syndrome.

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Evaluation of articular disc loading in the temporomandibular joints after prosthetic and pharmacological treatment in model studies

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Abstract

Background. Temporomandibular joint dysfunction is often related to excessive load in the stomatognathic system.

Objectives. The objective of the model tests, using numeric calculations, was to assess the articular disc loads in the temporomandibular joints after prosthetic and pharmacological treatment of functional disorders of the masticatory organ.

Material and methods. The study involved 10 patients, aged 21–48 years, of both sexes, randomly selected from a group of 120 patients treated with relaxation occlusal splints (60 patients, group I) and intramuscular injection of botulinum toxin type A (60 patients, group II), suffering from temporomandibular joint dysfunction with the dominant muscle component. In all subjects, a specialized functional examination was carried out. Treatment groups: occlusal splint therapy (group I) and intramuscular injection of botulinum toxin type A (group II). An assessment of the loads of 4 disc zones of the temporomandibular joints was carried out based on the results of clinical studies (phase I of the study), and numeric model tests (phase II). In the representatives of the study groups (5 patients in each group), measurements of occlusal forces and an evaluation of tension of the masseter and temporalis muscle were performed.

Results. The results of the average load values for all evaluated zones of the right and left articular disc differ in a statistically significant way in favor of group II, with the exception of the external mid part of the discs. In the case of the anterior of the right disc, the load was lower in patients belonging to group I than in those obtained in group II.

Conclusions. Botulinum toxin type A significantly reduces the loads within the temporomandibular joints, generated by masseter muscle hypertonia.

Key words: articular disk loading, temporomandibular joint dysfunction, botulinum toxin type A, prosthodontics treatment

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Temporomandibular joint dysfunctions, according to WHO reports, are the third of dental diseases in prevalence following dental caries and periodontal diseases.¹ The type of these disorders is determined by genetic, individual, environmental and psycho-emotional factors. It has been observed that an increasing level of stress leads to growth in harmful movement habits within the stomatognathic system, and a rapid increase in muscle tension adversely affects the functioning of the temporomandibular joints. Under physiological conditions articular load is favorable, enabling the correct functioning of the structure, but in the situation when the load is too high, there is often significant surface and disc damage within the joints.^{2–15}

The objectives of the model tests, using numeric calculations, was to assess the articular disc loads in the temporomandibular joints after prosthetic and pharmacological treatment of functional disorders of the masticatory organs.

The study involved 10 patients, aged 21–48 years, of both sexes, with full dental arches, randomly selected from a group of 120 patients treated with relaxation occlusal splints (60 patients - group I) and intramuscular injection of botulinum toxin type A (60 patients - group II). The group of 10 representative patients were created from 5 patients taken from group I and 5 from group II.

In all the patients, a specialized functional diagnostic procedure showed the presence of temporomandibular joint dysfunction with the dominant muscle component. The effects of excessive tension of the masticatory muscles were observed as symptoms in teeth and periodontium as well. In the representative group of 10 patients, there were no painful forms of dysfunction. The treatment was performed in the Prosthodontics Clinic of the Jagiellonian University Medical College in Kraków.

In all subjects, a specialized functional examination was carried out, according to the application form developed in the Consulting Room of Temporomandibular Joint Dysfunction of the Prosthodontics Department of Jagiellonian University. The form is based on the typical routine requirements of a functional evaluation of the stomatognathic system, such as: a rating of the mandibular range of movement, symmetry of mandible motion, deviation of opening path, occlusion condition, masticatory muscle sensitivity during palpation, pain of their attachments, and the presence of acoustic and pain symptoms in temporomandibular joints occurring during mandibular movement and palpation examination. Additional examination was concerned with an evaluation of pain intensity and imaging diagnosis.^{1,2} The patients were qualified for 2 prosthetic treatment groups: occlusal splint therapy (group I) and intramuscular injection of botulinum toxin type A with a total amount of 21 MU (mouse units) for each muscle (group II). The injections were done at the 3 points of vertices of a triangle (7 units in each point) along the largest cross-sectional of the

muscle, 1 time, using a 40 U insulin syringe. The pharmacological activity of the toxin lasts about 12 weeks.^{1,6}

The functional examination of the masticatory organ, masseter muscles and temporomandibular joint pain intensity and masseter muscle electrical activity were evaluated 4 times: before the prosthetic treatment began, after 10 days of the initiation of the 2 different treatment methods (splint and intramuscular injections) and after 14 and 22 weeks from the second examination.

An assessment of the loads of 4 disc zones of the temporomandibular joints was carried out based on the results of clinical studies (phase I of the project), and numeric model tests (phase II).^{1,17,18} In the representatives of the study groups (5 patients in each group), measurements of occlusal forces were performed using the method developed by professor W. Chladek as well as an evaluation of the masseter muscle tension and the anterior temporal muscles using a Bio Research EMG I and surface electrodes.^{10,17}

The maximum occlusal forces were evaluated around the central incisors and the first molars of the left and right side, using a special measuring device – developed for the needs of dental research in the Department of Mechanical Technology and Forming of Silesian Technical University in Katowice (patent no. P 334933). In order to calculate the maximum values of occlusal forces using the above method, the Mayer's formula was applied:

$$F = c \cdot d^n$$

where: c – a material constant; d – diameter of dents in the aluminum sample; n – Mayer's coefficient – 1.706.

The constant “ c ” and coefficient “ n ” appearing in the formula were specified for the series of aluminum samples applied in the research by measuring the diameters of the indentations formed during the calibration of the instrument in the universal testing machine. This formula determines the correlation of the resulting indentation size to pressure-gradient force penetrator, and hence the tooth pressure force on the measuring device. To measure the diameter of the dents in the disposable aluminum plates, a stereoscopic measuring microscope was used.^{10,11,18}

Since the direct measurement of loads in the temporomandibular joints in a living human is not possible, a computer model of the mandible, projecting masticatory organ functions, was constructed, which uses the following model assumptions:

1. For numeric calculations, the mandible was considered a non-deformable body, since the intended purpose of the study is not to evaluate the size of deformations of the mandible.

2. The studies assumed the average orientations of muscle forces, due to the fact that the muscle attachments are located in particular areas of the jaw or skull bones and can be “reduced” to the points in the model study, because the results of the clinical studies carried out on

various types of face – long and short – have shown the convergence of the lines of action of the muscle force vectors in the case of both types of face.

3. The loads occurring in the temporomandibular joints have been identified on the basis of a spatial model of the human masticatory system according to Margielewicz's concept.

4. Forces exerted on the temporomandibular joints were estimated based on the load acting on the articular discs. In the numeric calculations the replacement stiffness of discs was taken into account as the actual non-linear displacement-force characteristics make it difficult to perform numeric calculations.

5. The resilient properties of the muscles were projected using mechanical two-terminals, because the data characterizing the volumes of the cross-section of the muscle, its rest length, and the unit capacity are available.

Analytical dependency mapping replacement stiffness of the masticatory muscle fiber groups was calculated according to the following formula:

$$c_z = \frac{kA}{l}$$

where: k – the coefficient of unit performance of the cross section of the muscle equals 40 [N/cm²]; A – the maximum cross-section of the muscle; L – the rest length of the muscle.^{16,17}

From the point of view of biomechanics, one of the most important pieces of information about the masticatory organ muscles is the maximum forces generated by individual masseter muscles. They refer to the largest cross-sections of the muscles, therefore an important element for a model study of the stomatognathic system is the so-called coefficient of the unit performance of the cross section of the muscle. The value of this coefficient, according to Chladek, is 40–100 N/cm.^{2,10}

Proportions occurring between the maximum (limit) muscle forces and their bioelectric activity were calculated according to the formula:

$$\frac{EMG_{MP}}{F_{MP}} = \frac{EMG_{MG}}{F_{MG}}$$

where: EMG_{MP} – bioelectric activity of the surface fibers of the masticatory organ, EMG_{MG} – bioelectric activity of the medial pterygoid, F_{MP} – the maximum force generated by the surface fibers of the masticatory organ, F_{MG} – the maximum force generated by the medial pterygoid.

For the purposes of this study, the results of maximum occlusal forces, the electrical potential values of masseter and temporal muscles were discussed as well as the model tests following the prosthetic treatment carried out with the use of the two compared methods.

In the case of the clinical measures giving constant results, statistical analysis was based on the traditional

methods of calculation: mean values, standard deviation, minimal values, maximal values, standard error of the mean, variance analysis for dependent variables and post-hoc Tukey test for dependent variables being the statistical significance measure.

To compare the dependencies between the clinical results obtained in consecutive clinical tests, the non-parametric Friedman test, Kendall's W and Wilcoxon signed-rank test (comparing 2 related samples) were used. For the statistical studies, special STATISTICA 2010 computer software was used.

Only a few of the clinical studies were used in numeric model tests. The results of the disc loads in the temporomandibular joints in numeric model tests on the representatives of the study groups were statistically analyzed using the parametric Student's t -test and it was assumed that the results were statistically significant if $p \leq 0.05$.

Results

In the numeric model test, projecting the stomatognathic system activity, carried out on the representatives of the 2 groups, a significant difference in articular disc loads was observed between patients undergoing the splint therapy and botulinum toxin type A in favor of the second group.

The results of the clinical research of maximal bite forces (5 patients in group I and II) are presented in Fig. 1 and they were the basis for the work on the numeric tests. This was necessary for identification of the loads within the temporomandibular joints. Table 1 shows the results of the electromyographic measurements obtained in patients randomly selected from both groups evaluated for model tests in order to calculate the loads of the temporomandibular joints after the therapy using occlusal splints and after the application of botulinum toxin type A. The average values of the electrical potentials obtained during the clinical study of the right and left masseter and temporalis muscles were important to create the numerical model of a biocybernetic model of the masticatory system. The ranges of these values were 15.71–96.11 μV for both types of muscles. In this individual study, the numeric values of the replacement coefficients of articular disc zone rigidity were applied, calculated by Chladek et al.^{10,11}

The average values of loads (evaluated in newtons) acting on the individual disc zones of the left and right temporomandibular joint are summarized in Tables 2 and 3. The results of the average load values for all the evaluated zones of the right and left articular disc differ in a statistically significant way in favor of group II, with the exception of the external mid part of the discs. In the case of the anterior of the right disc, the load was lower in patients belonging to group I than in those obtained in group II.

Table 1. Average values of electrical potentials obtained during clinical studies in order to create the numeric model in microvolts [μ V]

Patient number		Masseter muscle surface fibers, EMG _{MP}		Temporal muscle anterior fibres, EMG _{TP}	
		right side	left side	right side	left side
G I	1	45.21	35.77	40.38	55.31
	2	113	85.22	96.11	114.57
	3	84.21	45.21	54.93	73.15
	4	21	15.71	21.88	39.07
	5	45.31	59.24	61.73	73.98
G II	1	45.68	39.21	48.98	55.98
	2	85.14	75.1	65.98	76.1
	3	24.32	32.14	28.9	39.17
	4	41.21	30.14	36.4	57.91
	5	19.27	24.19	38.1	47.91

Table 2. Average values of the forces acting on the right articular disc in [N] and statistical significance denoted by letter p

Articular disc zone	Group I	Group II	p-value
Anterior	6.8	10.76	0.00001
Posterior	119	123.7	0.00000
External mid	59.2	47.9	0.06000
Internal mid	62	63.5	0.000001

Table 3. Average values of the forces acting on the left articular disc in [N] and statistical significance denoted by letter p

Articular disc zone	Group I	Group II	p-value
Anterior	23.52	15.14	0.000016
Posterior	123.24	93.72	0.000011
External mid	33.58	23.16	0.000021
Internal mid	30.08	21.84	0.000010

Discussion

The main aim of the treatment of temporomandibular joint dysfunction is the remission of pain of the muscles and joints and restoration of the physiological norm for proper muscle tension and load of the joint. For the proper functioning of the stomatognathic system, it is very important to provide physiological stress on joints.

It ensures proper distribution of synovial fluid and blood supply, which is necessary within the articular surface.^{1,3,4} In the course of functional disorders, the masticatory system comes to excessive and highly damaging overloads of the joints and intra-articular discs, which is caused by a significant increase in masseter muscle tension.^{1,3,7,10,12,13,18,19}

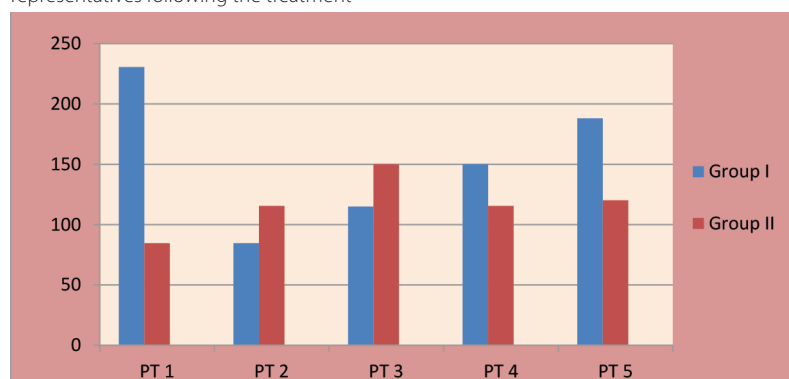
Occlusal splints, pharmacological treatment in the form of intramuscular injection and muscle exercises are methods that have been shown to have a positive effect on the state of masticatory muscle relaxation, which can be confirmed by objective testing instruments such as electromyography and occlusal evaluation using a computer program, and T-Scan device. Botulinum toxin is produced by Gram-positive *Clostridium* bacteria. In their natural environment, these bacteria form spores that release the neurotoxin (the strongest known to man) when they germinate. There are 6 serotypes of the toxin: A, B, C, D, E, F, and G. Botulinum toxin type A was first isolated in 1920s. Its therapeutic potential was put to the test for the first time then. All 7 serotypes are large proteins that act on cholinergic neuromuscular junctions to block transmission of synaptic vesicles.^{1,15} Edward Schantz and Alan Scott were pioneers of the therapeutic applications of the botulinum toxin used from the late 1970s to manage strabismus and hemifacial spasms at first and then eyelid spasm, uterine atony, and laryngological, nephrological, surgical and neurological therapies as well as aesthetic medicine. The medication is currently registered in approx. 120 countries.¹⁵

An assessment of disc loading within the temporomandibular joints of patients is not possible. In the stomatognathic system, direct measurement of living human articular disc loads and surface TMJ is not possible, therefore construction of a special model, which was based on the concept of one of the authors of this study, is an important instrument in establishing the initial orientation of the articular disc load changes depending on the state of muscle tension, and has not been used in the assessment of the effectiveness of treatment of the masticatory system by other authors at all.^{1,10,16–23}

Despite the rapid growth in model testing and biotechnological studies, only a few reports in the literature assess the loads within the temporomandibular joints. The assessment of the relationship between bite forces and occlusion, and the activity of muscle and loads acting on the temporomandibular joints is a difficult issue from a biomechanics perspective.^{17,18}

Results of the research available in the literature are mostly related to the evaluation of mechanical strain or anatomical changes in the properties of porcine discs, which are similar in structure to human discs. Experimental studies conducted on the intra-articular tissues derived from animals (cattle and pigs) for an assessment of the resistance to crushing or tensile strength have provided valuable information about the mechanical proper-

Fig. 1. Average values of the occlusal forces measured in groups' representatives following the treatment



ties of these structures, however, any changes in the in vitro environment had an important impact on the final value obtained.^{10,11}

Fernandez P. et al. conducted research of the dynamic compressive properties in 5 differentiations of the porcine temporomandibular joint disc which were investigated over a wide range of loading frequencies. The results of this investigation suggest that the dynamic viscoelastic compressive modulus is region-specific and depends on the loading frequency and has important implications for the transmission of load to the temporomandibular joints.²⁵ Model tests were also performed by Commisso et al., who evaluated an algorithm for adjusting the quasi-linear viscoelastic model to fit a material using a stress relaxation test and validating a protocol for performing such tests in temporomandibular joint discs.²⁶ Juran et al. assessed regional variation in the disc's shear load characteristics under a physiologically relevant load and to associate those mechanical findings with common clinical observations of disc fatigue and damage. The results of these studies suggest that the posterior region of the disc is the most often the zone in which fatigue occurs, what may lead to disc damage and perforation.²⁷

The numeric model tests, projecting stomatognathic system activities, carried out in the framework of this project, on the group of representatives treated with occlusal splints and intramuscular injection of botulinum toxin type A, show a significant difference in articular disc loads, demonstrating the positive impact of treatment using the pharmacological method.

Analyzing the results of the numeric model tests, higher load values in all the examined zones of the right and left discs were observed in the group of patients treated with occlusion splints (I), compared to the results obtained in the group treated with the injection of botulinum toxin (II), with the exception of the front zone of the right disc. The largest load values were obtained in model tests for the rear areas of both discs, because these values amount to 119 (right) and 123.24 N (left) in group I and, respectively, to 123.7 and 93.72 N in group II. Comparing the mean values for both discs for the rear discs zone – the difference

in the loads between the 2 groups was 27.79 N in favor of group II. The least significant load differences between groups were calculated for the right discs in the outer middle zone (2.4 N) and in the front zone. Chladek et al. notes that excessive static and dynamic TMJ load leads to rapid deformation of the articular disc and impairment of the flow of fluids in the disc and the zone outside the disc. These factors have a decisive influence on the development of dysfunctions within the joint system.^{10,11,28} Nickel et al., in the study carried out in 52 patients with the pain form of the functional disorders assessing the TMJ load, found that a decrease in

joint pain is closely related to reductions in the articular surfaces loads.¹⁹ Our results indicate lower loads occurring within the joints improve the clinical parameters of the mandible dynamics in the course of treatment with the botulinum toxin, as compared to the treatment with occlusal splints.

Summary

In the treatment of temporomandibular joint dysfunction, we use many diagnostic and therapeutic methods such as the use of occlusal splints, pharmacological treatment, physiotherapy and psychological support.^{1,5,6,12,14,20,23,29} The results of model tests indicate a more favorable impact using intramuscular injection of botulinum toxin type A on the load condition within the temporomandibular joint compared to the results obtained in patients with occlusal splint. The increase in loads beyond the physiological level within the joint structures is a factor in pain of high intensity and irreversible morphological changes of all its elements.^{1,11,16,17}

Conclusion

Botulinum toxin type A significantly reduces the loads of the disc within the temporomandibular joints generated by masseter muscle hypertonia.

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IL-22 modulates inflammatory properties of human primary aortic smooth muscle cells

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Conflict of interest

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Abstract

Background. IL-22 is expressed at barrier surfaces, which suggests its critical role in the maintenance of normal barrier homeostasis and tissue repair. IL-22 can both promote pathological inflammation and prevent the destruction of tissues. The functional outcomes of IL-22 on vascular smooth muscle cells, which are shown to regulate immune processes within the vascular wall and which are involved in certain pathologies, have not been analyzed.

Objectives. The effect of IL-22 on the expression of novel anti- and pro-inflammatory and barrier disrupting cytokines, apoptosis and the expression of adhesive molecules in human primary aortic smooth muscle cells (AoSMC) was investigated.

Material and methods. Human AoSMC were induced with IL-22 for 24 h in vitro. The influence of IL-22 on IL-35 subunits EBI3 and p35, IL-33, IFN- γ and VEGF mRNA expression in Ao-SMC were assessed using real-time PCR. Additionally, the surface expression of ICAM-1 and apoptosis of AoSMC were analyzed in the flow cytometer.

Results. IL-22 caused a 2- and 3-fold increase of mRNA expression of the EBI3 and p35 IL-35 subunits, and a 40% decrease of IL-33 mRNA expression in AoSMC. Additionally, IL-22 decreased ICAM-1 expression on the surface of AoSMC by 30%. However, IL-22 affected neither IFN- γ and VEGF mRNA expression in AoSMC nor their apoptosis and viability.

Conclusions. Our data suggest that IL-22, which is released by Th22 and NK cells, may be an agent affecting the inflammatory response of AoSMC, and thus it may regulate immune homeostasis of the vascular wall.

Key words: ICAM-1, IL-22, th22 cells, AoSMC

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Introduction

Interleukin-22 (IL-22) is a class 2 α -helical cytokine of the IL-10 family, which consists of IL-10, IL-19, IL-22, IL-24 and IL-26.¹ It is produced by cells of hematopoietic origin: Both the innate lymphocytes, NK cells, and the adaptive arm of the immune system, such as Th cells termed Th22 cells.² IL-22, which is preferentially released under Th-17-polarised conditions, signals through a class 2 receptor (IL-22R) composed of the subunits IL-22R1 and IL-10R2.^{3,4} The functional IL-22R is thought to be restricted to the nonhematopoietic cells of the skin, intestine, liver, lung and kidney.⁵

IL-22 expressed at barrier surfaces was shown to promote epithelial cell proliferation, survival and repair in the skin, airway and intestine. It induces the production of pro-inflammatory and remodeling cytokines, enhances proliferation and migration of colonic epithelial cells and promotes proliferative and anti-apoptotic pathways.^{2,6,7} This suggests its critical role in the maintenance of normal barrier homeostasis and tissue repair. However, IL-22 is involved in the development of chronic inflammatory conditions, such as psoriasis, inflammatory bowel disease, rheumatoid arthritis or asthma.^{8–10} Very recently, Th22 cells have been found in atherosclerotic plaques, which indicates the possible influence of IL-22 on vascular wall tissues, including vascular smooth muscle cells (VSMC).^{11,12}

VSMC, which comprise the medial layer of the artery wall, are one of the tissue factors that regulate physiological processes, contributing to the establishment of local homeostasis. As they serve as a strong source of pro-inflammatory cytokines and chemokines released to the

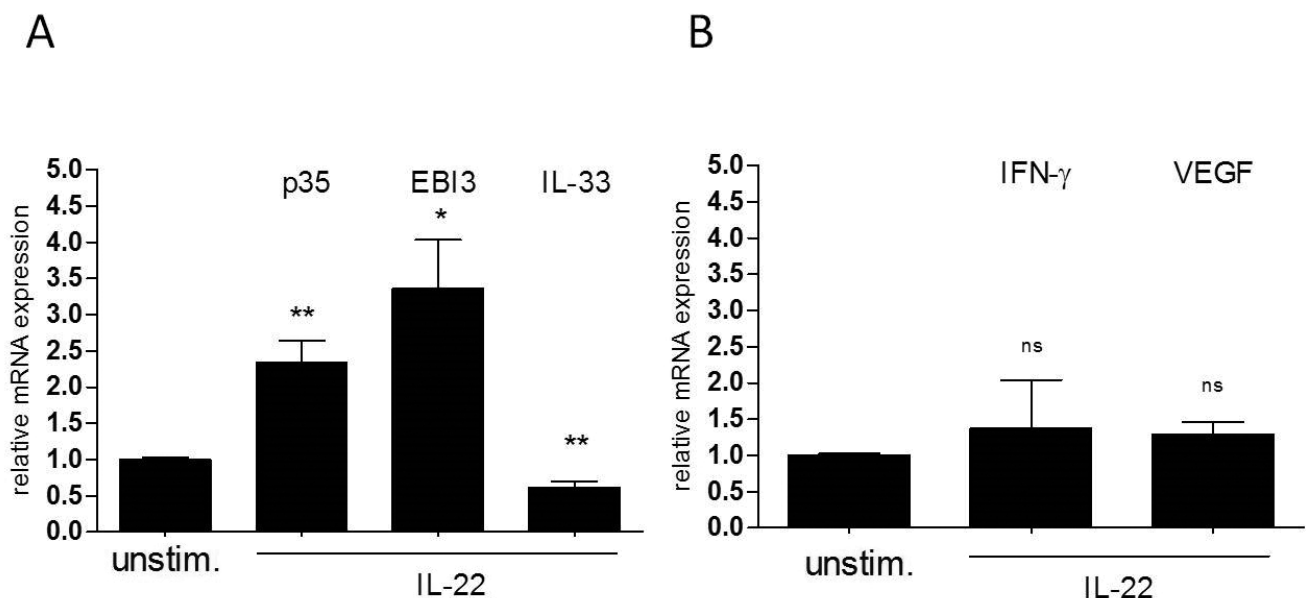
microenvironment in response to their activation, they may be involved in chronic inflammatory pathologies, i.e. atherosclerosis, where increased apoptosis of VSMC is observed as is the expression of adhesion molecules.^{13–15} Although Th22 cells have been found in the vascular wall, the effect of IL-22 on VSMC has not yet been investigated. Therefore, the aim of the present study was to assess the effect of IL-22 on the inflammatory profile of human primary aortic smooth muscle cells (AoSMC). For this purpose, we investigated the influence of IL-22 on the mRNA expression of novel, anti- and pro-inflammatory cytokines: IL-35 subunits and IL-33, respectively, which both may modulate the local immune environment and whose role in VSMC has not yet been described. Additionally, we analyzed the effect of IL-22 on the mRNA expression of IFN- γ and VEGF – well known endothelial barrier disrupting agents, surface expression of adhesive molecule ICAM-1 and apoptosis of AoSMC.^{16,17}

Material and methods

Cells

Human primary Aortic Smooth Muscle Cells (AoSMC) (Lonza, Clonetics, CC-2571, Basel, Switzerland) were expanded in Smooth Muscle Cell Basal Medium (Lonza, Clonetics, CC-3181, Basel, Switzerland), supplemented with SmGM-2 BulletKit (Lonza, Clonetics, CC-4149, Basel, Switzerland). After covering 80–90% of the base of a T-flask, the AoSMC were trypsinized with 0.05% trypsin with 0.02% EDTA (SAFC Biosciences, 59417C, Kansas, USA) for 4 min and neutralized using Trypsin

Fig. 1. The effect of IL-22 (1 ng/mL) on mRNA expression of A) anti- and pro-inflammatory cytokines and B) barrier disrupting agents in AoSMC (24 h) assessed by real-time pcr; (n = 6, from 3 independent experiments), (mean \pm SEM); unstim.- unstimulated control, *p < 0.05, **p < 0.01: statistically significant as compared to unstimulated control; ns – statistically not significant in comparison to unstimulated control



Neutralizing Solution (Lonza, Clonetics, CC-5002, Basel, Switzerland) for further experiments.

Cell culture

AoSMC were seeded on 12-well plates at a density of 50,000 cells per well in proper media. After reaching 80-90% confluence, AoSMC were induced with IL-22 (1 ng/mL) (Biolegend, 571302, San Diego, USA) for 24 h in order to measure the expression of particular cytokine mRNAs by real-time PCR as well as analyze their apoptosis, necrosis and viability, and ICAM-1 (CD54) surface expression by flow cytometry. The optimal concentration of IL-22 used in this study was determined in a set of pilot experiments.

Real time PCR

mRNA was isolated using the RNeasy Mini Kit (Qiagen, Hilden, Germany) kit, following the manufacturer's instructions. Potential genomic DNA contamination was removed with on-column DNase I digestion and 10 µg of mRNA was reverse transcribed with a High-Capacity cDNA Kit (Applied Biosystems, Foster City, USA). PCR was then carried out using an Applied Biosystems 9700HT Fast Real-Time PCR System (Applied Biosystems). The PCR mixture consisted of cDNA solution, SYBR-Green PCR Mastermix (Applied Biosystems) and both sense and antisense primers. The reaction was conducted as follows: 4 min at 95°C, followed by 40 cycles of 15 s at 95°C and 60 s at 60°C. EF1-α was amplified as a house-keeping gene. p35, EBI3, IL-33, IFN-γ and VEGF mRNA expression was normalized to EF1-α using $\Delta\Delta C_t$ calculation. Primers used for (1) p35 forward: TTC ACC ACT CCC AAA ACC TGC, reverse: GAG GCC AGG CAA CTC CCA TTA G; (2) EBI3 forward: CAG CTT CGT GCC TTT CAT AA, reverse: CTC CAA CTG CAC CTG TAG C; (3) IL-33 forward: TGA GTC TCA ACA CCC CTC AAA TG, reverse: GGC ATG CAA CCA GAA GTC TTT T; (4) IFN-γ forward: TCT CGG AAA CGA TGA AAT ATA CAA GTT AT reverse: GTA ACA GCC AAG AGA ACC CAA AA; (5) VEGF were forward: GAT TGA GAC CCT GGT GGA CAT C, reverse: CAC ACA GGA GGG CTT GAA GA. Those used for EF1-α were forward: CTG AAC CAT CCA GGC CAA AT, reverse: GCC GTG TGG CAA TCC AAT.

Apoptosis and ICAM-1 surface expression in AoSMC assessment in flow cytometry

AoSMC were trypsinized after 24 h of induction with IL-22 (1 ng/mL). Apoptosis was assessed in a Beckman-Coulter FC500 flow cytometer using Annexin V-FITC and propidium iodide (PI) co-staining (FITC-Annexin V Apoptosis Detection Kit, BD Pharmingen, 556547, San Diego, USA). Annexin-V(-) and PI(-) cells were considered

as living cells, Annexin-V(+) and PI(-) as early-apoptotic cells, Annexin-V(+) and PI(+) as late-apoptotic cells, and Annexin-V(-) and PI(+) as necrotic cells.

In order to assess ICAM-1 surface expression on AoSMC, PE anti-human CD54 antibodies (BioLegend, 322708) were used. For analysis, the percentage changes of Mean Fluorescence Intensity (MFI) of were used.

Statistical analysis

The results are presented as mean \pm SEM for variables with a normal distribution of values. The distribution of particular variables was verified by the Shapiro-Wilk W test, whereas the Levene test was performed to test homogeneity of variances. In the case of normal distribution and homogenous variance, the significance of differences between 2 groups was estimated using the Student t test for independent trials. However, if any of these criteria were not fulfilled, a Mann-Whitney U test was used for analysis of the differences between the 2 groups. A p-value < 0.05 was considered to be statistically significant. All analyses were conducted with STATISTICA v. 12.5 software (StatSoft, Inc., Tulsa, USA).

Results

The effect of IL-22 on anti- and pro-inflammatory cytokines: IL-35 subunits p35 and EBI3, IL-33 mRNA expression in AoSMC

The effect of IL-22 on the mRNA expression of anti- and pro-inflammatory cytokines in AoSMC was first investigated. EBI3 and p35 IL-35 subunits, IL-33 and IFN-γ mRNA expression in AoSMC was assessed in real-time PCR after 24 h of culture (Fig. 1A). In AoSMC induced with IL-22, an almost 2.5-fold increase of IL-35 p35 subunit mRNA expression was observed as compared to the unstimulated control ($p < 0.01$). Similarly, IL-35 EBI3 subunit mRNA expression was more than 3 times higher than that of unstimulated AoSMC upon IL-22 induction ($p < 0.05$). On the other hand, IL-33 mRNA expression was seen to halve upon stimulation of AoSMC with IL-22 as compared to the control ($p < 0.01$).

The effect of IL-22 on barrier disrupting cytokines: IFN-γ and VEGF mRNA expression in AoSMC

Next, we assessed the effect of IL-22 on the mRNA expression of cytokines involved in the regulation of barrier functions of tissues such as endothelium (Fig. 1B). For this purpose, IFN-γ and VEGF mRNA expression was analyzed after 24 h of culture of AoSMC with IL-22 (1 ng/mL). IL-22 did not change IFN-γ mRNA expression in AoSMC, as compared to the unstimulated cells

($p > 0.05$). Similarly, IL-22 did not significantly change VEGF mRNA expression in AoSMC, as compared to the unstimulated cells ($p > 0.05$).

The effect of IL-22 on AoSMC ICAM-1 (intercellular adhesion molecule-1) expression

For further analysis of the effect of IL-22 on the inflammatory profile of AoSMC, the surface expression of ICAM-1 on AoSMC was assessed in a flow cytometer after 24 h of culture, based on the percentage changes of Mean Fluorescence Intensity (MFI). Upon induction of AoSMC by IL-22 (1 ng/mL), a significant decrease of surface ICAM-1 expression was observed (mean: down to $69.2 \pm 7.2\%$; $p < 0.05$) as compared to unstimulated cells (Fig. 2A, B).

The effect IL-22 on AoSMC apoptosis and viability

In the next step, the effect of IL-22 on AoSMC survival was analyzed. The apoptosis and viability of AoSMC were assessed after 24 h of culture by flow cytometry (Fig. 3). IL-22 did not affect AoSMC apoptosis or necrosis, as the percentages of (Ax+Pi- plus Ax+Pi+) or Ax-Pi+ cells, upon induction with IL-22, were found to be comparable to those of unstimulated AoSMC (mean [Ax+Pi- plus Ax+Pi+] cells: $30.0 \pm 16.2\%$ versus $26.5 \pm 12.9\%$, respectively, $p > 0.05$) (Fig. 3A); (mean Ax-Pi+ cells: $2.5 \pm 1.1\%$ versus $2.9 \pm 1.4\%$, respectively, $p > 0.05$) (Fig. 3B). Similarly, IL-22 was not observed to have any effect on AoSMC viability: IL-22 did not change the percentages of Ax-Pi- cells, with mean values of $67.3 \pm 14.9\%$ respectively versus $70.6 \pm 12.4\%$ in controls ($p > 0.05$) (Fig. 3C).

Discussion

The study analyzes the influence of IL-22 on the inflammatory profile of VSMC. It can be seen that IL-22, which may be released by Th22 cells or NK cells penetrating the vascular wall, may affect the mRNA expression of certain cytokines and surface expression of adhesion molecules on VSMC.

As the biological effect of IL-22 on VSMC is unexplored, it was decided to investigate the influence of IL-22 on the mRNA expression of novel anti- and proinflammatory cytokines in human AoSMC. Firstly, it was observed that IL-22 induced mRNA expression of EB13 (Epstein-Barr virus-induced gene-3) and p35 - subunits of anti-inflammatory IL-35, a novel IL-12 family cytokine, relevant for coronary artery disease.^{18,19} IL-35, whose EB13 subunit is expressed in aortic smooth muscle cells, was shown to inhibit both Th1- and Th2-mediated chronic inflammatory conditions.^{20,21}

On the other hand, we have shown that IL-22 decreased mRNA expression of IL-33, a pro-inflammatory tissue-derived danger signal relevant in certain chronic inflammatory diseases, by roughly 40% in AoSMC.^{22–24} This suggests that IL-22 may diminish VSMC-derived IL-33-driven inflammatory processes in the vascular wall, such as survival and cytokine production by immune cells and the generation of Th-2 type immune responses.^{25–26} Thus, through the effect on IL-35 and IL-33 expression in AoSMC, IL-22 might affect VSMC to reduce local inflammatory processes within the vascular wall.

The endothelial barrier plays a key role in inflammatory processes. Both IFN- γ and VEGF are well known agents decreasing endothelial barrier functions.¹⁷ As VSMC are

Fig. 2. The effect of IL-22 on ICAM-1 expression on AoSMC. A) The effect of IL-22 (1 ng/mL) on the percentage change of surface ICAM-1 expression on AoSMC (24 h) by flow cytometry; (n = 4, from 4 independent experiments), (mean \pm SEM); * $p < 0.05$; (MFI - mean fluorescence intensity); B) Representative overlays of IL-22-induced changes of ICAM-1 surface expression on AoSMC, unstim. - unstimulated control; flow cytometry

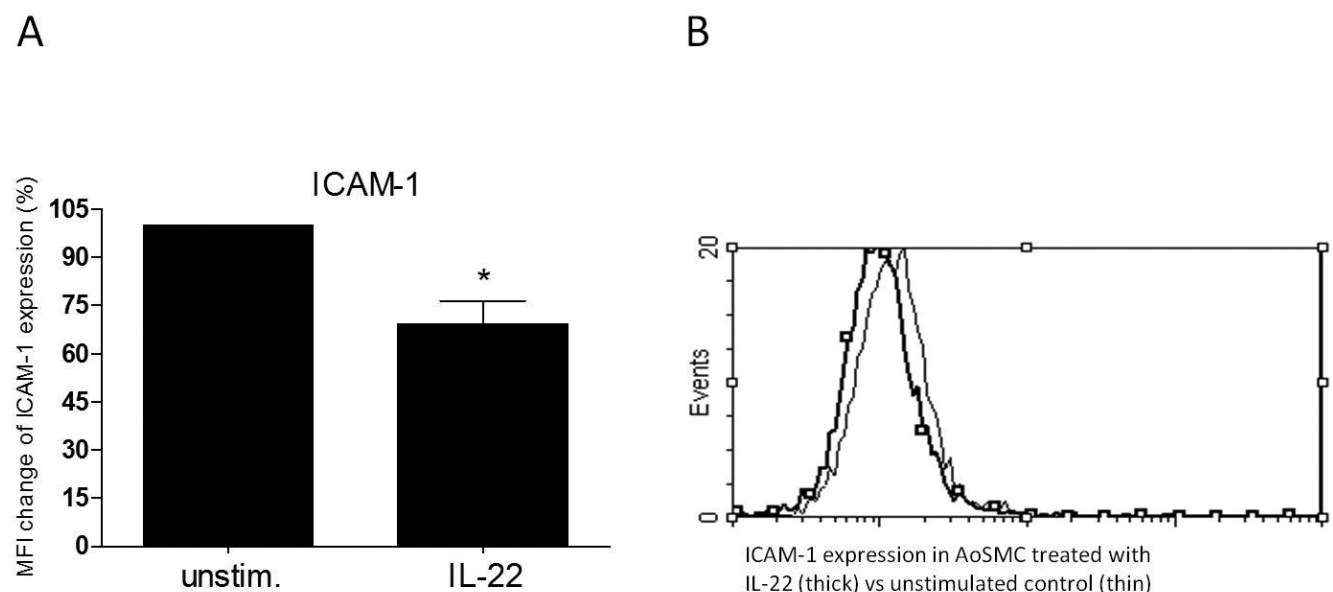
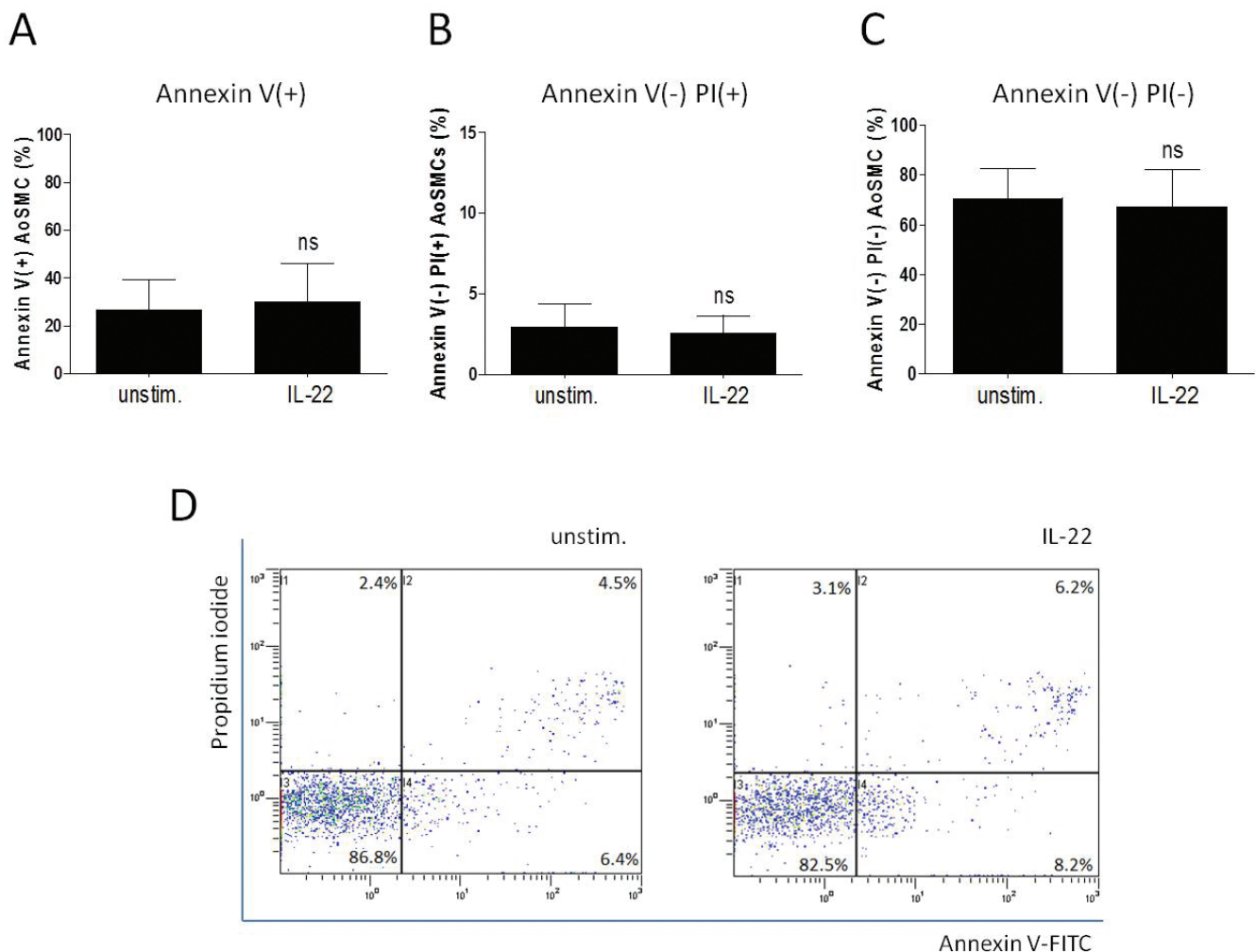


Fig. 3. The effect of IL-22 on AoSMC death and viability assessed by flow cytometry (24 h). The effect of IL-35 on A) apoptotic, B) necrotic and C) viable AoSMC percentages (24 h) ($n = 3$, from 3 independent experiments), (mean \pm SEM); (Annexin V(+) – Annexin V-positive AoSMC, PI(+) – Propidium iodide-positive AoSMC); (D) Representative dot blots of unstimulated (unstim.), IL-22 (1 ng/mL)-induced AoSMC; x axis – Annexin V-FITC, y axis – propidium iodide, flow cytometry



located in the endothelial layer neighborhood, they might affect its barrier functions via cytokine release to the microenvironment. The question that we raised concerned whether IL-22 may influence the mRNA expression of cytokines, decreasing barrier functions. However, according to our results IL-22 affected neither IFN- γ nor VEGF mRNA expression, in AoSMC.

Leukocyte-tissue interactions require the expression of ICAM-1, which may serve as an adhesion molecule for leukocytes and facilitate cell infiltration.²⁷ We observed that AoSMC weakly express ICAM-1. Nevertheless, ICAM-1 expression on AoSMC decreased by approximately 30% upon stimulation with IL-22, which suggests that IL-22 might slightly decrease the accessibility of arterial wall tissues for immune cells in contrast to other typical inflammatory cytokines, such as TNF- α , IFN- γ and IL-1 β , and affect VSMC migration.^{15,28,29}

Recent results describe the effect of IL-22 on apoptosis of the cells of certain tissues. Although IL-22 was seen, firstly, to prevent apoptosis of hepatic stellate cells, secondly, to induce the expression of antiapoptotic B-cell lymphoma cells and, finally, to demonstrate an ability to promote the survival of airway SMC, the effect of IL-22 on apoptosis of AoSMC has not been investigated.^{10,30} However, IL-22 was not observed to have any effect on apoptosis, necrosis or survival of resting AoSMC in normal conditions in our study. This data indicates that IL-22 does not affect apoptosis of VSMC.

To sum up, the results of our study showing certain effects of IL-22 on IL-35 subunits, IL-33 and ICAM-1 expression in AoSMC, support the assumption that IL-22, which may be released by Th22 and NK cells, may be an agent affecting vascular smooth muscle cell-orchestrated inflammatory processes, and thus may regulate the immune homeostasis of the vascular wall.

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A coronary proatherosclerotic marker: Pregnancy-associated plasma protein A and its association with coronary calcium score and carotid intima-media thickness

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Abstract

Background. Atherosclerosis, a chronic inflammatory disorder of the arteries, is responsible for the greatest number of deaths in westernized societies, with numbers increasing at a marked rate in developing countries. Coronary calcium score (CCS), carotid intima-media thickness (CIMT) and pregnancy-associated plasma protein A (PAPP-A) are predictors for the development of atherosclerosis.

Objectives. This study was aimed to investigate the relationship between CCS, CIMT and PAPP-A for earlier diagnosis of atherosclerosis.

Material and methods. A total of 99 patients were included in the study. Coronary computerized tomography (CT) angiography was performed on all patients. The calcium scoring technique was performed using a sequential scanning mode. CIMT measurement was done through the area 1 cm distal of the bulbous arteriosus with carotid Doppler ultrasound. PAPP-A values were analyzed by double immunoenzymatic technique.

Results. Out of 99 patients, 63 were found with coronary atherosclerosis using multislice computed tomography (MSCT) coronary angiography. When the cut-off point for CCS was taken to be 0.40, the sensitivity of this parameter was 97% and its specificity was 68.3%. When the cut-off point for CIMT was taken to be 0.60, the sensitivity and the specificity of these parameters were 75.0% and 87.3%, respectively, for the right measurements and 75.0% and 79.4%, respectively, for the left measurements.

Conclusions. This data support the conclusion that PAPP-A, like CCS and CIMT, is a parameter that can be used to detect subclinical atherosclerosis.

Key words: subclinical atherosclerosis, coronary calcium score, carotid intima-media thickness, pregnancy-associated plasma protein A

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Atherosclerosis is the most common cause of mortality and morbidity in the world. Estimation of the presence of atherosclerotic coronary artery disease (CAD) before its clinical presentation is very important in evaluating patients without any or with minimal symptoms. The likelihood of a future cardiovascular event in an individual can be calculated using risk factors, and treatment modalities are being developed to protect against such events. The coronary calcium score (CCS), carotid intima-media thickness (CIMT), and pregnancy-associated plasma protein A (PAPP-A) are predictors of the development of atherosclerosis.¹

In 1990, Agatston used cardiac scanning with high-resolution thin-section computed tomography (CT) and electrocardiography (ECG)-gating to develop a total calcium scoring method.² Today, the total calcium score is calculated with this method using EBT or 64/128-multislice computed tomography (MSCT). Several studies have demonstrated that CCS has great value in predicting cardiovascular outcomes in asymptomatic patients.³ A high calcium score increases the likelihood of sensitive plaque, but does not specifically detect sensitive lesions.⁴ It has been reported that the presence of point coronary calcium (smaller than 3 mm) more strongly predicts an acute coronary event than the calcium score.⁵

Measurement of CIMT with B-mode ultrasonography (US) has been approved by the American Heart Association (AHA) to assess the risk of atherosclerosis.⁶ The National Cholesterol Education Program Adult Treatment Panel III (NCEP-ATP3) has also stated that the CIMT can be used to assess the risk of CAD, together with other risk determinants.⁷

The timed disintegration of the extracellular matrix (ECM) is an important feature in certain physiological events, including normal development, morphogenesis, and tissue repair.⁸ The dysregulated coordination of this process may lead to myocardial and vascular-wall damage, such as atherosclerosis, left ventricle hypertrophy, heart failure, or aneurism.⁹ Matrix metalloproteinases are the enzymes predominantly responsible for the disintegration of the ECM, and may play a role in the pathogenesis of atherosclerosis and aneurism formation.^{10,11} Pregnancy-associated plasma protein A (PAPP-A) is a high-molecular-weight zinc-binding metalloproteinase, first detected in pregnant women.¹² It has been used to screen for fetal trisomy in the first trimester of pregnancy. Although it was first used in pregnancy, it has since become a prognostic and diagnostic marker of coronary disease.¹³ PAPP-A is produced in large amounts in eroded and ruptured plaques but in relatively lower amounts in stable plaques.¹³

In 2001, the presence of PAPP-A was shown for the first time in eroded and ruptured atherosclerotic plaques as well as increased levels in patients with acute coronary syndrome.¹³ Several studies have shown that the level of PAPP-A is higher in the presence of more complex and more widespread atherosclerosis in patients with stable CAD.¹⁴ Higher rates of multivascular disease have been

detected on the coronary angiography in people with higher PAPP-A values.^{15–17} Therefore, PAPP-A can be used for early diagnosis and prognosis of patients presenting with acute coronary syndrome, and high PAPP-A levels have been associated with poor prognoses in acute coronary syndrome.¹⁸ PAPP-A seems to be valuable in predicting the outcomes of patients admitted with high-risk NSTEMI-ACS or STEMI.¹⁹

The free fraction of pregnancy-associated plasma protein A (FPAPP-A) was found to be the PAPP-A form released to the circulation in acute coronary syndrome (ACS). We estimated the prognostic value of FPAPP-A vs total PAPP-A (TPAPP-A) concentrations in forecasting death and nonfatal myocardial infarction (combined endpoint) in patients with non-ST-elevation ACS.¹⁹

In this study we aimed to evaluate the value of PAPP-A in early diagnosis of atherosclerosis and its association with other well-known markers such as CIMT and CCS.

Patients and methods

Patient selection

The study population consisted of 99 patients who were clinically referred for noninvasive multislice computed tomography (MSCT) coronary angiography for CAD evaluation. The exclusion criteria for MSCT coronary angiography were acute coronary syndrome, known cardiomyopathy and congestive heart failure (ejection fraction < 50%), end-stage renal disease, liver failure, and patients with triglyceride levels above 400 mg/dL. Additionally, patients with hematologic disorders, acute or chronic infection or inflammatory conditions, severe chronic heart failure (New York Heart Association class 3), severe valvular heart disease, and a history of malignancy were excluded from the study. The study protocol was approved by our internal institutional review board.

Height and weight measured at the time of imaging were used to calculate body mass index (BMI). Hypertension was defined as systolic blood pressure \geq 140 mm Hg and/or diastolic blood pressure \geq 90 mm Hg or current antihypertensive treatment. Dyslipidemia was defined as total cholesterol \geq 200 mg/dL, low density lipoprotein (LDL) cholesterol \geq 130 mg/dL, high density lipoprotein (HDL) cholesterol < 50 mg/dL, or current lipid-modifying agent treatment. Diabetes mellitus was defined as fasting glucose \geq 126 mg/dL or current hypoglycemic treatment. Smoking was classified as current smoking if the patient smoked or quit within the last 30 days and not smoking if the patient never smoked or smoked in the remote past.

MSCT and CCS calculation

Patients were evaluated based on their pulse rates and clinical status. Those with a heart rate of \geq 70/min re-

ceived 50–100 mg of oral metoprolol succinate or 2.5–5 mg of intravenous metoprolol, according to cardiologist decision. They were included in the procedure when their heart rates became < 70/min. Coronary CT angiography was performed with a 64-slice consecutive MSCT scan (Philips Brilliance 64; Philips Medical System, Best, the Netherlands).

The calcium scoring technique was performed first, in the sequential scanning mode, after which 100 mL of iomeprol was infused via a vascular route at a speed of 5–6 mL/s. ECG editing was performed before reconstruction so that the data was not influenced by any arrhythmia.

CIMT measurement

The patients were examined with carotid ultrasound (US) using a Philips IE 33 echocardiography device with a 10 Mhz linear array transducer. The procedure was performed by 2 physicians. CIMT was measured through an area 1 cm distal to the bulbous.²⁰ Ultrasound images were acquired in semiautomatic mode of the device with dedicated software. Three measurements were performed by each physician separately and their mean values were recorded.

Carotid artery plaque (CAP) was defined as local thickening of the CIMT of 50% compared to the surrounding vessel wall, an intima media thickness > 1.5 mm, or local thickening > 0.5 mm.

The presence of plaque was recorded as “yes” or “no”.

PAPP-A measurement

Venous blood was drawn from an antecubital vein at the time of hospital admission before MSCT and 1 mL of serum obtained from this sample was placed in an Eppendorf +or plain tube, and stored at –80°C. After the blood samples were restored to room temperature, the PAPP-A levels were analyzed with a double immunoenzymatic technique (sandwich format) using the Beckman Coulter Unicel® Dxl 800 (Brea, California) device with a PAPP-A kit. The results are expressed in ng/mL. The median serum PAPP-A levels in male subjects [6.85 (undetectable (UD), 24.40) ng/mL] were significantly higher than that of female subjects [3.40 (UD, 36.7) ng/mL].²⁰ According to the manufacturer, the within-run coefficient of variation (CV) was 7.96% and the detection limit of the method was 0.019 ng/mL.

Data analysis

The normality of the distributions of the measured values was assessed graphically and with the Shapiro-Wilk test. Descriptive statistics are presented as means ± standard deviations (SD) or medians (interquartile ranges [IQRs]), depending on the normality of their distribution. Categorical variables are presented as numbers and percentages or in

Table 1. Baseline characteristics of patients in study group

Variables	N	%
Sex		
male	74	74.7
Carotid artery plaque		
yes	43	43.4
Previous history of CAD		
no	80	80.8
Medical therapy	5	5
Stent	9	9.1
Coronary bypass	5	5
CAD with MSCT		
normal coronary artery	36	36.4
mild stenosis (< 50%)	29	29.3
moderate stenosis (51–69%)	7	7.1
severe stenosis (> 70%)	27	27.3
Family history of CAD		
yes	24	24.2
Diabetes		
yes	17	17.2
Hypertension		
yes	39	39.4
Smoking		
yes	32	32.3
Hyperlipidemia		
yes	32	32.3
CIMT percentile		
< 25%	62	62.6
25–75%	31	31.3
> 75%	6	6.1
Statin using	19	19.1
ASA using	19	19.1

Variables	Min–Max	Median (IQR) /Mean ± SD
Age (years)	31.0–84.0	52.0 (21.0)
PAPP-A (ng/mL)	0.80–25.10	2.50 (1.38)
Coronary calcium score (agatston)	0.0–677.0	15.5 (139.8)
right CIMT (mm)	0.48–0.89	0.64 (0.18)
left CIMT (mm)	0.51–0.86	0.62 (0.16)
	107.0–304.0	188.5 ± 53.3
Total Cholesterol (mg/dL)		
HDL (mg/dL)	28.0–78.0	46.0 (20.0)
LDL (mg/dL)	55.0–218.0	124.5 ± 40.6
Triglyceride (mg/dL)	46.0–258.0	132.5 (88.0)
Body mass index (weight – kg/length – m ²)	23.3–33.6	25.1 (3.3)

Acetylcysteine (ASA), carotid intima–media thickness (CIMT), coronary artery disease (CAD), multislice computed tomography (MSCT), pregnancy-associated plasma protein A (PAPP-A), carotid intima–media thickness (CIMT), high-density lipoprotein (HDL), low-density lipoprotein (LDL).

cross-tables. Differences in continuous variables were calculated with Student's *t* test or the Mann–Whitney *U* test, depending on the normality of their distributions. Differences between categorical variables were analyzed with the *c*² test or the *c*² likelihood ratio. Correlations between PAPP-A, the calcium score, and carotid artery thickness

were analyzed with Spearman's rank correlation coefficient (r). A receiver-operating characteristic (ROC) curve was drawn to determine the cut-off points for PAPP-A, CCS, and CIMT based on the CT findings, and to calculate the sensitivity and specificity of the parameters. The areas under the curves (AUCs) and 95% confidence intervals were calculated. A logistic regression model using step-wise regression with the Wald statistic was established to determine the factors that might significantly affect plaque formation in the carotid artery and the degree of risk enhancement by these factors (odds ratio [OR]). Microsoft Excel 2003 and SPSS for Windows v. 15.0 (SPSS Inc., Chicago, USA) were used for the statistical analyses and calculations. $P < 0.05$ was considered to indicate that statistical decisions were significant.

Results

The study included 99 patients, of whom 25 were female (aged 32–86 years, median age 55.0 years [IQR 19.5]) and 74 were male (aged 31–87 years, median age 51.5 years [IQR 16.3]). The radiological and biochemical data for the patients are listed in Table 1. Certain characteristics of the patients are illustrated in Table 2.

The patients with CAP were older than those without CAP, and their PAPP-A, CCS, and CIMT values were also higher (Table 3). CAP was significantly more prevalent in patients with diabetes mellitus (DM) or hypertension (HT) (Table 4).

The prevalence of CAP differed significantly according to the percentile of CIMT ($c^2 = 12.331$, $p = 0.002$). When we investigated the class(es) in which this difference arose, we found that the differences between the $< 25\%$ CIMT group and the 25%–75% CIMT group ($c^2 = 8.032$,

$p = 0.005$) and between the $< 25\%$ CIMT and the $> 75\%$ CIMT groups ($c^2 = 6.478$, $p = 0.011$) were statistically significant. The difference between the 25%–75% percentile CIMT group and the $> 75\%$ percentile CIMT group was not statistically significant ($c^2 = 1.185$, $p = 0.276$).

PAPP-A, CCS, and CIMT (right and left) were determined and evaluated according to CAD (Table 5). The median PAPP-A, CCS, and left CIMT values were higher in the CAD-positive patients than in the CAD-negative patients ($p < 0.05$). The median CIMT (right) was higher in the CAD-positive patients than in the CAD-negative patients, but the significance of the difference was borderline ($Z = 1.937$; $p = 0.053$).

When we investigated the correlations between PAPP-A, CCS, and CIMT, there was a strong linear correlation between PAPP-A and CCS ($r = 0.805$; $p < 0.001$), moderately strong linear correlations between PAPP-A and right CIMT ($r = 0.532$; $p < 0.001$) and left CIMT ($r = 0.568$; $p < 0.001$), and strong linear correlations between CCS and right CIMT ($r = 0.616$; $p < 0.001$) and left CIMT ($r = 0.676$; $p < 0.001$).

The risk of developing CAP (OR = 6.944) was 6.944 times higher in the patients with DM than in those without DM. This risk was higher in patients with DM (95% confidence interval [CI]: 1.787–26.98; $p = 0.0057$). Similarly, the risk of developing CAP (OR = 2.810) was 2.810 times higher in patients with HT than in patients without HT. This risk was at the least 1.151 times higher and at most 6.860 times higher in patients with

Table 3. Distribution of CAP according to certain groups

Variables	CAP (–)		CAP (+)		p-value
	n	%	n	%	
Family history of CAD					
no	42	56	33	44	0.841
yes	14	58.3	41.7	24	
Diabetes					
no	53	64.6	29	35.4	< 0.001
yes	3	17.6	14	82.4	
Hypertension					
no	41	68.3	19	31.7	0.003
yes	15	38.5	24	61.5	
Smoking					
no	39	58.2	28	41.8	0.633
yes	17	53.1	15	46.9	
Hyperlipidemia					
no	40	59.7	27	40.3	0.362
yes	16	50	16	50	

Table 2. Results of radiology and biochemistry according to the presence of coronary artery plaque (CAP)

Variables	CAP (–)		CAP (+)		Test statistics
	min–max	median (IQR) mean \pm SD	min–max	median (IQR) mean \pm SD	p-value
Age	31–86	48.0 (12.0)	38–87	59.0 (15.0)	< 0.001
PAPP-A	0.80–18.40	1.80 (0.90)	1.30–25.10	2.70 (1.35)	< 0.001
Coronary calcium score	0.0–286.0	0.0 (0.0)	0.0–3100.0	57.0 (203.0)	< 0.001
CIMT – right	0.43–0.87	0.60 (0.13)	0.52–1.13	0.73 (0.19)	< 0.001
CIMT – left	0.43–0.95	0.60 (0.11)	0.57–1.03	0.73 (0.19)	< 0.001
Total cholesterol	107–309	206.9 \pm 41.8	119–304	195.6 \pm 42.2	0.209
HDL	27.0–82.0	42.0 (13.5)	27.0–70.0	45.0 (16.0)	0.289
LDL	55.0–209.0	133.8 \pm 39.5	51.0–218.0	118.2 \pm 38.6	0.068
Triglyceride	46.0–449.0	135.5 (78.3)	18.0–1062.0	145.0 (93.0)	0.691

Pregnancy associated plasma protein-A (PAPP-A); carotid intima-media thickness (CIMT); high-density lipoprotein (HDL); low-density lipoprotein (LDL).

Table 4. PAPP-A, coronary calcium score, and CIMT according to coronary artery disease (CAD)

Variables	No		Yes		Test statistic
	min–max	median (IQR)	min–max	median (IQR)	p-value
PAPP-A	0.80–25.10	1.90 (1.10)	1.30–24.00	2.80 (1.90)	0.001
Coronary calcium score	0.00–677.00	0.00 (44.50)	0.00–3100.00	123.00 (239.00)	< 0.001
CIMT right	0.48–1.13	0.62 (0.18)	0.52–0.89	0.70 (0.14)	0.053
CIMT left	0.51–1.00	0.61 (0.17)	0.57–1.03	0.71 (0.19)	0.013

Pregnancy-associated plasma protein A (PAPP-A); carotid intima–media thickness (CIMT).

HT, with a probability of 95% ($p = 0.023$). None of the other risk factors considered to affect the development of CAP increased or reduced the risk of CAP in this study ($p > 0.05$).

The sensitivity and specificity of PAPP-A, CCS, and CIMT were determined by recoding vascular stenosis as (1) normal or (2) stenotic, depending on the MSCT results. When the MSCT results for vascular stenosis (normal or stenotic) were determined using the PAPP-A values, the area under the ROC curve (AUC) was 0.813 (95% CI: 0.729–0.897), which was significantly higher than 0.50 ($p < 0.001$). Therefore, the most appropriate cut-off point for PAPP-A that could be used to predict the MSCT results was 2.35 ng/mL. Thus, a PAPP-A value > 2.35 indicates vascular stenosis and a value < 2.35 indicates a normal vascular structure. When the cut-off point for PAPP-A was taken to be 2.35, the sensitivity and specificity of this parameter were 94.3% and 63.9%, respectively.

When the MSCT results for vascular stenosis (normal or stenotic) were determined using CCS, AUC was 0.836 (95% CI: 0.758–0.913), which was higher than 0.50 ($p < 0.001$). Therefore, the most appropriate cut-off point for CCS that could be used to predict the MSCT results was 0.40. Thus, CCS > 0.40 indicates vascular stenosis and CCS < 0.40 indicates a normal vascular structure. When the cut-off point for CCS was taken to be 0.40, the sensitivity of this parameter was 97% and its specificity was 68.3%.

When the MSCT results for vascular stenosis (normal or stenotic) were determined using CIMT (right, left), AUC was 0.852 (95% CI: 0.773–0.932) for right CIMT and 0.848 (95% CI: 0.766–0.931) for left CIMT, which were both higher than 0.50 ($p < 0.001$). Therefore, the most appropriate cut-off point for CIMT that could be used to predict the MSCT results was 0.60. Thus, CIMT > 0.60 indicates vascular stenosis and CIMT < 0.60 indicates a normal vascular structure. When the cut-off point for CIMT was taken to be 0.60, the sensitivity and the specificity of these parameters were 75.0% and 87.3%, respectively, for the right measurements and 75.0% and 79.4%, respectively, for the left measurements.

Discussion

In this study, we showed that PAPP-A, CCS, and CIMT were significantly higher in CAP (+) patients than in CAP (–) patients. If CAP (+) patients are considered to have

subclinical atherosclerosis, it can be concluded that high PAPP-A, CCS, and CIMT are associated with subclinical atherosclerosis.

To our knowledge, this is the first study to evaluate PAPP-A levels on subclinical atherosclerosis with CCS and CIMT. Thus, this study

showed PAPP-A's stronger relationship with subclinical atherosclerosis.

Even though CCS and CIMT were known to be risk factors for subclinical atherosclerosis, the most important result of our study is to show that PAPP-A is also a risk factor for subclinical atherosclerosis.

Atherosclerosis develops during the lifespan of an individual and involves a number of steps: respectively, activation of the endothelium and recruitment of immune cells; monocyte differentiation and foam cell formation; development of fibrotic plaques due to the death of foam cells and migration and proliferation of smooth muscle cells (SMCs); plaque rupture and thrombosis. Both the innate and adaptive immune response in atherosclerosis is organized by a range of cytokines, which regulate all stages of the disease.²¹

Noninvasive imaging techniques can be used to detect and monitor preclinical atherosclerosis in human arteries. The use of CIMT and CCS to detect subclinical atherosclerosis has become a topical issue and many studies of this subject have been conducted. However, the results for the use of PAPP-A are so far contentious, and as yet, it has no place in clinical practice.

In daily practice, carotid US is used to explore the lesion obstructing the carotid artery. The measurement of CIMT using B-mode US has been approved by the AHA for the evaluation of atherosclerotic risk.⁶ Research has shown that CIMT is associated with myocardial infarction (MI), stroke, CVD, and death.²² Therefore, the use of CIMT as a marker for atherosclerosis is supported.²³ Carotid plaque is also valuable in assessing the risk of CVD, with an even higher positive predictive value than CIMT.²³

In this study, when the combined effects of the atherosclerotic risk factors on CAP formation were investigated, only the presence of DM and HT increased the risk of CAP, and no other factors did so. Although the prevalence of atherosclerosis is expected to be lower in females than in males, the incidence of CAP did not differ between the sexes in this study. This might be attributable to the advanced age of the women participating in the study. Other studies have demonstrated that the difference between females and males in the incidence of CAD disappears after menopause.²⁴

Our study showed that older subjects had a higher incidence of CAP, which is consistent with studies that have

shown an increase in atherosclerosis with age. Previous series of studies have proved the association of increased CIMT and the presence of carotid plaques with CAD. Ultrasonographic assessment of CIMT and carotid plaques is a valid and proven method for the early evaluation of cardiovascular disease.²⁵ This corroborates the hypothesis that CAP is an indicator of atherosclerosis, which is also consistent with the literature.

Several studies have demonstrated that high CCS is an indicator of atherosclerosis and that it is a valuable parameter for estimating cardiovascular outcomes in asymptomatic patients.^{2,26} CAC is always associated with mural atheromatous plaque.³

Our findings support the results of earlier studies that showed higher CCSs in patients with CAP (+).²⁷ In the present study, the prevalence of CAP was higher in patients with CAD and highest in those with advanced CAD when detected by MSCT. This indicates an association between the presence of CAP and the severity of CAD, which is consistent with the literature.

A study by Elesber et al. showed that patients who had ruptured plaques had higher serum PAPP-A levels than patients who had stable plaques.²⁸ However, Bayes-Genis et al. stated that PAPP-A levels are higher in the presence of more extensive and complex atherosclerosis in patients with stable CAD.¹³

A higher rate of multivascular disease was detected with coronary angiography in patients with higher PAPP-A values.¹⁴ It has also been reported that increased PAPP-A levels in patients with chronic stable angina pectoris predict all-cause mortality.¹⁶ Although PAPP-A has previously been reported as a predisposing factor for atherosclerosis, later studies have reported that PAPP-A is produced as compensatory in atherosclerosis.^{14,29} However, in both cases, PAPP-A is increased in atherosclerosis, so PAPP-A can be used as an indicator of subclinical atherosclerosis. In the present study, PAPP-A values were high in patients with CAP. Because the presence of CAP provides information about the presence of atherosclerosis, it can be concluded that PAPP-A is high in cases of atherosclerosis, which is consistent with the literature.

In this study, we found a strong linear correlation between PAPP-A and CCS, only a moderately positive linear correlation between PAPP-A and CIMT, and a strong linear correlation between CCS and CIMT. This information supports the conclusion that PAPP-A, like CCS and CIMT, is a parameter that can be used to detect subclinical atherosclerosis.

The cut-off values for PAPP-A, CCS, and CIMT were determined according to the presence or absence of CAD. Based on the results of this study, the most appropriate cut-off value for PAPP-A is 2.35 ng/mL. The sensitivity and specificity of this value in detecting CAD were 94.3% and 63.9%, respectively. When an Agatston score of 0.4 was taken as the cut-off value for CCS, the sensitivity and specificity of CCS in detecting CAD were 97.2% and 68.3%, re-

spectively. When 0.6 mm was taken as the cut-off value for the right CIMT, the sensitivity and specificity of the right CIMT in detecting CAD were 75% and 87.3%, respectively. When 0.6 mm was taken as the cut-off value for the left CIMT, the sensitivity and specificity of this parameter in detecting CAD were 75% and 79.4%, respectively.

In the process of selecting patients for stress tests and planning treatment strategies, these cut-off values could be used as reference parameters. For example, if PAPP-A is > 2.35 ng/mL, CIMT is over 0.6 mm, and CCS is over 0.4 in an asymptomatic patient with a moderate risk of CAD, then acetylsalicylic acid, beta blockers and statins are the options, of which one or more of them can be chosen as initial therapy. If the patient has atypical symptoms and the above-mentioned values are high, then the patient can be assigned to a stress test. If the patient is symptomatic and the above-mentioned values are high, then the patient can be assigned to conventional imaging methods without a stress test. In this way, the rates of false negatives and false positives of the stress tests can be reduced.

This study was conducted in a single center with 99 patients included. Multicenter studies in larger patient populations are required to clarify the values discussed above and to develop appropriate therapy modalities. In the analysis of CCS, the high-dose exposure of radiation due to MSCT is a disadvantage. For this reason, determining the CVD score seems to be a better parameter than CCS. Another limitation is that we measured total PAPP-A, not the free form. J. Lund et al. suggested that the free form of PAPP-A is a stronger predictor.¹⁸ The venous blood samples were obtained only once at clinical presentation and no other samples were taken for follow-up. We therefore cannot exclude whether follow-up PAPP-A levels have a better predictive value or not.

Another limitation of this study is that PAPP-A values also could not be evaluated in patients taking statins. The effects of statins may reduce the plaque burden, thus statins could also be an effect on PAPP-A levels. Whether statins could downregulate PAPP-A levels is unclear. A total of 19 of our patients were using statins. Because of the small number for evaluation, we thought this would not be healthy.

Statins have been widely used in cardiovascular diseases for their versatile function such as regulation of lipid level, anti-inflammation and endothelium repair. However, it remains unknown whether statins have established an effect in reducing PAPP-A. Miedema et al. reported that high-dose atorvastatin (80 mg/d) could decrease the serum PAPP-A in ACS patients after one month treatment.³⁰

In conclusion, PAPP-A elevations in circulation play an important role in the diagnosis of subclinical atherosclerosis. Although the sample size of our study was sufficient, there is a need for large-scale, randomized trials for clinical reflection. Larger clinical trials would enhance the diagnostic capability of this novel biomarker.

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Echocardiographic characteristics of pulmonary arterial hypertension in children with horizontally transmitted HIV

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Abstract

Background. The prevalence of primitive pulmonary arterial hypertension (PAH) in patients with human immunodeficiency virus infection (HIV) is estimated at approximately 0.5%, significantly higher than in the general population.

Objectives. This study aimed to assess the echocardiographic modifications in HIV-associated pulmonary arterial hypertension (PAH).

Material and methods. A group of 117 patients, aged under 16, with horizontally transmitted HIV staged according to the U.S. Center for Disease Control and Prevention criteria, were included in this prospective study, with echocardiographic abnormalities in 79 children. The study group consisted of 27 HIV-infected patients with PAH, while the control group consisted of 38 patients with normal ultrasound features. The diagnostic criterion for PAH was the presence of a mean pulmonary artery pressure above 25 mm Hg, determined at 2 consecutive measurements having at least 6 months distance between them. All subjects underwent a complex echocardiographic assessment, including assessment of left and right ventricular hypertrophy and evaluation of left ventricular function, associated with determination of the immunological stage.

Results. We recorded the presence of PAH in 27 patients (23.08%), in whom an average value of 31.48 mm Hg was recorded for pulmonary artery pressure. All patients had mild forms of PAH. Age, gender and immunological stage showed no significant differences in the PAH group compared to patients in the control group. Right ventricular hypertrophy was encountered in 95.23% and left ventricular hypertrophy in 88.88% of the patients with PAH. Left ventricular dysfunction, a complication of pulmonary hypertension, was relatively rare (11.11%).

Conclusions. In children with HIV infection, PAH is present in a relatively mild form and does not correlate with the clinical and immunological stage of HIV infection, evolving as a seemingly primitive condition.

Key words: children, pulmonary arterial hypertension, HIV, ultrasonography

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Introduction

The prevalence of primitive pulmonary arterial hypertension (PAH) in patients with human immunodeficiency virus infection (HIV) is estimated at approximately 0.5%, significantly higher than in the general population. The improvement in the survival rates of these patients, as a result of the introduction of highly active antiretroviral treatment (HAART) and of a better control of infectious intercurrents, increases the associated transmission risks and the importance of this pathology.¹ Similarly to primitive pulmonary arterial hypertension, the condition associated with HIV infection has a very complex pathogenesis, being characterized by abnormal vasomotor response of the pulmonary vascular bed, pulmonary angiogenesis disorders and disorders of cellular ion channels.² Restrictive and obstructive ventilatory dysfunction caused by recurrent pulmonary infections, hypoxia and secondary pulmonary hypertension, other viral infections, and cocaine and intravenous drug use can be added.³

According to European guidelines, PAH is defined as an increase in mean pulmonary arterial pressure above 25 mm Hg at rest, as assessed by cardiac catheterization. The present guidelines of the European Society of Cardiology indicate the invasive determination of pulmonary artery pressure using right heart catheterization as the gold standard for establishing the diagnosis of PAH.⁴ However, we should not forget that cardiac catheterization is associated with a significantly higher risk of HIV-infection transmission in this population, therefore the routine use of this method, especially for those patients, should be avoided. Instead, echocardiography (a non-invasive and repeatable method) presents numerous advantages (safe, non-invasive, painless, inexpensive), becoming an extremely useful tool not only for screening but also for monitoring the disease, while other non-invasive tests such as clinical examination, X-ray or ECG can only raise the suspicion of PAH.

The objective of this study was to assess the echocardiographic characteristics of PAH in children with horizontally transmitted HIV.

Patients and methods

Our study is a prospective, longitudinal and cohort study, conducted over a period of 5 years in the Infectious Diseases Clinic I, Tirgu Mures, Romania.

The study population was comprised of children with diagnosed HIV infection monitored by the aforementioned center. The inclusion criteria were the presence of HIV infection with documented horizontal transmission and age under 16 years. The exclusion criteria were the presence of congenital cardiac malformations, vertical transmission of the HIV infection and lack of cooperation with ultrasound examination during the study. The

patient population consisted of 117 HIV-infected children in whom cardiac ultrasound was performed at baseline and repeated during the 5-year follow-up (at 6-month intervals), divided into 2 groups: 79 children with echocardiographic abnormalities and 38 children without echocardiographic abnormalities. The study group consisted of 27 HIV-infected patients with PAH, while the control group consisted of 38 patients with normal ultrasound features.

The PAH study group was defined taking into account the dominant PAH pathology. Cases where PAH was secondary to other cardiac pathology were excluded.

The diagnosis of the HIV infection was confirmed based upon 2 second generation ELISA tests for HIV1 and HIV2 antibodies and confirmed by Western Blot.

Clinical and immunological staging, needed for stratification of the study population and for assessment of the correlations between the severity of cardiac involvement and the stage of the HIV infection, was done using the 1993 CDC Centers for Disease Control and Prevention (CDC) Atlanta criteria.⁵

The echocardiographic examination was performed using M mode (mono-dimensional), 2D (2-dimensional), spectral Doppler and color Doppler echocardiography. The following parameters characterizing the ventricular function and structure were monitored: left and right ventricular mass, left ventricular ejection fraction and fractional shortening, presence and severity of valvular regurgitation at aortic, mitral, pulmonary and tricuspid levels, ventricular wall thickness (tricuspid annular plane systolic excursion – TAPSE). Pulmonary artery pressure measurements in the study group were performed using Bernoulli's equation applied to the regurgitant flow across the tricuspid valve in systole. In the case of associated pulmonary regurgitation, color Doppler measurement of the regurgitant flow across the pulmonary valve made possible the assessment of diastolic pressure in the pulmonary artery. The ultrasound study of the pulmonary valve sometimes made possible diagnosis of PAH.

The diagnostic criterion for PAH was the presence of a mean pulmonary artery pressure above 25 mm Hg at rest, determined at 2 consecutive measurements having at least 6 months distance between them.⁴

We considered the measurements performed in the absence of acute or chronic respiratory infections when the restrictive or obstructive dysfunction of the pulmonary parenchyma may have led to hypoxia and changes in pulmonary vascular resistance. No adjustments were required for body weight criteria in children.⁶

Statistical analysis

Statistical analysis was performed with GraphPad In-Stat 2003 software. The data was considered statistically significant for $p < 0.05$. Mean, minimum, and maximum

values, standard deviation and confidence intervals were calculated for all the parametric data samples. Gaussian distribution was determined using the Kolmogorov-Smirnov formula, the value obtained having to be higher than 0.10. If the obtained value was lower, we changed the parameter data into nonparametric data or we used tests for nonparametric data (Mann-Whitney test) for the analysis of two independent samples.

For the statistical analysis of parametric values we used Student's t test to analyze 2 dependent or independent samples, and we used the t test with Welch's correction when significant differences were recorded in the standard deviations of the study.

Nonparametric data samples were statistically analyzed using Fisher's exact test for smaller groups and χ^2 test for larger groups, with or without Yates's correction, $p < 0.05$ being considered significant. Correlation between non-parametric unpaired samples was calculated using the Spearman correlation coefficient. In the case of paired data, the Wilcoxon test was used.

The study was approved by the institution's Ethics Committee.

Results

Epidemiological data

The gender distribution in the entire study group was balanced (49.58% males/50.42% females). The mean age of the children with HIV infection in the study group was 14.03 ± 0.23 years.

Analysis of the mean age of the children in the 2 groups did not show statistically significant differences between the groups (15.03 ± 0.28 years in the group with pulmonary hypertension vs 15.21 ± 0.41 years in the control group).

In the PAH group we found a higher prevalence of the disease in female patients (17 patients, 62.96%) than in male (10 patients, 37.03%). However, the gender distribu-

tion of patients did not differ significantly from the control group (24 patients, 63.16% and 14 patients, 36.84%) (Fisher's test $p = 1.00$).

Staging of HIV infection

According to CDC Atlanta criteria the distribution into groups of clinical and immunological severity staging (LT-CD4 depletion) did not show statistically significant differences between the groups ($p = 0.51$ for clinical staging and $p = 0.296$ for immunological staging).⁵

Echocardiographic changes in the study groups

The examinations in the study group revealed multiple echocardiographic aspects. The recorded changes were cumulative for the duration of the study and were not always evident on the first evaluation of the patient. We recorded echocardiographic abnormalities in a number of 79 children (67.52%) from the study group. As previously mentioned, the changes observed were left ventricular systolic dysfunction, left ventricular hypertrophy, pulmonary hypertension, right ventricular hypertrophy, dilated cardiomyopathy and pericardial collections. Thirty-eight (32.48%) patients had normal ultrasound features over the entire period and were designated as the control group.

Variable PAH were encountered in 16 other patients out of the 79 with echocardiographic abnormalities; all these patients had dilated cardiomyopathy. The diagnostic criteria in this lot and, respectively, the criteria for exclusion from the PAH study group were: ejection fraction below 40% and increased left ventricle end-systolic and end-diastolic diameters.⁶ Although the increase of the mean pressure in the pulmonary artery was significant in this lot (mean value 34.31 mm Hg), patients with dilated cardiomyopathy were not included in the PAH study group, as in their case the pressure change was considered to be a consequence of the systolic dysfunction pertaining to dilated cardiomyopathy (Fig. 1).

Fig. 1. Distribution of dominant ultrasound features

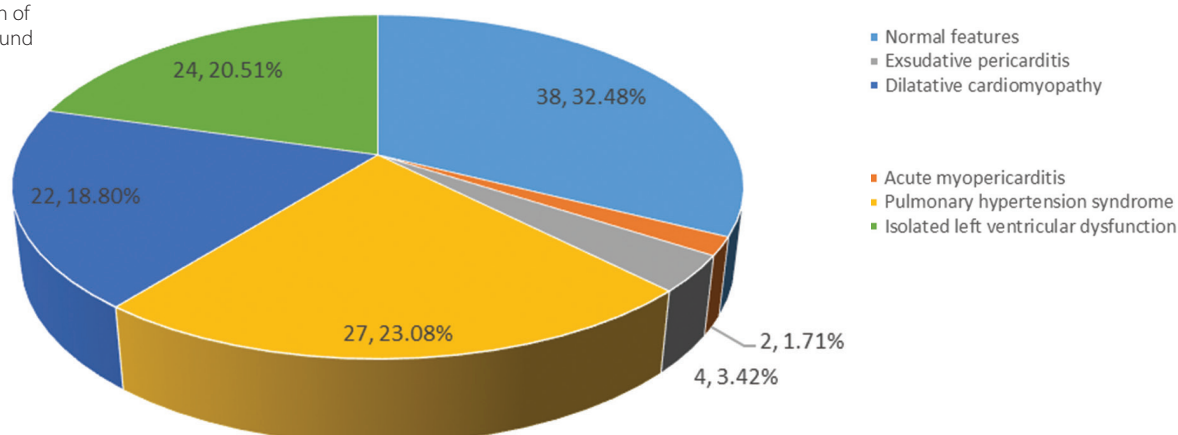


Table 1. Clinical and immunological staging of patients with PAH vs patients in the control group

Clinical and immunological categories	Clinical staging			Immunological staging		
	A	B	C	1	2	3
PAH group	14	12	1	14	11	2
Control group	16	18	4	21	10	7
Fisher's exact test	p = 0.51			p = 0.296		

Features of the PAH group

Twenty-seven patients, representing 23.08% of the entire pediatric group studied, constituted the subgroup of patients with PAH.

The evaluation of the clinical and immunological status of HIV infection in patients with PAH compared to the control group, did not show statistically significant differences regarding the clinical or immunological staging (Table 1).

The echocardiographic characteristics of patients in the group diagnosed with PAH and those in the control group are summarized in Table 2.

Mean pulmonary arterial pressure was 31.48 ± 1.74 mm Hg, compared to 17.33 ± 2.24 mm Hg in the control group ($p < 0.0001$).

Analyzing the severity of the pulmonary arterial hypertension syndrome in relation to the mean values of pulmonary arterial pressure (25–40 mm Hg = mild, 41–70 mm Hg = moderate, over 70 mm Hg = severe), we observed that all children with pulmonary hypertension associated with HIV infection showed mild forms. No more than 15 patients had values above 30 mm Hg, of which 8 had repeated mean values above 37 mm Hg.

Due to increased resistance in the pulmonary vascular bed, lower flow velocities were recorded in the pul-

Table 2. Echocardiographic characteristics in children and adolescents with horizontally acquired HIV infection and PAH vs. the control group

Measurement (PAH group/control group)	Result	Standard deviation	Confidence interval	n	Student t test
Mean PA pressure (mm Hg)	31.48	4.63	± 1.74	27	$p < 0.0001$
	17.33	3.61	± 2.24	24	
PA flow (m/s)	0.97	0.23	± 0.08	27	$p = 0.07$
	1.07	0.20	± 0.09	34	
LV diastolic diameter (cm)	4.28	0.44	± 0.18	23	$p = 0.961$
	4.27	0.52	± 0.17	34	
LV systolic diameter (cm)	2.58	0.64	± 0.20	23	$p = 0.163^*$
	2.8	0.37	± 0.12	34	
IVS diastolic thickness (cm)	1.02	0.27	± 0.11	22	$p = 0.478^*$
	0.98	0.14	± 0.04	36	
IVS systolic thickness (cm)	1.30	0.28	± 0.11	22	$p = 0.672^*$
	1.27	0.19	± 0.066	34	
LVPW diastolic thickness (cm)	0.97	0.29	± 0.12	22	$p = 0.241^*$
	0.89	0.15	± 0.05	36	
LVPW systolic thickness (cm)	1.26	0.33	± 0.14	22	$p = 0.560^*$
	1.31	0.3	± 0.1	34	
LA (cm)	3.07	0.49	± 0.26	13	$p = 0.158^*$
	2.81	0.53	± 0.213	24	
RA (cm)	4.66	0.56	± 0.31	27	$p < 0.0001$
	3.02	0.44	± 0.29	22	
RV (cm)	2.41	0.32	± 0.2	27	$p < 0.0001$
	1.78	0.29	± 0.12	23	
EF (%)	65.50%	8.38	± 3.35	24	$p = 0.937$
	69.88%	8.07	± 2.63	36	
FS (%)	33.52%	7.65	± 3.06	24	$p = 0.922$
	33.70%	6.29	± 2.11	34	
LV weight (g)	153.78	44.97	± 24.97	14	$p = 0.611$
	143.70	62.78	± 27.51	20	

PA – pulmonary artery; LV – left ventricle; IVS – interventricular septum; PW – posterior wall; LA – left atrial diameter; RA – right atrial diameter; RV – right ventricle diameter; EF – ejection fraction; FS – fractional shortening; *Welch correction.

monary artery trunk, the mean value in the study group being 0.97 ± 0.08 m/s vs 1.07 ± 0.09 m/s in the control group. However, the difference was not statistically significant because the pursued hemodynamic parameter is not important for a positive diagnosis or for the severity of the syndrome.

We did not note statistically significant differences between left ventricular diastolic diameter in children with pulmonary hypertension compared to the control group ($p = 0.9610$). Similarly, left ventricular systolic diameter did not differ significantly between the 2 groups ($p = 0.163$). The analysis of the left ventricular wall thickness in patients with HIV infection associated with pulmonary hypertension showed normal values. Thus, the mean diastolic interventricular septum thickness did not differ significantly between the 2 groups ($p = 0.478$) and neither did the mean systolic interventricular septum thickness differ significantly between the 2 groups ($p = 0.672$).

The posterior wall diastolic thickness of the left ventricle did not differ significantly between the 2 groups ($p = 0.241$) and neither did the systolic posterior wall thickness present statistically significant differences ($p = 0.560$).

The recorded diameter of the left atrium in the 2 groups indicated normal values and no statistically significant differences ($p = 0.158$).

Measurements indicated a greater size of the right atrium than normal, relative to body weight, in 16 of the patients in the study group, representing 59.25%. The mean diameter of the right atrium had higher values in patients with pulmonary hypertension than in those in the control group, and the difference was highly significant statistically ($p < 0.0001$).

Ultrasound examination revealed right ventricular dilatation in 25 of the children in the study group, representing 92.59%.

The mean diameter of the right ventricle was much greater in the group of children with PAH compared to those in the control group, and the difference was highly significant statistically ($p < 0.001$).

As a result of the absence of any morphological and functional alteration of the left ventricle in patients with pulmonary hypertension, there were no modifications observed at the level of the aortic valve (Table 3).

Thus, the aortic and mitral insufficiency did not differ significantly in frequency in the 2 groups studied (14.8% vs 10.52%, $p = 0.709$, and 7.40% vs 0%, $p = 0.168$, respectively). In all cases, color Doppler echocardiography revealed minor aortic insufficiency, with minimal regurgitation.

Increased pressure in the pulmonary vascular bed did not lead to a higher prevalence of pulmonary valvular insufficiency (40.74% vs 42.10%, $p = 1.00$). While pulmonary insufficiencies were mild in the control group, con-

sidered normal variants, we recorded 4 cases of moderate pulmonary insufficiency and 2 cases of severe pulmonary insufficiency (Table 3) in patients with PAH.

At least moderate tricuspid regurgitation was detected in the majority of patients with pulmonary hypertension associated with HIV infection (25 patients, 92.59%). The presence of a significant tricuspid regurgitation is an important sign of pulmonary hypertension and makes possible the measurement of the mean pressure in the pulmonary artery with the aid of the Doppler examination if there is any pulmonary valve stenosis. Differences between the groups in the severity of tricuspid regurgitation are highly significant statistically ($p < 0.0001$). (Table 3)

Table 3. The prevalence of valvular insufficiencies in patients with PAH vs. patients in the control group

Condition	n (%)		Fisher's test
	PAH group	control group	
Aortic insufficiency	4 (14.81)	4 (10.25)	$p = 0.709$
Mitral insufficiency	2 (7.40)	0	$p = 0.168$
Pulmonary insufficiency	11 (40.74)	16 (42.10)	$p = 1.00$
Tricuspid insufficiency	25 (92.59)	10 (26.31)	$p < 0.0001$

Patients with PAH syndrome showed no decrease in ejection fraction and the difference from the control group was not statistically significant in relation to ejection fraction ($p = 0.973$). In accordance with normal ejection fraction, the shortening fraction in patients with pulmonary hypertension associated with HIV infection was also within normal limits, differences from the control group being not statistically significant ($p = 0.922$).

Only 3 of the children with pulmonary hypertension, representing 11.11% of the population of the study group, had minimal left ventricular dysfunction (between 50 and 55%). These 3 children had mean values of pulmonary artery pressure between 35 and 38 mm Hg, the highest in the study group.

In terms of normal left ventricular morphological data, its mass did not show pathological values, the difference in mass being insignificant as opposed to the control group.

Discussions

Pulmonary hypertension had an increased prevalence in our study group, being encountered in 23.08% of patients. Literature data indicates a similar prevalence of seemingly primitive pulmonary hypertension associated with HIV infection: values of 27% reported by Cicalini et al. in patients without clinical symptoms suggestive of PAH, in whom, as in the present study, echocardiography was the only diagnostic method.⁷

In recent years, different new echocardiographic techniques, such as speckle tracking or tissue Doppler, have been introduced and have demonstrated their superiority in the complex assessment of ventricular global and regional function. Several parameters calculated with the

help of these new technologies, such as tricuspid annular plane systolic excursion (TAPSE), tricuspid annular systolic velocity (S'), right ventricular myocardial performance index (MPI) and right ventricular fractional area changes (FAC), have been demonstrated to be correlated with the severity of the PAH and the underlying heart disease, as indicators of the global function of the right ventricle, and are nowadays largely used for evaluation of children with PAH^{8,9} However, they might have a lower value for the assessment of the regional function in different segments of the ventricles. In such cases, information provided by the speckle tracking technology might help in identifying the pattern of ventricular wall motion, based on a determination of free wall longitudinal peak systolic strain and right ventricular global longitudinal peak systolic strain as markers of right ventricular deformation associated with progression of the PAH.^{10,11}

However, similar to our study, recently published literature data indicates that conventional echo parameters characterizing the right ventricle function, as determined with 2D echocardiography, proved to remain valuable to assess the severity of PAH in children with congenital heart diseases.¹² Since in our study the etiology of the PAH was related to the presence of HIV infection, we did not record major congenital heart diseases in these patients, therefore the assessment of the regional function of the right ventricle could be considered less important than the assessment of the global function. Therefore, the parameters determined by 2D echocardiography and routine Doppler assessment, like in our study, could be considered valuable enough to represent a standard routine test for screening of the presence of PAH in the HIV-infected children population.

Other studies report lower values of the prevalence of the disease: severe forms in 4.9% of cases, in a group of children with peripartum exposure to HIV infection.¹³ The prevalence of primitive PAH associated with HIV was estimated at 0.5% in the pre-HAART stage¹⁴, a level that remains unchanged at 0.46%, and post-HAART¹⁵, the data provided being highly suggestive of the ineffectiveness of this therapy regarding the incidence and severity of PAH associated with HIV infection.⁴ The most pertinent explanation regarding the high differences in the prevalence cited above, is the difference related to the diagnostic methods used.¹⁵

On the other hand, it is possible that the results of the present study on PAH in children and adolescents with HIV infection record differences relative to those reported in similar studies, due to the unique epidemiological characteristic of the studied group, which consisted of patients belonging to a pediatric group with horizontally acquired HIV infection at a young age, in the late 1980s, due to a well-known epidemiological accident in Romania. The infection of this pediatric group with the F1 subtype of HIV is also special, the subtype representing less than 1% of the strains circulating globally.¹⁶

In the present study, LT-CD4 depletion was not a risk factor for the development of PAH syndrome. The literature also indicates the lack of a statistically significant correlation between pulmonary hypertension associated with HIV infection and the stage of HIV cell immunosuppression, although some authors report the value of LT-CD4 as an independent factor in the prognosis of survival in PAH associated with HIV infection and a more severe evolution of the syndrome in patients in the AIDS stage of the disease.¹⁶

Ultrasound examination revealed right ventricular dilatation in 92.59% of patients in the PAH group, in whom the mean diameter of the right ventricle was significantly higher compared to those in the control group. This feature is part of the ultrasound picture characteristic of this condition, which does not present particularities in HIV infected children and adolescents. It is worth mentioning that in the study group there was a subgroup of patients with dilated cardiomyopathy or isolated right ventricular dilatation without elevated pressure in the pulmonary artery. On the other hand, out of 79 patients with echocardiographic abnormalities, there were patients with systolic dysfunction, dilated cardiomyopathy, and other dominant pathologies. Those patients can also manifest a degree of ultrasound-identifiable pulmonary hypertension.¹⁷

A careful reporting to body weight is required to establish normal or pathological left ventricular dimensions.⁶ Pathological values were recorded in 2 patients who had mild concentric hypertrophy of the posterior and septum wall. The data presented indicates a lack of association between pulmonary hypertension syndrome and left ventricular hypertrophy. This finding is important because left ventricular hypertrophy is one of the most important cardiac changes in HIV infection with well-studied pathogenetic mechanisms.¹⁸ The lack of this association most likely indicates the intervention of different pathogenetic mechanisms or an individual susceptibility to developing pulmonary hypertension.¹⁹ Some data in the literature indicates susceptibility determined by certain genes of the major complex of histocompatibility in causing pulmonary hypertension syndrome²⁰ in which pathogenesis is intricate and multifactorial, and with the most frequently-mentioned pathogenetic mechanisms responsible for being vasculitic changes caused by the mediators of the inflammation and by the interaction of viral proteins with the molecular structure of the host which stimulates the production of endothelin-1 and platelet growth factor.^{1,2}

We did not find alterations in the myocardial contractile function in the PAH group, despite the fact that the presumed pathogenetic mechanisms of both this syndrome and those of myocardial dysfunction have many common links, represented mostly by the immune response to the released inflammatory mediators in response to HIV infection.^{15,21} On the other hand, there are

studies in adults that report a prevalence of 25% of left ventricular dysfunction in patients with pulmonary hypertension associated with HIV infection.²²

An important limitation of this study is the lack of comparison between echocardiographic data and catheterization data, knowing that there is a variation between echocardiographically and hemodynamically assessed pulmonary artery pressure and that the diagnosis of PAH is established with the use of right heart catheterization according to current guidelines.⁴

Conclusions

Pulmonary hypertension syndrome in children with horizontally acquired HIV infection is common and it evolves as a seemingly primitive condition of mild severity without being influenced by the clinical and immunological stage of the HIV infection.

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Anti-enterococcal activities of pentacyclic triterpenes

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Abstract

Background. Asiatic (AA) and ursolic (UA) acids are widely studied phytochemicals, but their antimicrobial properties are still poorly understood. Therefore our research has focused on their activity against uropathogenic *Enterococcus faecalis* strains.

Objectives. The aim of this research was to determine the influence of AA and UA on the growth, cell morphology, virulence factors and biofilm formation by *E. faecalis* strains.

Material and methods. AA and UA were purchased from Sigma-Aldrich. *E. faecalis* strains were isolated from the urine samples of patients with urinary tract infections. The strains were checked for the presence of virulence genes using the PCR method. Their antimicrobial susceptibility was performed using the disc diffusion method. The MICs of triterpenes were determined using the broth microdilution method. The hydrophobicity of cells was established by salt aggregation test. Lipase and lecithinase activities were determined by using an agar medium containing egg yolk emulsion. DNase agar was used for the detection of DNase synthesis. Hemolytic activity was established using a sheep-blood agar. Todd-Hewitt agar medium containing gelatin was used for determination of gelatinase activity. The anti-biofilm activity of asiatic acid and ursolic acid was tested on polystyrene microtiter plates. It was examined using time-kill and biofilm assays.

Results. Reduction of growth and enzyme synthesis after exposure of *E. faecalis* to the acids was observed. None of the acids changed the hydrophobicity of bacteria. Stronger anti-biofilm activity was observed when the bacteria were incubated with AA. Thus, reduction of both the survival and the virulence factors will make bacteria less infectious.

Conclusions. Based on the results obtained, we can assume that the triterpenes investigated should be considered natural components of a human diet rather than as antibacterial agents used on their own.

Key words: *Enterococcus faecalis*, pentacyclic triterpenes, asiatic acid, ursolic acid, antimicrobial agents

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Pentacyclic triterpenes are secondary metabolites widely distributed in the plant kingdom. They are the subject of numerous phytochemical and pharmacological studies. Plants with a high content of pentacyclic triterpenes are often used in herbal medicine due to their valuable therapeutic properties.¹ Asiatic acid (2 α ,3 β ,23-trihydroxyurs-12-en-28-oic, AA) and ursolic acid (3 β -hydroxyurs-12-en-28-oic, UA) belong to the group of pentacyclic triterpenes. AA is a secondary metabolite first found in medicinal plant *Centella asiatica* L. (*Apiaceae*). The leaves of this herb are used in the treatment of diverse human disorders.^{2,3} UA is present in a wide variety of plants, such as: apple and grape peels, cranberries, lavender, rosemary, thyme, oregano, salvia, basil, leaves of yerba mate and many others.¹ AA and UA have similar chemical formulas (Fig. 1), but they differ in the number of CH₃ and OH groups.

Many studies have confirmed the antitumor, antioxidant, anti-inflammatory, antiviral, antiprotozoal, antidiabetic, antihyperlipidemic and anti-osteoporotic, as well as neuroprotective and hepatoprotective activities of pentacyclic triterpenes.^{2–6} However, there are only a few studies describing the antimicrobial effects of these compounds.^{7–9} Usually the studies focus on the determination of the minimal inhibitory concentration (MIC) values of the triterpenes. The influence of triterpenes on bacterial virulence factors has not yet been precisely described. Our previous studies have shown that AA and UA change the virulence of uropathogenic *Escherichia coli* strains.^{10–12} Impairment of the virulence factors decreases the pathogenicity of bacteria and thus reduces the development of infection.

Enterococcus faecalis is a gram-positive bacteria often associated with serious nosocomial infections of which urinary tract infections (UTIs) are the most widespread. These infections can be difficult to treat because of the

frequent resistance of *E. faecalis* to multiple antibiotics.¹³ For this reason, the need for alternative solutions for UTI prophylaxis and treatment nowadays is more important than ever.

Therefore, the aim of our study was to establish the effect of AA and UA on growth, virulence factors and biofilm formation by uropathogenic *E. faecalis* strains isolated from urine samples of patients suffering from recurrent UTIs.

Material and methods

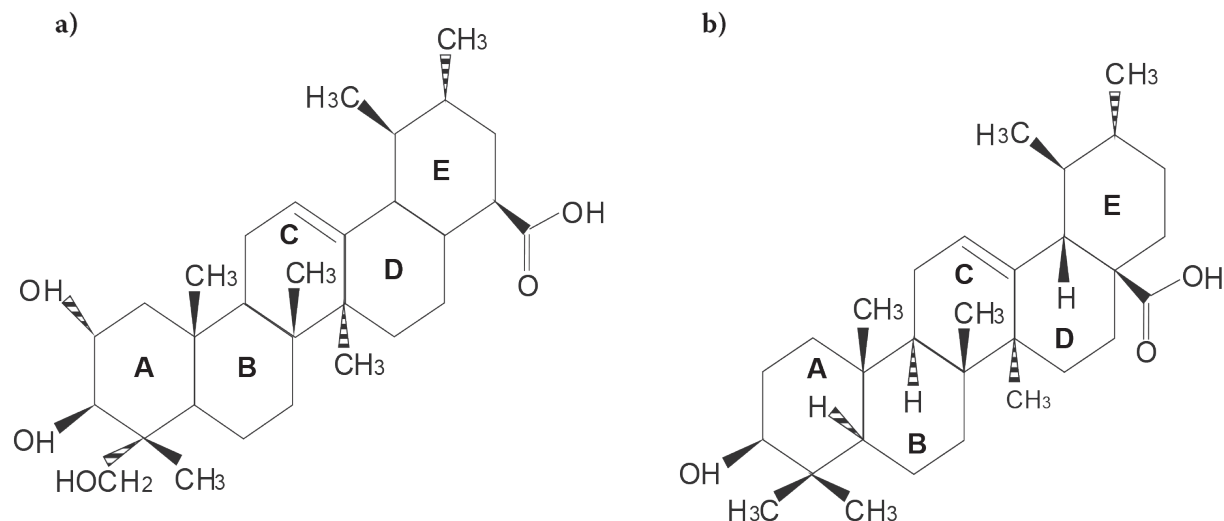
Isolation and identification of bacterial strains

E. faecalis strains (n = 10) were isolated from the urine samples of ambulatory patients with urinary tract infections. Bacteria were identified using the API ID32-Strep system (bioMérieux, Poland). The isolates were maintained on Mueller-Hinton agar slopes (Oxoid, UK) at 4°C. To confirm the membership of the tested strains to *E. faecalis* a PCR was performed with primers specific for the *E. faecalis* species.¹⁴

Antimicrobial susceptibility testing

Antimicrobial susceptibility of *E. faecalis* strains was performed using the disc diffusion method on Mueller-Hinton agar according to CLSI.¹⁵ The following antibiotics were tested: ampicillin (2 μ g), gentamicin (30 μ g), trimethoprim/sulfamethoxazole (trimethoprim 1.25 μ g and sulfamethoxazole 23.75 μ g), and nitrofurantoin (100 μ g). Bacteria were interpreted as susceptible or resistant according to the sensitivity zones of antibiotics as recommended by EUCAST.¹⁶

Fig. 1. Chemical structures of a) asiatic acid and b) ursolic acid. A, B, C, D, E – the names of rings in the molecules



Detection of virulence-related genes

Bacterial DNA was obtained by using GeneMATRIX Bacterial & Yeast Genomic DNA Purification Kit (EURx, Poland). The following virulence-related genes were amplified by PCR: gelE (gelatinase), esp (enterococcal surface protein), cylA (cytolysin activator) and cylB (cytolysin transporter), asa (aggregation substance) and ace (enterococcal adhesin to collagen). Specific primer sequences and expected amplicon sizes were described by Cosentino et al. and Creti et al.^{17,18} PCR amplification products were visualized and analyzed using the Quantity One Software (Bio-Rad, USA).

Pentacyclic triterpenes

Asiatic acid (AA, purity $\geq 97\%$) and ursolic acid (UA, purity $\geq 90\%$) were purchased from Sigma-Aldrich (Poznań, Poland) and dissolved in 96% ethanol to obtain stock solution. For all experiments, acids were diluted with Mueller-Hinton Broth (MHB; Biocorp, Poland).

Determination of minimal inhibitory concentration (MIC)

The MICs of AA and UA were determined by the broth microdilution method recommended by the CLSI.¹⁵

Effect of AA and UA on bacterial survival

The strains were grown overnight, and then transferred to MHB and incubated at 37°C for 30 min. Following incubation, the bacterial cells were centrifuged (4000 rpm/20 min) and suspended in PBS to reach the final density $1-2 \times 10^8$ CFU/mL. The bacterial suspension and AA or UA were mixed together to obtain $0.75 \times \text{MIC}$ of triterpene in each sample. Samples were incubated at 37°C for 24 h, then diluted and cultured on nutrient agar plates (Biomed, Poland). After 0, 2, 4, 6, and 24 h of incubation at 37°C, the number of CFU/mL was counted. Control samples contained no triterpenes.

Effect of triterpenes on bacterial morphology

E. faecalis strains were incubated at 37°C for 24 h with AA or UA at concentrations of $0.75 \times \text{MIC}$. The bacterial samples were then washed 3 times in PBS. The final pellets were Gram-stained and observed under microscope (Nikon Eclipse 400). The changes in bacterial cell morphology were recorded.

Effect of AA and UA on hydrophobicity of bacterial cells

The bacteria were grown overnight at 37°C in the presence of $0.75 \times \text{MIC}$ of AA or UA. Next, the bacteria were

harvested by centrifugation (4000 rpm for 20 min) and resuspended in PBS to obtain a final optical density (measured at 470 nm) of 1.0. The salt aggregation test (SAT) of ammonium sulphate was used.¹⁹ The lowest concentration of ammonium sulphate at which bacteria aggregated was determined. Based on the SAT values, the strains were classified as: 0.1–0.2 mol/L, very strongly hydrophobic; 0.4–1.0 mol/L, strongly hydrophobic; 1.2–1.6 mol/L, hydrophobic; 1.8–3.2 mol/L, hydrophilic.

Effect of AA and UA on enzyme activity

Lipase: Lipase activity was determined as described by Furumura et al.²⁰ The appearance of an opaque zone around the well indicated lipolytic activity.

Lecithinase: To determine lecithinase synthesis, 0.02 mL of bacterial suspension was inoculated onto plates with agar containing 10% egg yolk emulsion and incubated at 37°C for 24 h. The appearance of an opaque zone around the well indicated lecithinase activity.²¹

Hemolysins: The volume of 0.02 mL of bacterial suspension was spot-inoculated onto 5% sheep-blood agar plates and incubated at 37°C for 24 h. Hemolytic activity was confirmed by the appearance of a clear zone around the bacterial colonies.²²

Gelatinase: The volume of 0.02 mL of bacterial suspension was spot-inoculated onto Todd-Hewitt agar medium containing gelatin. Gelatinase production was confirmed by the appearance of a turbidity zone around the bacterial spots.²³

DNase: For detection of DNase synthesis, 0.02 mL of bacterial suspension was cultured on plates with DNase agar (Oxoid, UK). After 24 h of incubation at 37°C, the plates were flooded with 1 mol/L HCl. Transparent zones around the colonies indicated DNase activity.²⁴

Effect of AA and UA on biofilm production

The biofilm production assay was performed according to O'Toole and Kolter with slight modifications.²⁵ Briefly, diluted cultures ($1-2 \times 10^8$ CFU/mL) were inoculated into a polystyrene plate's wells containing 0.2 mL of MHB and AA or UA at a concentration of $0.75 \times \text{MIC}$. After incubation for 1–10 days at 37°C, the wells were rinsed. Bacterial cells attached to the plate's walls were stained with 1% (w/v) crystal violet (Sigma-Aldrich, Poland) and rinsed. The dye bound to bacteria was resolubilized with 95% (v/v) ethanol. The optical density (OD) was measured at 590 nm (Infinite® 200 PRO, TECAN, Switzerland). The ODC value was defined according to Stepanovic et al.²⁶ In the current research, the ODC was 0.047. *E. faecalis* strains were classified as follows: $\text{OD} \leq \text{ODc}$, no biofilm producer; $\text{ODc} < \text{OD} \leq 2 \text{ODc}$, weak biofilm producer; $2\text{ODc} < \text{OD} \leq 4 \text{ODc}$, moderate biofilm producer; $\text{OD} > 4 \text{ODc}$, strong biofilm producer.²⁶

Bacterial survival in biofilm in the presence of AA or UA

This experiment was done according to the method by Di Bonaventura et al.²⁷ Bacterial survival in biofilms was assessed after 1–10 days. Bacterial cultures were washed to remove non-adherent cells. Biofilms were scraped and transferred into microtubes containing PBS and centrifuged to separate the cells from the biofilm matrix. Bacteria were plated onto nutrient agar plates and the CFU/mL was counted.

Statistical analysis

All values are expressed as a mean \pm SD of 3 independent experiments. Statistical differences between bacterial strains exposed to triterpenes and unexposed (controls) were analyzed by non-parametric Kruskal-Wallis test followed by a Dunn's multiple comparison test. All statistical analysis was performed using STATISTICA v. 12.0 (StatSoft, Poland). Values of $p < 0.05$ were considered statistically significant.

Results

In our study, ten clinical *E. faecalis* strains possessed virulence-related genes: gelE, esp, cylA, cylB, asa, and ace, which are important in the pathogenesis of enterococcal infections.²⁸

All tested strains of *E. faecalis* demonstrated high level resistance to gentamycin (HLGR), 60% were resistant to nitrofurantoin, 50% to ampicillin, and 40% to trimethoprim/sulfamethoxazole (Table 1).

The MICs of AA and UA against *E. faecalis* isolates are presented in Table 2. The MICs of AA were 64 and

128 $\mu\text{g/mL}$. The MICs of UA were in the range of 32–512 $\mu\text{g/mL}$. No correlation was noticed when the susceptibility pattern and MIC values of pentacyclic triterpenes were compared.

In the current study, the effect of AA and UA on the growth of planktonic forms of *E. faecalis* was examined. The anti-growth effect of both triterpenes was observed after 2, 4, 6 and 24 h of incubation (Fig. 2). The greatest activity of AA was noticed after 24 h (Kruskal-Wallis test, $H = 17.841$, $p = 0.0001$; Dunn's Multiple Comparison test, $p = 0.00007$). The survival of bacteria was decreased 250-fold in comparison to the control sample (control – 37.5×10^7 CFU/mL; AA – 0.15×10^7 CFU/mL). The statistically significant bactericidal activity of UA was noticed after 2, 4 and 6 h of incubation (Kruskal-Wallis test, $H = 17.841$, $p = 0.0001$; Dunn's Multiple Comparison test, $p = 0.00007$). The most reduced survival was observed after 6 h of incubation. The CFU/mL were 32.7×10^7 and 0.042×10^7 in the control and the sample with UA, respectively.

We also found alterations of the size and arrangement of bacterial cells in samples containing AA (Fig. 3). *E. faecalis* cocci cultured in presence of AA were bigger than those incubated in UA, and formed aggregates instead of chains, in comparison to untreated bacteria.

In the current study, we demonstrated that all clinical *E. faecalis* strains possess a very strong hydrophobic cell surface, which confirms the important role of this trait in non-specific mechanism of adhesion. Unfortunately, our experiments have shown that none of the acids changed the hydrophobic nature of the *E. faecalis* isolates.

We also determined the effect of AA and UA on the ability of *E. faecalis* strains to synthesize lipase, lecithinase, DNase, hemolysin and gelatinase.

As shown in Fig. 4a, AA significantly reduced the synthesis of lipase in 4 *E. faecalis* strains (Dunn's Multiple Comparison test, $p < 0.05$), and UA in 6 cases (Dunn's Multiple Comparison test, $p < 0.05$). One of the tested strains significantly increased lipase production after the treatment by UA. The opaque zone diameters became wider in comparison with untreated bacteria (Dunn's Multiple Comparison test, $p < 0.001$).

In our study, 8 out of 10 strains synthesized lecithinase in control conditions. It is worth noting that AA significantly decreased production of this enzyme in 5 *E. faecalis* strains (Dunn's Multiple Comparison test, $p < 0.05$) and completely inhibited its secretion in one case (Dunn's Multiple Comparison test, $p < 0.001$) (Fig. 4b). Two strains of cocci grown in the presence of UA completely stopped the synthesis of lecithinase (Dunn's Multiple Comparison test, $p < 0.001$).

As shown in Fig. 4c, UA decreased the secretion of hemolysins in 4 *E. faecalis* strains (Dunn's Multiple Comparison test, $p < 0.05$) and completely inhibited hemolytic activity in 3 cases (Dunn's Multiple

Table 1. Susceptibility pattern of *E. faecalis* strains

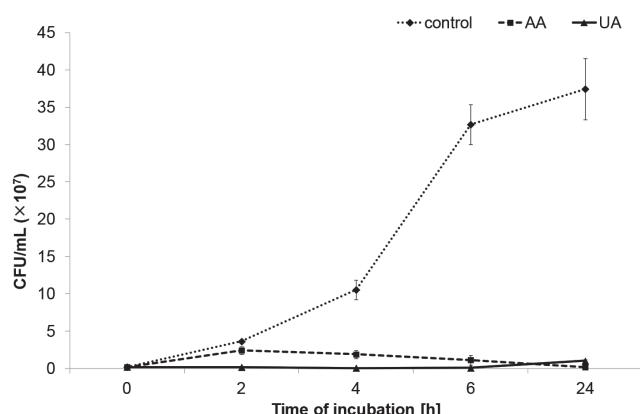
	<i>E. faecalis</i> strain No.									
	3	4	9	11	38	51	69	71	80	83
Ampicillin	R	S	S	S	R	R	R	S	S	R
Gentamycin	R	R	R	R	R	R	R	R	R	R
Trimethoprim/ Sulfamethoxazole	S	S	R	S	S	R	S	S	R	R
Nitrofurantoin	R	R	R	R	S	S	R	S	S	R

R – resistant; S – sensitive.

Table 2. The MICs of AA and UA against *E. faecalis* strains

	<i>E. faecalis</i> strain No.									
	3	4	9	11	38	51	69	71	80	83
AA ($\mu\text{g/mL}$)	128	64	64	128	64	64	64	64	64	64
UA ($\mu\text{g/mL}$)	64	64	64	64	512	256	128	32	128	64

Fig. 2. Effect of asiatic acid (AA) and ursolic acid (UA) on the growth of the planktonic forms of *E. faecalis* clinical strains. Values represent the mean \pm SD of three separate experiments for ten strains



Comparison test, $p < 0.001$). The anti-hemolytic activities of AA were significant in 5 *E. faecalis* strains (Dunn's Multiple Comparison test, $p < 0.05$ or $p < 0.001$).

In our study, 8 out of 10 strains synthesized gelatinase in control conditions (Fig. 4d). The anti-gelatinase effect of triterpenes was noted in the case of 5 strains incubated with AA and 2 strains incubated with UA (Dunn's Multiple Comparison test, $p < 0.05$ or $p < 0.001$).

The results of the current study showed that only 3 *E. faecalis* strains produced DNase. The synthesis of this enzyme was completely suppressed only in 1 isolate treated with UA (Dunn's Multiple Comparison test, $p < 0.001$).

The results showing the influence of AA and UA on biofilm formation by *E. faecalis* strains are given in Fig. 5a. The cultures of untreated *E. faecalis* strains incubated up to 10 days produced weak ($0.047 < OD \leq 0.094$) or moderate ($0.094 < OD \leq 0.188$) biofilm. The reduction of biofilm was observed when bacteria were incubated with AA during the whole time of observation. The mean OD values were ≤ 0.047 . There were significant differences between OD values of biofilm in the presence of AA and control samples after the 1st, 7th and 10th days of incubation (Dunn's Multiple Comparison test, $p < 0.05$ or

$p < 0.001$). A weaker effect on the biofilm formation ability was exerted by UA. The OD values were lower compared to the control samples with the exception of the 2nd, 3rd and 8th day. Bacteria incubated in the presence of UA still produced weak or moderate biofilm. The mean OD value was 0.039. There were no significant differences between the OD values of biofilm in the presence of UA and control samples (Dunn's Multiple Comparison test, $p > 0.05$).

In our study, the bacterial survival in biofilm mass was determined after each time of incubation. As shown in Fig. 5b, the number of bacterial cells per mL has been decreased in cultures containing both AA or UA compared to control samples. In the biofilms formed in the presence of AA, the CFU/mL ranged from 0.003×10^7 to 0.107×10^7 . The values of CFU/mL in the biofilms treated with UA was much higher and ranged from 0.130×10^7 to 35.9×10^7 . There were significant differences between the CFU/mL in the biofilms treated with AA and control samples (Dunn's Multiple Comparison test, $p < 0.001$). Statistically significant differences in CFU/mL were present between the biofilms treated with UA and untreated after the 1st, 2nd and 3rd day of incubation (Dunn's Multiple Comparison test, $p < 0.05$).

Discussion

The search for alternative methods of treatment of infections caused by antibiotic-resistant bacteria is a major challenge of medicine today. Currently, an increased interest can be observed in plant compounds that can be used to support the standard therapy or to prevent bacterial and fungal infections. Among others, pentacyclic triterpenes are being intensively examined due to their multiple pharmaceutical activities combined with relatively low toxicity to eukaryotic cells.^{2–6}

The MICs of AA were 64 or 128 $\mu\text{g/mL}$ for all studied strains. Similarly, Taemchuay et al. showed that the

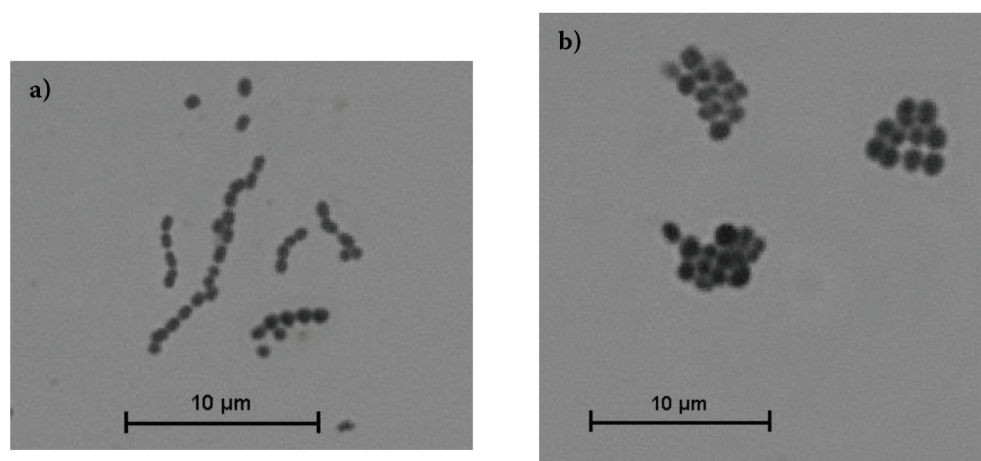
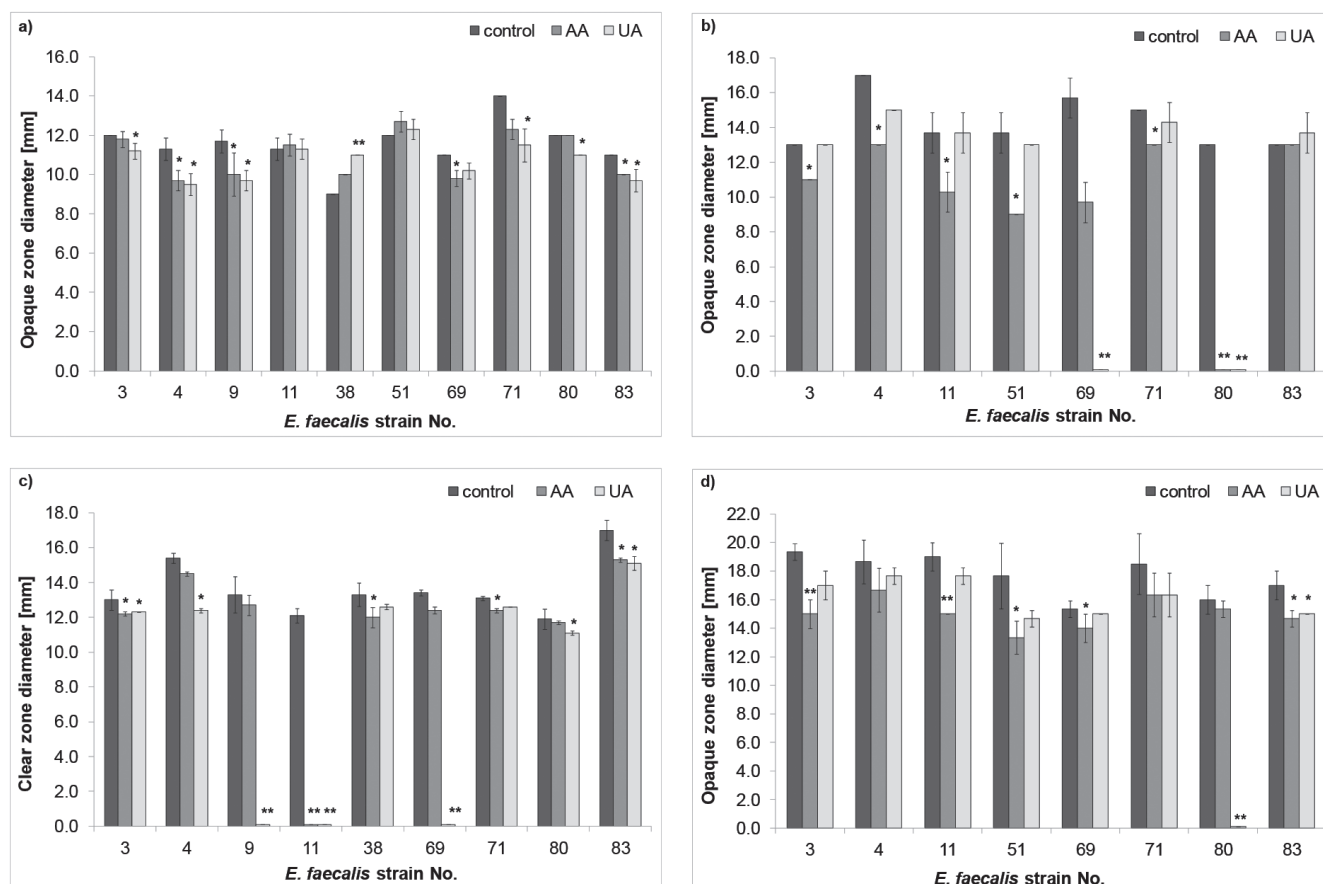


Fig. 3. Effect of asiatic acid on size and arrangement of the cells of *E. faecalis* strains; a) bacteria untreated, b) bacteria treated with AA. Magnification $\times 1000$

Fig. 4. Effect of asiatic acid (AA) and ursolic acid (UA) on synthesis of a) lipase; b) lecithinase; c) hemolysin; d) gelatinase by *E. faecalis* clinical strains. Dunn's Multiple Comparison test, * $p < 0.05$; ** $p < 0.001$



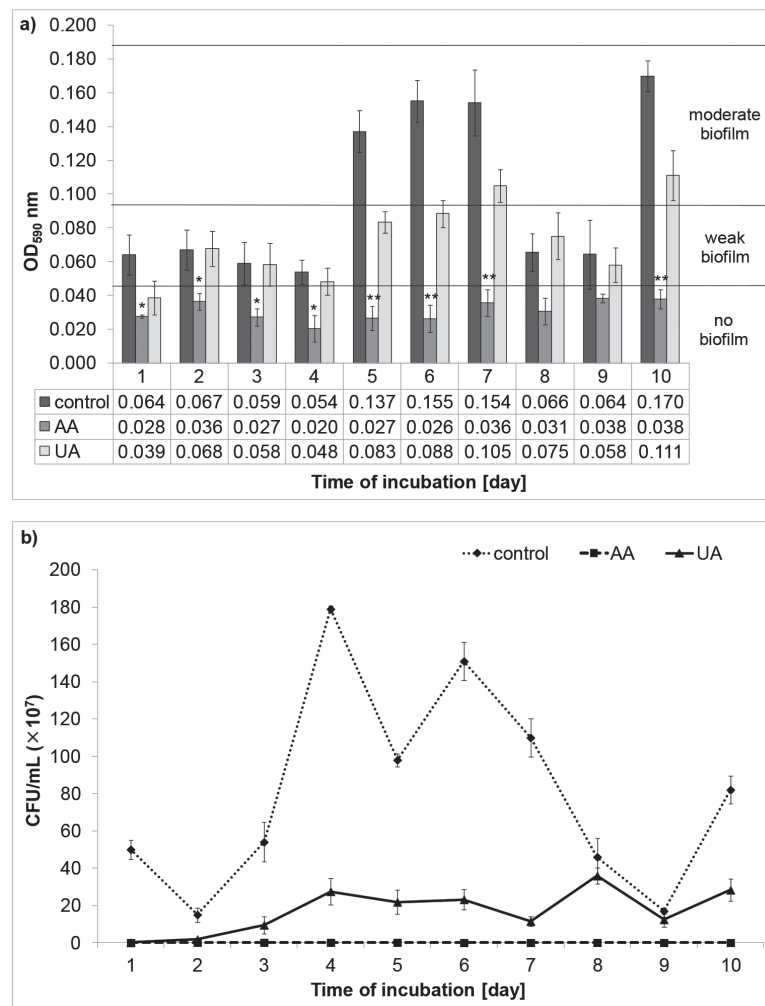
MIC values of AA against *Staphylococcus aureus* ranged from 20 to 160 $\mu\text{g}/\text{mL}$.²⁹ In contrast to our results, Liu et al. showed the MICs of AA against gram-positive (*E. faecalis*, *S. aureus*, *Listeria monocytogenes* and *Bacillus cereus*) and gram-negative (*Escherichia coli*, *Salmonella typhimurium* and *Pseudomonas aeruginosa*) bacteria were significantly lower and ranged from 20 ± 2 to 36 ± 4 $\mu\text{g}/\text{mL}$.³⁰ The differences in MICs may be caused by differences in methods used for MIC value determination, different species of tested bacteria and various sources of origin of these microorganisms. In contrast to results obtained by Horiuchi et al., the MIC values of UA in our study were much higher and for different strains ranged from 32 to 512 $\mu\text{g}/\text{mL}$.³¹ Our results are similar to those published by Fontanay et al., who established that the MIC values of UA against clinical strains of *E. faecalis* and *S. aureus* were ≥ 256 $\mu\text{g}/\text{mL}$.³² However, the results of Horiuchi et al. as well as our previous studies have shown that the MICs of AA and UA against gram-negative bacteria were higher than the MICs for gram-positive strains.^{10–12,31} Such discrepancy is associated with the structural differences between the cell walls of these 2 types of bacteria. Gram-negative bacteria have an outer membrane with lipopolysaccharide in its

outer leaflet and phospholipids in the inner leaflet. This specific structure is an effective barrier protecting gram-negative rods from chemical factors.³³

Our results showed that UA has better anti-growth activity to planktonic forms of *E. faecalis* than AA. Kim et al. have also demonstrated that UA used in a sub-inhibitory concentration ($0.5 \times \text{MIC}$) reduced the survival of planktonic forms of the oral *Streptococcus sobrinus* strain.³⁴ Such activity of UA may be related to the differences in the chemical structure of the examined triterpenes (Fig. 1). The number of hydroxyl and methyl groups is different in AA and UA. It should be noted that AA has 6 methyl groups attached to C4, C8, C10, C14, C19, and C20. UA contains one more methyl group (in the C4 position), therefore is more hydrophobic than AA. Moreover, Broniatowski et al. have found that pentacyclic triterpene acids interact with the phospholipids of bacterial membranes.³⁵ In particular, UA disintegrates cardiolipin-rich domains present in membranes, which can lead to the destruction of bacterial cells and their death.

The alterations of the size and arrangement of bacterial cells found in our research were similar to those observed by Ramirez-Arcos et al. for *E. faecalis* strains with a mutation in a gene encoding for a protein responsible for cell di-

Fig. 5. Effect of asiatic acid (AA) and ursolic acid (UA) on a) biofilm formation and b) bacterial survival. Values represent the mean \pm SD of 3 separate experiments for ten *E. faecalis* clinical strains. Dunn's Multiple Comparison test, * $p < 0.05$; ** $p < 0.001$



vision and chromosome segregation.³⁶ It was demonstrated that abnormal chromosome segregation and disruptions of cell division can cause the phenotypic changes in the cells morphology e.g. increase the cell diameter and the occurrence of irregular groupings of cells. Therefore, it can be assumed that AA interferes with cell division processes.

The first step in the pathogenesis of UTIs is adhesion of the microorganism to host tissues. One of the important factors involved in this process is the hydrophobic character of the bacterial cell surface.¹⁰ The current research has shown no activity of both pentacyclic triterpenes on cell surface hydrophobicity. Our previous study showed that the impact of AA and UA on this virulence factor of clinical *E. coli* rods was very weak.¹⁰

Another important mechanism in the pathogenesis of UTIs is very likely associated with the secretion by bacteria of the host's tissue-damaging extracellular enzymes (e.g. lipases, lecithinase, gelatinase, DNase) and toxins (e.g. hemolysins).³⁷ They belong to proteins which can be transported across the plasma membrane.³⁸ Liu et al. established that AA destroys the cell membrane integrity

and causes its dysfunction.³⁰ Also, Broniatowski et al. indicated that UA may incorporate into the bacterial membrane leading to its structural and functional alterations.³⁵ Cell membrane damage may impair the transport of enzymes and toxins from the bacteria to the external environment. Therefore, our results showed that secretion of lipase, lecithinase, gelatinase, DNase and hemolysin by *E. faecalis* strains decreased after incubation with AA or UA. No other articles have been published regarding the antibacterial activity of pentacyclic triterpenes on bacterial protein secretion, therefore there was no possibility to compare our results with others.

Pathogenic microorganisms rarely exist as single cells. Bacteria usually form biofilms consisting of exopolysaccharide-surrounded microcolonies. Pathogens growing in a biofilm mass are a serious threat to human health because of their resistance to immune system factors and antibiotics. The better anti-biofilm activity of AA than UA might be associated with the chemical nature of these acids. AA possesses 3 hydroxyl groups (C2, C3, C23) that make it hydrophilic. UA has only one hydroxyl group (C3) and therefore is hydrophobic. Probably due to its hydrophilic nature, AA better penetrates into biofilm structures. The influence of UA on biofilm formation has been reported by other researchers.^{8,9} Ren et al. found that UA inhibited biofilm formation in gram-negative *E. coli*, *Pseudomonas aeruginosa* and *Vibrio harveyi* strains when added to inoculum or to 24 h biofilms.⁸ Zhou et al. noticed that 0.25 MIC of UA

affected biofilm production by gram-positive *Streptococcus mutans* and *Streptococcus gordonii*, while lower concentrations of this pentacyclic triterpene (0.125 \times MIC and 0.0625 \times MIC) weakly inhibited biofilm development.⁹

The survival of *E. faecalis* in biofilm mass decreased after the treatment in both triterpenes. Garo et al. evaluated that AA applied alone did not reduce the cell viability of *P. aeruginosa* biofilms. However, this pentacyclic triterpene increased the susceptibility of biofilm bacteria to antibiotics.³⁹

Based on the results obtained, we can assume that the triterpenes investigated should be considered natural components of a human diet rather than as antibacterial agents used on their own.

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Effect of BMI on quality of life and depression levels after bariatric surgery

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Abstract

Background. Studies conducted in Poland have found that 1% (~300,000) of Polish adults are obese. The degree of weight loss and reduction of discomfort associated with severe obesity are used to evaluate bariatric surgery outcomes. From the patient's point of view, QoL and mental health are the most important determinants of successful surgery, which is why interest in QoL assessment has increased.

Objectives. To assess the effect of BMI on quality of life and depression levels depending on the type of bariatric surgery.

Material and methods. The group included 57 women and 43 men aged 20–60 years (mean age 40 years) with BMI from 36 to 40 (31%) and > 40 (69%). Twelve patients (12%) underwent laparoscopic adjustable gastric banding (LAGB), 58 (58%) sleeve gastrectomy, and 30 (30%) Roux-en-Y Gastric Bypass (RYGB). The Bariatric Analysis and Reporting Outcome System (BAROS) was used to assess QoL. The severity of mood disorders was assessed using the Self-Rating Scale of Depression and Anxiety.

Results. Six months or 1 year after bariatric surgery, the number of patients with BMI > 40 had decreased from 69 to 14%. We found that the time since bariatric surgery contributed to a significant ($p < 0.01$) difference in BAROS outcomes. In the long-term perspective, we observed better quality of life.

Conclusions. MA-QoL II is a useful tool in assessing bariatric surgery, including quality of life. Long-term monitoring will be essential in determining psychological changes and the degree of weight loss.

Key words: quality of life, depression, anxiety, bariatric surgical procedures

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In recent years, the number of people with obesity has significantly increased worldwide.¹ Recent data from the World Health Organization (WHO) indicate that 1.6 billion people are overweight and 400 million are obese (BMI > 40 kg/m²).² The results of a nationwide Polish study, conducted under the NATPOL, Household Food Consumption and Anthropometric Survey (HF-CAS), and WOBASZ programs, have shown that 1% of Poles have obesity (BMI > 40 kg/m²), which is approximately 300,000 people. In selected rural populations, this percentage increased to 2.8%, and in urban areas to 3%.^{3–6}

Obesity leads to serious medical complications, such as type 2 diabetes, cardiovascular and respiratory diseases (e.g. coronary artery disease, myocardial infarction, hypertension, Chronic Obstructive Pulmonary Disease, embolism), as well as certain hormone-dependent cancers or colon cancer, osteoarthritis and infertility in women, and impotence in men.^{6–8} The psychological consequences of obesity include low self-esteem, anxiety and depression.⁹

In addition to the physical consequences, obesity causes psychosocial disability and decreases quality of life (QoL). Patients have significant limitations in performing many daily tasks. Most have professional problems and difficulty finding a job due to appearance, the high cost of treatment, and frequent absenteeism.¹⁰ Social isolation and addiction are widespread and many patients die prematurely.^{9–12}

Conservative treatment based on a low-calorie diet, lifestyle modifications, increased physical activity, and drug therapy is not always effective. Currently, the best long-term results are achieved through bariatric surgery.^{13–16} The degree of weight loss and reduction of discomfort associated with severe obesity are used to evaluate bariatric surgery outcomes.¹⁷ The main evaluation results of bariatric surgery have traditionally focused on the degree of weight loss and control of comorbidities.^{17,18} From the patient's perspective, an important element in assessing the effectiveness of surgery is an improvement in psychosocial functioning and quality of life.¹⁹

The aim of this study was to assess the effect of BMI on QoL and depression levels depending on the type of bariatric surgery.

Material and methods

The study was conducted at the Department of General and Endocrine Surgery, Medical University of Białystok, Poland, on 100 patients after bariatric surgery. The research was conducted using the prospective-cohort method. A sample of patients was recruited with consecutive admission to the hospital.

The group included 57 (57%) women and 43 (43%) men aged 20–60 years (mean age 40 years) with BMI from 36 to 40 (31%) and > 40 (69%). Comorbidities included:

hypertension (35%), type 2 diabetes (28%), cholelithiasis (22%) and lower limb varicose veins (8%). Nine patients underwent *Helicobacter pylori* eradication therapy before surgery. The reason for bariatric surgery in 72% of the patients was health problems. People with secondary (35%) and higher education (32%) more frequently decided to undergo surgery.

Twelve patients (12%) underwent laparoscopic adjustable gastric banding (LAGB), 58 (58%) sleeve gastrectomy, and 30 (30%) Roux-en-Y Gastric Bypass (RYGB).

Measurement

The Bariatric Analysis and Reporting Outcome System (BAROS) was used to assess quality of life. BAROS was developed by psychologists Moorehead and Ardelt-Gattinger for patients with severe obesity.^{20,21} BAROS evaluates the percent of excess weight loss (%EWL) and excess BMI loss (EBL) calculated using the formula: %EBMIL = 100 - [(current BMI - 25 / initial BMI - 25) × 100], improvement and/or control of comorbidities, and 5 aspects of QoL (self-esteem, physical, social, professional and sexual activity, complications and reoperations). The final score is based on improvement, deterioration or no change in the 5 aforementioned aspects. Each aspect is rated as: much worse, worse, unchanged, improved or very improved, scored, respectively: -0.5, -0.25, 0, +0.25 and +0.5.

The severity of mood disorders (depression, anxiety) was assessed using the Self-Rating Scale of Depression and Anxiety developed by Kokoszka.²² It is possible to record the following on the 10-point scale: mood, energy, strength of interests, experiencing pleasure, speed of thought and action, anxiety, mental tension, nervousness, fear of a specific threat, fear of what might happen, feelings of physical stress, and avoiding anxiety-inducing situations. Internal consistency of the scale for anxiety and depression is 0.96. Each statement can receive from 0 to 10 points; the maximum number of points in each subscale is 100. In interpreting the results, we assumed that 0–8 points meant that the existence of depressive and/or anxiety disorders was low; 9–27 points meant an average possibility of a depressive and/or anxiety disorder. The higher the score from 28 to 100 points meant the greater the severity of the disorders. The results are translated into a sten score. The results from 0 to 8 points (sten score 1–4) should be interpreted as the existence of depressive and/or anxiety disorders is low, results from 9 to 27 points (sten score 5–6) as average, results from 28 to 100 points (sten score 7–10) as high.²² We compared well-being before and after surgery.

An analysis of the medical records of the patients who underwent bariatric surgery consisted of obtaining information on the occurrence of postoperative complications and the severity/amelioration/regression of comorbidities.

The study was conducted in the years 2013–2014. Patients were examined 6 months or 1 year after surgery during a check-up at the Outpatient Surgery Clinic.

The study was approved by the Bioethics Committee of the Medical University of Białystok (number 123-24579P).

Inclusion criteria

Patients included in the study met the guidelines developed by the National Institute for Health and Clinical Excellence (NICE) for bariatric surgery. These include: BMI > 40 or BMI > 35 with at least 1 comorbidity (e.g. cardiovascular diseases, sleep apnea, type 2 diabetes, musculoskeletal diseases caused by obesity impairing typical physical activity, etc.).^{3,15}

Exclusion criteria

The study excluded patients with contraindications to anesthesia administration.^{3, 15}

Surgical procedures

Laparoscopic adjustable gastric banding (LAGB) was performed by placing a silicone band around the upper part of the stomach. The diameter of the band was controlled by a liquid feed through a port implanted under the skin above the left costal arch and connected by a drain with the band. The band divides the stomach into two parts: an upper pouch with a volume of about 25–40 mL, and a much bigger bottom section. Sleeve gastrectomy (SG) was done laparoscopically by total vertical stomach resection of the greater curvature.

The Roux-en-Y gastric bypass (RYGB) consisted of 3 steps: stomach reduction, gastroenterostomy, and then enteroenterostomy. All procedures were performed laparoscopically.

Table 1. BMI of patients before and after bariatric surgery

BMI (kg/m ²)	Patients before surgery n (%)	Patients after surgery n (%)	p-value
25–30	–	13 (13)	0.001
31–35	–	38 (38)	
36–40	31 (31)	35 (35)	
> 40	69 (69)	14 (14)	

Statistical analysis

Data analyses were conducted using the Statistical Package for Social Sciences (SPSS) v. 20.0. Questionnaire results are presented in the Table and Figures.

An analysis of the differences of the results of the brief Self-Rating Scale of Depression and Anxiety and BAROS was performed using the nonparametric test Wilcoxon Signed Ranks Test. A level of $p < 0.05$ was considered statistically significant.

Results

Six months or 1 year after bariatric surgery (LAGB, SG, RYGB), the number of patients with BMI > 40 had decreased from 69% to 14% ($p < 0.001$). Weight loss decreased BMI to 25–30 in 13% and 31–35 in 38% of the patients. Such BMI had not been observed before the treatment (Table 1).

Analyzing the occurrence of depression and anxiety by using the Self-Rating Scale of Depression and Anxiety after bariatric procedures, it was found that 16% of subjects had a low level of depression and anxiety. Almost half (48%) of those surveyed had an average severity of depression and anxiety and a group of 36% of the respondents had high levels (Fig. 1).

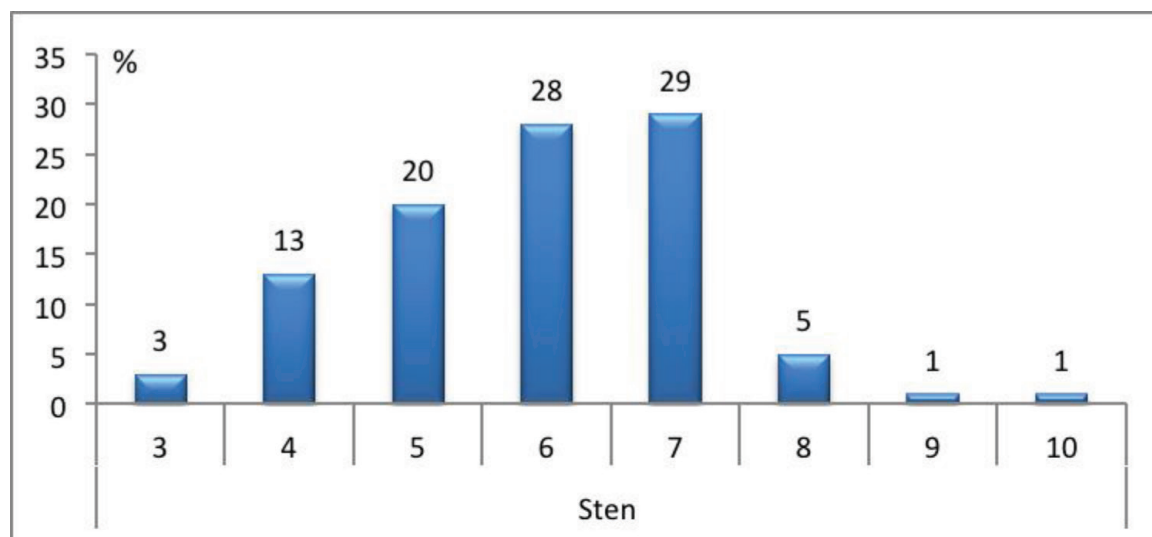


Fig. 1. Depression and anxiety severity among patients after bariatric procedures

Results from sten score 1–4 means a low, 5–6 means an average, and 7–10 means a high existence of depressive and/or anxiety disorders.

Final evaluation of QoL on BAROS showed 62% of patients had good, very good or excellent bariatric surgery outcomes (Fig. 2). Eighteen percent of patients did not have positive outcomes.

The time since surgery had no significant effect on the results on the Self-Rating Scale of Depression and Anxiety in the group of treated patients. However, we found that the time since bariatric surgery contributed to a highly significant ($p < 0.01$) difference in BAROS outcomes (Fig. 3). We found that the longer since surgery the higher the final BAROS score, from an average of 1.95 (median 2) for up to 6 months, through an average of 2.74 (median 3) in the period from half to 1 year, up to an average of 3.3 (median 3.5) for over 1 year.

In this study, there was no evidence that surgery type (LAGB, SG, RYGB), age, or sex had any effect on the re-

sults of the symptoms of depression and anxiety, and were independently associated with the QoL assessed on BAROS (Figs. 4–6).

Discussion

Bariatric surgery is now the last chance for good long-term outcomes when conservative and pharmacological therapy do not produce results.

BAROS, introduced by Oria and Moorehead in 1998, is an established and recognized tool for QoL evaluation in people with obesity.^{20,21} Bobowicz et al. used BAROS to assess sleeve gastrectomy (SG) outcomes in 84 patients 5 years after surgery.²³ Positive changes were achieved in QoL in the physical, social and professional domains; and moderate improvement was noted in the sexual domain. An overall very good result was achieved in 30% of patients, whereas no effects were reported by 13% of respondents. Better results are achieved by women, who had 46.5% excess weight loss (EWL) compared with 35.3% for males.

Ribaric presented a 3-year follow-up HRQoL on BAROS of patients operated on in France using the Swedish adjustable gastric band (SAGB) method.²⁴ The results were evaluated in the preoperative period and 1, 3, 6, 12, 18, 24 and 36 months after surgery. It was found that weight loss resulted in improved QoL over the 3 years of observation. The overall BAROS score increase from 1.4 preoperatively to 3.6 (2.2, $p < 0.001$) after 3 years.

Zuger et al. evaluated RYGB and LAGB as treatment methods for obesity.²⁵ After 5-year observations, they accepted 52% EWL after LAGB and 79% EWL after RYGB as effective criteria.

We found that the time since bariatric surgery, regardless of type, contributed to a highly significant ($p < 0.01$) difference in BAROS outcomes. Also, the longer since surgery the higher the final BAROS assessment.

Fig. 2. BAROS evaluation of bariatric surgery outcomes

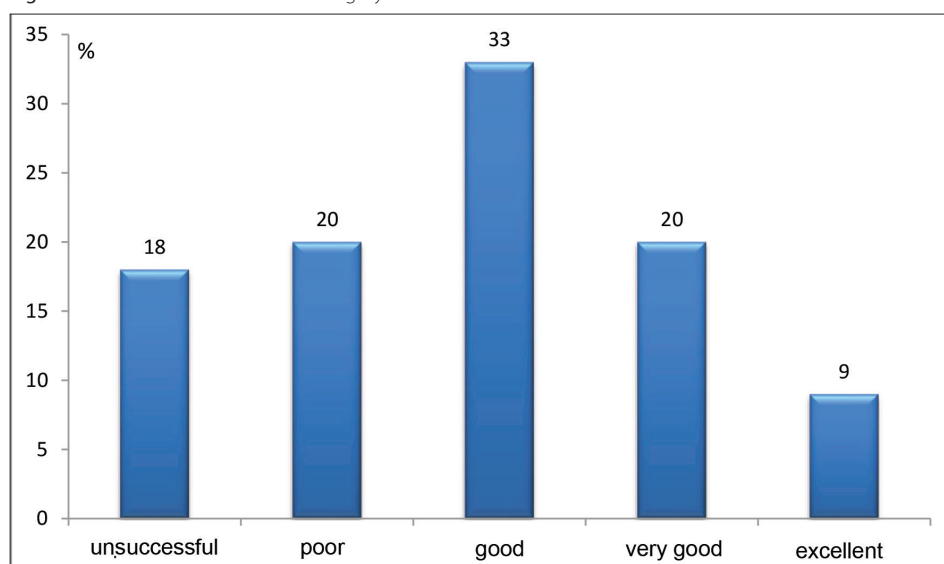
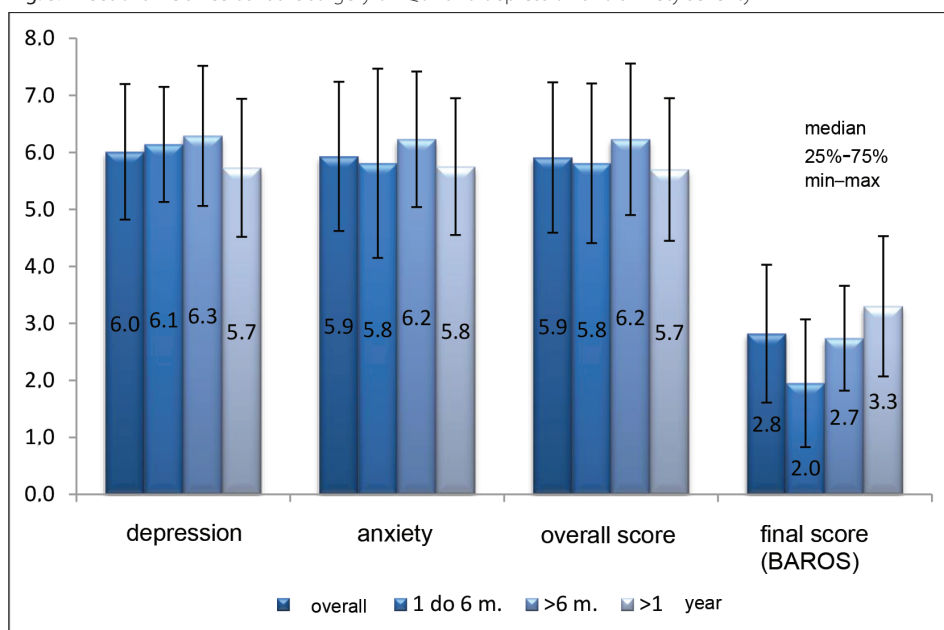


Fig. 3. Effect of time since bariatric surgery on QoL and depression and anxiety severity



More than half (62%) of the patients assessed the surgery outcome as good, very good or excellent. Eighteen percent of patients did not have positive outcomes. Similar studies were conducted in the same center by Dadan et al., who evaluated patient QoL 6 months after LAGB and RYGB.²⁶ They found that in both groups (LAGB and RYGB), QoL was evaluated as much better by 55% of the patients, better by 42%, and unchanged by 3%. Sierżantowicz et al. found a highly significant difference ($p < 0.0001$) in BMI changes depending on surgery type.²⁷ A higher reduction in BMI was achieved in RYGB surgery than LAGB and sleeve gastrectomy. Six months after surgery, body weight was reduced by an average of 30% EBL.

To assess the QoL of patients after bariatric surgery

in the physical dimension, it is necessary to analyze laboratory parameters, including ghrelin, insulin, glucose, triglycerides, HDL and LDL cholesterol. Abnormal values will help to determine the risk factors or types of comorbidities. Effective bariatric treatment reduces excess weight, improves the patient's general condition, and normalizes metabolic parameters, which significantly improves QoL. These assumptions have been confirmed by research conducted by Hady H.R. et al.^{28,29}

Given the multidimensional nature of QoL, in addition to the clinical evaluation of patients after bariatric surgery, psychological assessment is equally important. Burgmer et al. conducted a study with the aim to assess QoL and identify psychological problems.³⁰ Symptoms of anxiety and depression were analyzed using a questionnaire (Hospital Anxiety and Depression Scale – HADS) 1 and 2 years after bariatric surgery. The study showed significant differences between baseline assessment and the 2-year follow-up, where the severity of depressive symptoms in 40.5% of patients decreased to 16.4%. No changes were observed in the evaluation of anxiety in the studied group.

Prior to surgery, 48% of the patients indicated average depression and anxiety levels measured on the Self-Rating Scale of Depression and Anxiety. Repeated evaluation 6 months or 1 year after bariatric surgery showed a downward trend of depression and anxiety symptoms. There were no statistically significant differences between surgery type, sex or age.

Fig. 4. Effect of depression, anxiety and QoL quality of life according to of the type of bariatric procedure

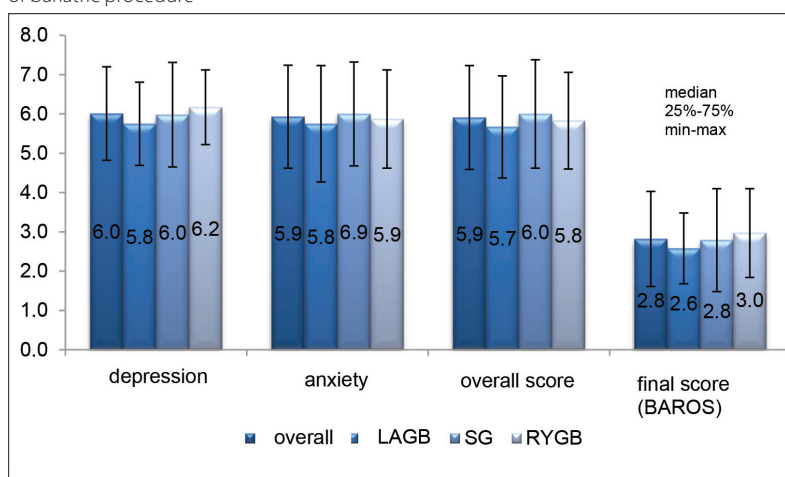


Fig. 5. The level of depression, anxiety and QoL in relation to age

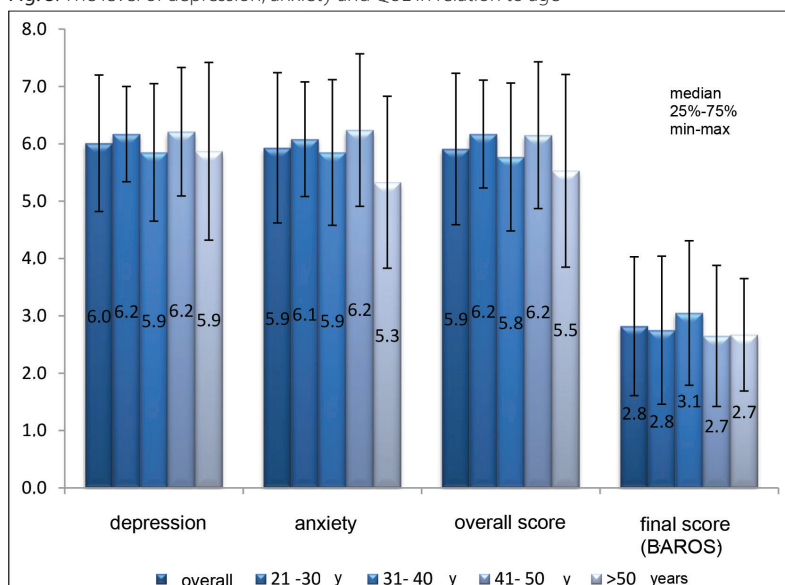
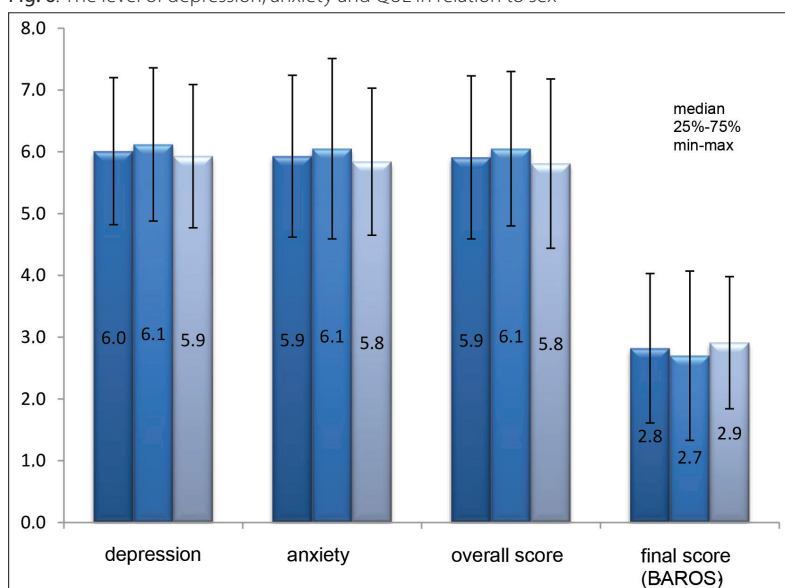


Fig. 6. The level of depression, anxiety and QoL in relation to sex



Study limitations

The study should be repeated 2–5 years after bariatric surgery to assess QoL and depression levels.

In conclusion, our results are consistent with those studies that found improvement but not in all aspects of HRQoL. The time since surgery had the greatest impact on QoL after bariatric surgery, regardless of type. We have come to the conclusion that MA-QoL II is a useful tool in assessing bariatric surgery, including QoL. Long-term monitoring will be essential in determining psychological changes and the degree of weight loss.

The individually selected bariatric surgery procedure determines the effectiveness of treatment by average weight loss and long-term maintenance. This includes improving health, control of comorbidities, reduction of psychological problems, and high QoL. Currently, the laparoscopic method dominates, and multidisciplinary teams are more and more appreciated in perioperative care, guaranteeing the safety and effectiveness.

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Trends in the prevalence of autoimmune thyroiditis in the leading private health-care provider in Poland

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Abstract

Background. Autoimmune thyroiditis (AT) is one of the most common endocrine disorders affecting a significant percent of the population, especially women. It may have serious and long-lasting consequences. The etiology of AT is multifactorial and it arises from an interplay between environmental and genetic factors. Tendencies in AT prevalence and incidence are unclear. In Poland there are no national registers covering the data on AT prevalence.

Objectives. The aim of the investigation was to assess changes in diagnosing AT in the largest chain of outpatient medical centers in Poland.

Material and methods. We compared frequency at which AT and hypothyroidism diagnoses were made during endocrinology consultations in the period 2006–2013. The data was extracted from the database of LUXMED (part of BUPA).

Results. Within 8 years, the prevalence of newly diagnosed AT dropped from 10.4% to 4.8% ($p < 0.001$) alongside with a decrease in the prevalence of newly diagnosed hypothyroidism from 17.8% to 7.7% ($p < 0.00001$). AT was widespread in young women aged 20–39. There were relatively more cases in the southern areas of Poland.

Conclusions. The analyzed data does not support a hypothesis indicating a growing incidence of AT in the last years. Detailed epidemiological studies would be helpful in designing screening strategies for patients with this common disorder.

Key words: prevalence, hypothyroidism, autoantibodies, autoimmune thyroiditis

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Autoimmune thyroiditis (AT) is a common disorder of the thyroid gland. It is usually diagnosed when thyroid autoantibodies (TPOAbs/TGABs) are detected in patients with hypothyroidism or goiter. It is assumed that thyroid autoimmunity may affect 2% of males and more than 10% of females, while thyroid dysfunction due to AT occurs in 2% of the population.¹

The etiology of AT is complex and not fully explained.² The disease arises as a result of an interplay between genetic and environmental factors. Among genes that may contribute to AT one can count: HLA-DR3, HLA-DRβ1-Arg74, HLA-DR pocket-sequence variant and non-MHC genes e.g. CD40, CTLA-4, PTPN22, thyroglobulin and TSH receptor genes. The environmental influences comprise: smoking, stress, iodine intake, medications, bacterial and viral infections, irradiation, pollutants and pregnancy. It is emphasized that epigenetic effects may play an important role in thyroid autoimmunity.^{3,4}

Common experience suggests that the incidence of AT is increasing. Although some studies point to such a trend in the incidence and prevalence of AT, real patterns of the disorder are not easy to trace and compare.^{5–8}

The aim of the present study was to evaluate whether there was any change in the prevalence of AT in a sample of Polish population consulted in LUXMED centers in the period 2006–2013.

Material and methods

In order to assess the prevalence of AT a search of LUXMED database was performed. LUXMED (part of BUPA) is the leading private healthcare provider in Poland offering services through a chain of 140 company's medical centers. The company's services are offered primarily to people paying a monthly fee (corporate clients), but single, paid consultations are provided as well. It is estimated that the company's database comprised around 230,000 patients (mainly corporate) in 2006 and 1,200,000 (including 800,000 corporate) in 2013. The annual number of consultations in the company's clinics exceeds 3,000,000 including more than 155,000 endocrinology consultations. It can be assumed that LUXMED corporate patients are professionally active (the majority between 20–50, practically all under 65), work in the private sector (or are self-employed), earn more than average, their jobs do not involve much physical activity and are not significant exposed to chemicals/toxins/radiation. Most of them reside in cities/towns rather than in villages.

The main search term used in the present investigation was E06.3 (autoimmune thyroiditis, AT). The diagnosis of AT required the presence of autoantibodies (TPOAbs/TGABs) in association with goiter/thyroid atrophy/typical ultrasound findings or hypothyroidism. It was made solely during endocrinology consultations. Each entry

was counted once for a specific patient. The acquired data was compared with the number of endocrinology consultations in the given year and the number of patients in whom the diagnosis of hypothyroidism (E03 with exclusion of E03.2, E03.5 and E03.3) was made. There were no other exclusion criteria used. Both corporate and one-time patients were counted. The data was available for the period 2006–2013.

Statistical analysis was performed using PQStat (v. 1.4.2.324). The number of patients with the diagnosis of AT in consecutive years was assessed with the χ^2 test and the χ^2 test for the trend. The Pearson's coefficient of correlation and the Spearman's rank correlation coefficient were also employed. The prevalence of men and women with AT in respective age groups were compared with the Mann-Whitney U test. The prevalence of patients with AT diagnosed in different centers in Poland in respective age groups were compared with Kruskal-Wallis test. The associations between sex and age groups for patients with AT were analyzed with the χ^2 test and the χ^2 test for the trend. A p-value less than 0.05 was considered significant and less than 0.01 as highly significant.

Results

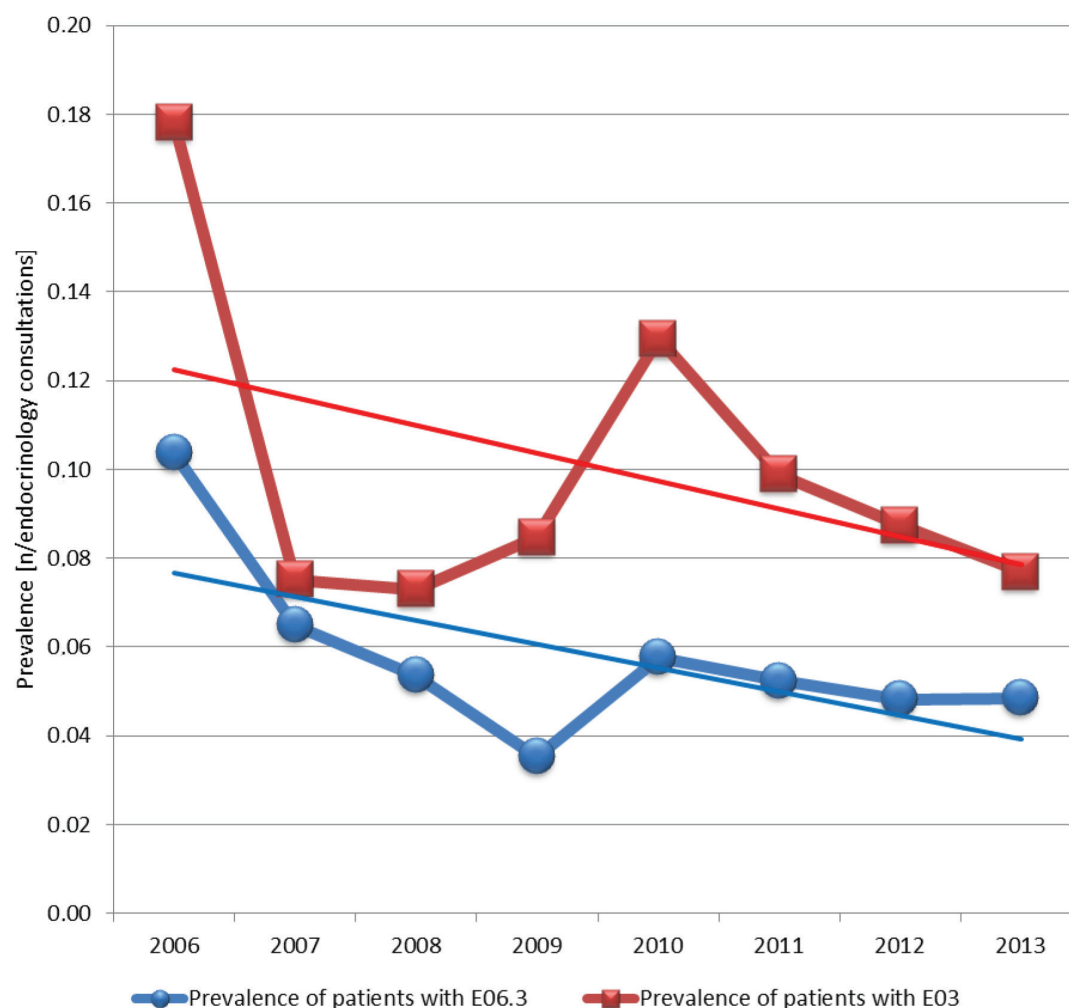
In the studied period a highly significant trend in the prevalence of AT was observed ($\chi^2 = 0.85$, $df = 1$, $p < 0.00013$) and there was a highly significant association ($\chi^2 = 908.17$, $df = 7$, $p < 0.0001$) between the number of patients diagnosed with AT and specific years (Fig. 1, Table 1).

Between 2006 and 2007 a steep decrease in AT frequency from 10.4% to 6.5% was noted. In the next two years the frequencies were respectively above 5 and 3%.

Table 1. Numbers of patients diagnosed with autoimmune thyroiditis (E06.3) and hypothyroidism (E03) and relative frequencies of prevalence (at endocrinology consultations EC) in the consecutive years

Year	E06.3 (n)	E03 (n)	EC (n)	E06.3/EC (%)	E03/EC (%)
2006	656	1127	6326	10.37	17.82
2007	953	1104	14686	6.49	7.52
2008	999	1362	18662	5.35	7.30
2009	2482	5952	70216	3.53	8.48
2010	4885	10961	84687	5.77	12.94
2011	5716	10786	109117	5.24	9.88
2012	6144	11148	127567	4.82	8.74
2013	7540	11942	155442	4.85	7.68

Fig. 1. Prevalence of autoimmune thyroiditis (E06.3) and hypothyroidism (E03) diagnosed during endocrinology consultations in the consecutive years



In 2007 a transient increase of AT frequency to 5.8% was followed by a decrease to the level below 5% in the last years.

The analysis of correlation confirmed the presence of linear ($r = -0.9816$, $p < 0.00001$) and rank correlation ($r = 1.0000$, $p < 0.00001$) between the number of AT patients and the consecutive years.

A highly significant trend ($\chi^2 = 476.64$, $df = 1$, $p < 0.00001$) and association ($\chi^2 = 2657.79$, $df = 7$, $p < 0.0001$) was also found for the diagnosis of hypothyroidism (E03). In 2006 the prevalence of hypothyroid patients was very high, close to 18%, however it dropped to 7.6% in 2007. In the following years the number of patients diagnosed with hypothyroidism at endocrinology consultations fluctuated between 7.3 and 12.9% and settled at 7.7% in 2013. The analysis of correlation coefficients showed linear ($r = -0.9433$, $p = 0.0004$) and rank ($r = 0.9524$, $p = 0.0003$) associations between the number of patients with hypothyroidism and the consecutive years.

Endocrinologists diagnosed AT most often in subjects aged 30–39. The second group in which AT prevalence was high was the group between 20 and 29 years of age. The

diagnosis was made significantly more often in women than in men in 2 age groups: 20–29 ($p = 0.0005$) and 30–39 ($p = 0.0186$). In other age groups there were no considerable differences of AT prevalence between sexes (Fig. 2).

A comparison of data from 4 distinguished areas of the country: north (Olsztyn, Trójmiasto, Szczecin), south (Katowice, Tychy, Opole, Kraków, Dębica, Zabierzów), middle (Warszawa, Łódź, Aleksandrów Łódzki, Bydgoszcz) and west (Wrocław, Poznań, Gorzów Wielkopolski) revealed geographical differences of the prevalence of AT in all age groups apart from patients under 19 and aged 30–39 (Fig. 3).

The number of AT in the age group 20–29 was lowest in the north and highest in the south of Poland ($p = 0.0007$). To the contrary, the prevalence of AT was lowest in the south of Poland in patients aged 40–49, 50–59 and over 60 (Table 2, Fig. 4).

There was a statistically significant difference of the prevalence of AT depending on the area of the country ($\chi^2 = 104.93$, $df = 3$, $p < 0.0001$). The diagnosis was made most frequently in the west (0.0544) and least frequently – in the south of Poland (0.043).

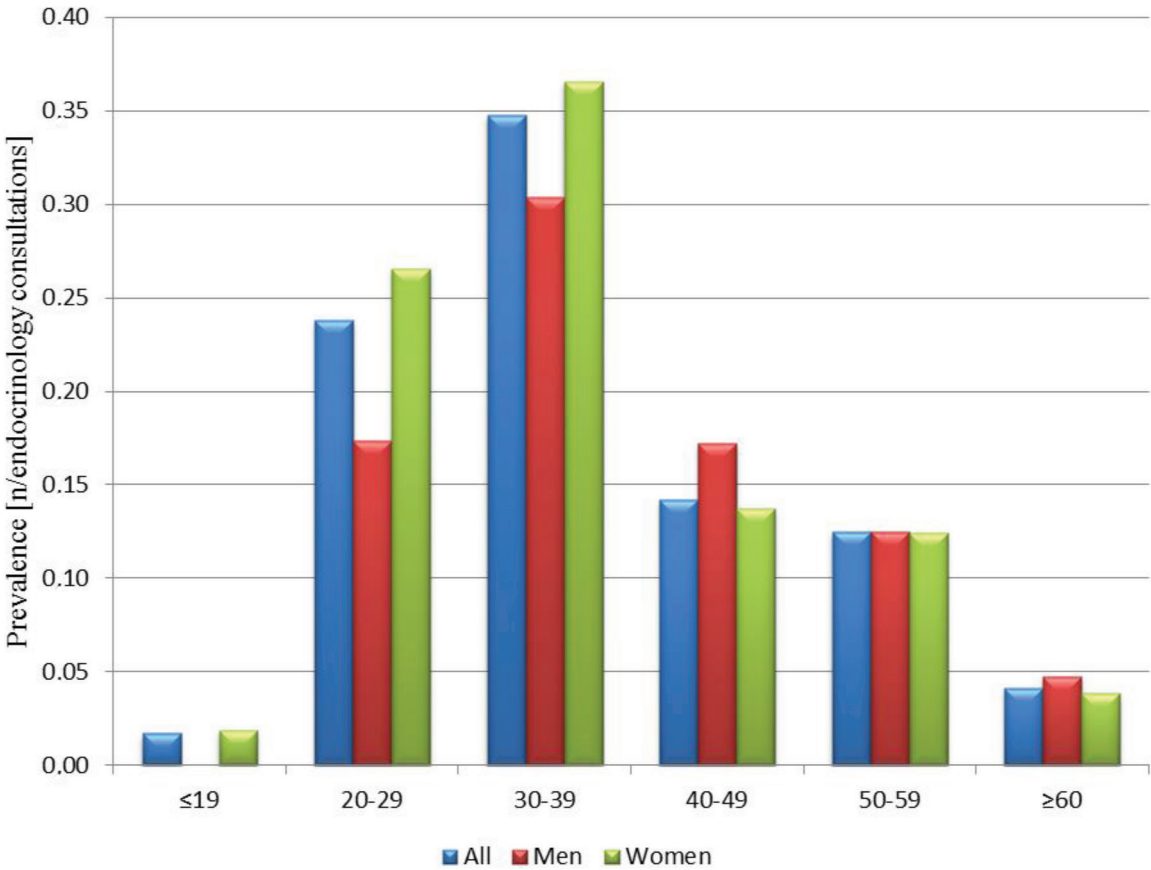


Fig. 2. Frequencies of patients diagnosed with E06.3 in age/sex groups

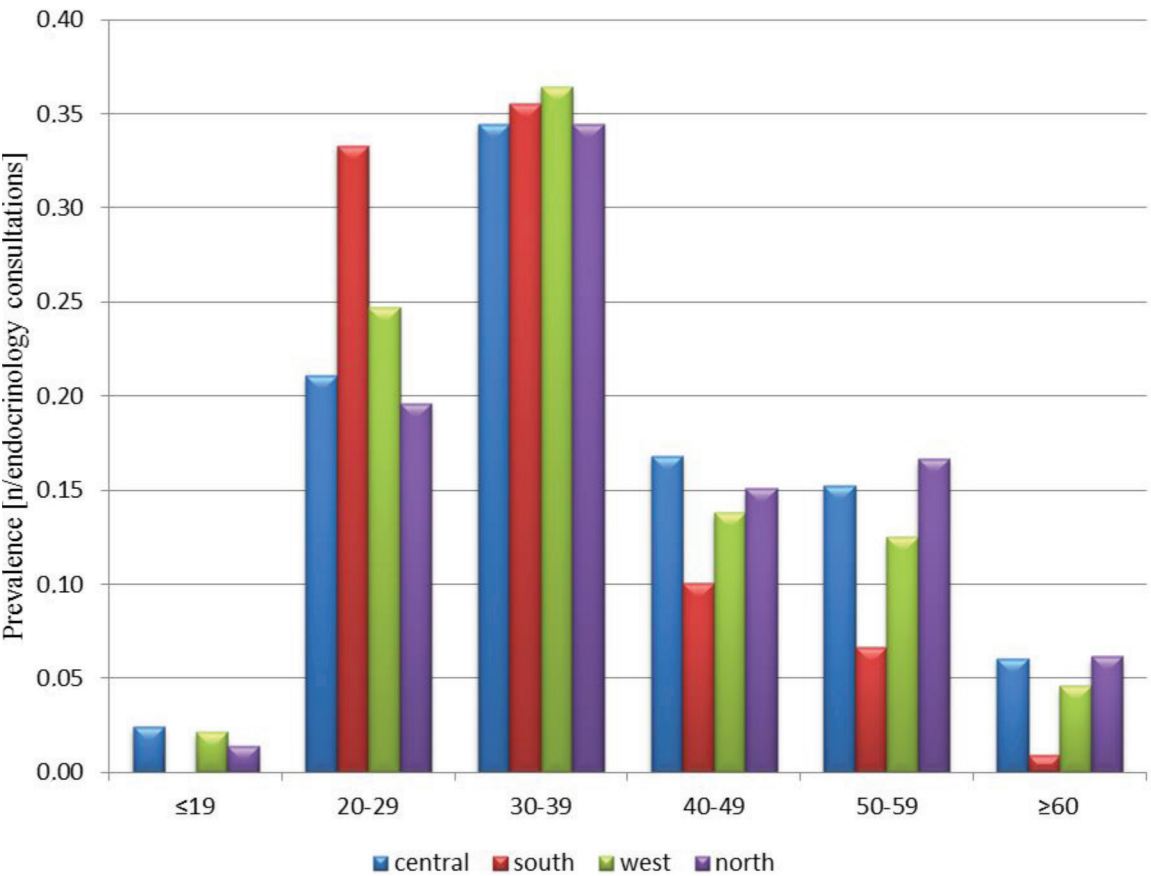


Fig. 3. Prevalence of patients diagnosed with E06.3 in 4 distinguished regions (data presented for age groups)

Discussion

Prevalence of the thyroid autoantibody positivity is relatively high worldwide. It is well-known that in iodine-sufficient areas there is a higher rate of AT prevalence than in iodine-deficient ones (9). Iodine intake is probably one of the most important factors that affects thyroid autoimmunity and the incidence of AT.¹⁰

In a random sample of general, euthyroid population living in Holland ($n = 2703$) the prevalence of the TPOAbs positive cases was 8.4%.¹¹ In Denmark among 4649 randomly selected subjects between 18 and 65 years of age 13.1% were TPOAbs positive and 18.1% had increased concentrations of either TPO or TG autoantibodies.¹² In the United States, TPOAbs were positive in 11.3% and TGABs in 10.4% in a population sample of 13,344 subjects. In Japan among 1818 adults 31.4% of women and 17.7% of men were positive for TgAbs or TPOAbs.¹³ In another study from the same country presence of TPOAbs or TGABs was reported in 12.8% of the studied cases.¹⁴ A very similar number (12.4% TPOAb positivity) was noted also by Australian authors.¹⁵ In India TPOAbs were detected in more than 13% subjects from a group of 4409 adults and 22% from a sample of 5376 adults.^{16,17} Generally, TPOAbs are present in 12–26% of euthyroid subjects, more often in women.¹⁸

These observations are in line with the data on the prevalence of hypothyroidism. In Europe the prevalence of undiagnosed and diagnosed hypothyroidism is estimated to be between 3.05 and 4.94%.⁸ In the United States 4.6% of the population has hypothyroidism.¹⁹ In Japan overt or subclinical hypothyroidism is noted in 6.5% of the population, while in India subclinical hypothyroidism is diagnosed in 15.9% of men and 21.4% of women (overall prevalence 19.3%).^{13,17}

In Poland information on thyroid function/thyroid autoimmunity was gathered, e.g. in 4190/1594 subjects over 55 years of age in the framework of the PolSenior study. In this cross-sectional investigation 17.4% of subjects were TPOAb positive and 7.9% could be classified as hypothyroid. The prevalence of TPOAb seropositivity was higher in females than in males (27 vs 15%) (20).

As expected, also in our sample more women than men were diagnosed with AT (and hypothyroidism, data not presented). Such results were concordant with observations of hypothyroidism from Poland and other countries.^{8,19,20}

It is more difficult to say whether there is a rising trend in AT occurrence over the years. Such an impression emerges from an analysis of studies conducted in the United Kingdom, Slovenia and Denmark.⁷ In Australia thyroid autoantibodies were detected in 9.8 of women and 2.8% of men in 1975 and in 1981 these ratios changed into respectively 17 and 6.8%.^{7,21} A hypothesis of an increasing incidence of thyroid autoimmunity is also supported by results of an Italian investigation performed in a rural community of 1411/1148 subjects. In this study

a clear rise in the number of thyroid autoantibodies positive cases was noted between 1995 and 2010. TPOAbs positivity rose from 12.6 to 19.5% ($p < 0.001$) and AT from 3.5 to 14.5% ($p < 0.0001$).¹⁰ In concordance with the above findings are observations of Danish cohorts from 1997–1998 and 2004–2005. The positivity for TPOAbs changed from 14.3 to 23.8% and for TGABs from 13.7 to 19.9% after introducing mandatory iodization of salt.²² Also in Poland, obligatory iodine prophylaxis (which started in 1997) was associated with a clear increase in the percent of TPOAbs positive individuals from 3.8 to 11.8%. Over the period of 10 years (1989–1999) the prevalence of hypothyroidism ratios in the city of Kraków increased insignificantly in women and men from respectively 1.4 to 2.1% and from 0 to 0.3%.²³

Our data does not confirm increasing trends in AT occurrence. They correspond to some extent with the results of a comparison of two population surveys (33,917/49,180 individuals) performed in 1995–1997 and 2006–2008 in Norway. The Scandinavian authors noted a decrease in the prevalence of overt hypothyroidism in women from 0.75 to 0.12% (84%) and in men from 0.21 to 0.12% (43%). The prevalence of subclinical hypothyroidism changed from 3.0 to 1.1% (64%) in women and from 2.1 to 1.0% (54%) in men. Conversely, the prevalence of treated hypothyroidism among women increased by 60% from 5.0 to 8.0% in men by 100% from 1.0 to 2.0%. It must be admitted that the prevalence of all forms of hypothyroidism did not change remarkably, as it was found in 9% of women and 3% of men.²⁴

The strength of the present study is a relatively high number of observations made in clinics from all over the country. Unlike other medical conditions (e.g. neoplasms, contagious diseases) thyroid pathology is not covered by any public database of a national scale. According to the report of the Central Statistical Office (http://www.stat.gov.pl/gus/5840_12706_ENG_HTML.htm), the number of endocrinology consultations in outpatient clinics in Poland (public sector) was around 4,000,000 in 2012. The consultations covered by our study (127 567 in 2012) would constitute 3.2% of the whole number of endocrinology consultations.

The data was acquired from an ethnically homogenous population from an area with sufficient iodine intake dating back to 1997.²⁵ Although the criteria of AT (or hypothyroidism) could not be imposed, it should be stressed that the diagnoses were made only by endocrinologists.

A limitation of the investigation is the fact that the majority of the subjects from the sample live in urban environments, are employed and under 65. The lifestyle parameters may not reflect the situation of the general population. One may assume that the economical status of the consulted patients is higher than average. The latter may be associated with e.g. diet/eating habits. The presented results could not be controlled for age, smoking status, pregnancy, concomitant ailments or received medications either.

Table 2. Prevalence of patients diagnosed with E06.3 in the 4 distinguished areas.

Age of patients/area of the country		Descriptive statistics							Kruskal-Wallis test
		mean	standard deviation	minimum	lower quartile	median	upper quartile	maximum	
≤ 19	central	0.0259	0.0202	0.0000	0.0151	0.0244	0.0383	0.0741	H = 7.56 p = 0.0559
	south	0.0488	0.1691	0.0000	0.0000	0.0000	0.0236	1.0000	
	west	0.0324	0.0367	0.0000	0.0000	0.0214	0.0501	0.1250	
	north	0.0717	0.2121	0.0000	0.0000	0.0144	0.0462	1.0000	
20–29	central	0.2399	0.2050	0.0000	0.1644	0.2109	0.2469	1.0000	H = 17.08 p = 0.0007
	south	0.3710	0.2731	0.0000	0.2000	0.3333	0.4615	1.0000	
	west	0.2537	0.1874	0.0000	0.1970	0.2474	0.3000	1.0000	
	north	0.1761	0.1159	0.0000	0.1111	0.1962	0.2615	0.4000	
30–39	central	0.3392	0.1619	0.0000	0.3041	0.3446	0.3902	1.0000	H = 0.31 p = 0.9571
	south	0.3339	0.2412	0.0000	0.1818	0.3555	0.4923	1.0000	
	west	0.3680	0.2263	0.0000	0.2929	0.3645	0.3930	1.0000	
	north	0.3563	0.2473	0.0000	0.2632	0.3447	0.3775	1.0000	
40–49	central	0.1851	0.1240	0.0000	0.1388	0.1682	0.2077	0.6667	H = 13.12 p = 0.0044
	south	0.1161	0.1703	0.0000	0.0000	0.1008	0.1538	1.0000	
	west	0.1650	0.1265	0.0000	0.0984	0.1381	0.1913	0.5000	
	north	0.1420	0.1154	0.0000	0.0000	0.1508	0.1905	0.4444	
50–59	central	0.1457	0.0702	0.0000	0.1241	0.1527	0.1766	0.3333	H = 21.48 p = 0.0001
	south	0.0968	0.1809	0.0000	0.0000	0.0667	0.0946	1.0000	
	west	0.1394	0.1200	0.0000	0.1057	0.1250	0.1600	0.5000	
	north	0.1562	0.1397	0.0000	0.1111	0.1667	0.2000	0.6667	
≥ 60	central	0.0642	0.0507	0.0000	0.0244	0.0605	0.0926	0.1923	H = 12.05 p = 0.0072
	south	0.0334	0.0576	0.0000	0.0000	0.0091	0.0435	0.3000	
	west	0.0414	0.0390	0.0000	0.0000	0.0459	0.0524	0.1594	
	north	0.0978	0.1562	0.0000	0.0000	0.0617	0.1000	0.6000	

Conclusions

It seems there is a downward trend in the prevalence of autoimmune thyroiditis (AT) in privately insured patients in Poland over the years. Whether this is true for the whole population needs to be confirmed.

Young female patients aged 20–39 suffer from AT most often. There are relatively more cases in the southern areas of the country.

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Correlation between the state of periodontal tissues and selected risk factors for periodontitis and myocardial infarction

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D – writing the article; E – critical revision of the article; F – final approval of article

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Abstract

Background. The current level of knowledge indicates a relationship between periodontitis and diabetes and/or cardiovascular diseases (CVD). Periodontitis can be not only a risk factor for these diseases, but also a condition modifying other primary risk factors associated with the occurrence of cardiovascular complications (lipid disorders, arterial hypertension, etc.) or diabetes.

Objectives. The aim of the study was an analysis of the correlation between the state of periodontal tissues and selected risk factors for myocardial infarction (MI) in patients after recent myocardial infarction.

Material and methods. The study included 417 patients (92 women, 325 men) hospitalized due to recent MI. The inclusion criteria were MI history and age below 70 years. The state of periodontal tissues (plaque index, bleeding on probing, pocket depth and clinical attachment loss, CPI index) and selected risk factors for periodontitis and CVD were recorded.

Results. An analysis of the results showed no statistically significant correlation between the depth, the number, percentage of periodontal pockets and the average clinical attachment level on one hand and BMI on the other hand. Whereas a statistically significant correlation was observed between tobacco smoking and the degree of severity of periodontal diseases measured by the average pocket depth, the number and percentage of pockets above 4 mm and the average clinical attachment loss, as well as between hypertension and the state of oral hygiene and between diabetes and the number of preserved teeth and the number of pockets above 4 mm.

Conclusions. The degree of severity of periodontal disease can impact hypertension and diabetes, which could potentially influence the occurrence and course of CVD.

Key words: risk factors, periodontitis, cardiovascular diseases

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Introduction

The current level of knowledge indicates a relationship between periodontal disease and systemic diseases, including diabetes, cardiovascular diseases, stroke, premature birth and low birth weight, as well as Parkinson's disease, Alzheimer's disease and pancreatic cancer. Periodontitis can constitute not only a risk factor for these diseases, but also a condition modifying other primary risk factors associated with the occurrence of cardiovascular complications (lipid disorders, arterial hypertension, etc.) or diabetes.^{1,2}

Cardiovascular diseases are the main cause of premature death in most European countries. This situation is closely associated with lifestyle and the influence of risk factors on the occurrence and course of cardiovascular diseases.

Risk factors beyond our control include age, gender, genetic load. Those on which we have an influence include cigarette smoking, arterial hypertension, lipid disorders, diabetes and obesity, bad eating habits, stress.

The results of studies in recent years also suggest that in the future periodontal disease may be considered one of the many risk factors for cardiovascular diseases.³

Research has shown that the same risk factors as well as the same pathophysiological processes are the underlying cause for the destabilization of atherosclerotic plaques and the destruction of periodontal tissues. Particular attention should be paid to the interactions that could potentially occur between periodontal diseases and other risk factors, i.e. the concentration of lipoproteins LDL and HDL, or arterial hypertension, due to their joint participation in the induction of oxidative stress in the circulatory system.^{2,4}

Oxidative stress, generally coexisting with pathogens associated with periodontal disease, which are often detected in atherosclerotic plaque, accelerates apoptosis, and increases inflammation. This process can initiate the erosion of atherosclerotic plaque and raise its vulnerability to rupture, which is a high risk of thrombosis and acute coronary events.^{5,6}

The prevalence of periodontal disease in our civilization and the positive results of periodontal therapy necessitate a deeper examination of the pathogenetic mechanisms linking periodontitis with atherosclerosis and consequently with the resulting cardiovascular diseases.^{7,8}

Therefore, the objective of this study was to assess the state of periodontal tissues and to analyze the correlation between the state of periodontal tissues and selected risk factors for myocardial infarction in patients after acute myocardial infarction.

Material and methods

The study was conducted in the Department of Periodontology and in the Clinic and Department of Cardiology of five medical universities in Poland (Warszawa, Szczecin, Wrocław, Lublin, Białystok) in 2010–2014.

The study included 417 patients hospitalized with recent acute myocardial infarction (MI). The inclusion criteria were MI history and age below 70 years. The patients agreed to participate in the study by signing a declaration approved by the Bioethics Committee in Medical University of Warszawa (opinion: KB-145/2011). Patients diagnosed with cancer, rheumatic disease, autoimmune disease, chronic liver disease, chronic renal disease stages 4 and 5, stroke history and individuals receiving periodontal treatment or systemic antibiotic therapy in the preceding 6 months were excluded from the study.

The study group included 417 patients (92 females, 325 males) aged 25 to 69 years.

Social enquiry and general medical history

All participants of the study were interviewed, which included a collection of the following data:

- General patient data: first name, family name, gender, date of birth, phone number, place of residence;
- Education, defined as primary, secondary and higher education;
- Socio-economic status, determined on the basis of income per family member per month: < 800 PLN, 800–1500 PLN, > 1500 PLN;
- Cigarette smoking, defined as: current (smoking of 10 or more cigarettes a day continuously for at least 5 years), smoking in the past and never;
- Identification of risk factors for cardiovascular disease: arterial hypertension, diabetes, as well as BMI and WHR.

Physical examination

Physical measurements were performed in accordance with applicable guidelines. All the study participants were evaluated for:

- Weight [kg], height [cm], waist circumference [cm] and hip circumference [cm], which allowed for the calculation of each patient's body mass index (BMI was calculated by dividing the body weight [kg] by the square of the body height [m²]) and WHR (WHR was calculated by dividing the waist circumference by the hip circumference). BMI 25–29.9 kg/m² was defined as overweight, and BMI ≥ 30 kg/m² was defined as obesity. Abdominal obesity was diagnosed when waist circumference (WC) was ≥ 80 cm in women and ≥ 94 cm in men.
- Blood pressure, which was measured in a sitting position after several minutes' rest, using a sphygmomanometer. Arterial hypertension was defined as systolic blood pressure ≥ 140 mm Hg or diastolic blood pressure ≥ 90 mm Hg or use of antihypertensive drugs.

Dental and periodontal examination

The dental examination took place within 6 weeks after the myocardial infarction. This examination was carried out in an artificial light, using a dental mirror and a peri-

odontal probe (Hu-Friedy PCPUNC 15). The number of teeth, the number of roots with pulp necrosis present in the mouth and edentulousness rate were determined. The examination did not include third molars. The periodontal examination covered:

- Dichotomous Plaque Index (PI) by O’Leary⁹ on 4 surfaces of the tooth (mesial, distal, lingual and buccal); the presence or absence of plaque was determined;

- Bleeding on Probing (BoP) index by Ainamo and Bay¹⁰ where the examination was conducted at 4 sites around the tooth: mesial buccal (MB), buccal (B), distal buccal (DB) and lingual (L), only the presence or absence of bleeding from gingiva while probing the pocket was determined;

- Pocket depth (PD) at 4 sites around the tooth: MB, B, DB, L, which was defined as the distance from the gingival margin to the bottom of the pocket determined by the probe;

- The number of active (bleeding) pockets above 4 mm in depth;

- Clinical attachment loss (CAL) at 4 sites around the tooth: MB, B, DB, L, which is defined as the distance between the bottom of the pocket determined by the probe and the cemento-enamel junction.

The periodontal status of each patient was determined on the basis of the CPI index definition¹¹, with the following categories:

- CPI-0 – no inflammatory symptoms,
- CPI-1 – presence of bleeding on probing,
- CPI-2 – presence of supra- and/or subgingival calculus or filling overhangs,
- CPI-3 – presence of pathological periodontal pockets from 3.5 to 5.5 mm deep,
- CPI-4 – the presence of pathological periodontal pockets 5.5 mm and deeper.

Statistical analysis

Statistical analysis was performed using PQStat v. 1.4.4 software. The Mann-Whitney and Kruskal-Wallis tests were used to assess the significance of differences between 2 and more groups. The χ^2 test was used to search significant differences between the frequencies of the analyzed data. Correlation between variables were measured by the Spearman rank correlation. P-value lower than 0.05 was decided as significant differences.

Results

Characteristics of the study group

The age of patients ranged from 25 to 69 years, with the median at 57 years, and with significantly more men than women (77.9% vs 22.1%).

Most of the patients came from large cities (223 individuals, 56.9%), the least - 69 patients (17.6%) - lived in the country outside urban areas.

Within the study group the largest subgroup were individuals with a secondary education 53.9%, the least numerous - with incomplete university education or full university education (15.7%).

Analyzing the income per family member it was observed that more than half had an income ranging from 800 to 1500 PLN per family member (Table 1).

The results of the periodontal examination showed an average of 12 preserved teeth in women and 18 in men (total median: 16 preserved teeth). Mean values of plaque index (PI) were high in both females and males (76.9% and 78.2% respectively). Also the BOP index was high, regardless of gender (44.6%). The number of pockets > 4 mm was significantly higher in men (Table 2).

Characteristic of risk factors and correlations between variables

Analyzing risk factors for heart diseases, overweight (BMI 25-30 kg/m²) or obesity (BMI \geq 30 kg/m²) were observed in almost 80% of patients. Most of the patients were current or past smokers of tobacco (almost 80%), arterial hypertension was present in 90.1% of individuals in the study group, diabetes in almost 25%, dyslipidemia in more than half of the patients with myocardial infarction (Table 3).

Table 1. Characteristics of the study group

Patients' overall profiles		Total
Number of patients		417
Percentage of the study group		100.0%
Age	median	57
	Q1-Q3 range	52–61.25 25–69
Number and percentage of females		92 22.1%
Place of residence	city	223 56.9%
	town	100 25.5%
	village	69 17.6%
Education	primary	122 30.4%
	secondary	216 53.9%
	licentiate	10 2.5%
	Master's degree	53 13.2%
Income per month	< 800 PLN per person	101 25.5%
	[800; 1500] PLN per person	212 53.5%
	> 1500 PLN per person	83 21.0%

Table 2. Full-mouth dental and periodontal status

Periodontal parameters		Females (n = 92)	Males (n = 325)	Comparison (Mann-Whitney test)	Total
Number of teeth (median; Q1–Q3)		12 (4–18)	18 (9–23)	p = 0.0007	16 (8–22)
Number of lost teeth (median; Q1–Q3)		16 (10–24)	10 (5–19)	p = 0.0007	12 (6–20)
Number and percentage of edentulous patients		15 (16.3%)	30 (9.2%)	p = 0.0007	45 10.8%
Applies only to patients with preserved own teeth (n = 372)	PI (mean (SD))	76.9% (25.4)	78.2% (21.4)	p = 0.8178	77.9% (22.3)
	BOP (mean (SD))	45.0% (30.8)	44.5% (28.6)	p = 0.8917	44.6% (29.0)
	PD (mean (SD))	2.8 (1.3)	2.8 (1.0)	p = 0.6169	2.8 (1.1)
	number and percentage of patients with pockets ≥ 4 mm*	67 (87.0%)	270 (92.8%)	p = 0.1050	337 (91.6%)
	PD ≥ 4 mm – number (median; Q1–Q3)	6 (2–15)	11 (5–21.75)	p = 0.0041	10 (4–20)
	PD ≥ 4 mm – percentage (mean (SD))	24.8% (25.1)	27.4% (24.6)	p = 0.2508	26.8% (24.7)
	CAL (mean (SD))	3.9 (2.0)	3.9 (2.2)	p = 0.8814	3.9 (2.1)
CPI (median; Q1–Q3)		3 (3–4)	4 (3–4)	p = 0.0511	4 (3–4)

* χ^2 test**Table 3.** Values medical risk factors for periodontitis

Risc factors	Subgroups	Values
BMI	mean (SD)	28.7 (4.9)
	underweight and emaciation (BMI < 18.5)	4 (1.0%)
	optimal weight	83 (20.1%)
	overweight (25 \leq BMI < 30 kg/m ²)	179 (43.3%)
	obesity (BMI \geq 30 kg/m ²)	147 (35.6%)
WHR (mean (SD))		0.98 (0.10)
Tobacco smoking	presently	204 (49.3%)
	in the past	115 (27.8%)
	never	95 (22.9%)
Arterial hypertension		309 (90.1%)
Diabetes		100 (24.2%)

An analysis of the relationship between periodontal status and sociological parameters demonstrated a statistically significant correlation between the number of lost teeth, and age, gender, education and income, a similar association was observed between CPI median and education and income of patients.

An inverse correlation between BOP and PI on one hand and education and income on the other hand was also observed (Table 4).

The conducted study showed a correlation between age, place of residence, education and income on one hand and average pocket depth, number and percentage of pockets above 4 mm and mean CAL on the other one (Table 5).

No correlation was observed between BMI and activity and severity of periodontitis or the state of oral hygiene and CPI median.

The number of lost teeth, BOP and CPI correlated with smoking. The number of lost teeth and the plaque index showed a statistically significant positive correlation with the cumulative effects of tobacco and its combustion products in the form of the number of pack-years (Table 6).

An analysis of the results showed no relationship between the depth, number and percentage of periodontal pockets and the clinical attachment level on one hand and BMI on the other hand, whereas a certain correlation was observed between tobacco smoking on one hand and the average depth of pockets, the number and percentage pockets > 4 mm and the average clinical attachment loss on the other hand.

Own research also shows that a great majority, 97% of individuals with heart attack history, are past or present cigarette smokers. Actually smokers had significantly deeper periodontal pockets and clinical attachment loss. There was significant correlation between CAL and body weight measured only by WHR (Table 7).

The number of teeth in patients with diabetes was significantly lower than among non-diabetic group (Table 8).

Table 4. Relations between sociodemographic variables and periodontal status (number of teeth, PI, BoP and CPI indices)

Median (Q1; Q3) Mean \pm SD		Number of teeth	PI	BoP	CPI median
Age (Spearman's rank correlation test)		$r = 0.37$ $p < 0.0001$	$r = 0.07$ $p = 0.1763$	$r = 0.02$ $p = 0.6986$	$r = 0.07$ $p = 0.1707$
Gender	females	16 (10; 24)	76.9% \pm 25.4 p.p.	45.0% \pm 30.8 p.p.	3 (2; 3)
	males	10 (5; 19)	77.6% \pm 22.2 p.p.	44.2% \pm 28.6 p.p.	3 (2; 4)
Mann-Whitney U test		$p = 0.0007$	$p = 0.7310$	$p = 0.9595$	$p = 0.5283$
Place of residence	city	11 (5; 20)	78.3% \pm 21.6 p.p.	43.7% \pm 29.1 p.p.	3 (2; 4)
	town	15 (8; 23)	77.5% \pm 22.4 p.p.	49.4% \pm 28.9 p.p.	3 (2; 4)
	village	11 (6; 17)	83.0% \pm 18.8 p.p.	49.1% \pm 28.5 p.p.	3 (3; 3)
Spearman's rank correlation test		$r = 0.04$ $p = 0.4125$	$r = 0.06$ $p = 0.2705$	$r = 0.10$ $p = 0.0677$	$r = 0.05$ $p = 0.3254$
Education	primary	16 (9; 23)	86.8% \pm 17.8 p.p.	57.1% \pm 29.5 p.p.	3 (3; 4)
	secondary	12 (6; 20)	77.6% \pm 21.0 p.p.	42.7% \pm 27.4 p.p.	3 (2; 4)
	BA	10 (8; 12)	41.1% \pm 28.1 p.p.	26.0% \pm 22.8 p.p.	2 (1; 3)
	MA	6 (3; 14)	70.2% \pm 21.6 p.p.	36.3% \pm 27.7 p.p.	2.5 (2; 3)
Spearman's rank correlation test		$r = -0.26$ $p < 0.0001$	$r = -0.31$ $p < 0.0001$	$r = -0.28$ $p < 0.0001$	$r = -0.26$ $p < 0.0001$
Income per month	< 800 PLN per person	15 (9; 23)	87.4% \pm 17.5 p.p.	56.2% \pm 29.1 p.p.	3 (3; 4)
	[800; 1500] PLN per person	12 (8; 21)	77.9% \pm 21.1 p.p.	44.6% \pm 28.2 p.p.	3 (2; 4)
	> 1500 PLN per person	6 (3; 12)	71.2% \pm 22.9 p.p.	37.4% \pm 27.4 p.p.	3 (2; 3)
Spearman's rank correlation test		$r = -0.30$ $p < 0.0001$	$r = -0.27$ $p < 0.0001$	$r = -0.24$ $p < 0.0001$	$r = -0.22$ $p < 0.0001$

Discussion

Age and gender are an important, non-modifiable risk factor for both periodontal disease and cardiovascular disease. Numerous epidemiological studies confirm a higher incidence of periodontitis in the elderly.^{12,13} Studies conducted by Persson et al.¹⁴ showed that both of these diseases may occur simultaneously, particularly in individuals over 60 years of age. The occurrence of periodontal disease was observed in about 50% of those aged over 60 years, and 55% were overweight or with an episode of stroke or acute coronary syndrome.¹⁴

Our study also showed a higher incidence of myocardial infarction, as well as periodontal disease in men. Male predilection for the occurrence of periodontal disease and cardiovascular disease was also observed by other authors.^{13,15,16}

An analysis of modifiable factors, which depend on us, included, among other things, education and the degree

of wealth. Our study showed a lower number of individuals with higher education and higher income among patients after myocardial infarction. Zhang et al.¹⁷ demonstrated that education lasting at least 6 years significantly affects the decreased incidence of advanced periodontal disease and impacts the course of treatment and survival of patients after ACS.

Individuals with higher education usually have a permanent job, which is associated with a regular income and frequent access to dental care. They also have higher health awareness.

Our results are also confirmed by other authors, indicating that the level of wealth is an important modifiable risk factor for periodontal disease.¹³ These results can also indicate that individuals with a low income lose their own teeth earlier, due to the limited possibility of receiving treatment. These results also showed a high proportion of toothlessness, which occurred earlier in individuals with a lower income after myocardial infarction.^{12,18}

Table 5. Relations between sociodemographic variables and periodontal status (PD, CAL)

Median (Q1; Q3) Mean \pm SD		Mean PD	PD \geq 4 mm – number	PD \geq 4 mm – percentage	Mean CAL
Age (Spearman's rank correlation test)		$r = 0.10$ $p = 0.0677$	$r = -0.03$ $p = 0.5966$	$r = 0.11$ $p = 0.0283$	$r = 0.18$ $p = 0.0007$
Gender	females	2.8 ± 1.3	6 (2; 15)	$24.8\% \pm 25.1$ p.p.	3.9 ± 2.0
	males	2.8 ± 1.0	11 (5; 22)	$27.3\% \pm 24.6$ p.p.	3.9 ± 2.2
Mann-Whitney U test		$p = 0.6146$	$p = 0.0039$	$p = 0.2599$	$p = 0.8270$
Place of residence	city	2.7 ± 0.9	9 (3; 21)	$25.2\% \pm 23.5$ p.p.	3.8 ± 2.1
	town	3.1 ± 1.1	9 (5; 16)	$31.3\% \pm 28.8$ p.p.	4.6 ± 2.1
	village	3.1 ± 1.4	11 (6; 22)	$28.4\% \pm 24.1$ p.p.	4.0 ± 2.0
Spearman's rank correlation test		$r = 0.13$ $p = 0.0184$	$r = 0.07$ $p = 0.2099$	$r = 0.09$ $p = 0.0937$	$r = 0.09$ $p = 0.0859$
Education	primary	3.1 ± 1.0	11 (6; 24)	$33.6\% \pm 26.3$ p.p.	4.6 ± 2.2
	secondary	2.9 ± 1.1	10 (4; 20)	$26.5\% \pm 23.9$ p.p.	3.9 ± 2.0
	BA	2.2 ± 0.6	3.5 (1; 9.5)	$8.8\% \pm 9.7$ p.p.	1.6 ± 1.4
	MA	2.5 ± 0.9	6 (2; 16)	$19.2\% \pm 23.9$ p.p.	3.3 ± 1.8
Spearman's rank correlation test		$r = -0.23$ $p < 0.0001$	$r = -0.16$ $p = 0.0031$	$r = -0.24$ $p < 0.0001$	$r = -0.26$ $p < 0.0001$
Income per month	< 800 PLN per person	3.1 ± 1.1	10.5 (5; 23)	$33.0\% \pm 26.4$ p.p.	4.5 ± 2.2
	[800; 1500] PLN per person	2.9 ± 1.2	10 (5; 19)	$28.4\% \pm 25.6$ p.p.	4.2 ± 2.1
	> 1500 PLN per person	2.4 ± 0.7	8 (2; 18.5)	$18.0\% \pm 19.0$ p.p.	2.9 ± 1.7
Spearman's rank correlation test		$r = -0.22$ $p < 0.0001$	$r = -0.07$ $p = 0.1661$	$r = -0.21$ $p = 0.0001$	$r = -0.24$ $p < 0.0001$

Studies by Bertoldi et al.¹⁵ indicated that a higher income correlated with a lower number of lost teeth, but also with a better condition of periodontal tissues. People with a lower socio-economic status had fewer teeth, most likely due to the fact that tooth extractions are less expensive and less time consuming, and persons with higher status had more teeth, perhaps because they more often opted for conservative treatment.

Analyzing the group of patients after myocardial infarction, it clear that the number of preserved teeth and the condition of periodontal tissues is much worse compared to epidemiological studies on randomly selected Poles of the same age group.¹³ Indeed, the average number of preserved teeth in the group after myocardial infarction was 12 in women and 18 in men (mean 16).

Studies by Górski et al.¹⁹ showed an average of 24 teeth in the control group of the same age. In the group of patients after myocardial infarction also the percentage of

edentulous individuals was high at 16.3% in women and 9.2% in men (mean 10.8%), in the control group examined by the above-mentioned author the number of edentulous patients was 2.5%.

A research by Desvarieux et al.²⁰ on the relation between the number of teeth and progression of atherosclerosis and risk of myocardial infarction showed plaque in carotid arteries in 46% of individuals who had lost from 0 to 9 teeth and in 60% of individuals who had lost more than 10 teeth. Also studies by Schillinger et al.²¹ indicated that toothless patients had more advanced atherosclerotic lesions in carotid arteries.

Holmlund et al.²² reported that individuals with more than 10 teeth demonstrated a 7-fold increase in the risk of mortality from heart attacks compared to persons with more than 25 teeth, as well as more advanced atherosclerotic lesions in carotid arteries in individuals with fewer teeth.

Table 6. Relations between general health parameters and periodontal status (number of teeth, PI, BoP and CPI indices)

Median (Q1; Q3) Mean \pm SD		Number of teeth	PI	BoP	CPI median
BMI	underweight and emaciation (BMI < 18.5)	11 (3; 18)	72.0% \pm 33.1 p.p.	37.7% \pm 33.0 p.p.	3 (2; 4)
	optimal weight	14 (8; 21)	78.4% \pm 24.0 p.p.	46.9% \pm 32.3 p.p.	3 (2; 3)
	overweight (25 \leq BMI < 30 kg/m ²)	10 (5; 19)	75.6% \pm 23.7 p.p.	41.8% \pm 28.7 p.p.	3 (2; 4)
	obesity (BMI \geq 30 kg/m ²)	11 (7; 20)	79.1% \pm 21.0 p.p.	46.5% \pm 27.6 p.p.	3 (3; 4)
Spearman's rank correlation test		r = -0.03 p = 0.6007	r = 0.02 p = 0.7601	r = 0.06 p = 0.2171	r = 0.07 p = 0.1961
WHR (Spearman's rank correlation test)		r = 0.05 p = 0.3231	r = -0.01 p = 0.8888	r = 0.02 p = 0.7217	r = 0.10 p = 0.0827
Tobacco smoking	presently	14 (7; 21)	80.4% \pm 20.5 p.p.	48.0% \pm 28.7 p.p.	3 (3; 4)
	in the past	11 (5; 20)	74.1% \pm 23.4 p.p.	41.1% \pm 29.1 p.p.	3 (2; 3)
	never	10 (5; 17)	75.1% \pm 26.0 p.p.	40.6% \pm 28.6 p.p.	3 (2; 4)
Kruskal-Wallis ANOVA		p = 0.0138	p = 0.0583	p = 0.0350	p < 0.0001
Number of pack-years (Spearman's rank correlation test)		r = 0.29 p < 0.0001	r = 0.18 p = 0.0196	r = 0.02 p = 0.7817	r = 0.13 p = 0.0972

The state of periodontal tissues evaluated by the number and percentage of patients with pockets above 4 mm and the average CAL was higher compared to the control group in the study by Górski et al.¹⁹, respectively 14.3 vs 8.5 and 4.2 vs 2.5.

Our study showed no correlation between BMI and the average pocket depth, the number and percentage of pockets above 4 mm, or the average clinical attachment loss. However, a statistically significant correlation was observed between WHR and the number of pockets above 4 mm and the average CAL. The BMI data is not a confirmed association between obesity and periodontitis by other authors.^{23,24} Al-Zahrani et al.²⁵ showed that there is an association between high BMI and WHR on the one hand and an increase of typical periodontal disease indicators: CAL, PD and BOP. Similar observations were made by Dalla Vecchia et al.⁶, who evaluated the association between overweight, obesity and periodontitis. The meta-analysis of Chaffee and Weston²⁶ demonstrated a stronger obesity- periodontitis association in younger persons, women and non-smokers than in the general population.

In our study we observed a correlation between smoking and average pocket depth, the number and percentage pockets above 4 mm and an average loss of clinical attachment loss. The study also shows that most people who experienced a heart attack were past or present cigarette smokers. Hyman et al.²⁷ demonstrated that smokers

with concomitant advanced periodontitis were at 8-fold higher risk of myocardial infarction. Tobacco smoking is undoubtedly the most important risk factor for both cardiovascular²⁸ and periodontitis. Studies have shown that in the case of death of a smoker before 50 years of age due to CVD, there is a 80% probability that this death was caused by the addiction.²⁹ Results obtained by other authors^{16,30} confirm our data.

Another modifiable risk factor for both diseases is diabetes. Our own study shows that 25% of patients after myocardial infarction also suffered from coexisting diabetes. Woźakowska-Kapłon et al.³¹ observed diabetes in 34% of MI patients younger than 60 years old. The risk of periodontitis progression in patients with poorly controlled diabetes is 11 times higher than in healthy control.³² Studies by Kannel et al.³³ and McGee et al.³⁴ showed that diabetes increases the risk of fatal cardiovascular complications by 1.7 times in men and 2.1 times in women. The INTERHEART study showed that diabetes increases the risk of myocardial infarction by 3.08 times³⁵ and of mortality in patients with acute coronary syndrome.³⁶ On the other hand, studies by Gerstein et al.³⁷ indicated that the risk of MI rises with values of HbA1C > 5.4%, and any growth in hemoglobin concentration by one percentage point independently increases the risk of MI by additional 19%.

In our study group, more than 90% of patients with myocardial infarction had arterial hypertension, which is

a very important risk factor for cardiovascular disease, and recent studies increasingly indicate a relationship between the state of marginal periodontal tissues and blood pressure values.³⁸ This correlation appears to result from the outflow of bacteria associated with periodontitis,

which may be responsible for the increase in both systolic and diastolic blood pressure through an immune reaction by activating T lymphocytes. The immune reaction may cause increased sensitivity of the body to the action of angiotensin II.³⁹

Table 7. Relations between general health parameters and periodontal status (PD, CAL)

Median (Q1; Q3) Mean \pm SD		Mean PD	PD \geq 4 mm – number	PD \geq 4 mm – percentage	Mean CAL
BMI	underweight and emaciation (BMI < 18.5)	3.1 \pm 1.5	10 (5; 18)	34.2% \pm 37.9 p.p.	4.0 \pm 3.3
	optimal weight	2.9 \pm 1.0	9 (4; 18)	27.9% \pm 23.8 p.p.	3.9 \pm 2.2
	overweight (25 \leq BMI < 30 kg/m ²)	2.8 \pm 1.0	10 (3; 22)	26.4% \pm 25.3 p.p.	3.8 \pm 2.2
	obesity (BMI \geq 30 kg/m ²)	2.9 \pm 1.1	10 (5; 20)	26.6% \pm 24.4 p.p.	4.0 \pm 2.0
Spearman's rank correlation test		r = 0.02 p = 0.6621	r = 0.06 p = 0.2542	r = 0.02 p = 0.7447	r = 0.06 p = 0.2764
WHR (Spearman's rank correlation test)		r = 0.07 p = 0.2040	r = 0.11 p = 0.0544	r = 0.09 p = 0.1015	r = 0.13 p = 0.0235
Tobacco smoking	presently	3.1 \pm 1.2	12 (6; 22)	32.8% \pm 27.0 p.p.	4.5 \pm 2.0
	in the past	2.6 \pm 0.9	7 (2; 17)	22.0% \pm 22.5 p.p.	3.2 \pm 2.2
	never	2.6 \pm 0.7	9 (3; 18)	20.1% \pm 19.1 p.p.	3.3 \pm 1.9
Kruskal-Wallis ANOVA		p < 0.0001	p = 0.0039	p = 0.0001	p < 0.0001
Number of pack-years (Spearman's rank correlation test)		r = 0.07 p = 0.3625	r = 0.12 p = 0.1382	r = 0.13 p = 0.0934	r = 0.13 p = 0.0964
Arterial hypertension	yes	3.0 \pm 1.1	10 (5; 20)	29.3% \pm 25.4 p.p.	4.2 \pm 2.0
	no	2.8 \pm 0.9	8 (3; 21)	23.4% \pm 21.9 p.p.	4.1 \pm 1.8
Mann-Whitney U test		p = 0.3546	p = 0.488	p = 0.1985	p = 0.5376
Diabetes	yes	2.8 \pm 0.9	7 (2; 14)	25.2% \pm 23.7 p.p.	4.3 \pm 2.0
	no	2.9 \pm 1.1	11 (4; 22)	27.4% \pm 25.1 p.p.	3.8 \pm 2.2
Mann-Whitney U test		p = 0.8718	p = 0.0121	p = 0.4631	p = 0.0904

Table 8. Differences between periodontal status (number of teeth, PI, BoP and CPI indices) in patients with arterial hypertension and diabetes

Median (Q1; Q3) Mean \pm SD		Number of teeth	PI	BoP	CPI median
Arterial hypertension	yes	12 (7; 20)	78.5% \pm 22.4 p.p.	46.1% \pm 28.8 p.p.	3 (2; 4)
	no	12 (6; 17)	86.0% \pm 17.9 p.p.	50.5% \pm 30.5 p.p.	3 (2; 4)
Mann-Whitney U test		p = 0.1527	p = 0.0618	p = 0.3864	p = 0.6304
Diabetes	yes	16 (7; 22)	79.7% \pm 20.5 p.p.	46.3% \pm 31.6 p.p.	3 (2; 4)
	no	11 (6; 20)	76.5% \pm 23.6 p.p.	43.3% \pm 27.9 p.p.	3 (2; 4)
Mann-Whitney U test		p = 0.0132	p = 0.3665	p = 0.5923	p = 0.844

Conclusions

Summing up, it must be said that in the light of modern research the role of risk factors for the occurrence and course of periodontal diseases and cardiovascular diseases is unquestionable, but whether the list of known risk factors for CVD will be supplemented by periodontitis is a matter of time and research.

Our own studies have confirmed that periodontitis and the degree of its severity have an impact on hypertension and diabetes, WHR, which potentially could influence the occurrence of cardiovascular diseases, which could lead to myocardial infarction.

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Energy exchangers with LCT as a precision method for diet control in LCHADD

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Abstract

Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHADD) is a rare genetic disease. The LCHADD treatment is mainly based on special diet. In this diet, energy from long-chain triglycerides (LCT) cannot exceed 10%, however energy intake from the consumption of medium-chain triglycerides (MCTs) should increase. The daily intake of energy should be compatible with energy requirements and treatment should involve frequent meals including during the night to avoid periods of fasting. In fact, there are no recommendations for total content of LCT in all of the allowed food in the LCHADD diet. The aim of the study was to present a new method of diet composition in LCHADD with the use of blocks based on energy exchangers with calculated LCT content. In the study, the diet schema was shown for calculating the energy requirements and LCT content in the LCHADD diet. How to create the diet was also shown, based on a food pyramid developed for patients with LCHADD. The blocks will make it possible, in a quick and simple way, to create a balanced diet which provides adequate energy value, essential nutrients and LCT content. This method can be used by doctors and dietitians who specialize in treating rare metabolic diseases. It can also be used by patients and their families for accurate menu planning with limited LCT content.

Key words: diet, long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHADD), long-chain triglycerides (LCT), intake of energy

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LCHADD (long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency) is a rare genetic disease, inherited in an autosomal recessive pattern. The disease is caused by 3-hydroxyacyl-CoA dehydrogenase deficiency, an enzyme complex taking part in mitochondrial β -oxidation of fatty acids.¹ Deficiency of the enzyme contributes to accumulation of toxic metabolites which can lead to metabolic decompensation, associated with damage to the mechanisms of the body homeostasis. This results in the occurrence of life-threatening disorders in the functioning of one or more organs. Symptoms of metabolic decompensation are: myopathy, cardiomyopathy, hepatitis, peripheral neuropathy and retinitis pigmentosa, as well as hypoketotic hypoglycemia.^{2,3} Clinical symptoms result from a significant reduction in energy production in the long-chain triglycerides (LCT), the toxic effects of carnitine acyl derivatives and the incidence of hypoglycemia.⁴ These symptoms occur or worsen due to dietary mistakes and in a time of increased catabolism during a course of fasting, an infectious disease, stress or vaccinations.⁵

It is estimated that in Poland about 400 newborns are born each year with metabolic defects.⁶ The expected incidence of LCHADD in Poland is around 1 in 120,000 newborns. It most frequently occurs in Pomeranian Voivodeship (1 in 16,900 births).⁷ Since 2014, testing aimed at detection of 21 inborn metabolic defects, including LCHADD, has been applied to all newborns in Poland.⁸ Presently, thanks to neonatal screening, it is possible to diagnose the disease in children before symptoms occur. In this case, application of the appropriate pharmacological and dietary treatment prevents or reduces the risk of the occurrence of clinical symptoms of the disease, its complications and sudden death.

Table 1. Estimated rest periods between meals depending on the age of children with LCHADD⁵

Age	Rest periods between meals	
	break per day	break per night
Infants	3 h	night feeding ²
< 6 months	4 h	night feeding ²
> 6 months ¹	4 h	6–8 h ³
> 12 months ¹	4 h	10–12 h

¹ For each age group, a break in the night feeding is estimated. In children with the risk of complications, the interval should be shorter and individualized.

² In properly fed infants, fasting during the night may be extended up to 6 h from 3 months of age.

³ In properly fed infants, fasting during the night may be extended up to 8 h from 6 months of age.

The purpose of the study

The aim of the study is to present a new method for preparing the menu in LCHADD using blocks based on energy exchangers with calculated LCT content. Currently, there are no dietary computer programs or scientific studies determining the total amount of LCT in all products allowed in the LCHADD diet. Patients should not have the option of self-modifying their own diet because of the probability of making mistakes. Blocks will allow a faster and easier way to prepare a balanced menu in terms of energy value and essential nutrients, as well as the LCT content in the diet.

Nutrition in LCHAD deficiency

Patients with LCHADD require application of a recommended dietary caloric intake with limited LCT.

Energy requirements

In patients with LCHADD, it is important that the energy nutrient intake from the diet is correct and fasting is avoided by taking frequent meals, including at night, especially in the infancy period and during infection.^{9,10} On the other hand, a large energy nutrient supply is also unfavorable as it leads to development of overweight and obesity in patients.⁹

Table 2. The energy requirements for healthy infants in the first year of life¹⁴

Boys		Girls	
age (months)	daily energy requirement (kcal/kg/d)	age (months)	daily energy requirement (kcal/kg/d)
0–1	113	0–1	107
1–2	104	1–2	101
2–3	95	2–3	94
3–4	82	3–4	84
4–5	81	4–5	83
5–6	81	5–6	82
6–7	79	6–7	78
7–8	79	7–8	78
8–9	79	8–9	78
9–10	80	9–10	79
10–11	80	10–11	79
11–12	81	11–12	79

Table 1 presents estimated rest periods between meals depending on the age of children with LCHADD, as developed by Spiekerkoetter et al.⁵ The authors recommend that in states of exacerbation, rest periods between meals should be shorter.

Energy requirements should be set individually for each patient with LCHADD, taking into account age, weight, height and physical activity.¹¹ In determining the energy requirements in the LCHADD diet it is recommended that Polish norms or rules on the nutrition of children

and adults are used, taking into account the recommendations of the World Health Organization (WHO).^{12–14} Table 2 shows the energy requirements for healthy infants in the first year of life while Table 3 shows the energy needs for healthy children between 1 and 10 years of age according to the WHO.¹⁴ Table 4 presents the energy requirements for healthy adults with moderate physical activity developed on the basis of Polish norms.¹²

Although the daily energy requirements given in the standards and recommendations are determined for healthy people, these values can be used for preparing a diet for patients with LCHADD, including monitoring the energy supply from the diet and the patient's health and controlling body weight.

The structure of the diet

The optimal LCHADD diet is considered to be norm-caloric, norm-protein and high-carbohydrate with a limited content of LCT – up to 10% of the energy nutrient intake in daily food rations.^{9,13} Due to the reduced percentage of LCT in the diet, the intake of medium chain fatty acids (medium-chain triglycerides, MCT) should be increased up to 10–25%. The appropriate distribution of the meals throughout the day should also be taken into account (Table 1).

In the case of overweight and obesity in children with LCHADD, a higher intake of protein and a proportional reduction in the calorie intake from carbohydrates is recommended in order to maintain proper metabolic balance.^{9,15} Gillingham et al. observed a positive impact of higher protein intake in the diet on energy balance and metabolic control in patients with LCHADD.¹⁵ However, more studies are needed to confirm these observations. Table 5 shows the structure of the diet in LCHADD developed on the basis of the current scientific findings.

MCT oil

In the treatment of LCHADD, the use of MCT oil reduces the risk of catabolism caused by peri-

Table 3. The energy requirements for healthy children between 1 and 10 years of age¹⁴

Boys			Girls		
age (months)	weight (kg)	daily energy requirement (kcal/kg/d)	age (months)	weight (kg)	daily energy requirement (kcal/kg/d)
1–2	11.5	82.4	1–2	10.8	80.1
2–3	13.5	83.6	2–3	13.0	80.6
3–4	15.7	79.7	3–4	15.1	76.5
4–5	17.7	76.8	4–5	16.8	73.9
5–6	19.7	74.5	5–6	18.6	71.5
6–7	21.7	72.5	6–7	20.6	69.3
7–8	24.0	70.5	7–8	23.3	66.7
8–9	26.7	68.5	8–9	26.6	63.8
9–10	29.7	66.6	9–10	30.5	60.8

Table 4. The energy requirements for healthy adults with a moderate physical activity (PAL = 1.6)¹²

Boys			Girls		
age (years)	weight (kg)	daily energy requirement (kcal/d)	age (years)	weight (kg)	daily energy requirement (kcal/d)
19–30	60	2600	19–30	50	2000
	70	2800		60	2200
	80	3100		70	2650
31–50	60	2500	31–50	50	2000
	70	2700		60	2100
	80	2800		70	2250
51–65	60	2300	51–65	50	1900
	70	2450		60	2000
	80	2550		70	2100

Table 5. Estimated energy structure of the diet in LCHADD developed on the basis of the current scientific findings^{5,13,15,24,25}

Protein (% total energy intake)		12–15%*		
Carbohydrate (% total energy intake)		60–67%*		
Fat (% total energy intake)		20–30%		
MCT10 – 15% of energy intake	LCT 5 – 10%. of energy intake including:			
	DHA		NNKT 1–4 %	
	weight < 20 kg	weight > 20 kg	LA (C18:2; omega – 6)	ALA (C18:3; omega – 3)
	60 mg/d	120 mg/d	2–4 %	~ 0.5 %

*Some authors suggest a higher intake of protein and a proportional reduction in the calorie intake from carbohydrates in patients who have problems with maintaining a healthy weight.

ods of fasting, and has a beneficial effect on improving the fatty acids profile and acylcarnitine concentration in the blood.⁹ The MCT oil should cover 10–20% of the daily caloric intake.¹³ The study of Gillingham et al. in children with LCHAD deficiency whose caloric intake of LCT was $\leq 10\%$ of the total calories in daily food rations and the intake of MCT was $\geq 10\%$ of the calories in daily food rations, indicated lower concentrations of the toxic acyl derivatives of carnitine.¹⁶

Medium chain fatty acids play an important role during physical effort in patients with LCHADD and can provide an alternative source of energy for muscular work. Insufficient calorie intake both before and during doing physical exercise can lead to cardiomyopathy and rhabdomyolysis.^{17,18} Some authors suggest that supplementation of MCT with a dose of 0.5 g per kg of the non-fat body mass during increased physical activity can prevent the occurrence of metabolic disorders in children with LCHADD.⁹ A study by Gillingham et al. demonstrated that supply of MCT immediately before exercise improved exercise tolerance and reduced the risk of rhabdomyolysis in 6 out of 10 patients with disorders of fatty acid oxidation disorders (FAODs).¹⁹ A study by Behrendt et al. indicated that the intake of MCT before doing exercise increased medium chain fatty acid oxidation, caused a reduction in glucose oxidation and a reduction of the burden on the heart during exercise in 8 of 11 patients with LCHADD, compared to providing exclusively carbohydrates.¹⁷ However, the study's authors point out that, in practice, MCT intake (0.3–0.4 per kg) before physical effort contributes to their greater use, especially when the MCT are given with a beverage containing carbohydrates. This allows patients with FAODs to safely perform the training for up to one hour of moderate intensity (60–70% of maximum heart rate, HRmax).

An example of food for a special medical purpose (FSMP) which can be administered prior to the scheduled physical effort is MCT Procal. 1 portion (16 g) provides 105 kcal, 10 g of MCT and 0.14 g of LCT.²⁰ Fantomalt is an example of a FSMP containing a mixture of carbohydrates in which the complex ones constitute 90%. Three measuring spoons of this preparation (3×5 g) provide 57 kcal. Fantomalt in its composition consists mainly of maltodextrin with less maltose and glucose. The preparation can be diluted with water or juice or added to a ready dish. The number of portions of these FSMP should be selected individually for each patient depending on their physical activity.

Linoleic acid (LA, C18:2, omega-6) and α -linolenic acid (ALA, C18:3, omega-3)

Adequate intake of fatty acids LA and ALA with the diet is particularly important and should be included in the total consumption of LCT with the diet. LA and ALA prevent diseases of the retina, peripheral neuropathy, growth deficit and dermatitis.^{9,13} According to the recommendations made by the European Food Safety Authority (EFSA) of 2013, the demand for fatty acids LA and ALA in healthy children up to 12 months of age, is 4 and 0.5% of the total energy intake, respectively.^{21,22} However, according to the recommendations of German, Austrian and Swiss Experts (DACH), intake of LA with the diet for children over 4 months of age up to 12 months of age should constitute 3.5% of the energy requirements. In the diet of children between 1 and 4 years of age and between 4 and 10 years of age the intake of LA should constitute 3% and 2.5% of the total energy requirements, respectively.²³ Because of the risk of deficiency of essential fatty acids (EFA), their intake in the diet in LCHADD should be similar to the dietary intake of healthy children and adults. However, some authors suggest that the supply of essential fatty acids in patients with LCHADD should be from 1 to 2% of the total energy intake, and their main source from food should be vegetable oils, such as linseed oil, rapeseed oil, walnut oil, and safflower oil.^{24–26}

The use of linseed oil, camelina oil or walnut oil, in comparison with rapeseed oil may reduce the accumulation of the various acyl-CoA intermediates, preventing peripheral neuropathy.⁹ Spiekerkoetter et al. recommend the use of walnut oil, soybean oil or wheat germ oil for an optimal ratio of omega-6 to omega-3.⁵ However, in the studies of Charles et al. in patients with the β -oxidation of fatty acids disorder, it has been indicated that the combination of linseed oil and walnut oil is more favorable than rapeseed oil.²⁷ It is associated with a higher proportion of essential fatty acids (EFAs) in linseed oil and walnut oil, compared to rapeseed oil. Table 6 presents the recommended daily dose of the vegetable oils mentioned above, depending on the age of the patient. Table 7 shows the contents of LA, ALA and LCT in vegetable oils and lin-

Table 6. The recommended daily amount of vegetable oils such as soybean oil, wheat germ oil and walnut oil, depending on the age of the patient⁵

The patient's age	Portion of oil [g/d]
0–4 months	3.5
4–12 months	5.0
1–4 years	6.0
> 4 years	10.0

seeds in one energy exchanger (1 EE \approx 50 kcal, discussed later in the paper).

In connection with the recommendations concerning adequate dietary intake of essential fatty acids in LCHADD, the market offers food for special medical purposes, which in their composition in addition to MCT also contain EFAs, e.g. Lipistart.²⁸

Supplementation with DHA acids

Opinions regarding the dietary intake of DHA in LCHADD are divided. Some authors recommend DHA acid as a dietary supplement, due to its negligible dietary intake with the intake in the diet with limited LCT. In the research studies of Gillingham et al. of children with LCHADD (n = 14), the only source of DHA in the diets were dietary supplements.¹⁶

Supplementation with DHA ethyl esters, about 100 mg/day can be considered, due to the possibility of improving visual acuity.⁹ Spiekerkoetter et al. recommend dietary supplementation with DHA at a dose of 60 mg per day for children weighing less than 20 kg and 120 mg per day for children weighing more than 20 kg.⁵

Cornstarch

The use of raw corn starch in the diet of patients with LCHADD reduces the risk of nocturnal fasting.⁹ Consumption of raw starch before bedtime prevents the morning hypoglycemia after an overnight break in nutrition.^{9,24} Corn starch in the diet of children with LCHADD below 2 years of age is recommended in doses of 1–1.5 g per kg of body mass. At the age of 2 years of age, the dose is gradually increased (1.75–2.5 g per kg of body mass).^{9,24,25,29}

Spiekerkoetter et al. believe that routine enriching of the diet with corn starch and glucose polymers is not recommended, yet may be a part of an oral prophylaxis and treatment in emergency cases related to the risk of clinical symptoms caused by too long a night break.⁵ The oral intake of raw corn starch is not recommended for infants less than 9 months of age, due to the insufficient action of pancreatic enzymes.³⁰

Carnitine supplementation

For patients with carnitine deficiency, supplementation of carnitine may be considered.⁹ Gillingham et al. found no sign of toxicity during application of carnitine at a dose of 50 mg per kg of body mass.²⁴ However, there is no published data concerning the potential benefits of supplementation with long term carnitine in LCHADD. It is forbidden to use it during acute metabolic decompensation.^{5,9}

Intake of glucose intravenously

The occurrence of metabolic decompensation in a patient with LCHADD frequently requires hospitalization and ensuring the adequate intake of glucose as an energy source. When the oral intake of carbohydrates before hospitalization does not give the expected results, the patient receives an intravenous infusion of glucose in amounts of 8–10 mg/kg of body mass/min. The intake

Table 7. The contents of LA, ALA, LCT and the ratio of omega-6 to omega-3 in vegetable oils and linseeds in one energy exchanger (1 EE \approx 50 kcal)

Grams of selected fatty acids and ratio of omega-6 to omega-3 in 1EE = in some oils	Flaxseed oil (1EE = 6.0 g)	Walnuts oil (1EE = 6.0 g)	Soybean oil (1EE = 6.0 g)	Wheat germ oil (1EE = 6.0 g)	Canola oil, cold-pressed (1EE = 6.0 g)	Linseed (1EE = 9.0 g)
1EE \approx 50 kcal [g]	6.0 ¹	6.0 ¹	6.0 ¹	6.0 ¹	6.0 ¹	9.0 ¹
LA (omega-6) [g]	0.9	3.2	2.5	3.3	1.1	0.5
ALA (omega-3) [g]	3.2	0.6	0.4	0.4	0.6	2.1
LCT [g]	5.7	5.7	5.7	5.6	5.7	3.6
omega-6/omega-3	0.3	5.1	6.1	7.9	1.9	0.3

¹ 6.0 g of vegetable oil is about 1 teaspoon, and 9.0 g of flaxseed is less than 1 spoon, however in LCHADD, all food products should be weighed.

Table 8. The energy requirements of the patient converted to the amount of energy exchangers and LCT content (g/d)

Daily energy requirement	Amount of EE	LCT content depend on daily energy requirement and share of dietary energy consumption from LCT (4-10%)						
kcal/d	EE/d	4%	5%	6%	7%	8%	9%	10%
500	10	2.2	2.8	3.3	3.9	4.4	5.0	5.6
600	12	2.7	3.3	4.0	4.7	5.3	6.0	6.7
700	14	3.1	3.9	4.7	5.4	6.2	7.0	7.8
800	16	3.6	4.4	5.3	6.2	7.1	8.0	8.9
900	18	4.0	5.0	6.0	7.0	8.0	9.0	10.0
1000	20	4.4	5.6	6.7	7.8	8.9	10.0	11.1
1200	24	5.3	6.7	8.0	9.3	10.7	12.0	13.3
1500	30	6.7	8.3	10.0	11.7	13.3	15.0	16.7
1800	36	8.0	10.0	12.0	14.0	16.0	18.0	20.0
2000	40	8.9	11.1	13.3	15.6	17.8	20.0	22.2
2200	44	9.8	12.2	14.7	17.1	19.6	22.0	24.4
2500	50	11.1	13.9	16.7	19.4	22.2	25.0	27.8
2800	56	12.4	15.6	18.7	21.8	24.9	28.0	31.1
3000	60	13.3	16.7	20.0	23.3	26.7	30.0	33.3

of glucose stimulates insulin secretion and inhibits peripheral lipolysis. However, some patients with abnormal response to insulin should additionally be supplemented with insulin therapy without diminishing the adequate dietary intake of glucose.^{9,31}

Supplementation with vitamins and minerals

Intake of certain vitamins and minerals in the form of dietary supplements, especially fat-soluble vitamins, is recommended in the case of low dietary intake or documented deficiency.⁵ It is important that fat-soluble vitamins were served with meals containing fat, in this case MCT, to be absorbed better.

The method developed for dietary control in LCHADD

LCHADD treatment is based mainly on dietary therapy. Parents or guardians of a sick child should have

knowledge concerning the allowed and forbidden products. The aim of dietary therapy in LCHADD is to adequately balance the diet as regards energy values and the diet structure, i.e. adequate intake of proteins, fats and carbohydrates and the content of LCT. The available Polish studies give the nutritional value of foods and specify the composition of fatty acids in food products. However, the calculation of the sum of LCT, which is important in preparing a menu in LCHADD, has to be done independently and it is rather time consuming.³²

Due to the lack of a clear and easy method for preparing the diet in LCHADD, this paper presents a new method for preparing the menu in this rare metabolic disease.

The basis for dietary calculations in LCHADD is correctly calculating the energy requirements individually for each patient. Meals should be spread evenly throughout the day and with adequate caloric content, in order to prevent a starvation period. Parents and caregivers of children with LCHADD often make dietary mistakes. The most common error, resulting from the fear of the occurrence of fasting and symptoms connected with it, is to give an excessive amount of simple carbohydrates,

which can lead to overweight or obesity in a patient. Treatment of obesity or overweight in patients with LCHADD is difficult and may be limited due to the possibility of the occurrence of disease symptoms resulting from catabolism. In the body of a patient with LCHADD, at the time of fasting there is no production of ketone compounds, which substitute a substrate for the brain in the case of a deficiency of glucose. Therefore controlling energy intake, the LCT content in daily food rations and an appropriate diet structure in LCHADD are important elements in preventing both catabolism and obesity.

The developed block method of preparing a menu in LCHADD applies energy exchangers (EE). One energy exchanger is the weight of the product expressed in g, which provides 50 kcal (210 kJ). LCT content was calculated with regard to the energy exchangers of various food products. The values of energy exchangers and LCT are based on data from the Polish “Tables of Composition and Nutritional Value of Food”, published in 2005.³²

Energy requirements calculated as the number of energy exchangers are shown in Table 8. The dose of LCT

(g/day) sufficient for energy requirements was calculated, depending on the desired LCT energy input in the diet.

In the Polish study, the total LCT value is not calculated, yet the contents of each of 26 individual fatty acids for particular food products are given.³² On the basis of the tables, LCT calculations were made by summing up the fatty-acids containing 12 and more carbon atoms in the chain in different food products. Then the LCT content in 100 g of food was converted into the LCT content in a portion of products including 50 kcal.

Safe products which can be used in the diet in LCHADD were regarded to be all the products in which the LCD content does not exceed 2 g in 100 g. Other products have been conventionally classified as hazardous. Unsafe products are marked in red, and some of them are presented in the form of blocks as some of them may be used in limited and controlled amounts in the LCHADD diet, e.g. linseed oil, walnut oil, soybean oil and beef.

Selection of food products allowed in LCHAD deficiency is important due to the proper balance of the diet in terms of the caloric content, LCT input in daily food

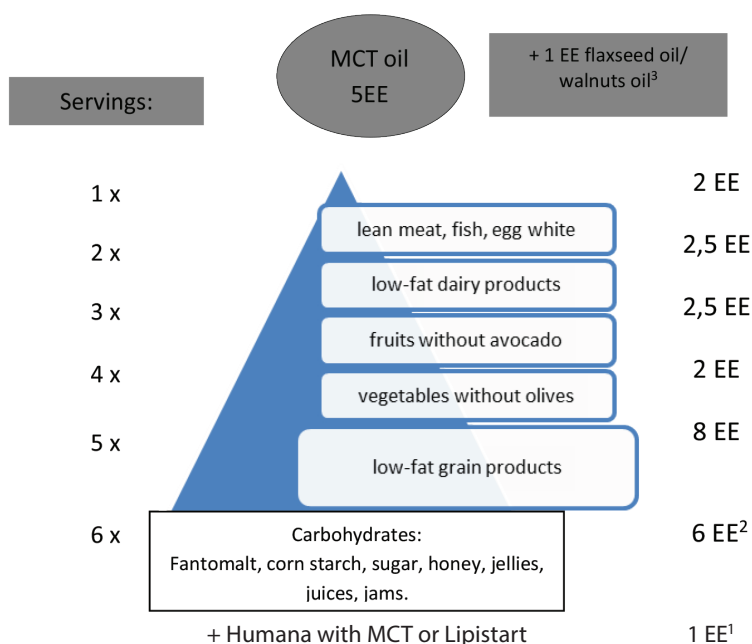
Table 9. Description of blocks in the developed method in LCHADD

Number of blocks	Type of blocks	Description of blocks	Block color
1, 2	medical: - MCT milk, MCT oil, - mixture of carbohydrate	food for special medical purposes for use under medical supervision	light blue (doctor's or dietitian's face on block)
3	emergency	food products with high energy density and small amount of LCT	gold
4, 5	allowed	food products which do not contain LCT	dark green
6	meat	food products which contain safe amount of LCT and are good source of protein	white
7	fish		
8	dairy ≤ 0.5% fat		
9	bread	food products which contain safe amount of LCT and are good source of protein and carbohydrates	
10	other grain products		
11, 12, 13	fruits	fresh fruits which contain safe amount of LCT	yellow
14, 15	fruits, emergency	dried fruits, jam, marmalade and fruit syrups containing a small amount of LCT	orange
16, 17, 18, 19, 20	vegetables	mainly fresh vegetables	green dark blue
21	spice	spicy vegetables (e.g. garlic) and processed products (e.g. ketchup) giving the dishes more intense flavor	pink
22	LCT bombs – vegetable oils	vegetable oils which are source of EFAs and containing a lot amount of LCT	red – amount of food products in this group is very limited
23	LCT bombs – meat	beef, veal, turkey and variety meats containing a lot of LCT which are good source of certain nutrients (eg. vitamin B12)	
24, 25	sports	the blocks defining the various sport activities and their energy expenditure (- EE)	black

Table 10. The energy expenditure (-EE) of various physical activities calculated for a person weighing about 60 kg^{33,34}

Type of physical activity		Energy expenditure kcal/h	Energy expenditure 50 kcal [min]
Tennis		204	15
Baseball		247	12
Cycling		264	11
Lawn tennis		365	8.5
Canoeing 6 km/h		370	8.5
Walk 4 km/h		172	17.5
March 6 km/h		257	12
Playing football		471	6.5
Mountain climbing		529	6
Running	run 9 km/h	540	6
	cross country running	587	5
	run 11 km/h (on a treadmill)	736	4
	long-distance running	793	4
	run 14 km/h (on a treadmill)	818	3.5
	run 18 km/h (on a treadmill)	1036	3
	run (sprint)	1985	1.5

Fig. 1. Food pyramid for patients with LCHADD for 1500 kcal (30 EE)



rations and proper diet structure. Blocks created based on EE and LCT content are assigned to the appropriate groups of food products according to the nutrition pyramid in LCHADD (Fig. 1). Food products are situated on 5 walls rows of blocks and the LCT content in energy exchangers is given. Each block is positioned with a drawing on its upper surface, and adjusted to a given group of food products. 23 food blocks and 2 sports blocks have been developed in the new method. A description of the blocks is presented in Table 9.

In the sports blocks the concept of energy expenditure (-EE) has been introduced, which is defined as the amount of energy expended by the human body during a given activity at a certain time. One energy expenditure (-EE) is the amount of time the activity is done expressed in min and s, which corresponds to the energy expenditure of 50 kcal (210 kJ). In contrast to energy exchangers, the shortcut of energy expenditure has a minus sign, and all the squares of energy expenditure are marked in black. Each side of the square in the sports block shows the energy expenditure of 50 kcal (210 kJ). Energy expenditures were calculated for a person weighing about 60 kg. Table 10 shows the energy expenditure of various physical activities.^{33,34}

Adequate increase of caloric content of the diet in LCHADD in relation to the planned additional vigorous exercise is one of the basic elements of the diet to prevent metabolic decompensation. For this purpose, the relevant measures of specific nutritional purposes with MCT are used and should necessarily be administered before and during exercise. Sports blocks in the form of energy expenditure will allow a quick way to determine if the daily

¹ It can use more EE from low-fat dairy products or lean meat, fish and egg white whereas the amount of food for a special medical purpose (FSMP), like Humana with MCT or Lipistart, should be proportionally reduced.

² It can use more EE from low-fat grain products whereas the amount of carbohydrate-containing foods should be proportionally reduced.

³ Flaxseed oil or walnut oil should not be used during acute metabolic decompensation.

Table 11. Nutrition plan for 6-year-old patient with LCHADD (1500 kcal = 30EE)

Time of meal	Food product	Energy exchangers			Food preparation
		EE	serving	LCT	
7:00	wheat flour type 500	1	15 g	0.17 g	Mix quark fresh cheese with egg white, flour and MCT oil and then blend it. Fry pancakes on a frying pan for pancakes. Pour honey over pancakes. Prepare apple juice with flaxseed oil to drink (blend flaxseed oil with orange juice). *Instead of using 6 mL flaxseed oil, you can use 3 mL walnuts oil and 3 mL flaxseed oil.
	egg white	1/2	26 g	–	
	quark fresh cheese, low-fat	1	51 g	0.21 g	
	honey	1/2	7.5 g	–	
	MCT oil	1	6 mL	–	
	orange juice	1 3/4	204 g	0.21 g	
	flaxseed oil*	1	6 mL	5.7 g	
8:00	apple	1	109 g	0.15 g	An apple between meals.
10:30	semolina	1	14 g	0.13 g	To boiling milk (approx. 120 mL) with water (approx. 50 mL) add semolina and stir thoroughly for several min. Pour porridge into a bowl. Cut orange into small pieces and add it to semolina. Also add a pinch of cinnamon or pinch of gingerbread spice and honey. Humana with MCT to drink.
	milk, 0.5% fat	1	128	0.51 g	
	orange	1/2	57 g	0.06 g	
	honey	1/2	7.5 g	–	
	Humana with MCT	1	11 g	0.73 g	
14:00	celery	1/4	59.5 g	0.09 g	Cream soup of white vegetables: peel and cube vegetables to small pieces. Put the pieces of celery and parsley root into the pot with hot MCT oil. Fry them for about 2 min then add water (approx. 300 mL) and spices (salt, pepper, allspice, bay leaf). After 15 min add the pieces of turnip and cauliflower, cook for another 20–25 min, until vegetables are tender. Remove allspice and bay leaf. Blend the soup to a smooth cream, add spices (e.g. a pinch of nutmeg). To prepare cream add toast bread, prepared earlier on a dry frying pan.
	turnip	1/4	48 g	0.10 g	
	parsley root	1/2	66 g	0.24 g	
	cauliflower	1/4	57 g	0.08 g	
	plain bread	1	20 g	0.27 g	
	MCT oil	1	6 mL	–	
15:30	cod	1 1/4	80 g	0.35 g	Cook white rice in salted water, add two pinches of curcumin to the rice. Season cod with pepper, brush each side of the cod with MCT oil by using a pastry brush. Fry the cod on a hot grill pan about 3 min on each side, then add salt. Cook broccoli and serve with yoghurt and MCT oil (6 mL) or blend broccoli with yoghurt and MCT oil (6 mL). Water to drink.
	white rice	2	30 g	0.18 g	
	broccoli	1/2	92.5 g	0.20 g	
	plain yoghurt, 0% fat	1/2	26 g	0.03 g	
	MCT oil	2	12 mL	–	
17:30	apple	1	109 g	0.15 g	An apple between meals.
19:30	French baguette	3	54 g	0.87 g	Bake french baguette at about 200 degrees Celsius for 1 min, remove baguettes from oven, rub a clove of garlic, add MCT oil, add ham and roast for about 3 min. Pour yoghurt or ketchup over ready baguettes and season to taste. Serve cherry tomatoes with baguettes.
	turkey ham	1/2	30 g	0.48 g	
	plain yoghurt 0% fat	1/2	26 g	0.03 g	
	ketchup	1/4	13.5 g	0.10 g	
	cherry tomatoes	1/4	83 g	0.16 g	
	MCT oil	1	6 mL	–	
23:00	corn starch	3	42 g	–	3 EE of corn starch mix with cold water (approx. 150 mL) and give before bedtime.
Sum		30	–	11.2 g	

The structure of diet: 16% energy from proteins, 60% energy from carbohydrates, 24% energy from fats, 6.7% energy from LCT, including 3.8% energy from EFAs (ALA + LA); 100 mg of DHA.

energy requirement in a patient with LCHADD should be increased depending on their physical activity.

The scheme for preparing a menu using energy exchangers with LCT

Below, the description shows the exact diet nutrition plan to propose a menu for a particular patient with LCHADD using energy exchangers with LCT.

Knowing the basic data of a patient with LCHADD, i.e. age, height, body weight and level of physical activity, the energy requirement should be determined using Tables 2–4. Example of patient data: a boy with LCHADD, 6 years of age, weighing 20.8 kg, height of 120 cm, moderate level of physical activity. The daily energy requirement calculated from Table 3 for a 6 year-old-boy was 1508 kcal (\approx 1500 kcal).

The energy requirements of the patient converted to the energy exchangers and LCT content (g/d) should be read in Table 8. The energy exchangers given value for 1500 kcal is 30 EE. Daily energy intake with LCT in the diet in LCHADD should not exceed 10% of the total energy intake and should be determined individually by the patient's doctor. The given value in Table 8 for 10% of energy with LCT equals 16.7 g. This means that the LCT content in the diet should not exceed a sum of 16.7 g in a boy with LCHADD whose energy requirement equals 1500 kcal.

The intervals between meals and their number should be consulted with a doctor and a nutritionist (Table 1).

In some cases, as a night meal, a patient can be given raw corn starch prepared e.g. with cold water. The amount of corn starch depends on the weight and age of the patient. For a 6-year-old patient, weighing 20.8 kg, the starch content of the night meal is 42 g (3 EE), assuming 2 g/kg of body mass/meal.

The next stage is preparing the menu using the blocks based on EE with the calculated LCT content. The number of portions from a given group of food products should be read in the food pyramid for LCHADD patients in Fig. 1. A one-day menu for the 6-year-old boy with LCHADD (30 EE) is described in the Table 11.

Summary

The presented method of composing a menu in LCHADD with 25 blocks with LCT allows the preparation of a menu for a sick child or an adult in a very fast and precise way. This method can prove to be effective in the hands of both the doctor and the nutritionist, dealing with the dietary therapy of inborn defects of metabolism, but above all it makes it easier for the patients themselves and their families to plan and precisely control the diet in LCHADD every day.

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Intravitreal ocriplasmin: A breakthrough in the treatment of vitreomacular traction?

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Abstract

Vitreoretinal interface pathologies, such as vitreomacular traction syndrome, epiretinal membranes and macular holes are sight-threatening conditions and one of the important causes of vision defects and vision loss. To this date, vigilance with observation of how the vitreomacular traction resolves, or vitreoretinal surgery in more severe cases, were the only treatment options. Recent rapid progress in ophthalmology, especially in diagnostic and visualization techniques, provided better insight into the mechanisms taking place on the vitreoretinal surface, which enabled a more accurate selection of treatment options. Development of ophthalmic pharmacological procedures, such as treatment of vitreomacular traction syndrome with ocriplasmin, constitutes an innovative breakthrough in ophthalmology. The enzyme is a genetically engineered form of human plasmin, a component of blood coagulation cascade that has been envisioned for human therapy since 1950s. It has never been used for vitreolysis in ophthalmology before. The aim of this review is to analyze and compare therapeutic options for symptomatic vitreomacular adhesion and vitreoretinal traction, with particular emphasis on microplasmin. We reviewed the results of recent studies comparing ocriplasmin to other widespread treatment options, such as pars plana vitrectomy.

Key words: vitreoretinal surgery, optical coherence tomography, microplasmin, vitreous body, vitreous detachment.

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Rapid progress in retinal visualization techniques taking place during the last decade facilitated accurate diagnosis and selection of appropriate treatment methods. Introduction of optical coherence tomography (OCT) to the panel of standard retinal diagnostic tests with no doubt represents a milestone in ophthalmology. OCT, also referred to as optical biopsy, provides detailed insight into the retinal structure and vitreoretinal interface. As a result, various abnormalities that previously could not be visualized by means of ophthalmoscopy, such as vitreoretinal traction syndrome, can be expeditiously and accurately diagnosed. This is of vital importance as persistent adhesion and partial detachment of the posterior vitreous cortex that take place on the vitreoretinal surface are the leading cause of many vitreoretinal pathologies.

Vitreous body and vitreoretinal interface pathologies

Interaction between the retina and vitreous body vary by location. The vitreoretinal interface “glue like” proteins, i.e. laminin and fibronectin, fix collagen fibrils of the posterior vitreous body to the internal limiting membrane (ILM) of the retina.¹ At the vitreous base, vitreous collagen fibers pass through the ILM and communicate directly with retinal collagen.²

Posterior vitreous detachment (PVD) from the ILM of the retina is a common condition in an ageing eye. Vitreous body liquefaction and its collapse that occur during the course of the ageing process lead to complete vitreoretinal separation in the majority of patients. PVD is a natural process and does not contribute to vision loss.^{3,4}

Vitreous is most firmly attached to the retina at the vitreous base, optic disc, fovea and along the major retinal blood vessels.⁵ Perifoveal detachment with residual vitreofoveal adhesion is considered the first stage of PVD.⁶ Due to an incomplete posterior vitreous separation, some residual sites of persistent vitreoretinal adhesion (VA) can still be observed.

Vitreomacular adhesion (VMA) is defined as a perifoveal vitreous separation with remaining vitreomacular attachment and unperturbed foveal morphologic features. As long as adhesions of the vitreous to the retina do not transform into a pathological vitreoretinal traction (VRT), the condition is asymptomatic and does not result in any malformation or damage of the retinal architecture.^{4,7,8}

Vitreomacular traction (VMT) is associated with anomalous posterior vitreous detachment accompanied by anatomic distortion of the fovea and intraretinal structural changes. The Vitreomacular Study Group proposed a classification of vitreomacular adhesions based on the diameter of the vitreous attached to the macular surface measured by OCT. Attachment of 1500 μm or less is defined as a focal and attachment of more than 1500 μm

as broad.⁹ VRT occurs whenever the detaching posterior vitreous surface produces pulling forces on the areas of the retina with interface pathologies. Macula and fovea are particularly sensitive. VMT may cause microscopic damage of the retinal surface, which induces the healing process along with the formation of fibroglial scar tissue, proliferation of myofibrocytes and astrocytes. This eventually leads to the thickening of the ILM and epiretinal membranes.^{10–12}

VMT is a potentially sight-threatening condition, manifesting itself as metamorphopsia, photopsia, blurred vision, decreased visual acuity and optical distortion. Escalating VMT may lead to various macular defects, such as foveal pseudocysts, macular edema (ME), macular holes (MH), accumulation of subretinal fluid and more or less intense bleeding from damaged retinal vessels, retinoschisis or even tractional retinal detachment. It may also be associated with age-related macular degeneration (AMD).¹³ Furthermore, it has been suggested that VMA may be associated with the progression of diabetic retinopathy.¹⁴

MH is a full-thickness break in the retinal fovea, involving retinal layers from internal limiting membrane to outer segments of photoreceptors.

Diagnosis and treatment of vitreoretinal interface pathologies

Diagnosis of vitreoretinal interface pathologies includes ophthalmoscopy, biomicroscopy, visual function tests (such as Amsler test, Watzke-Allen test, laser beam test, microperimetry), USG A and B. Crucial state-of-the-art imaging technologies, such as spectral domain OCT (SD-OCT) and spectral OCT imaging with confocal scanning laser ophthalmoscope (SLO) (SD-OCT/SLO), are the most sensitive diagnostic tools that improve visualization of the vitreoretinal surface.

Currently, a standard approach to the treatment of vitreomacular pathologies, such as symptomatic VMA and VMT, includes observation and watchful waiting or surgery. According to the general recommendations that have been published in 2014 and onwards, *inter alia* by Shao Lei and Wei Wenbin, patients with asymptomatic VMT should be observed for at least 2-3 months to see if a spontaneous PVD occurs, and surgery is recommended in the case of significant VMT-related disorders, such as ERM or MH.¹⁵

Typically observation and waiting are continued until VMT resolves spontaneously or until visual acuity of the patient decreases, visual symptoms become intolerable and macular defects progress. Under such circumstances, a decision to perform the intraocular surgery is made by a vitreoretinal surgeon.

However, other studies demonstrated that delayed vitrectomy and too long observation of patients with MH

and VMT might result in a worse outcome than after a quick surgery. In a paper published in 2005, Jaycock et al. reported that a shorter time between the diagnosis and surgery of MH is associated with better postoperative visual acuity and anatomical closure rates: 95.2% for MH of less than 6 months duration, 91.7% for those from 6 to 12 months duration, and 47.4% for those persisting longer than 1 year.¹⁶ According to Theodossiadis et al., persistent traction on the macula may induce irreversible damage to the structure of retinal layers and induce ERM formation. This may be the reason behind incomplete functional and anatomical recovery of the retina after a delayed surgery.¹⁷

Pars plana vitrectomy (PPV) with membrane peeling relieves traction and leads to normalization of the macular architecture and function.^{8,13} Reconstitution of the macular neurosensory layers observed on post-operative OCT was identified as the most relevant parameter for visual improvement.¹⁸

The aim of the PPV procedure is to surgically detach the posterior surface of the vitreous from the retina, perform vitrectomy, ILM peeling, and thereby relieve vitreoretinal tractions.

Despite the significant progress in vitreoretinal surgery since its introduction in early 1970s, it is still not free from severe complications. Use of modern equipment, smaller gauge systems, sutureless techniques, high-speed vitrectomes and chromovitrectomy make it possible to treat the majority of pathologies located at the vitreoretinal interface.¹⁹ However, although this technique is highly effective, represents a breakthrough in ophthalmology and as such is now commonly performed in ophthalmological centers, PPV always requires extreme caution and may be associated with complications.

The most serious adverse events associated with vitrectomy include endophthalmitis (observed in 0.018–0.23% of the cases), bleeding (0.14–0.17%), retinal detachment (in up to 10.9% of patients subjected to 20- or 23-gauge PPV), infrequent progression of a cataract that may develop in 42.5% of the vitrectomized patients, macular edema (5.5%) and retinal breaks.^{20–25} Noticeably, surgical removal of macular tractions associated with mechanical induction of the posterior hyaloid face (PHF) separation represents an important risk factor for development of iatrogenic retinal breaks. The incidence of this complication is reported at up to 18.2% in the case of 23-gauge PPV.²⁶ This may be associated with the presence of VMT-related abnormalities at the vitreoretinal interface.

Pharmacological treatment of symptomatic VMA

Research on simpler and more effective preventive measures that could be implemented at earlier stages of vitreoretinal junction pathologies stimulated prog-

ress in pharmacotherapy. Pharmacological induction of PVD represents a breakthrough in minimizing the need for surgical procedures and the risk of related complications.

On October 17, 2012, the United States Food and Drug Administration (FDA) authorized ocriplasmin for the treatment of symptomatic vitreomacular adhesion, and on March 13, 2013 this agent was granted marketing authorization for the European Union states. Ocriplasmin is the first injectable drug approved to treat symptomatic VMA. But does it constitute a true breakthrough in ophthalmology?

Ocriplasmin, also referred to as microplasmin, is a truncated form of plasmin, an enzyme that has been envisioned for human therapy since 1950s and is a component of the blood coagulation cascade. Plasmin and ocriplasmin have been studied to be effective in vitreolysis.^{27,28} Administration of ocriplasmin into the vitreous cavity was shown to result in a dose- and time-dependent posterior vitreous detachment.²⁹

Ocriplasmin, a recombinant human protein derived from the cells of yeast *Pichia pastoris* with a recombinant DNA technology, is a variant of human plasmin with retained protease activity. It is active against the substrates that play an important role in the vitreous structure and vitreoretinal interface, such as collagen, fibronectin and laminin. Laminin and fibronectin are involved in attaching the posterior vitreous to the retina. Microplasmin belongs to the serine protease family; it degrades the protein scaffold linking the vitreous to the retina, induces vitreous liquefaction and vitreoretinal separation.^{28,30} When administered at a therapeutic dose, ocriplasmin does not induce any morphological or functional changes of the retina.

The enzyme consists of 2 polypeptide chains linked by sulfide bonds. The longer chain has 4 additional disulfide bonds. Due to its proteolytic and autolytic properties under physiological pH, microplasmin is only moderately stable upon injection and undergoes a rapid degradation. Therefore, the time of its activity is fairly limited.^{27,28} Its biological activity within the vitreous cavity can be observed no longer than several days post-injection.³¹

The drug is available in solution for injection. It is designed for intravitreal use under aseptic controlled conditions. The recommended dose is 0.125 mg administered by pars plana intravitreal injection to the affected eye as a single injection. Ophthalmological monitoring for potential side effects should follow the procedure. Administration to both eyes, as well as repeated administration to the same eye, is not recommended, as there is insufficient evidence of the safety of such approach.

Although the drug seems to be highly promising as a treatment for VMA that may restore anatomical and functional conditions with a minimally invasive and less traumatic procedure than vitrectomy, its use still raises some controversies.

Ocriplasmin is indicated for the treatment of vitreomacular traction (VMT) in adults, also associated with a macular hole of ≤ 400 microns in diameter. Patients with smaller baseline diameters of VMA (≤ 1500 microns) are more likely to benefit from the treatment than those with diameters of > 1500 microns.³² Analysis of the vitreoretinal interface abnormalities, conducted within the framework of 2 phase III studies on intravitreal ocriplasmin, demonstrated that ERM may decrease the efficacy of ocriplasmin efficacy, perhaps due to the increase in the strength of fibrocellular organization and contraction.^{28,32–34} The best outcomes are observed in younger (< 65 years) phakic patients, as well as in individuals in whom VMT is associated with MH of < 250 microns in diameter.³² Consequently, indications for ocriplasmin treatment are currently limited, and appropriate qualification of patients represents a key to a successful outcome.

In a controlled randomized multicenter pivotal study on enzymatic vitreolysis with ocriplasmin for VMT and MH demonstrated that vitreomacular adhesion resolved in 26.5% and 10% of ocriplasmin- and placebo-injected eyes, respectively. Total PVD occurred in 13.4% and 3.7% of ocriplasmin- and placebo-injected eyes, respectively, and vision improved by 3 or more lines in 12.3% of ocriplasmin-injected eyes. Nonsurgical closure of MH by day 28 was achieved in 40.6% of ocriplasmin-injected eyes and in only 10.6% of eyes injected with placebo.²⁸ Although these outcomes are highly impressive, the results of recent research on the application of ocriplasmin for the treatment of stage 2 MH imply that the closure rates may be lower and sporadically the holes that failed to close after ocriplasmin injection may even enlarge.³⁵

The results of phase III trials imply that ocriplasmin is usually well tolerated. The main adverse events, such as vitreous floaters, were reported in 16.8%, progression of cataract in 8.2%, and mild or transient intraocular inflammation in about 7% of ocriplasmin-treated patients.²⁸ Also other transient mild ocular adverse events, such as eye pain and decreased visual acuity, were observed after the drug was administered. The incidence of post-injection retinal detachment and retinal tear was similar as in the placebo group.²⁸

Owing to the proteolytic properties of microplasmin, this enzyme may also target other intraocular structures that contain fibronectin, laminin or collagen IV, e.g. the lenticular zonules. This may result in lens subluxation and phacodonesis. Although studies using scanning an electron microscope did not reveal any post-injection damage to the ciliary body and zonules, a single case of lens subluxation following intravitreal ocriplasmin injection has been reported in a participant of a phase II trial.^{27,36,37}

Ocriplasmin may also exert a diffuse enzymatic effect, which is not limited solely to the macular region but involves the entire retina, including photoreceptors and retinal pigment epithelium (RPE). Importantly, mi-

croplasmin's target, laminin is also present in multiple retinal layers, including photoreceptors and retinal pigment epithelium. This may explain the etiology of a few reported cases in which an acute panretinal dysfunction and vision loss with severely reduced electroretinography responses were observed after intravitreal ocriplasmin injection.^{38,39}

Furthermore Silva et al. reported 2 cases in which the formation of retinal breaks leading to retinal detachment (involving macula in one patient) was documented on continued follow-up. In both these cases, retinal breaks did not result from incorrect ocriplasmin injection, but rather were the consequence of successful releasing VMT and remaining VRT on the periphery of the retina.⁴⁰

To this date, we lack sufficient evidence regarding the application of microplasmin in some specific clinical situations, e.g. concomitantly to anti-VEGF therapy, in patients with large-diameter macular holes, individuals with high myopia, aphakia, history of rhegmatogenous retinal detachment, lens zonule instability, recent ocular surgery, proliferative diabetic retinopathy, ischemic retinopathies, retinal vein occlusions, exudative AMD, vitreous hemorrhage, infants and children scheduled for vitrectomy. Therefore, ocriplasmin is not recommended in such cases and considered solely as an experimental treatment.

Owing to the innovative character of the treatment and the fact that ocriplasmin is a subject of many ongoing and planned clinical trials, this agent is still monitored by various bodies, including the European Medicines Agency.

The high cost of ocriplasmin therapy may also constitute a significant obstacle on the route to considering it a widespread standard treatment. Considering the fact that VMA resolved in only 26.5% of ocriplasmin-treated patients participating in clinical trials and the drug itself is costly, PPV may turn out to be more cost-effective.²⁸

Conclusion

Despite all the concerns regarding ocriplasmin treatment, it doubtlessly constitutes an alternative to vitrectomy and expectant management in patients with vitreomacular interface pathologies. Nonsurgical introduction of PVD with microplasmin is less traumatic and may eliminate major risks inherent to surgical approach. It also provides a more physiological state of the retina without exposing it potential side effects, such as retinal breaks and iatrogenic macular damage that may occur during the ILM peeling and introduction of PVD. Furthermore, enzymatic vitreolysis also allows for earlier intervention and prevents consequences of VMT. Although further research is still needed, the discovery and introduction of ocriplasmin treatment constitute a significant step forward toward a minimally invasive surgery and a breakthrough in the treatment of VMT.

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Zirconium: The material of the future in modern implantology

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Abstract

The authors present the contemporary state of knowledge concerning alternative materials for dental implantology. First of all, factors influencing osseointegration are stated. The most important factors seem to be the type of implant surface. Among the numerous parameters describing them, the most important are: average roughness and porous density. Some studies proved that materials with comparable surface roughness provide similar osseointegration. In modern implantology titanium is the material still considered as a “gold standard”. However, aesthetic features of titanium still bear several disadvantages, especially in the case of periodontium with a thin biotype in the anterior, aesthetic sensitive area of the jaw. If a titanium implant is used in such a case, the mucosa at the implant’s neck may become grayish and, consequently limits the success of the overall treatment. That was the reason for seeking alternative materials to manufacture dental implants. Initiated by general medicine, mainly orthopedics, the search led to the discovery of zirconium dioxide used in dental implantology. A small number of complications, good chemical parameters, anticorrosion, mechanical strength, elasticity module close to the one of steel, and especially biocompatibility made zirconium a perfect material for this purpose, although this material presents several problems in achieving optimal roughness. In this overview one of the probable methods, a process of partial sinterization, is presented.

Key words: literature review, zirconium, dental implantology

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Previously used materials in implantology

People have been trying to recreate lost tooth structures for ages. The first attempts to implant teeth were made as early as in ancient Egypt, and the first “implants” had been animal teeth. Usually, they were made of mechanically shaped parts of ivory. On the basis of other, later excavations, it was claimed that similar implantation procedures were used not only by the ancient Egyptians, but were also found in South America and Europe. The Mayans used volcanic glass, shaped like a tooth root, to achieve immediate implantation of a lost tooth. Also animal shells were used as implant material.^{1–3} The first modern xenogenic material used in implantology was gold. In 1809 the Italian dentist Maggiolo applied immediately after extraction one-part structures whose root shape matched the tooth socket.⁴ Materials used in implantology changed over the decades and in the nineteenth century, apart from gold, other metals and materials started to be used: platinum, iridium, lead, rubber and porcelain. In 1934 the Bulgarian surgeon Hans Abel was the first to use an implant made of ferrous alloy.⁴ Three years later Adams was the first to patent two-phase implants, in which, after healing, supra-gingival retaining elements were fixed to the root with the use of a screw.⁴ The first implant that looked like modern implants was created by Strock in 1983, which was made of cobalt-molybdenum alloy.⁵

Nowadays, the most significant metal used in dental implantology is titanium. It became so popular because of its features: biocompatibility, mechanical strength, ability to osseointegrate and the fact that it is easy to produce. The number of recorded treatments with titanium implants combined with long observation periods of them was also important for approving titanium implants as a standard procedure in implantology.⁶ However, the aesthetic features of titanium still bear several disadvantages especially in the case of periodontium with a thin biotype in the anterior, aesthetic sensitive area of the jaw. If a titanium implant is used in such a case, the mucosa in the implant's neck area may become grayish and, consequently, limit the success of the overall treatment. Titanium implants may also require additional surgical procedures concerning soft tissue augmentation, e.g. connective tissue drafting, which aims to widen and thicken calloused gum or bone augmentation due to an age-related bone shift and atrophic and resorptive processes after tooth loss, resultant from a lack of strain, as an important functional stimuli for preservation and remodeling of the alveolar bone.⁷

Materials alternative to titanium

In order to improve aesthetics, white materials started to be used to produce implants. Due to its good osseointegrative properties, the first ceramic material used in

implantology was aluminum oxide.^{8,9} In follow-up examinations after 10 years, the success of those implants was between 87 and 92.5%.^{9,10} Systems based on aluminum oxide were used for immediate implantation in cases of single tooth loss in the jaw, in the area of incisors, canines and premolars, i.e. in the areas where chewing forces are relatively weak. Even though implants were used in the above-mentioned clinical circumstances, there were cases of damage caused by chewing functions, and by reason of inadequate mechanical strength, aluminum-oxide implants were no longer in use.^{11–14} Another alternative material was introduced by general medicine, mainly orthopedics. Since the 1970s, zirconium has been used to reconstruct hip joints.

To this day over 1,000,000 treatments using zirconium to replace hip joints have been performed. A small number of complications, good chemical parameters, anticorrosion, mechanical strength, elasticity module close to the one of steel, and especially biocompatibility made zirconium the perfect material for implantology.^{15,16} Zirconium's biocompatibility was thoroughly examined in relations to all cell lines existing in a potential contact with a dental implant. Research conducted by Dion et al. and Li et al. on fibroblast cell lines and epithelial cells, with the use of human cord blood, did not show any cytotoxicity of zirconium.^{17,18} The mentioned researchers added pure zirconium powder after contact and then performed cell proliferation and differentiation with immunofluorescent methods. Ko et al. conducted detailed research on human osteoblast cell lines, in which they compared the population of human osteosarcoma cells on the surfaces of zirconium and pure titanium.¹⁹ Osteoblast cell lines were incubated on a specially prepared medium, consisting of pure titanium and zirconium. Observations after 6, 24, 48 and 96 h were performed with the use of a scanning electron microscope and then cell proliferation was measured as mRNA concentration function with the use of PCR method. The investigation showed the comparative ability of osteoblasts, cultured in contact with both, pure titanium and zirconium, to proliferate and differentiate towards osteocytes. Osteoblasts adhered even better to zirconium surfaces.

The role of implant surface nanostructure in osseointegration

As far as dental implantology is concerned, the main factor in achieving treatment success is implant osseointegration, i.e. functional incorporation of the implant into the human organism. On microscopic scale, osseointegration appears when there is contact between the implant and the bone (BIC – bone to implant contact).

The main reason why zirconium could not be used as the most common material in dental implantology was

the fact of its high resistance to physicochemical processes and that its surface cannot be modified to the same extent as titanium surfaces can. Rather than other parameters, e.g. the implant length, the success of osseointegration strongly depends on the implant surface structure, which may be described by many parameters out of which the main 3 are:

a) average roughness (Ra), which is an average measure of values of vertical deviations of a chosen plane from the particular surface points. Mathematically, it is described by the following formula:

$$R_a = \frac{1}{L} \int_0^L |y(x)| dx$$

where l is a measure of the distance and y -function is the value of profile deviations;

b) the distance between particular deviations (S) is mathematically:

$$\frac{1}{n} \sum_{i=1}^n S_i, S_i = \frac{S_1 + S_2 + \dots + S_n}{n}$$

where S_i is a distance between two local peaks and n is the number of distances evaluated on a given distance;

c) porous density (PD).^{20,21}

Oshid et al., in their studies, could prove that for optimal osseointegration the Ra parameters of the implant surface have their highest and lowest restrictions. If the roughness is too high (Ra below 1 μm) microleakages occur and chemical compounds are released to the external environment.^{22,23} Apart from microleakages, it is stated that excessive roughness of the implant surface represents a mechanical obstacle for culturing the implant surface with cells. Moreover, excessively rough surfaces with too many structural processes of small sizes are surfaces with relatively low integrity. On the other hand, a surface which is too smooth is not an optimal environment to be cultured with osteoblasts. Another problem in this context seems to be the roughness of the transmucosal part of the implant. Due to the fact that the extrinsic fiber cementum on the implant surface is missing, no fiber attachment is possible at the implant neck area, which was why primarily highly polished titanium surfaces were preferred to improve soft tissue attachment in this area, which on the other hand, bears the risk of bone loss and of pronounced epithelial down-growth.^{24,25}

In the studies by Matsuzaka et al., it was proved that on a cellular and subcellular level the characteristics of the surface corrugation of the material implanted in the bone tissue influence patterns formed by cells populating this area.²⁶ On a rough surface (Ra: 1–2 μm) osteoblasts were able to fully fill the surface hollows after 8 days of incubation, whereas during the same observation period on a smoother or rougher surface, cell clusters could only be seen at the hollow edges. At a subcellular level, it was proved that increased amounts of endoplasmic reticulum (RE) causes increased metabolic activity of the cells lo-

cated in the most internal layers, making the mentioned surface significantly predisposed.

Zinger et al. and Deligianni et al., in their experiments on human osteoblastic sarcoma cell lines, could show that the most preferential diameter of micropores for cell proliferation is 30 μm .^{27,28} With these pore parameters, cells easily adapted to a 3-dimensional medium. Mediums with micropores with diameters of 10 μm were not recognized by the cells, whereas in bigger micropores (100 μm) cells did not find mechanical retention and were rinsed. These authors also proved the influence of medium size pores on cell differentiation after observing osteoblast filopodia creation.

De Oliveira and Nanci used immunohistochemical methods to prove the increased metabolism of osteoblasts that manifests itself in significantly higher secretion of osteopontin (OPN) and bone sialoproteins (BSP) to extracellular matrix.²⁹ It is worth noting that in the mentioned studies there were no statistically significant differences in the synthesis of other protein matrixes: fibronectin, tubulin and type I collagen. However, increased biosynthesis of BSP and OPN was proved, which was a pioneering proof of selective and precise influence of the type of the medium's nanostructure on gene expression, metabolism direction and, as a result, potentially bone tissue cells differentiation. The presence of extracellular matrix, typical for bone tissue, is a factor that differentiates mesenchymal cells towards osteoprogenitor cell lines. Both these processes: primary, resulting from the influence of the medium, and secondary, resulting from the composition of the primary matrix, act additionally in the processes of osteogenesis. Mentioned viewpoints are similar to the results of Delignani et al., who proved the importance of the medium surface to start osteogenesis.²⁸ Biological activity and cell adhesion to the medium surface was measured in these studies by checking the alkaline and fibronectin phosphatases activity. According to them, the factor influencing this is the strength of osteoblasts binding to the surface of dental implants.

Anselme and Bigerelle created their own method of evaluating the strength of osteoblast binding to the surface.³⁰ After a period of 7, 14, 21 days of incubation, cultured osteoblasts were rinsed with EDTA. The number of cells was counted after 5, 10, 20, 30 and 60 min of centrifuging and measured in a flow cytometer. On the basis of the results, a graph line was created which resembled normal distribution with its maximum around average values (10–30 min). The greater the deviation towards 60 min, i.e. the longer the cells adhered to the medium surface, empirically the stronger was the binding with the medium. The highest results were obtained for the medium of Ra = 0.76 μm and 6.25 μm .³⁰ On this basis, the term "adhesive power" (AP) was suggested, which is a measure of the deviation from the standard cytometric curve of a normal distribution.

Osseointegration of zirconium implants

The topic of the histological evaluation of zirconium implants is still relatively new. The only studies that may be taken into consideration are those in which implant/bone contact indicator value (BIC) was evaluated, the studies which included a greater number of implants, conducted according to the standards of statistical analyses and those which included the evaluation of titanium implant control group as well. In the current literature there are only a few studies in accordance with these criteria.^{31–40}

First, in a time dependent studies about the osseointegration of $n = 156$ zirconium implants with a Ra of $0.9 \mu\text{m}$ the BIC-values (bone-implant contact) reached 86% after 8 weeks of observation.³¹ However, so far this has been the only study, in which such high BIC-values of osseointegration of zirconium implants were found. In other studies the BIC-values for the above mentioned implants reached 45 and 65%.^{32,33} In a study of Stanic et al. on 28 zirconium implants with a Ra of $1.26 \mu\text{m}$ the average BIC-value was 56% after 60 days of observation.³⁴ These studies provide unstable results, where the difference between the highest and the lowest BIC-value was about 32%. Scerano et al. as well showed in a rat model that direct bone to zirconium implant contact exists.³⁵ In 4-week observations on 20 implants they stated that the BIC-value was 68%. With the same assumptions, Aldini et al. evaluated a BIC of 55% ($\pm 27\%$) after an observation period of 60 days.³⁶

Whereas in the last 2 mentioned studies the surface nanostructure features were not taken into consideration, studies of Senerby et al. show quite precise analysis of the relation between the degree of roughness of zirconium implants and the results of osseointegration.³⁷ In their study, zirconium implants were divided into 3 groups: the first group included implants with a surface roughness of $0.75 \mu\text{m}$, in the second group the surface roughness was $1.24 \mu\text{m}$, and in the third $0.93 \mu\text{m}$. The research was conducted in a rabbit model where implants were placed in the bone of the tibia and femur. Resultant BIC-values differed depending on the place of implantation. For implants placed in the bone of the femur more referential results were obtained: 46% for the first group, 60% for the second and 70% for the third group, whereas BIC-values for implants placed in the bone of the tibia were much lower: 19, 31 and 22%, respectively. It may be considered that the most preferential degree of roughness of dental implants made out of zirconium is slightly different from the one of titanium implants.

There are also studies showing much worse osseointegrative features of zirconium implants. In Deprich's study on 48 zirconium implants (Ra = $0.598 \mu\text{m}$) placed in the tibia of 12 minipigs, the BIC-value reached 18% after 3 months of observation.³⁸ Moreover, it is difficult to dis-

cuss the osseointegration of zirconium implants, because not only is the number of studies on this topic small, but they also differ in methodology and consequently in results. For example, osseointegration was evaluated on the basis of observations that included a time span of 2 weeks up to 24 months. Implants were also evaluated on the basis of different animal models and placed in different anatomical structures. Another matter was implant loading. In most studies implants were not loaded, but there are also a few studies in which they had been loaded.

Surprising results were obtained while studying the osseointegration of ceramic implants. Measured BIC-values varied from 2 to 86.8%.^{31,32} Additionally, not all studies include the evaluation of titanium implants as a control group, which makes it difficult to draw conclusions. Titanium implants, as a reference, were used only in studies conducted by Dubruille et al. and Kohal et al.^{33,39}

Based on these studies, it can be concluded that with the osseointegration of zirconium implants the bone tissue behaves the same way without any worse features in comparison to conventionally used titanium implants. Currently, there are only 5 studies comparing the osseointegration of ceramic and titanium implants after loading histologically.^{37–40} The first research with such defined methodology was conducted on a monkey model by Kohal et al.³⁹ The investigation period was about 9 months and the implants were loaded after 5 months. In this study, it was proved that osseointegration of ceramic and titanium implants is comparable with an equal BIC of 68% for ceramic implants.

In other studies, negative behavior of bone tissue in the presence of zirconium implants after loading was observed.⁴⁰ In these cases, immediately loaded zirconium implants (one-phase loading) were used, along with unloaded ones (two-phase loading). Despite generally good BIC-values (around 70% in all studies), after loading, alveolar marginal bone loss could be detected. Hence, with ceramic implants a 2-phase implantation method seems to be preferable.

In a study by Sennerby et al. that followed these strict criteria, osseointegration of titanium implants and 3 groups of zirconium implants with different types of surface modification were compared.³⁷ Osseointegration was evaluated histomorphometrically as well as with the help of SEM imaging and biochemical tests. Referring to these studies, it may be concluded that in the case of zirconium and titanium implants with the same or comparable roughness, the obtained osseointegration is comparably good. In the case of zirconium implants, the method of modifying the surface to obtain such characteristics is a challenge. Methods known for titanium implant surface modification (e.g. acid-etching and sand-blasting) are not successful in the case of zirconium implants, because of the physicochemical parameters of that material.

It seems that it is worth seeking methods to modify zirconium implants surfaces in such a way that they would

have features and characteristics of ceramic material surfaces. One of the probable methods is a process of partial synerization, in which the implant is covered with a mixture of two powders: binding and structural. Both powders bind to the implant surface, but only the structural one does so in a durable way. Binding powder is supposed to only block potential binding places for structural powder, stochastically. After that, it is removed, whereas remaining structural powder that is bound to the surface creates processes making the surface rougher. Such modified zirconium implants are being thoroughly studied in cell and animal models.

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The role of human papillomavirus in oncogenic transformation and its contribution to the etiology of precancerous lesions and cancer of the larynx: A review

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Abstract

Human papillomavirus (HPV) belongs to the *Papillomaviridae* family and infects squamous cells and mucous membranes of humans. Various studies conducted over the last years have shown a correlation between HPV infection and carcinogenesis process. The DNA of the virus is detected in approximately 20% of cancers of the upper respiratory tract. The presence of HPV in cancerous lesion of the larynx varies depending on the procedure applied for sample collection and the viral DNA detection method. The high variance in the frequency of HPV detection is observed even among results obtained with the use of PCR reaction. It varies between 3 and 85%. HPV is also the etiological factor of laryngeal papillomas in both children and adults. However, a considerable amount of research demonstrates that 1–7% of the larynx papillomas in adults undergo transformation into squamous cell carcinoma. The aim of the study was to summarize the current state of knowledge regarding the presence of the HPV virus in the larynx as well as its participation in malignant transformation.

Key words: human papillomavirus, larynx cancer, laryngeal papillomatosis, laryngeal neoplasm, tumorigenic transformations

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Various studies conducted over the last years have shown a correlation between human papillomavirus (HPV) infection and the carcinogenesis process. The virus may cause neoplastic lesions, especially in the mucous membrane of the cervix, but also in other anatomic sites. The most important parts of the body toward which the virus shows the highest invasiveness include the reproductive tract, skin, as well as oropharynx and larynx.¹ With the use of various molecular biology methods, one can detect the DNA of HPV in benign papillomatosis, in carcinoma verrucosum and malignant lesions in carcinoma planoepitheliale of the larynx.² However, the virus's role in the development of laryngeal cancer has not yet been fully elucidated. The data is usually acquired from retrospective studies, which are based on formalin-fixed and paraffin-embedded material. Moreover, research is conducted on small groups often counting less than 100 patients.³ The aim of the study was to summarize the current state of knowledge regarding the presence of the HPV virus in the larynx as well as its participation in malignant transformation.

The role of HPV in oncogenic transformation

Virus biology

HPV taxonomically belongs to *Papillomaviridae* (previously *Papovaviridae*) family. HPV, however, infects skin squamous epithelial cells and mucous membranes in humans. Papillomaviruses diameter is 55 nm, they do not have lipid envelope and their genome is protected by protein capsid of icosahedral symmetry that is built out of L1 protein (55 kDa) in 80% and of L2 protein (70 kDa) in 20%.⁴⁻⁷ There are about 150 different types of HPV, which are well characterized.⁸ L1 capsid protein coding sequence has to differ in at least 10% in each newly identified virion in order to classify it as a new type of HPV virus.^{4,8} The development of molecular biology techniques contributed to the rapid isolation and identification of new HPV types. Since 2004, it has been possible to identify about 30 new types of human papillomavirus.⁴

The genetic material of HPV is circular, double-stranded DNA molecule consisting of about 8000 bp (Fig. 1). Whole genetic information of a virus is encoded on one of the DNA strands, which is transcriptionally active. The genomic sequence of HPV contains 8 open reading frames (ORFs), which encode virus proteins. ORFs may be divided into E – early and L – late. The first of them is necessary for the replication of a virus genome. Those are 6 open reading frames encoding E1, E2, E4, E5, E6 and E7 proteins. The other 2 ORFs encode L1 and L2 capsid proteins.⁷ In the HPV genome, there is also a long control region (LCR), which is associated with the control of rep-

lication and transcription of virus genes. It contains *cis* elements, into which transcription factors bind.⁸ Functions of HPV proteins are shown in Table 1.

HPV penetrates into the organism via micro-injuries of mucosa membrane, which allows the basal layer of the epithelium to be reached. The virus adsorbs and infects dividing cells. HPV attaches itself to the cell membrane with the use of epidermal growth factor receptors and α -6 integrins. It may infect cells of the basal and para-basal layer of stratified squamous epithelium of oral cavity and pharynx.^{2,9,10} In the infected cells of the epithelium basal and para-basal layer, only E HPV genes encoding early viral proteins are expressed. However, HPV goes through a full development cycle only in differentiated keratinocytes that produce capsid proteins and make infectious progeny virions.¹¹

The mechanism of oncogenic transformation of cells

The DNA of human papillomavirus, following its penetration into the cell, functions as an additional, autonomous molecule, so-called episomal form. In this format it is found in primarily infected and undifferentiated epithelial cells.¹² During its developmental cycle in the host cell, HPV can enter into the lysogenic cycle, which means connection of virus DNA with genome of infected cell. Such integration is crucial for HPV-dependent carcinogenesis. Integration of genetic information occurs at ORF E1 and E2 site within the virus's nucleotide sequence, wherein products of those ORFs control level of expression of virus E6 and E7 oncoproteins.¹³ The consequence of virus DNA integration is sequence disruption, E2 deletion and/or inactivation resulting in the inhibition of gene function and an increase of expression of E6 and E7 oncogenes.¹²

Three of the early genes, E5, E6 and E7, are associated with cancer development. Proteins E6 and E7 cause dysregulation of a cell cycle, whereas E5 is associated with the carcinogenesis process, as it protects cells against apoptosis, participates in the inhibition of antigen presentation by virus-infected cells and disrupts communication

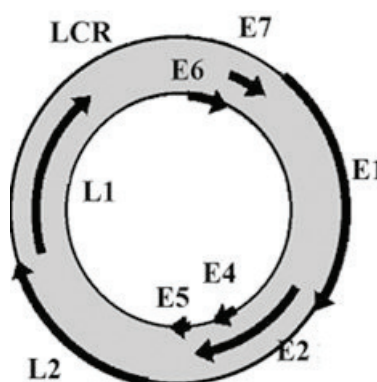


Fig. 1. Genome of human papillomavirus types 31. LCR – control region, E – early region (E1-E2, E4 – E7), L – late region (L1, L2)⁸

Table 1. Proteins function of human papillomavirus – HPV⁵

Protein	Function
E1	<ul style="list-style-type: none"> • necessary for replication of viral DNA • connections to the initiation site of viral DNA replication • forms with E2 complex, which has a helicase activity
E2	<ul style="list-style-type: none"> • it is a major transcription regulator of the virus gene • combines the LCR sequences and activates or represses transcription • participates in the replication of viral DNA • facilitates the attachment of E1 to origin of replication
E4	<ul style="list-style-type: none"> • links to keratin filaments, causing changes in the keratinocytes • facilitates the submission of virus particles and release them during keratosis • expressed mainly in the late stage of the virus cycle in differentiated keratinocytes • causes cell arrest at the interface of the G2 / M in the differentiated keratinocytes
E5	<ul style="list-style-type: none"> • induces uncontrolled cell proliferation • inhibits apoptosis • disrupts intercellular exchange • activates receptors for growth factors such as EGFR
E6	<ul style="list-style-type: none"> • induces DNA synthesis • causes the activation of telomerase (immortalization of cells) • prevents cell differentiation • interacts with the four classes of cellular proteins including: transcriptional co-activators, proteins associated with the polarity of the cell, tumor suppressor proteins (mainly p53) and apoptosis inducers, factors involved in the replication and repair of DNA
E7	<ul style="list-style-type: none"> • induces uncontrolled cell proliferation • interacts with the tumor suppressors (especially Rb)
L1	<ul style="list-style-type: none"> • the major capsid protein • reacts with cell receptors (this cells that are susceptible to HPV infection)
L2	<ul style="list-style-type: none"> • additional viral capsid protein • facilitates the assembling of the capsid • it may react with DNA and cellular receptors

between cells.^{7,14} HPV-infected epithelium is the only site of expression of this protein, which is not observed following the development of neoplastic lesion. The most accepted model of E5 contribution into carcinogenesis is endocytic ATPase inhibition resulting in the prevention of endocytes acidification. It leads to abnormal signaling pathways of epidermal growth factor receptors (EGFRs) endocytic exchange.¹⁴ E5 participates also in EGFR activation. Overexpression of this receptor in laryngeal cancer is a prognostic factor of tumor aggressiveness and invasiveness. EGFR increase is observed in keratinocytes and cells of laryngeal papillomas infected with HPV. It is believed that viral E5 protein may have an impact on the increased EGFR expression in cells. However, an increase in the number of receptors may result from E6 and E7 oncoprotein-dependent mechanism, which causes the transcription of a certain group of genes to be activated.¹⁰ An

additional, newly discovered function of E5 is cell fusion triggering. Studies indicate that cell fusion may play an important role in neoplastic transformation of cells. Other viruses, which, similarly to HPV, show cell fusion and are related to cancers, are: HBV, HCV and Epstein-Barr.^{14,15}

The E6 protein of these high-risk viruses is also involved in a multistep process of neoplastic transformation of cells. It reacts with various cell proteins involved in apoptosis, transcription regulation, the maintenance of chromosomal stability, epithelium organization, differentiation, adhesion, polarization, and the control of cell proliferation.¹⁶ Moreover, E6 oncoprotein causes an increase of hypoxia-inducible factor-1 α (HIF-1 α) and VEGF expression, which may be important in the angiogenesis process.¹⁷

The best-known mechanism involving E6 is ubiquitin-dependent degradation of p53 suppressor protein.¹⁸ P53 is a transcription factor, which plays many important cellular functions, such as cell cycle regulation, DNA-repair activation and induction of apoptosis. The expression level of this protein in normal cells is low; however, it increases in response to DNA damage and viral infections.^{11,18} E6 oncoprotein causes the abolition of p53 protein activity, consequences of which may be impaired regulation of cell proliferation (facilitation of cell division), frequent occurrence of spontaneous mutations and the stabilization of chromosomal instabilities.¹⁹ In normal cells, p53 suppressor protein degradation occurs with the participation, inter alia, of Mdm2 ligase. In virus-infected cells proteolysis depends on E6 oncoprotein, which integrates with E6AP ligase. Both proteins form stable complex into which p53 joins, thus enabling its degradation. The effectiveness of p53 degradation depends on the strength of association of suppressor protein, E6 oncoprotein and E6AP ligase. This process depends on the type of virus that has infected the cell, because there are differences in binding both p53 and E6AP ligase between high and low risk HPV. E6 HPV 16 and 18 proteins bind stronger in comparison with HPV 11.^{19,20}

E6 oncoprotein influences also other cellular mechanisms. p53 deregulation is one of the possible methods for preventing apoptosis of the infected cell. E6 may also destroy other pro-apoptotic factors such as Bak protein, fast-associated death domain protein (FADD) and procaspase-8. Inhibition of programmed cell death may also occur through NF- κ B transcription factor activation leading to the increase of expression of inhibitor of apoptosis 2 (IAP-2).¹⁹ E6 associates also with tumor necrosis factor receptor 1 (TNF R1).¹⁶ Apoptosis may be also inhibited by E7 oncoprotein, which blocks expression of pro-apoptotic factor Mcl-1.²¹

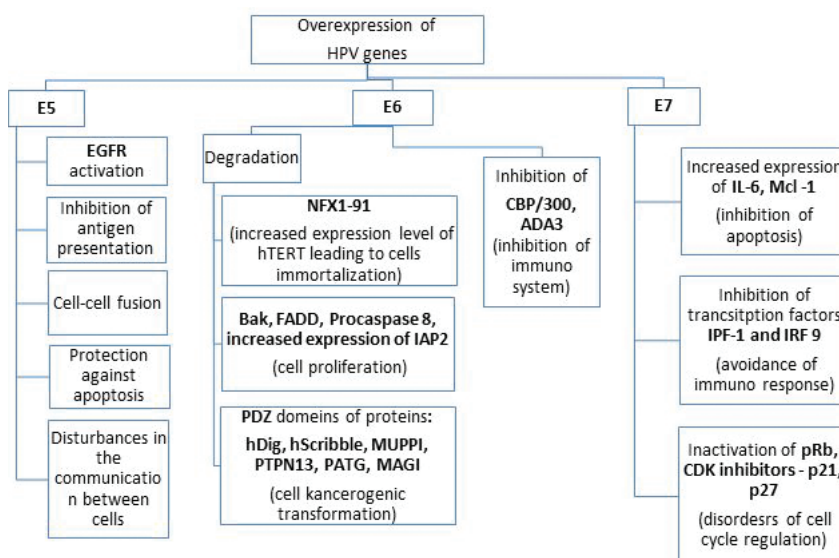
Another feature of E6 protein important for carcinogenesis is its ability to immortalize the infected cell. Host cells immortalization by high-risk HPV is possible thanks to telomerase activation. In normal cells this enzyme is inactive. Increased telomerase activity was shown in can-

cer cells.²² E6 HPV 16 protein affects the activity of this enzyme in a number of ways. One of them is an increase of the expression of hTERT gene, the product of which is enzyme catalytic subunit. E6 and E6AP ligase complex affects the level of expression of this gene. E6 may also interact with c-Myc or other transcription factor located at the hTERT promoter, for example with Sp1. Another way is the degradation of NFX1-91, which is hTERT gene repressor.¹⁷

High-risk human papillomavirus E6 oncoprotein has also additional functions due to its ability of association and degradation of proteins that have characteristic PDZ domain. They are responsible, inter alia, for transcription regulation, cell polarity, as well as for intercellular signaling. The group of proteins that associate with E6 oncoprotein includes human homologs of DLG and Scrib suppressor proteins, MUPP1, MAGI 1-3, PTPN13 phosphatase and PATG.^{17,19}

The most important mechanism impairing cell cycle, involving the E7 protein, is the association and degradation of proteins from retinoblastoma family (pRb) and related p107 and p130. pRb protein binding by E7 releases transcription factor from E2F family and activates the transcription of certain group of genes, which regulate cellular proliferation. This causes a disruption of the cell cycle, promotion into S phase of a cycle and stimulation of amplification of its genes, as well as virus genes.¹¹ On the other hand, E2F influences the increase of the expression of proteins, such as, e.g. p16 and MCM 7. In HPV-dependent laryngeal cancer increased level of cyclin D1 is observed, which is also associated with pRB pathway.^{23,24} Another method by which the virus promotes the amplification of its own genes is the association of E7 with cyclin-dependent kinase inhibitors (CKI), such as, e.g. p21 or p27.²⁰ Interactions of E5, E6 and E7 proteins and other factors are shown in Fig. 2.

Fig. 2. E5, E6 and E7 proteins overexpression of high-risk HPV and their interaction with different host cells factors¹⁶



Virus detection

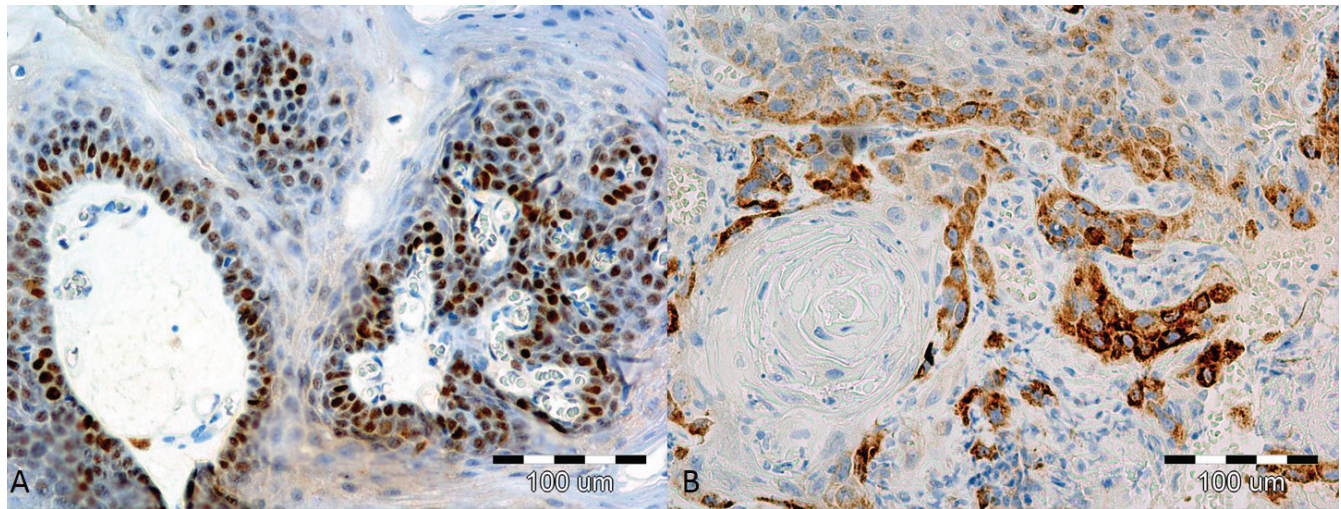
There are many methods of HPV detection in abnormal tissues collected from the head and neck region, such as the analysis of tissue material (biopsies), cellular material (cytology – washings, brush tip swabs) and serum from patients.²⁵ Certain morphological features of HPV infection are visible in microscopic images. Infected cells look like koilocytes with a characteristic paranuclear clear zone, the so-called halo, as well as a clearly marked cell membrane, which results from HPV propagation. The cell nucleus is also morphologically changed; it is small and deformed, with abnormal staining. The presence of koilocytes and extensive vasculature is called “koilocytosis florida” and it coexists mainly in low-risk HPV infections – 6 and 11.²⁶

However, only molecular diagnostic techniques provide a definitive diagnosis of HPV infection. The most frequently used methods include polymerase chain reaction (PCR), in situ hybridization (ISH) and immunohistochemical method (IHC) evaluating expression level of p16 protein. The most frequently used method in molecular biology is PCR technique, however it is not routinely used in each laboratory. It has some constraints as it is believed to be sometimes too sensitive and prone to contamination.²⁷ The most of PCR reactions is carried out with the use of a pair of primers GP5+/GP6+, MY09/11 and SPF10. It allows for the detection of a broad range of different types of HPV. The set of GP 5/6 primers was initially designed in order to detect HPV 6, 11, 16, 18, 31 33, but later it was shown that they may be used for the detection of up to 27 types of HPV. All of them target highly conservative DNA fragment of HPV L1 gene and are able to detect a number of HPV types during one PCR reaction.

The sensitivity of the reaction is increased by an additional nested-PCR with the set of MY09-MY11 primers, followed by GP 5+/6+. However, one should bear in mind the fact that in some cases the result of PCR reaction with the use of the abovementioned primers may be false negative. This is because of the fact that L1 gene-containing a part of HPV genome might be lost during the integration of viral genetic material with the genome of the host cell. For this reason PCR methods have been developed that allow the amplification of E6 and E7 gene-containing fragments of the genome. Both of those genes contain highly-conservative regions and should be taken into account in the case of PCR reactions with false negative results.²⁸

The in situ hybridization method allows for the detection of HPV localization in the nuclei of infected cells, es-

Fig. 3. A positive nuclear reaction immunohistochemistry (IHC) performed using an antibody directed against the protein p16 on paraffin sections of laryngeal papillomas (A) and IHC positive cytoplasmic reaction in squamous cell carcinoma of the larynx (B). In both cases revealed the presence of HPV also using the PCR method



pecially cancerous ones. However, ISH is characterized by the limited sensitivity and higher costs in comparison with traditional IHC method. For in situ hybridization reaction, chromogen or fluorine-labelled probes are used, which allow for the detection of a broad range of HPV types, especially in paraffin-sections. ISH allows for the detection of HPV's DNA integrated with the genome of the host cell, which is the case for cancer cells. Therefore, it may be a tool for the detection of mucous membrane cells cancerous transformation process at its early stages.

Immunohistochemical technique (IHC) is a method commonly used in the diagnostic laboratories. In particular, the use of IHC for the evaluation of p16 expression level is recognized as a surrogate marker used in the screening for transcriptionally active infections (Fig. 3).^{27,28}

It is believed that high-risk HPV E7 oncoprotein associates with factors from retinoblastoma family (Rb), which causes E2F transcription factor release from the complex. This factor activates transcription of multiple genes, including p16-INK4a and proteins from minichromosome maintenance proteins (MCMs) family. The consequence of this reaction is an increase of p16 and MCM expression in cells infected with high-risk HPV. For this reason p16 and MCM proteins could be supportive marker indicating HPV infection.^{24,29} Moreover, recent studies have shown that p16 expression correlates with better survival of patients with squamous cell head and neck cancer.²⁷

HPV in laryngeal pre-neoplastic and neoplastic lesions

Among various types of human papillomavirus, there are about 40 types, which attack genitals and are present in biopsy material from uterine cervix cancers. The DNA of the same virus types is found in nearly 20% of upper

respiratory track cancers. Currently, it is believed that exactly those HPV types may contribute to the immortalization and oncogenic transformation of the infected cells. Those viruses are considered as high-risk HPV. The most representative among this group are HPV types: 16, 18, 31, 33, 39, 45, 52, 58 and 69.¹⁹ The other group of viruses is low-risk HPVs, which are associated with the aetiology of, e.g. cutaneous warts, condyloma acuminata or recurrent laryngeal papillomatosis. This group comprises HPV 6, 11, 42, 43 and 44.^{11,19} Infection with oncogenic types of HPV is one of the conditions required for the development of cervical cancer. Virus DNA is found in 99% of the analyzed neoplastic lesions of uterine cervix.¹⁹ HPV DNA is found in the lower percent of analyzed laryngeal squamous cell carcinoma cases (from 7 to 59%) in comparison to uterine cervix cancers. HPV 16 accounts for 74% and HPV 18 for 16% of types detected in this cancer. In laryngeal neoplastic lesions it is, similarly to uterine cervix cancer, the most frequently occurring type of human papillomavirus.⁵

HPV infection is a factor enabling the neoplastic transformation of epithelial cells. Usually other stimuli are present but they are not necessary for cancer development.³⁰ In the case of laryngeal cancers, such factors include smoking and consuming excessive amounts of alcohol, incorrect diet, as well as gastroesophageal reflux disease (GERD) and laryngopharyngeal reflux (LPR).^{31–33} The occurrence of permanent genetic changes is also important for cancer development, leading to the impairment in the control of cell division, growth and differentiation.^{11,20}

Laryngeal epithelium is also susceptible to HPV infection. Transmission route for the virus are most often sexual contacts. In the case of the head and neck, oral contact with infected genitals is an important way of infection. It has been shown that there is a direct dependency between

an infection of genital and oral cavity.^{34,35} Georgiewa S. and Iordanov V. described the case of a woman found with the presence of HPV 16. The woman was also diagnosed with 3 cancers of uterine cervix, external genital organs and larynx, which were developing simultaneously. Researchers considered all of the cancers to be related to the same, HPV16, types of the virus.³⁶ Children can be also infected with the virus from the mother during labor. Perinatal transmission of the infection with oncogenic types of human papillomavirus occurs not only in the case of vaginal birth. Vertical transmission is a result of contact of a fetus with HPV infected cervical cells. It was observed that a mother's higher viral load is an important and determining factor in the transmission to their infants. HPV infections are rather rare during pregnancy and labor complications as well, and they concern less than 1% of new born children. Additionally, the HPV virus was also detected in samples from breast milk. There is a theoretical possibility to infect a child via breast-feeding.^{37,38}

Papillomas usually develop at the interface between stratified squamous epithelium and multi-row respiratory epithelium, but they may occur also few centimeters away from this area. In the laryngeal respiratory epithelium, HPV induces proliferation of reserve cells with stem potential. This process may lead to the thickening of squamous epithelium layers and to the development of papilloma, as well as to metaplasia outbreaks.²

HPV in laryngeal papillomas

Laryngeal papillomas are benign epithelial lesions. This term is used to describe 2 diseases that differ in clinical course, etiology and prognosis. One of the diseases is an adult type and the second is children type of laryngeal papillomatosis. HPV type 6 and 11 infection is an etiological agent of both types of laryngeal papillomas. The first of abovementioned types of virus is more frequent among adult patients. Vocal folds are the most common place for the emergence of lesions. Laryngeal papillomas found in children are characterized by the disease peak in the 5th year of life, while the adult type papillomas – between 20–30th year of life. In adulthood the diseases are more often found in men, while no such correlation was found in children.^{1,39}

Malignant transformation of laryngeal papillomas occurs very rarely. Many studies have shown that 1–7% of laryngeal papillomas transform into squamous cell carcinoma.^{3,39} Although HPV is an essential factor for the establishment of papillomas, an infection alone is not sufficient for their development. The presence of HPV has been found also in normal cells of laryngeal epithelium.⁴⁰ The most researchers in their reports have shown that HPV11 type is more aggressive and causes severe papillary lesions, especially in children.⁴¹ The mechanism of this transformation is not yet fully known and explained.

Some of the authors believe that both integration of HPV's DNA with host cell DNA, as well as p53 gene mutation is necessary for oncogenic transformation.¹⁹ It is also suggested that low-risk HPV infections (HPV 6 and 11) may cause oncogenic transformation in combination with other co-factors, such as nicotine and alcohol. However, some studies have shown the integration of HPV 6 DNA with host cell's DNA in case of tonsil cancer, which is similar to the infection with high-risk HPV.²⁶ The host's immune response is also an important factor for malignant transformation. HPV-infected cancer cells may also evade immunological response. E7 protein associates with interferon regulation factor IRF1 and IRF9 and therefore leads to their inactivation and blockage of interferon alpha (INF α) signaling pathway.⁴²

Papillomas, although they are non-malignant lesions, may cause airway obstruction, thus posing a risk for life. They require multiple hospitalizations, surgical procedures and pharmacological treatment. The main method of treatment of laryngeal papillomatosis is surgical removal using a CO₂ laser. The children are also treated by alpha-interferon in the case of an aggressive course of the disease. The results of therapy are not satisfactory, because the disease often recurs.³⁹ An interesting phenomenon is the fact that for some patients infected with HPV 6 and 11, immune responses have been shown, which encourages the maintenance of chronic HPV infection. A characteristic feature of the diseases caused by HPV, such as recurrent papillomatosis of upper respiratory track, skin papillomas or uterine cervix cancer, is clear lack of HPV-specific cytotoxic T lymphocytes (CTL) and Th1 lymphocytes releasing inflammatory cytokines, such as IFN- γ , IL-2 and TNF- α .⁴³

HPV in laryngeal cancer

Head and neck cancer is the fifth most common cancer in the world.⁴⁴ Every year, there are more than 640,000 cases of this cancer reported and it causes over 350,000 deaths.²⁸ Squamous cell carcinoma is the most frequent type of neoplastic lesions affecting the head and neck area.³⁴ On the other hand, laryngeal squamous cell cancer is the most common among head and neck neoplasms and it accounts for about 60% of all cancers in the head and neck area.⁴⁴

HPV infections occur often in the larynx. In a vast majority of cases, the virus is related to benign, single papillomas or cancers. In those lesions abnormalities in maturation and differentiation of epithelial cells occur, which is characteristic for HPV infections. Once cells of basal layer of epithelium are infected, HPV may enter a latency stage and give no symptoms. Then no histological changes in epithelium are observed. Such a situation may last for several months or even years. However, persistent HPV infection may cause neoplastic transformation of cells.⁴⁴

Table 2. Prevalence of HPV in verrucous carcinoma²

Source	Publication date	Sample size	Detection technique	HPV + (%)
Abramson, et al.	1985	5	IHC, ISH	5 (100%)
Kasperbauer, et al.	1993	20	PCR ISH	17 (85 %) 0 (0%)
Fliss, et al.	1994	29	PCR	13 (45 %)
Lopez-Amado, et al.	1996	10	PCR	4 (40 %)

Laryngeal cancer may result from late complication of squamous cell papilloma (SCP), although most of those malignant changes develop without papillomas. Generally, squamous cells laryngeal cancer development begins based on dysplastic changes, intraepithelial neoplasia and pre-invasive cancer (carcinoma in situ) occurring within epithelium of mucosa membrane lining the organ.¹

The clinical importance of HPV infection and its association with head and neck cancers development is well documented and confirmed by multiple studies.⁴⁵ Within this group of cancers, oropharyngeal and laryngeal carcinomas are the most dependent on HPV. However, HPV involvement in laryngeal cancer etiology has not yet been fully evaluated.^{2,46} Many studies related to this subject have been conducted within the past decade. The presence of HPV

in cancerous lesion of larynx varies depending on the procedure applied for samples collection and viral DNA detection method. High variance in the frequency of HPV detection is observed even among results obtained with the use of PCR reaction. It varies between 3 and 85%. The differences depend on primers used for the reaction, genomic localization and the length of the product amplified by PCR, conditions of the reaction and false positive results obtained due to the sample contamination with viral particle.⁴⁶

Squamous cell laryngeal cancer accounts for more than 90% of malignant lesions of this organ. A high prevalence of this virus among such cancers is noted in verrucous carcinoma. It is a rare variant of low malignancy, highly differentiated LSCC.^{3,47} In verrucous carcinoma, the dominant types of HPV are 16, 18, 11 and 6. Most of the studies on the presence of the virus in this type of cancer were carried out in the nineties. They revealed a fairly strong relationship between HPV and this rare cancer; however, the research was based on small study groups. The results of the studies are summarized in Table 2. In verrucous carcinoma, the relationship between the presence of HPV and a high stage of cancer were observed. However, no correlation was reported between the presence of HPV in cancer and the age of patients, tumor localization or response to radiotherapy.²

Nevertheless, most of the research is carried out on typical laryngeal squamous cell carcinoma. Kreimer et al. showed meta-analysis of 5,046 cases of head and neck cancers from all over the world, 1,435 of which were laryngeal cancers tested for the presence of human papillomavirus with the use of PCR detection. In 35 publications related to the cancer of larynx, HPV was shown in 24% of cases. Kreimer et al. paid particular attention to the geographical localization of the conducted studies and they confirmed that the prevalence of HPV occurrence in cases of head and neck cancers depends on the region of the world inhabited by the patients. The HPV prevalence in case of larynx infection was highest in Asia and accounted for 38.2% of squamous cell cancers.⁴⁷

Based on the research conducted over recent years, one can conclude

Table 3. Selected research on the prevalence of HPV in LSCC²

Source	Publication date	Sample size	Detection technique	HPV + (%)	Genotypes
Syrjänen, et al.	1987	116	ISH	12.9%	11, 16
Salam, et al.	1995	87	genotyping RFLP	22.2%	6, 11, 16
Fouret, et al.	1997	103	PCR	6.8%	16
Ma, et al.	1998	102	genotyping southern blot hybridization	58.8%	6, 11, 16, 18, 33
Gorgoulis, et al.	1999	91	Genotyping Nested PCR	21%	6, 16, 18, 33
Gillison, et al.	2000	86	ISH	19%	16
Almadori, et al.	2001	42	PCR	35.7%	16, 18
de Oliveira, et al.	2006	110	multiplex PCR	37.3%	16, 18
Gungor, et al.	2007	95	genotyping multiplex PCR	7.4%	6, 11, 16
Applebaum, et al.	2007	93	ELISA	13%	–
Koskinen, et al.	2007	108	PCR	3.3%	6, 11, 16, 33
Morshed, et al.	2008	93	genotyping INNO-LIPA	35.5%	16, 18, 33
Fakhry, et al.	2008	–	PCR ISH	40%	16, 33, 35

that there is a high probability of HPV 16 (and less frequently detected HPV 18, 31 and 33) contribution to the etiology of laryngeal cancers – at least a certain group of them. Table 3 shows some of the results of research conducted on groups of about one hundred patients.

The overview of clinical and epidemiological data demonstrates the presence of 2 subtypes of head and neck cancers. One of them is cancer depending on the exposition to tobacco. On the contrary, the other group of neoplasms develops in association with HPV infection. In science, the idea of HPV-dependent and HPV-independent cancer is commonly accepted.¹ Cancers associated with viral infections show some common features. In comparison to HPV-independent group, they have less DNA mutations and single, small chromosomal aberrations. They are characterized by long promotion period, resulting from latent, persistent infection.³⁴ In this group of laryngeal cancers, contribution of addictions considered to be the most important risk factors, i.e. smoking and alcohol consumption, is significantly lower.⁴⁷ Also, HPV-dependent head and neck cancers have less severe disease course and are characterized by longer patient survival.³⁴ Moreover, Gillson and co-workers showed that the risk of death was significantly decreased (from 60 up to 80%) in patients with HPV infected oropharyngeal squamous cell cancers in comparison with control group. Schwarz et al. also confirmed longer survival in case of HPV infected patients with oral squamous cell cancer. The better response for radiotherapy and chemotherapy was described by a few researchers. This better response may be the reason for lower mortality in patients with HPV infections. Currently, knowledge about the reasons for better response to the treatment of patients with HPV related laryngeal cancer is poor. Further research on this subject is needed. Isayeva et al. published data concerning the prognostic significance of HPV in squamous cancer of the oral cavity, larynx, sino-nasal tract and nasopharynx. They found no association between HPV infections and treatment outcome.^{48–51}

It seems important to also raise the issue of preventive vaccines against this virus. Currently, there are 2 types of commercially available vaccines, the bivalent (targeted to HPV 16 and HPV18) one and the tetravalent (against HPV 16, 18, 11 and 6) one. The vaccines were designed to induce the production of antibodies against the viral protein and do not have viral DNA. Thus far, only girls have been included in the preventive vaccines plan due to high virus detection in mucosal membrane of uterine cervix in various pre-cancerous and cancerous lesions. According to The Polish Gynecological Society, girls aged 12–13 years, who have had no sexual contact, may be vaccinated. In view of many studies regarding the relationship between HPV infection and head and neck cancers there are suggestions that, perhaps, boys also should be included in vaccination schedules.

Summary

The role of human papillomavirus in the etiology of pre-cancer laryngeal lesions and larynx cancer is unquestionable. HPV is one of the laryngeal cancer risk factors. Laryngeal cancer associated with viral infections shows a long promotion period, resulting from a latent, persistent infection. The contribution of addictions in the group of laryngeal cancers with HPV infections, i.e. smoking and alcohol consumption, is significantly lower. HPV-dependent head and neck cancers have less severe course of the disease and are characterized by longer patient survival. There is little research on this question in laryngeal cancer. However, according to current knowledge, there is no data suggesting that HPV related laryngeal cancer is milder and leads to higher survival rates. Further research on this subject is needed.

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Prognostic factors in pulmonary arterial hypertension: Literature review

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Abstract

Pulmonary arterial hypertension is a disease that has a bad influence on the patient's prognosis. Recently, the possibility of therapy has dramatically changed. Nowadays, the treatment of this disease is concerned mainly with the pathophysiological target. In clinical practice, it is important to start therapy at the appropriate time, when the patient is qualified because of an unsatisfactory examination result or improve therapy when the patient is getting worse. The understanding of prognosis factors in pulmonary arterial hypertension is necessary, because it is used to determine the length of patients' life expectancy. In September of 2015, new guidelines of ESC concerning the diagnosis and treatment of pulmonary hypertension have been presented. In our article we centered on the prognosis factor in pulmonary arterial hypertension. This document is a continuation of ESC guidelines. Many of the most contemporary articles are here summarized.

Key words: pulmonary arterial hypertension, prognosis, risk factor, mortality

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Pulmonary hypertension means an abnormal increase in pulmonary arterial pressure, which may occur in various heart, lung, and pulmonary vascular diseases. Pulmonary hypertension is defined as the mean pulmonary arterial pressure ≥ 25 mm Hg at rest as assessed by right heart catheterization (RHC).¹ Values obtained during exercise are of no diagnostic significance.

The current classification includes 5 etiologic categories of pulmonary hypertension, and in the present review we focused on the first group, i.e. pulmonary arterial hypertension (PAH).¹ This group includes patients with idiopathic PAH, hereditary forms of PAH (e.g. due to BMPR2 gene mutation), pulmonary hypertension due to connective tissue disease, HIV infection, or portal hypertension, and patients with uncorrected congenital heart disease (most commonly Eisenmenger syndrome).

RHC is required to confirm the diagnosis of pulmonary hypertension, and pressure measurements include the assessment of pulmonary arterial wedge pressure (PAWP) to differentiate between precapillary and postcapillary pulmonary hypertension. PAWP values below 15 mm Hg indicate that a left heart disease is an unlikely cause of pulmonary hypertension. It should be noted that the lack of standardization of PCWP measurements has contributed to differences between various centers when measuring this parameter.² The most recent guidelines recommend calculating the diastolic pressure gradient (DPG) as a difference between pulmonary artery diastolic pressure and PAWP in order to differentiate between isolated postcapillary pulmonary hypertension and combined pre- and postcapillary pulmonary hypertension.¹ DPG values below 7 mm Hg (with PAWP > 15 mm Hg) indicate the presence of isolated postcapillary pulmonary hypertension, most commonly due to a left heart disease.

Prognosis

Prognosis in PAH is severe and when untreated it is a potentially fatal disease that rapidly leads to disability and premature mortality. Evaluation of disease severity, including clinical assessment, imaging and haemodynamics, during follow-up visits is essential when selecting patients for specific drug therapy or its intensification. Knowledge of prognostic factors associated with poor outcomes is helpful in deciding on the correct treatment plan.

The type of disease underlying PAH has an important role when determining prognosis in these patients. In patients with congenital heart disease, prognosis is better compared to those with idiopathic PAH.³ In patients with congenital cardiac shunt disease who have not been treated surgically, a right-to-left shunt reduces right ventricular pressure, which has a protective effect on the right ventricular function. Recent studies indicate a very poor prognosis in patients with PAH that developed fol-

lowing surgical correction of a congenital heart disease compared to patients with Eisenmenger syndrome.⁴

Prognosis is also very poor in patients with systemic sclerosis in whom pulmonary vascular smooth muscle proliferation and constriction leads to PAH.⁵ Pulmonary hypertension is a major cause of death in this group in addition to pulmonary fibrosis. Before the era of vasodilating drug therapy, the median survival of patients with systemic sclerosis and PAH was 1–3 years.⁵ Concomitant interstitial lung disease adds to this poor prognosis, resulting in a 5-fold increase in mortality. One-, 2-, and 3-year survival in patients with PAH and interstitial lung disease was 82, 46, and 39%, respectively, compared to 87, 79, and 64% in patients without interstitial lung disease.⁶

Prognostic factors

To analyze the effect of age and gender on prognosis in patients with PAH, Corciova et al. performed a prospective study that included 553 patients diagnosed with pulmonary hypertension, including idiopathic PAH.³ There were no differences in mortality in relation to gender in this group. Also patient age, which was on average 64.7 years at the time of death, was not found to be a risk factor for mortality. In contrast, another study showed that male gender and older age had a significant influence on survival.⁷ Patients with idiopathic PAH below 14 years of age and above 65 years of age have worse prognosis compared to those aged 14–65 years. However, children seem to show increased vasodilatation in response to a rise in pulmonary arterial pressure and thus are characterized by a better prognosis.⁸

Mortality risk is closely related to the disease severity as categorized using the World Health Organization (WHO) functional classification and is highest in the WHO class IV.⁹ Before the era of specialist treatment, the mean survival was about 6 months in the WHO class IV, 2.5 years in the WHO class III, and 6 years in the WHO class I. The difference in survival between WHO class III and IV patients was significant ($p < 0.001$). Progression to a higher WHO class is one of the strongest adverse prognostic factors and should prompt a search for a reason of such worsening.¹⁰

In addition to the WHO class, exercise tolerance in patients with PAH is assessed on the basis of the 6-min walking distance (6MWD). During this test, aside from the walking distance, dyspnea severity by the Borg scale, heart rate, systemic blood pressure, and oxygen saturation are also evaluated. Savarese et al. performed a meta-analysis of 22 randomized clinical trials to determine the relationship between 6MWD and the risk of a combined clinical endpoint that included death, hospitalization due to PAH, and the need for lung or lung and heart transplantation.¹¹ This analysis included studies with documented 6MWD at baseline and at follow-up or at

the time of clinical endpoint occurrence. No association was found between the change in 6MWD at follow-up compared to baseline and the risk of clinical endpoint occurrence.

Performing a 6-min walking test is a useful way to evaluate treatment effectiveness. The prognostic value of this parameter lies not in the change of 6MWD in response to treatment, but most of all in its absolute value, particularly if it is lower than 250 m.⁷ There was significantly lower mortality in patients with 6MWD higher than 440 m.¹² A number of studies showed that an improvement in the 6-minute walking test was parallel to the improvement of hemodynamic parameters as assessed during cardiac catheterization.¹¹ A significant association between the change in 6MWD and the change in pulmonary vascular resistance measured during RHC was found.¹¹ However, monitoring PAH on the basis of 6MWD is significantly limited by lack of standardization, suboptimal measurement reproducibility, and the effect of gender, height, age, and patient motivation on 6MWD values. Despite this, evaluation of 6MWD is the only exercise test to assess the effectiveness of PAH treatment that was approved by the Food and Drug Administration (FDA) and the European Medicines Agency (EMA).

Echocardiography is commonly used for diagnostic and treatment monitoring purposes in patients with PAH due to its wide availability, non-invasive nature, and reproducibility. An adverse prognostic sign is the presence of pericardial effusion, which accumulates due to impaired venous and lymphatic drainage caused by right ventricular systolic dysfunction and a subsequent increase in right atrial pressure.²¹ However, the threshold amount of pericardial fluid, which is associated with significantly worsened prognosis in patients with PAH, has not been determined yet.

A parameter that correlates with right ventricular ejection fraction is tricuspid annular plane systolic excursion (TAPSE).²² TAPSE values below 18 mm indicate right ventricular systolic dysfunction and impaired remodeling, leading to worse outcomes.²² Of note, the evaluation of TAPSE is not useful when a significant tricuspid regurgitation is present. In the study by Ghio et al. performed in a group of patients with PAH, TAPSE below 15 mm was associated with higher mortality compared to patients with TAPSE above 15 mm.²³

An increase in the Tei index of myocardial performance, calculated as the sum of isovolumic contraction and relaxation times divided by the right ventricular ejection time, is observed alongside the decline in the right ventricular function. The Tei index is significantly increased in patients with PAH, and its values of 0.83 or higher indicate poor prognosis.²⁴ However, an evaluation of the Tei index for the right ventricle has some limitations such as high dependence on the volume status, poor measurement reliability during tachycardia, and pseudonormalization in

very advanced right ventricular failure, which limits the ability to use this parameter in all patients.

Another study that included 72 patients with idiopathic PAH, Ghio et al. showed that mortality was higher in patients with the right ventricular dimension > 36.5 mm by echocardiography compared to those with lower values ($p = 0.044$).²⁵ Right ventricular free wall thickness was not found to have an influence on survival. However, when both these parameters are taken into account, the right ventricular dimension > 36.5 mm combined with the right ventricular free wall thickness > 6.6 mm was not associated with a worse prognosis. This may be explained by the fact that the risk is reduced if right ventricular dilatation, indicating poor prognosis, is accompanied by an increase in wall thickness. In other words, an increase in the right ventricular wall thickness is protective when the right ventricle is dilated, as myocardial wall tension is then reduced. Better right ventricular function, and thus better prognosis, is also indicated by an increase in the right ventricular systolic pressure by 30 mm Hg during stress echocardiography, indicating preserved right ventricular contractility reserve.²⁶

In turn, Bustamante-Labart et al. showed a relationship between the right atrial size and the risk of death or the need for heart or heart and lung transplantation in patients with idiopathic PAH.²⁷ Death or transplantation occurred in 81% of patients with the right atrial area above 27 cm², including all patients with the right atrial area higher than 34 cm². Right atrial area > 26 cm² was also indicated in the most recent guidelines as associated with worse prognosis.¹

Biochemical markers are also used to evaluate prognosis in patients with PAH. Their advantages include easy repeatability and a non-invasive nature. Serum uric acid level was found to be of a significant prognostic importance in patients with heart failure, as it reflects the degree of aerobic metabolism impairment in ischemic tissues. Bendayan et al. showed a correlation between serum uric acid level and the severity of pulmonary hypertension in patients with PAH.¹³ A positive association was found between serum uric acid concentration and the severity of heart failure by the WHO classification ($p < 0.01$), and a negative association for the 6-min walk test: the higher was serum uric acid level, the lower was 6MWD. Serum uric acid concentration was also found to be significantly higher in those patients who died during the follow-up compared to survivors. However, no association was found between serum uric acid level and patient age, nor the mean pulmonary arterial pressure, cardiac output, and mixed venous oxygen saturation assessed during RHC. Nagaya et al. showed a positive correlation between serum uric acid concentration and an increase in pulmonary vascular resistance.¹⁴ In addition, a trend for serum uric acid level lowering was seen in response to vasodilating drug therapy. In contrast, no association was found between arterial oxygen saturation and

serum uric acid concentration, despite the fact that other studies indicated the activation of the purine degradation pathway in ischemic tissues, resulting in the overproduction of uric acid.¹⁵ Clearly, the effect of other factors on serum uric acid level should be taken into account when evaluating prognosis in patients with PAH, such as concomitant diseases (chronic obstructive pulmonary disease and chronic kidney disease), diet, and medications (diuretics, allopurinol).

Brain natriuretic peptide (BNP) or inactive, more stable N-terminal pro-B-type natriuretic peptide (NT-proBNP) are also parameters of prognostic importance in patients with heart failure. BNP and NT-proBNP are released by right and left ventricular cardiomyocytes in response to an increase in wall tension. In a group of patients with idiopathic PAH, Nagaya et al. showed that BNP concentration above 150 pg/mL was associated with adverse prognosis.¹⁶ For NT-proBNP level, the threshold value for adverse prognosis was 1400 pg/mL.¹⁷ In the study by Blyth et al., cardiac magnetic resonance imaging (MRI) was performed and NT-proBNP concentration was measured in a group of 25 patients with pulmonary hypertension (including 19 with PAH).¹⁸ A negative relationship was found between NT-proBNP level and right ventricular ejection fraction. NT-proBNP level equal to or higher than 1685 pg/mL was nearly 100% sensitive and specific for right ventricular systolic failure. Similarly, NT-proBNP concentration in the study by Fijałkowska et al. was found to correlate with hemodynamic and echocardiographic parameters of right ventricular systolic dysfunction.¹⁷ In early stage of the right ventricular failure, no signs may be identified during physical examination. Natriuretic peptide level measurements in patients with PAH may lead to earlier institution of specific drug therapy and thus prevent progression to severe disease. In clinical practice, NT-proBNP levels are measured more frequently than BNP levels, as NT-proBNP is more stable both in vivo and in vitro.

Torbicki et al. evaluated high-sensitivity troponin T (hs-TnT) concentration in 56 patients with pulmonary hypertension (including 51 with PAH). The mean hs-TnT level was 0.034 ng/mL (range 0.010–0.077 ng/mL).¹⁹ Hemodynamic parameters evaluated during cardiac catheterization did not differ between patients with elevated or normal hs-TnT levels, but patients with higher hs-TnT concentrations were characterized by a significantly higher heart rate, lower mixed venous oxygen saturation, higher NT-proBNP concentration, and lower exercise tolerance during the 6-min walking test. During 2-year follow-up, 63% patients with elevated hs-TnT level died compared to 15% among those with normal hs-TnT concentration. These studies indicate the importance of hs-TnT level measurements during follow-up visits in patients with PAH.

Takeda et al. evaluated the effect of hepatic dysfunction on prognosis in patients with PAH.²⁰ Hepatic dysfunction

often accompanies right ventricular failure and results from passive hyperemia and reduced hepatic blood flow. In the cited study that included 62 WHO class II–IV patients with PAH (idiopathic or due to connective tissue disease), an elevated bilirubin level was found to be associated with higher disease severity. Patients with elevated total bilirubin level remained in a higher WHO class and had higher NT-proBNP levels and higher right atrial pressures as measured during cardiac catheterization. In addition, bilirubin was found to be a more sensitive marker of right ventricular failure compared to transaminase levels.

Cardiac MRI is increasingly commonly used in patients with PAH, particularly for monitoring the disease course. This modality allows highly precise three-dimensional evaluation of cardiac morphology and unlike echocardiography, it is not limited by suboptimal acoustic windows. Unfortunately, limitations of MRI include low availability and a relatively high cost. In the study by Bradlow et al. the right to left ventricular mass ratio as evaluated by MRI was associated with survival in patients with PAH.²⁸ The ventricular mass index (VMI) or the end-diastolic ratio of right to left ventricular mass higher than 0.7 and an increased right ventricular mass were indicators of poor prognosis. In the study by van Wolferen et al., adverse prognostic MRI parameters included right ventricular dilatation, reduced right ventricular ejection fraction and ejection volume, and reduced left ventricular end-diastolic volume.²⁹

Summary

Pulmonary arterial hypertension is a condition associated with particularly poor prognosis. Thus, identification of its prognostic factors is of major importance for therapeutic decision making, including treatment intensification.

Evaluation of prognosis in patients with PAH has been discussed in the most recent 2015 European Society of Cardiology/European Respiratory Society guidelines on the diagnosis and management of pulmonary hypertension.¹ Depending on one-year mortality risk, patients with PAH were divided into 3 groups characterized by a high (> 10%), intermediate (5–10%), or low risk (< 5%). A number of factors including the clinical presentation of PAH, 6MWD, BNP/NT-proBNP levels, and echocardiographic, spiroergometric, and RHC parameters are taken into account when evaluating prognosis.

As indicated by the authors, rapid disease progression, disease severity (WHO class IV), clinically overt right ventricular failure, 6MWD < 165 m, and syncope are all associated with an increased mortality risk (> 10% at one-year follow-up). Adverse prognostic factors also include BNP level > 300 ng/L or NT-proBNP level > 1400 ng/L, pericardial effusion, right atrial size > 26 cm² by

echocardiography, peak oxygen consumption (VO_2max) $< 11 \text{ mL/min/kg}$ ($< 35\%$ of the predicted value) by the spiroergometric testing, and right atrial pressure $> 14 \text{ mmHg}$, cardiac output index $< 2 \text{ L/min/m}^2$ and mixed venous oxygen saturation $< 60\%$ by RHC.

In contrast, better prognosis (one-year mortality risk $< 5\%$) is indicated by a lower disease severity (WHO class I–II) and stable clinical course, no signs of right ventricular failure in the clinical examination, $6\text{MWD} > 440 \text{ m}$, BNP level $< 50 \text{ ng/L}$ or NT-proBNP level $< 300 \text{ ng/L}$, no pericardial effusion, normal right atrial size ($< 18 \text{ cm}^2$) by echocardiography, $\text{VO}_2\text{max} > 15 \text{ mL/min/kg}$ ($> 65\%$ of the predicted value) by the spiroergometric testing, and normal right atrial pressure ($< 8 \text{ mmHg}$), cardiac output index $> 2.5 \text{ L/min/m}^2$ and mixed venous oxygen saturation $> 65\%$ by RHC.

Knowledge of prognostic factors in PAH allows for the identification of therapeutic targets in this patient group. Treatment efforts in a patient with PAH should aim at obtaining the above defined low risk clinical profile, which indicates better outcomes and thus better effectiveness of the therapy that has been instituted.

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History of discovery of polycystic ovary syndrome

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Abstract

Stein and Leventhal are regarded to have been the first investigators of polycystic ovary syndrome (PCOS); however, in 1721 Vallisneri, an Italian scientist, described a married, infertile woman with shiny ovaries with a white surface, and the size of pigeon eggs. It was not until the early 1990s at a National Institute of Health (NIH) sponsored conference on PCOS that formal diagnostic criteria were proposed and afterwards largely utilized. Many scientists tried to explain the pathophysiology of PCOS and many studies were made. It is now accepted that it is multifactorial, partly genetic; however, a number of candidate genes have been postulated. Insulin resistance has been noted consistently among many women with PCOS, especially in those with hyperandrogenism, but it is not included in any of the diagnostic criteria. Now there is strong evidence that cardiovascular disease risk factors and disturbances in carbohydrate metabolism are all increased in patients with PCOS compared to the healthy population. The criteria established by a group of experts during a conference in Rotterdam held in 2003 are obligatory (The Rotterdam ESHRE/ASRM – Sponsored PCOS Consensus Workshop Group). The subsequent “Rotterdam criteria” incorporated the size and morphology, as determined by an ultrasound, of the ovary into the diagnostic criteria.

Key words: polycystic ovary syndrome, infertile, hyperandrogenism

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Although Stein and Leventhal are regarded as the first investigators of polycystic ovary syndrome (PCOS), it was Vallisneri, an Italian medical scientist, physician and naturalist, who in 1721 described a married, infertile woman with shiny ovaries with a white surface and the size of ovaries as pigeon eggs.¹ Another report can be found in 1844, when Chereau and Rokitsansky described fibrous and sclerotic lesions in the ovaries of a degenerative character with hydrops follicle.^{2,3} Bulius and Kretschmar described hyperthecosis for the first time.⁴ In 1879 Lawson Tait presented the need for bilateral oophorectomy for the treatment of symptomatic cystic degeneration of the ovaries.⁵ Partial resection of the ovaries was soon proposed.⁶ In 1902 von Kahlden published a review on the pathology and clinical implications of these ovaries.⁷ Because of many critical voices regarding ovarian resection, John A. McGlinn in 1915 suggested puncturing "those cysts which are upon the surface" rather than resorting to ovarian resection.⁸ In 1935 Stein and Leventhal presented a group of 7 women with common features: menstruation disturbances, hirsutism and enlarged ovaries with the presence of many small follicles.⁹ They were also the first to describe the lack of menstruation in women with increased volume of ovaries and to suggest using ovarian wedge resection. After this surgical intervention regular menstrual cycles returned in all 7 patients and 2 of them became pregnant. After a bilateral ovarian wedge resection, menstruation returned in almost 90% of women and 65% of them became pregnant.¹⁰ However, as medical treatment became available with the use of clomiphene citrate, follicle stimulating hormone (FSH) and urinary source, surgical treatment became less often used.^{11–13}

PCOS was described as a distinct masculinization and theca luteinization syndrome.^{14,15} Many scientists tried to explain etiology of cystic ovaries. Fogue and Massabau proposed 3 potential mechanisms: inflammation, congestion and dystrophy.¹⁶ Stein and Leventhal in their original report thought that bilateral cystic ovaries result from abnormalities in hormonal stimulation, which was confirmed later.^{9,17} Plate suggested that source of androgens in women may not be only adrenals but ovaries also.^{18–20} Regardless of the source of androgens in PCOS, scientists in 1953 proposed to use cortisone therapy or to treat sclerocystic ovaries with exogenous testosterone.^{21–23} In 1958, 3 investigators were the first to describe an increased level of luteinizing hormone (LH) and 17-ketosteroids in the urine of women with bilateral cystic ovaries.^{24,25} Increased LH and testosterone levels were regarded to be of key importance in diagnosing PCOS.^{17,26} Later, abnormal release of gonadotropins, LH/FSH ratio and androgens were confirmed.²⁷ Finally the condition of abnormal concentrations of gonadotropins for the diagnosis of PCOS was rejected.²⁸ However, following the description of a method of testosterone level measurement in plasma in 1961, increased circulating level of androgens in women with PCOS was demonstrated shortly thereafter.^{29,30} Because

of the limitations of laboratory tests in measuring the total androgenic hormone levels, many women met the clinical criteria of PCOS, without confirmation of hormones secretion disorders in laboratory tests.³¹ Secretion of hormones released by the pituitary gland and gonads is pulsative, so maximal and minimal concentrations may differ significantly during the day, which is why a single determination may be misleading, especially in women with rather low androgens levels compared to men. Researchers were looking for such a diagnostic tool which would replace roentgenography or reconnaissance laparotomy used before to diagnose polycystic ovaries. Surgical treatment of resistant anovulation has had a resurgence with the laparoscopic method popularized by Gjoanness H.³² Ultrasound examination of the reproductive system was a great progress in the clinical practice. Benefits of this research method, including its non-invasive character, repeatability, its simplicity in use and precision in assessing the ovary stroma and ovary follicles were immediately appreciated. Swanson was the first who described a structure of ovaries in women with PCOS using ultrasonography.³³ The improved technology and utilization of ultrasound in medicine led to the ultrasound definition of polycystic ovaries, defined primarily on the morphology and the number of small antral follicles. A study performed by Fox in 1991, aimed at comparing the use of transvaginal and transabdominal ultrasound, proved the presence of falsely negative results in the case of examination through the abdominal lining in case of as many as 30% examined women.³⁴ Progress made in the ultrasound diagnose enabled to verify of the ultrasound criteria.³⁵ It seemed that the ovarian stroma area to total area ratio had been the best condition of a PCOS diagnosis. Almost one quarter of the population had the appearance of polycystic ovaries when examined ultrasonically, but more than half of these had no clinical signs or symptoms. These women are referred to have polycystic ovaries.

The list of the various names of the same disorder which can be found in the literature are the following: polycystic ovaries disorder, a syndrome of polycystic ovaries, functional ovary androgenism, hyperandrogenic, chronic anovulation, polycystic ovarian syndrome, ovarian dysmetabolic syndrome, sclerotic polycystic ovary syndrome, polycystic ovary syndrome.³⁶

It was not until the early 1990s at a National Institutes of Health (NIH) sponsored conference on PCOS that formal diagnostic criteria were proposed and afterwards were largely utilized. These criteria, known as "the NIH criteria", were published as the conference proceedings and received large scale of acceptance in the research and clinical communities. According to these criteria, PCOS is defined as unexplained hyperandrogenic anovulation. PCOS can be diagnosed in women if the following criteria are found: symptoms of excess of androgens (clinical or biochemical), rare ovulations, exclusion of other disorders with similar clinical symptoms.³⁷

Thus, PCOS remains a diagnosis of exclusion. In the light of many later research studies, modification of the definition seemed to be necessary. The 2004 criteria established by a group of experts during a conference in Rotterdam in the Netherlands held in 2003 are obligatory (The Rotterdam ESHRE/ASRM – Sponsored PCOS Consensus Workshop Group). The subsequent “Rotterdam criteria” incorporated the ultrasound determined size and morphology of the ovary into the diagnostic criteria.³⁸ According to them the presence of 2 out of 3 following criteria are necessary to make a PCOS diagnosis:

1. rare ovulations or lack of ovulations,
2. excessive activity of androgens confirmed by a clinical or laboratory examination,
3. features of polycystic ovaries in the ultrasound after the exclusion of other pathologies characterized by hyperandrogenism, such as adrenocorticotrophic hormone-dependent or independent hypercortisolemia, thyroid gland disorders, a classical and non-classical form of congenital adrenal glands hypertrophy, tumors of adrenal glands or ovary tumors producing androgens, as well as the influence of received medication.

Clinical features of hyperandrogenemia are: hirsutism assessed according to Ferriman-Gallwey score (giving the points according to the scheme), seborrheic skin disease, androgenic balding and symptoms of virilization in the form of clitoris overtrophy or lowered tone of voice. An analysis of concentrations of testosterone, 17-OH progesterone, cortisol, sex hormone-binding globulin, albumins and hormones released from the pituitary gland including thyroid-stimulating hormone, prolactin, are useful in the assessment of hyperandrogenism. Ultrasound criterion is diagnostic if made by using a transvaginal ultrasound, performed during follicular phase, more than 12 follicles of the diameter of < 10 mm are visible or an increased ovary volume is a value > 10 mL. What is important, these lesions do not have to be bilateral.

The Rotterdam definition is much wider and includes more patients, in particular those without clinical or biochemical hyperandrogenism, while into the NIH definition biochemical hyperandrogenemia was necessary for making the PCOS diagnosis. The Rotterdam criteria have been criticized for including more mild phenotypes, especially for the combination of polycystic ovaries with oligomenorrhea. Critics of this Rotterdam definition are of the opinion that the results obtained on the basis of examinations in patients with an excess of androgens cannot be extrapolated to normoandrogenic patients. These additional phenotypes may lead to the generalization of clinical trials to treat PCOS and may also elevate the prevalence of PCOS in the general population.

In 2006 the Androgen Excess Society (AES) issued a statement – criteria attempted to establish hyperandrogenism as a sine qua non diagnostic condition in combination with other signs of the syndrome.³⁵ The focus on hyperandrogenism was to eliminate milder phenotypes

(without excessive amount of androgens, with menstruation disorders and a typical ultrasound image PCOS) and based on evidence that hyperandrogenism tends to track with both reproductive (i.e. acne, hirsutism, and androgenic alopecia) and metabolic (i.e. insulin resistance, dyslipidemia, and elevated cardiovascular risk) symptoms of the syndrome. However, it was also emphasized, that further work on defining PCOS is necessary for the appropriate progress in medicine, research studies and the treatment of patients, as PCOS not only causes menstruation disorders, infertility, obstetric complications and hyperandrogenism, but also increases the risk of more frequent occurrence of cardiovascular diseases and cancers of the reproductive system.

Many years have passed since the first publication concerning PCOS, but the etiology of PCOS is still puzzling. It is now accepted that it is multifactorial and partly genetic; however, a number of candidate genes have been postulated. Insulin resistance has been noted consistently among many women with PCOS, especially in those with hyperandrogenism, but it is not included in any of the diagnostic criteria. Now there is strong evidence that cardiovascular disease risk factors and disturbances in carbohydrate metabolism are all increased in patients with PCOS compared with the healthy population. The other very important point that has been made is that the basis of treatment is the modification of lifestyle. As the primary biochemical abnormality is insulin resistance, metformin can be used in the treatment. There have been a number of recommendations for the use of insulin sensitizing agents not only to restore ovulation but to facilitate weight loss, counteract androgenic symptoms, prevent long-term complications, decrease the risk of early pregnancy loss, decrease the risk of ovarian hyperstimulation syndrome, and even improve the outcome of in vitro fertilization therapy. There is still research conducted on improving therapy in PCOS women.

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