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Original papers

Reviews

The protective action of tocopherol and acetylsalicylic acid on the behavior of rats treated with dioxins

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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 the article

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Abstract

Background. Dioxins contribute to neurological disorders in humans and animals, causing also neurological disorders in offspring during prenatal and postnatal periods. These compounds significantly affect the development of the central nervous system (CNS) structures, which results in behavioral changes. Tocopherol (TCP) and acetylsalicylic acid (ASA) may provide protective measures to reduce the inflammatory effects in the CNS associated with free radicals generated by 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), thus contributing to the reduction of the negative effects of dioxin.

Objectives. The main objective of this study was to determine the influence of dioxin on rats and their behavioral functions, and to ascertain whether a combined administration of TCP and ASA to rats treated with TCDD shows the possibility of potential protective effect on the functioning of the CNS.

Material and methods. Experiments were performed on 75 female and 12 male Buffalo strain rats, which are offspring of females from particular study groups. TCDD was used in the experiments, TCP and ASA were administered orally every day for 3 weeks. Animals were subjected to behavioral testing: the tail and swimming tests.

Results. During the observation of the offspring of both sexes born to females exposed to TCDD, males did not demonstrate any attempt to swim, whereas in females, the immobility time was significantly extended. Assessing the response times from the tail test in the animals treated with dioxins in relation to the control group, it was demonstrated that the response time was extended in the 3rd measurement in both females and males.

Conclusions. Dioxin is characterized by neurotoxic effect causing behavioral disorders associated with prolonged response times. The use of TCP after the administration of dioxins causes a significant reduction and improvement of reflex response times. In contrast, ASA reduces the reflex response times also in the offspring of females exposed to TCDD and ASA.

Key words: central nervous system, 2,3,7,8-tetrachlorodibenzo-p-dioxin, tocopherol, acetylsalicylic acid, behavioral functions

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Introduction

Dioxins due to lipophilicity are mostly deposited in adipose tissue and nerve tissue. The rationale for the study was to ascertain depression in patients intoxicated with dioxins, treated with antidepressants.[1] In experimental studies on animals treated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), behavioral disorders connected with reduced activity and apathy were observed.[2–6]

Dioxin and related compounds are the cause of neurological disorders in humans and experimental animals as well as their offspring in the perinatal period after exposure to these compounds through the placenta and milk in prenatal and postnatal periods.[7–9] Dioxins significantly affect brain development in the prenatal and postnatal periods associated with the time of breastfeeding by mothers intoxicated with dioxins.[10–13] Changes caused by dioxins in the central nervous system (CNS) in pre- and postnatal periods lead to later changes in life. Functional disorders of the brain structures, including the cerebellum and diencephalon, should also be associated with changes in thyroid hormone levels and estrogen receptors (ER) induced by dioxins.[9,14–17]

It was shown that dioxins lead to the underdevelopment of the bones and teeth in both humans and animals as a result of mineralization disorders.[18–20] It was found that after birth there is a significant concentration of dioxins in the breast milk, correlating negatively with a newborn head circumference. This indicates that the brain development of the fetus can be significantly affected by maternal exposure to TCDD, which, together with milk, provides a substantial amount of dioxins to the offspring.[13,21,22] In patients intoxicated with dioxins, there were observed: severe depression, apathy and reduced physical activity.[23,24] This should be associated with the anti-estrogenic effects of TCDD.[25] Experimental studies in animals treated with TCDD by other authors also found behavioral disorders associated with decreased motility, apathy and circadian rhythm disruption.[26–28] Studies in rainbow trout revealed that exposure to TCDD induces biochemical and structural changes in the eye and brain, leading to behavioral deficits and reduced efficiency.[29]

Tocopherol (TCP) reduces the contact of TCDD with the aryl hydrocarbon receptor (AhR) blocking the formation of *CYP1A1*, thereby reducing the amount of generated free radicals. In this case, a TCP beneficial effect is manifested in selected biochemical indicators of the rats blood exposed to TCDD in which TCP is used in high doses.[14,30,31] According to Hassoun et al., there are areas in the brain which are less or more reactive to the active oxygen species.[32,33] Areas sensitive to the effects of free radicals generated by TCDD include the cerebral cortex, hippocampus, cerebellum, and brain stem. It was shown that administration of vitamin E reduced the secretion of reactive oxygen species (ROS) induced by TCDD; at the same time it showed the protective properties of the brain structures.[34,35] A significant increase in tumor necrosis factor alpha (TNF-α) in mice treated with TCDD was also noted. In turn, the increase in the level of TNF-α is responsible for lethargy and anorexia. In experiments where high doses of TCP were used, the concentration of TNF-α significantly decreased, regardless of experimentally induced pleuritis.[11,14,31]

Acetylsalicylic acid (ASA), in addition to the antiprostaglandin effect in inflammatory reactions, decreases the production of free radicals, nitric oxide synthesis, and inhibits the production of proinflammatory cytokines such as TNF, IL-1, IL-6 through the inhibition of nuclear factor kappa β (NF-κB). ASA also blocks COX-1 and COX-2.[36–38] It was found that ASA also protects the brain structures against ROS. A study conducted by Maharaj et al. confirmed that the use of ASA in a daily dose of 100 mg per 1 kg body weight (b.w.) inhibited the production of superoxide anions and lipid peroxidation products, thereby protecting the hippocampal neurons of a rat.[39] The studies by McDonald et al. showed that salicylamide reduces the binding of TCDD to cytosolic AhR.[37,38] It is concluded that this agent is a potent inhibitor of AhR and it blocks signal transduction initiated by the exposure to TCDD. The reason for the use of ASA as a protective substance in dioxin intoxication may be supported by the results of other studies, which found that ASA through the release of glutamate from nerve terminals has a presynaptic effect in the tail test, associated with the reduction of subsequent times. This leads to the phenomenon known as facilitation associated with facilitating neuromuscular transmission, and thus leading to faster reflex responses.[40]

The aim of this study was to determine the effect of dioxins on rats behavioral functions and to check whether the combined administration of TCP and ASA to rats treated with TCDD shows the potential of protective action of these compounds on the functioning of CNS in relation to the effects achieved by a single application of each of these compounds in the evaluation with the use of behavioral testing.

Material and methods

Animals

Experiments were performed on 117 rats: 75 females and 12 males of Buffalo strain, weighing 140–160 g, 8–10 weeks of age, and 30 females aged 6 weeks and weighing 120 g which were offspring of females from particular study groups listed below. All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals (NRC 2011). All experiments were performed according to guidelines for the experimentation on animals. The study was approved by the Local Ethics Council for Animal Experiments (No. 38/2009). Animals were housed in air-conditioned rooms characterized

by 15 air changes per hour at a temperature of 22°C, humidity of 55% and a 12/12 cycle of daylight. The rats were kept in polystyrene cages (6/cage), with free access to water and nutritious food Labofeed H dedicated for laboratory and breeding animals.

TCDD administration

In the experiments, 2,3,7,8-tetra-p-dioxin (Sigma Chemical Co. – Sigma-Aldrich Ltd., Poznań, Poland) was used, administered at a dose of 5 μg/mL b.w. and 12.5 μg/kg b.w. dissolved in a 1% solution of dimethyl sulfoxide (DMSO) at a concentration of 1 μg/mL. TCDD was administered intramuscularly in the hind limb muscles in a volume dependent on the body weight, i.e., 0.7–0.8 mL, or 1.6–1.8 mL for administration of TCDD in a dose of 12.5 μg/kg b.w. The studies used α-TCP acetate in an oily solution, administered daily for 3 weeks at a dose of 30 mg/kg b.w, subcutaneously (s.c.) in a volume of 0.2 mL and ASA administered orally (gavage) in the form of suspension in the starch solution at a dose of 50 mg/kg b.w., in a volume of 0.5 mL daily for 3 weeks. Induced pleurisy was induced with a single injection of 1% carrageenan solution into the pleural cavity between 5th and 6th right intercostal space in a volume of 0.15 mL in the experimental model worked out previously.[41]

Behavioral tests

Animals were subjected to behavioral tests, such as the tail test and the swimming test, performed in accordance with the applicable procedures. In both tests, the response time was measured with the use of calibrated metal electronic stopwatch Spokey Quary 2.

The swimming test involved placing a rat in a cylinder shaped container of 40 cm height and a diameter of 18 cm filled with water at 25°C for 5 min. The aim of the study was to measure the time of immobility, i.e., the time that the test rat spends without any movement and to describe intensity of swimming scoring from 0 to 3 points: no swimming – 0 points, intensive swimming – 3 points.

The tail test consisted in placing the tail of the test subject in a plastic tube with a hole for a tail, which was then immersed in a glass container filled with water at a constant controlled temperature of 58°C. The time which was measured was the time from the moment of tail insertion to its drawing from water by the rat. The longer the response time, the less energetic and active a rat was. To determine the dynamics of rat responses, the measures of response times in the tail test in each animal were performed 3 times at intervals of 60 s.

The swimming test and the response time were performed in the following groups:

- 1A – a control group C (F) of 6 females not exposed to effects of any agents;
- 1B – a control group C (M) of 6 males not exposed to effects of any agents;
- 2 – TCDD group (F) of 6 females treated with TCDD (i.m.) 3 weeks prior to the study (F);
- 3 – TCDD group (M) of 6 males treated with TCDD (i.m.) 3 weeks prior to the study;
- 4 – TCDD + TCP group (F) of 6 females treated with TCDD (i.m.) and α-TCP acetate 3 weeks prior to the study;
- 5 – TCDD + ASA group (F) of 6 females treated with TCDD (i.m.) and ASA 3 weeks prior to the study;
- 6 – TCDD + TCP + ASA group (F) of 6 females treated with TCDD (i.m.) 3 weeks prior to the study and daily with α-TCP acetate and ASA;
- 7 – TCP group (F) of 6 females treated daily with α-TCP acetate for the period of 3 weeks;
- 8 – ASA group (F) of 6 females treated daily with ASA for the period of 3 weeks.

The tail test, which measured the response times, was also used in the study of 6 weeks old offspring born to females from the above groups:

- 1P – a control group PC of 6 randomly selected females from offspring born to 6 females from C group (F);
- 2P – PTCDD group of 6 randomly selected females from offspring born to 6 females from TCDD group (F);
- 3P – PTCDD + TCP group of 6 randomly selected females from offspring born to 6 females from TCDD + TCP group (F);
- 4P – PTCDD + ASA group of 6 randomly selected females from offspring born to 6 females from TCDD + ASA group (F);
- 5P – PTCDD + TCP + ASA group of 6 randomly selected females from offspring born to 6 females from TCDD + TCP + ASA group (F).

Statistical analysis

The data was processed with the use of EXEL spreadsheet and the package STATISTICA PL v. 9. The results of the swimming test measurements and response times in particular groups of the studied subjects were presented as mean values with standard deviations. The analysis of hypothesis on normality of distribution of variables was verified with the use of the Shapiro-Wilk test and David-Hellwig test at the significance level of 0.05. The obtained values from the 2 subgroups were compared using the Student's t-test for unmatched pairs. The level of significance was set at $p = 0.05$. The influence of one factor on the results of performed measurements was examined using the analysis of variance by repeated measures ANOVA. If the analysis of variance did not reveal the significance in differences between the analyzed mean values in subgroups, no further tests were carried out. When the null hypothesis of equality of means in subgroups was rejected in the analysis of variance ($p < 0.05$), the significance of differences between the means of particular subgroups was studied using post-hoc tests (multiple comparisons) – NIR test (least significant differences) and Duncan test.

If the distribution of the studied parameter in one of the subgroups did not meet the assumptions of ANOVA, non-parametric alternative to ANOVA test was applied, the Kruskal-Wallis test.

Results

Results of the tail test in adult subjects

A comparison of subsequent response times in the control group C (F) to the response times in TCDD group (F) did not show statistically significant differences in the first 2 measurements; this difference occurred in the 3rd measurement. The statistical evaluation of response times from the control group of females C (F) showed that the response time evaluated with a tail test in 3 subsequent measurements did not change significantly. The reaction time in the control group C (M) of males slightly shortened with each measurement. In the 1st measurement of response times in control groups C (F) and C (M), there appears a statistically significant difference associated with a longer reaction time in males. In the 2nd measurement, the response time in the group C (M) is longer than in C (F); however, this difference is not statistically significant. Mean response times for the control group of females and males C (M) and C (F) showed no statistically significant difference (Fig. 1, 2).

A comparison of the control group C (M) with the TCDD (M) group showed a statistically significant difference only in the 3rd measurement, in which the response time in animals treated with a dioxin was extended. In TCDD (F) group, the extension of subsequent response times was observed (Fig. 1), where the 3rd measurement revealed a significant statistical difference in relation to the 1st measurement. The similar dynamics was noted in TCDD (M) group, however, with no statistically significant difference (Tables 1–3). A comparison between the obtained times from different groups TCDD (F) and TCDD (M) showed no statistically significant differences (Fig. 1).

The conclusion after comparing the response times of TCDD (F) group to TCDD + TCP (F) and TCDD + ASA (F) groups was that all response times in these groups were shortened, but a statistically significant difference occurred only in the 3rd measurement in both groups. The conclusion after comparing the response time of TCDD (F) group to TCDD + TCP + ASA (F) group was that all response times in these groups were shortened; however, a statistically significant difference occurred in the 2nd and 3rd measurement in both groups. The conclusion after comparing the response times of TCDD + TCP + ASA (F) group to TCDD + TCP (F) and TCDD + ASA (F) groups was that a statistically significant reduction in response time was observed in the 1st measurement for TCDD + TCP (F) group. Comparing the response time of K (F) group to the response times of TCP (F)

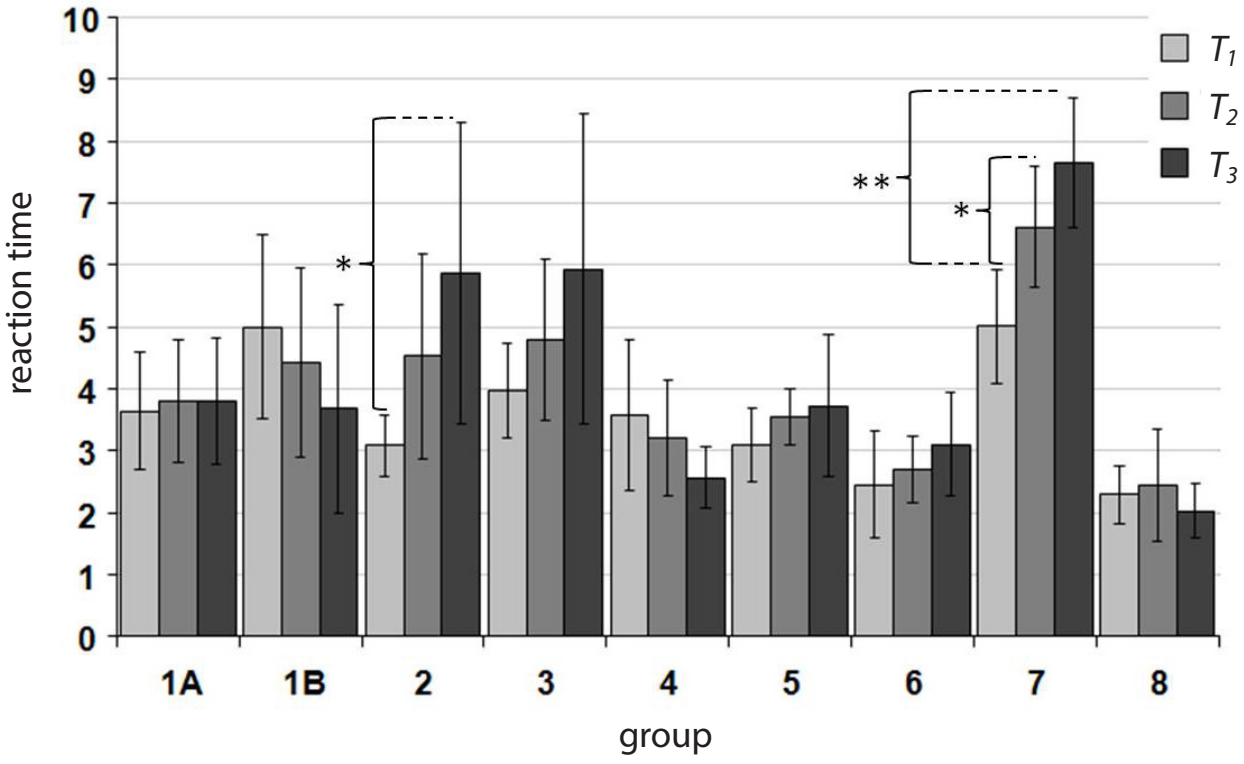

Fig. 1. Response times [s] of 3 subsequent measurements in particular groups in the tail test

Table 1. Significance of differences between the groups in the T_1 measurement of the tail test (p-values)

Study group	1A	1B	2	3	4	5	6	7	8
1A	–	0.013	0.297	0.518	0.904	0.330	0.037	0.012	0.009
1B	0.013	–	0.001	0.059	0.010	0.001	0.000	0.985	0.000
2	0.297	0.001	–	0.095	0.356	0.983	0.258	0.001	0.114
3	0.518	0.059	0.095	–	0.444	0.116	0.008	0.057	0.001
4	0.904	0.010	0.356	0.444	–	0.390	0.048	0.009	0.012
5	0.330	0.001	0.983	0.116	0.390	–	0.270	0.001	0.128
6	0.037	0.000	0.258	0.008	0.048	0.270	–	0.000	0.756
7	0.012	0.985	0.001	0.057	0.009	0.001	0.000	–	0.000
8	0.009	0.000	0.114	0.001	0.012	0.128	0.756	0.000	–

Fisher's least significant difference post hoc test; $p < 0.05$.

Table 2. Significance of differences between the groups in the T_2 measurement of the tail test (p-values)

Study group	1A	1B	2	3	4	5	6	7	8
1A	–	0.336	0.266	0.131	0.352	0.705	0.108	0.000	0.028
1B	0.336	–	0.878	0.574	0.062	0.198	0.014	0.001	0.002
2	0.266	0.878	–	0.682	0.044	0.153	0.009	0.002	0.001
3	0.131	0.574	0.682	–	0.017	0.071	0.003	0.006	0.000
4	0.352	0.062	0.044	0.017	–	0.608	0.462	0.000	0.210
5	0.705	0.198	0.153	0.071	0.608	–	0.234	0.000	0.086
6	0.108	0.014	0.009	0.003	0.462	0.234	–	0.000	0.680
7	0.000	0.001	0.002	0.006	0.000	0.000	0.000	–	0.000
8	0.028	0.002	0.001	0.000	0.210	0. 086	0.680	0.000	–

Fisher's least significant difference post hoc test; $p < 0.05$.

Table 3. Significance of differences between the groups in the T_3 measurement of the tail test (p-values)

Study group	1A	1B	2	3	4	5	6	7	8
1A	–	0.883	0.018	0.015	0.151	0.934	0.436	0.000	0.030
1B	0.883	–	0.012	0.010	0.196	0.954	0.522	0.000	0.043
2	0.018	0.012	–	0.945	0.000	0.019	0.003	0.042	0.000
3	0.015	0.010	0.945	–	0.000	0.016	0.003	0.049	0.000
4	0.151	0.196	0.000	0.000	–	0.196	0.546	0.000	0.498
5	0.934	0.954	0.019	0.016	0.196	–	0.504	0.000	0.047
6	0.436	0.522	0.003	0.003	0.546	0.504	–	0.000	0.203
7	0.000	0.000	0.042	0.049	0.000	0.000	0.000	–	0.000
8	0.030	0.043	0.000	0.000	0.498	0.047	0.203	0.000	–

Fisher's least significant difference post hoc test; $p < 0.05$.

group, a statistically significant increase in 3 response times in a group TCP (F) was observed both in relation to the control group C (F) and subsequent measurements in this group (Fig. 1; Tables 1–6). A statistically significant reduction in all response times in ASA (F) group in comparison to the control group C (F) was observed (Fig. 3). On the other hand, a statistically significant increase in all response times in TCP (F) group in comparison to the control group C (F) was observed (Fig. 4).

Results of the tail test in offspring groups

The analysis of response times was conducted with the use of the tail test on 6 weeks old female offspring born to females treated with dioxins: TCDD (F) group, the group in which TCP was used: TCP + TCDD (F), and the group also treated with ASA: TCDD + ASA (F).

The offspring response time in the tail test in PTCDD group in the 2nd measurement was significantly extended

and characterized by a high standard deviation, however PTCDD + TCP, and PTCDD + ASA and PTCDD + TCP + ASA were characterized by a reduced response time in 3 subsequent measurements, which statistically differed significantly from the control group C and PTCDD (Tables 4–6; Fig. 5, 6). The corresponding groups of females which gave birth to the studied offspring TCDD + TCP (F), TCDD + ASA (F) and TCDD + TCP + ASA (F) in the 2nd and 3rd measurement of response times its reduction was observed, similarly to the response of their offspring described above (Fig. 5, 6). The response time in *T1* offspring of rats from the control group C and PTCDD group is significantly longer than in other groups ($p < 0.001$). The response time in *T2* offspring of the rats from the control group C and PTCDD group is significantly longer than in other groups ($p < 0.05$). The response time *T3* offspring of the rats from the control group C and PTCDD group is significantly longer than in other groups ($p < 0.05$).

Analyzing the response time, based on the tail test, in the offspring born to females exposed to TCDD which were given ASA (PTCDD + ASA), for 3 weeks prior to pregnancy, similarly to adult females from this group, a statistically significant reduction of response time was found in each of the 3 measurements in relation to the offspring group born to females treated with TCDD only. This response is similar to that observed in the offspring group born to females from TCDD + TCP group.

The analysis of the combined use of ASA and TCP in the tail test on animals exposed to TCDD, which induced the inflammatory reaction, showed a reduction in response times at the 2nd and 3rd measurement in relation to TCDD (F) group. As to TCDD + TCP (F) group, the response time was reduced in the 1st measurement, but a general conclusion after analyzing the response times in this group is that they were shorter than in TCDD + TCP (F) and TCDD + ASA (F) groups. The offspring group of females exposed to TCDD, which were treated with TCP and ASA before pregnancy, also demonstrated a reduction of 3 response times in relation to all studied offspring groups. The swimming test in TCDD + TCP + ASA (F) group showed that, at the beginning, the swimming was accelerated and the immobility period in this group was significantly longer than in TCDD + TCP (F) and TCDD + ASA (F) groups. The immobility period, however, was shorter and intensity of swimming was higher compared to TCDD (F) group.

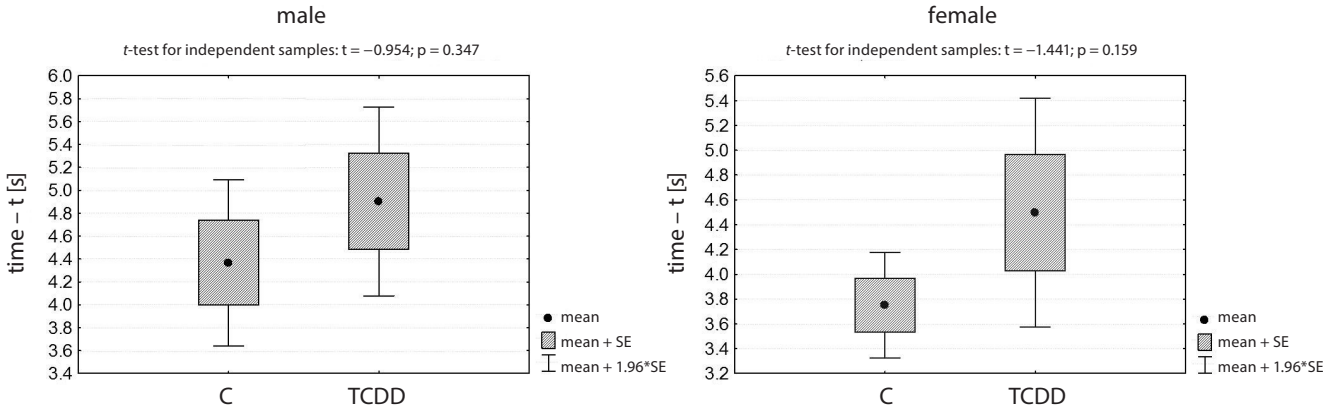

Fig. 2. Statistical analysis of mean response times [s] obtained between the groups of males and females from the control group and after TCDD administration

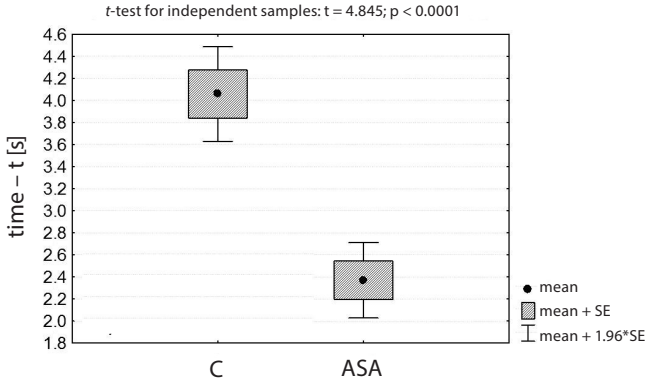

Fig. 3. Statistical analysis of mean response times [s] obtained between the control group C (F) and ASA (F)

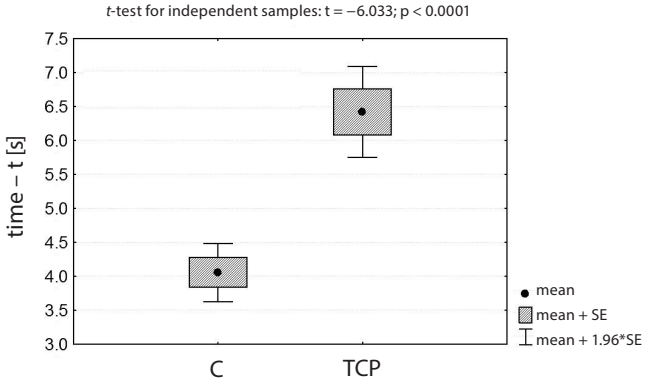

Fig. 4. Statistical analysis of mean response times [s] obtained between the control group C (F) TCP (F)

Results of the swimming test

The swimming test in the control group C (F) and (M) showed a statistically significant difference between males and females, finding an extended immobility period in females. In animals treated with dioxins, the comparison between a group of TCDD males (M) and TCDD females (F) indicates statistically significant differences. Males from this group TCDD (M) did not make attempts to swim, showing a tendency to drown. In females from TCDD (F) group, the immobility time was significantly extended in relation to TCDD + TCP (F) and TCDD + ASA (F) groups. The results in this group showed a greater degree of deviation from the mean value in relation to the control group C (M). Analyzing the swimming test results in TCDD + ASA (F) group, there was no period of immobility observed and the swimming activity was intensified. The comparison of the control group C (F) to ASA (F) group and TCP (F) shows a statistically significant reduction in immobility periods in TCP (F) and ASA (F) groups. The swimming time in TCDD + TCP + ASA (F) group was reduced in relation to TCDD (F) group (Table 6; Fig. 7, 8).

The evaluation of rats swimming intensity in a performed test showed that the control group C (F) was characterized by moderate intensity, while in K (M) group, the response was diversified (Table 6). In animals treated with dioxins, TCDD (F) group demonstrated a weak and moderate response, while in TCDD (M) group, there was no swimming response. In TCDD + ASA (F) group and TCDD + TCP + ASA (F) group the swimming response was intensified in relation to TCDD (F) group, in TCDD + TCP (F) group the response was moderate with a slight tendency to intensify. Comparing the control group C (F) to ASA (F) group, a significant intensification of response was observed after ASA administration. Administration of TCP in TCP group (F) induced weaker dynamics of response (Table 6).

Discussion

In our study, behavioral changes were observed in rats exposed to dioxins in terms of reduced physical activity, apathy and reduced food intake, often leading to cachexia and reduction in the concentration of plasma proteins. These findings are confirmed by studies of other authors.[41–43]

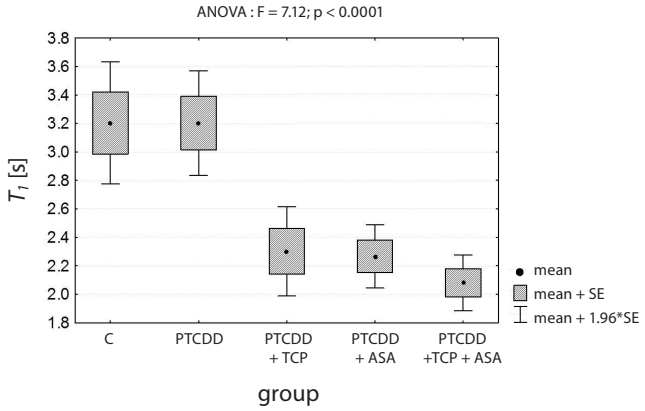

Fig. 5. Comparison of response times [s] from particular groups in the T_1 measurement

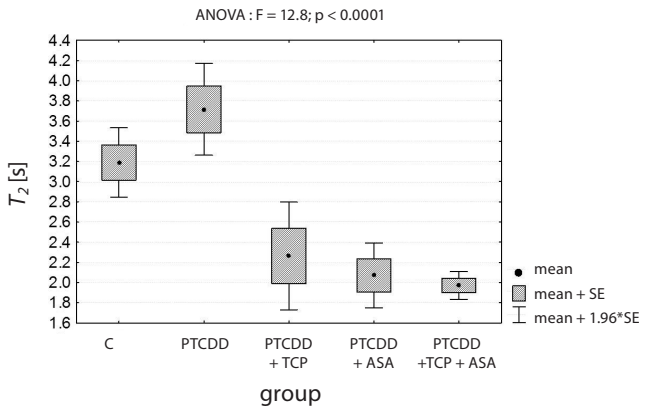

Fig. 6. Comparison of response times [s] from particular groups in the T_2 measurement

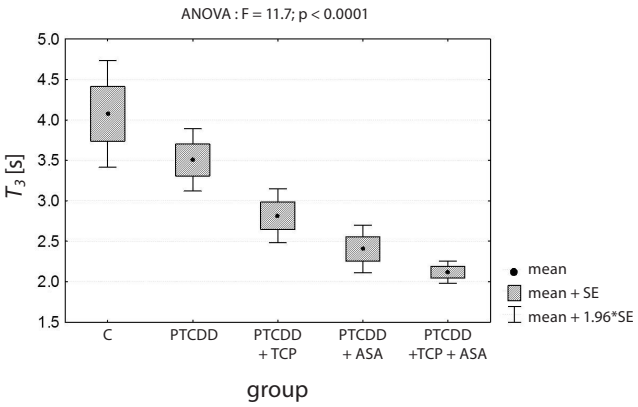

Fig. 7. Comparison of response times [s] from particular groups in the T_3 measurement

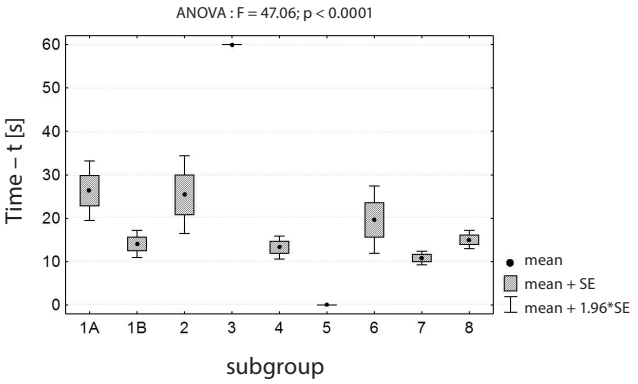

Fig. 8. Dynamics of the measured immobility time [s] of rats in the swimming test between particular groups

This type of behavior was also observed in the offspring of rats born to females treated with TCDD.[18,20] During the observation of offspring of both sexes born to females exposed to TCDD, males did not demonstrate any attempt to swim, whereas in females, the immobility time was significantly extended. This behavior supports the occurrence of depressive changes, and is associated with the lack of motivation to swim. Assessing the response times from the tail test in the animals treated with dioxins in relation to the control group, it was demonstrated that the response time was extended in the 3rd measurement in both females and males, which is the indicator of the extension of time and route of nerve impulses conduction. This is also supported by the extension of subsequent response times. Both reflex and behavioral function disorders should be explained by the formation of free radicals affecting the CNS and higher levels of pro-inflammatory interleukins (IL-1 and II-6), and especially TNF, whose increased concentration in inflammation induces appetite disorders and apathy which was proven in numerous studies.[2,42,44,45]

Table 4. Significance of differences in response times from the T_1 measurement in the offspring born to females from different study groups (p-values)

Study group	{1} = 3.20	{2} = 3.20	{3} = 2.30	{4} = 2.27	{5} = 2.08
{1} C (F)	–	0.996	0.004	0.001	0.001
{2} PTCDD	0.996	–	0.004	0.001	0.001
{3} PTCDD + TCP	0.004	0.004	–	0.913	0.566
{4} PTCDD + ASA	0.001	0.001	0.913	–	0.603
{5} PTCDD + TCP + ASA	0.001	0.001	0.566	0.603	–

Fisher's least significant difference post hoc test; $p < 0.05$.

Table 5. Significance of differences in response times from the T_2 measurement in the offspring born to females from different study groups (p-values)

Study group	{1} = 3.19	{2} = 3.51	{3} = 2.82	{4} = 2.40	{5} = 2.12
{1} C (F)	–	0.143	0.027	0.005	0.007
{2} PTCDD	0.143	–	0.000	0.000	0.000
{3} PTCDD + TCP	0.027	0.000	–	0.576	0.474
{4} PTCDD + ASA	0.005	0.000	0.576	–	0.793
{5} PTCDD + TCP + ASA	0.007	0.000	0.474	0.793	–

Fisher's least significant difference post hoc test; $p < 0.05$.

Table 6. Significance of differences in response times from the T_3 measurement in the offspring born to females from different study groups (p-values)

Study group	{1} = 4.08	{2} = 3.51	{3} = 2.82	{4} = 2.40	{5} = 2.12
{1} C (F)	–	0.076	0.001	0.000	0.000
{2} PTCDD	0.076	–	0.019	0.000	0.000
{3} PTCDD + TCP	0.001	0.019	–	0.183	0.059
{4} PTCDD + ASA	0.000	0.000	0.183	–	0.395
{5} PTCDD + TCP + ASA	0.000	0.000	0.059	0.395	–

Fisher's least significant difference post hoc test; $p < 0.05$.

Unusual behavior resulting from the influence that dioxins exert on the body, comparable with the results obtained in our study, was found in polar bears. Studies by Sonne et al. and Verreault et al. revealed that the cause of numerous disorders in the reproductive tract of both sexes is a significant concentration of dioxins accumulated in the bodies of mammals.[46,47] Many studies found that estrogens affect cognition, memory, mood, behavior, emotional reactions, pain, posture, balance, and movement.[7] In view of the above, it should be believed that the unusual behavior of people intoxicated with dioxins (rats in this study, studies on polar bears) is associated with disturbances in the metabolism of estrogen, as it was also observed in the studies by other authors.[27,28,48,49]

The evaluation of response time of animals treated with dioxins and TCP: TCDD + TCP (F) showed the reduction in the response time in the tail test in relation to the group which was given only TCDD (F). Measurements of the 3 subsequent response times in TCDD + TCP (F) group demonstrated a reduction of all 3 times against each other, implying the improvement of reflex responses.

Measurements of response times in the tail test of an offspring 6 weeks of age born to females exposed to TCDD (PTCDD + TCP), which were given TCP for 3 weeks before pregnancy, also showed the same change trends as their mother, which demonstrated a statistically significant reduction in responses in all 3 measurements.

These observations suggest that TCP administered to females before pregnancy induces lower intoxication of their offspring with this dioxin from mothers, and less damage to the CNS in the offspring.

Similarly, the swimming test in female subjects exposed to TCDD and treated with TCP showed, in TCDD groups, in terms of intensity level, a significant reduction in the immobility period, which proves the improvement of reflex reaction and reduction of depressive reaction. The improving effect obtained after a long-term use of TCP in both tests can be justified by a protective role of this vitamin on the phospholipids of cell membranes, mitochondrial membranes, as well as reduced activity of free radicals and nitric oxide, and lower level of TNF concentration, which is also confirmed by studies of other authors.[50–52]

Administration of ASA in ASA (F) group also resulted in shorter response times, which confirms the positive effect of ASA on neurotransmission. Similar results are presented in the studies by Gong et al., Wang and Lu et al.[40,53,54] This study confirms

that ASA is an antagonist of the glycine receptor and it reduces the inhibitory effect of GABA, releases glutamate from presynaptic terminals, resulting in the facilitation effect, which induces the hyperreactivity. This can be beneficial both in subjects with delayed response times and depressive type disorders that occur in dioxin intoxication, as has been manifested in the swimming test with no attempts to swim or with extended immobility in swimming.

The obtained response time results suggest that the administration of ASA and TCP in mothers treated with TCDD before pregnancy eliminated the negative effects of dioxin on CNS functioning in offspring compared to the offspring born to the females treated with TCDD only.

The analysis of the swimming test in rats exposed to dioxins, which are treated with ASA, showed a lack of immobility period and intensified continuous swimming. This type of reaction also occurred in S (F) group. These results support the idea that ASA has a strong anti-inflammatory effect, eliminating the negative effects of the proinflammatory action of TCDD connected with generating a large number of free radicals, TNF and prostaglandins which affect the functioning of the CNS, induce depression, apathy and lethargy (Fig. 2). These findings can be confirmed by similar results obtained in studies by other authors.[45,55–57] In addition, our most recent studies with the use of histopathological and ultrastructural analysis of the hippocampus in rats found that TCDD contributes to atrophy of estrogen receptors, in which also destructive and inflammatory changes were found along with demyelination of myelin sheaths. It was determined a total protective action of TCP and ASA towards CNS functions that was characterized by poorly expressed degenerative changes and smaller inflammatory reactivity.[16]

Conclusions

Dioxin has an angiotoxic, neurotoxic and glycotoxic effect, which induces behavioral disorders associated with prolonged response times. Administration of TCP causes a significant reduction and improvement of reflex response times, which is also confirmed in the offspring born to females exposed to TCDD and treated with TCP. The use of ASA also reduces the reflex response times in the offspring of females exposed to TCDD. Based on the results, it can be concluded that the combined use of TCP and ASA significantly reduces the response time in the tail test and increases the intensity of the swimming activity.

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Comparison of polypropylene and silicone Ahmed® glaucoma valves in the treatment of neovascular glaucoma: A 2-year follow-up

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Abstract

Background. Inflammation associated with biomaterials of Ahmed® glaucoma drainage devices may cause the formation of a capsule around the device and can thus have a significant influence on the level of intraocular pressure reduction.

Objectives. The objective of this study was to compare the clinical outcomes after the implantation of a polypropylene or silicone Ahmed® glaucoma valve in patients with neovascular glaucoma.

Material and methods. In the study, 27 eyes with neovascular glaucoma (group 1) received silicon Ahmed® valves and 23 eyes (group 2) received polypropylene valves. The best corrected distance visual acuity (BCDVA), intraocular pressure (IOP) and number of anti-glaucomatous drugs were recorded preoperatively and during a follow-up period of 24 months after surgery. Success was defined by the following criteria: 1) intraocular pressure in the rage of 6–21 mm Hg; 2) IOP reduction of at least 30% relative to preoperative values. All complications were registered.

Results. One month postoperatively, the mean BCDVA increased significantly in both groups compared to preoperative values ($p < 0.001$). These values did not change during the 24 months of follow-up examinations. The probability of success defined by criterion 1 at 24 months of observation was 66.7% for silicone and 27.3% for propylene valves group ($p < 0.007$). According to criterion 2, the difference in success between the groups was not statistically significant. The total number of complications that occurred in both groups during the 24 months of follow-up examinations was similar, except for a higher occurrence of Tenon's cyst formation in the group with a polypropylene valve (18% vs 35%; $p < 0.04$).

Conclusions. In patients with neovascular glaucoma, the implantation of a silicone valve is associated with a significantly higher probability of long-term reduction of IOP below 21 mm Hg and with a lower risk of valve encapsulation in comparison to polypropylene valves. The obtained results suggest that silicone Ahmed® valves are more effective in the treatment of patients with neovascular glaucoma.

Key words: inflammation, neovascular glaucoma, glaucoma drainage implants, biocompatible materials

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Introduction

Glaucoma drainage devices are increasingly used in the surgical glaucoma treatment. The surveys of the American Glaucoma Society indicate that a selection of aqueous shunts, as the preferred surgical approach, increased from 17.5% in 1996 to 50.8% in 2008.[1] Glaucoma drainage devices are a good treatment option in the cases involving refractory glaucoma, e.g., neovascular or uveitic glaucoma. The devices drain the aqueous humor from the anterior chamber through a tube to the posterior plate inserted under the conjunctiva and Tenon's capsule. Aqueous fluid is then removed by venous capillaries or lymphatics.[2] One of most frequently used devices is Ahmed® Glaucoma Valve (New World Medical Inc., Rancho Cucamonga, USA). Two types of Ahmed® glaucoma valves are available: polypropylene (S2) and silicone (FP7). In experimental studies, the different materials showed varying degrees of postoperative inflammation and pseudocapsule formation.[3] Inflammation associated with biomaterials may contribute to the failure of the glaucoma treatment by drainage devices. The risk of such inflammation is high, especially in patients with neovascular glaucoma.

In the literature, there are few studies suggesting that clinical outcomes are better after the implantation of an Ahmed® glaucoma valve with a silicone plate in comparison to a polypropylene one.[4–6] Our study was designed to compare the clinical outcome 2 years after the implantation of polypropylene or silicone Ahmed® glaucoma valves in patients with neovascular glaucoma.

Patients and methods

In the study, 50 eyes of 50 patients with diabetic neovascular glaucoma were treated by the implantation of Ahmed® valves – 27 eyes of 27 patients, with a mean of age of 61.8 ±11.8 years, received silicone valves (model FP7 – group 1) and 23 eyes of 23 patients, mean of age 61.2 ±13.7 years, received polypropylene valves (model S2 – group 2). Inclusion criteria were as follows: diagnosed diabetic neovascular glaucoma and elevated intraocular pressure (IOP) that was not responsive to conventional medical and surgical therapy. Exclusion criteria included patients younger than 18 years, eyes requiring combined surgery, eyes with previous cyclodestructive treatment, silicone oil treatment or previous glaucoma drainage device implantation.

Both models of Ahmed® glaucoma valves had the same plate surface area (184 mm²) and a similar design and shape. The surgery was performed by one of the 2 experienced glaucoma surgeons (WL, LK) under Möller HI-R 900 microscope with magnification of ×5. The choice of the Ahmed® glaucoma valve – polypropylene (S2) or silicone (FP7) – was done randomly for each patient. The same surgical implantation procedure was used for both implants. After regional anesthesia, a fornix-based conjunctival-subtenon flap was created in the supratemporal quadrant. The anterior edge of the plate was fixed with 9–0 nylon sutures to the sclera 8 mm from the limbus. A 2/3 thickness limbal-based scleral flap was made and the valve tube was inserted into the anterior chamber through a 23-gauge needle puncture under the flap. The scleral flap and then Tenon-conjunctiva flap were sutured using 10–0 nylon sutures. The anterior chamber was reformed with balance salt solution through paracentesis. After surgery, topical antibiotics and steroids were installed for a period of 2 months. To achieve the desired IOP reduction, antiglaucomatous drops were added as required.

The best corrected distance visual acuity (BCDVA – Snellen chart) , IOP and number of used antiglaucomatous drugs were recorded preoperatively, on postoperative days 1 and 7, and in postoperative 1, 3, 6, 12, and 24 months after surgery. Any complications were registered.

Surgical success was assessed using 2 different criteria. Criterion 1 was IOP in the rage of 6–21 mmHg, with or without the use of additional antiglaucomatous drops. Criterion 2 was IOP reduction of at least 30% relative to preoperative values. Eyes requiring additional antiglaucomatous interventions (including cyclophotocoagulation) or the removal of the implant, or eyes that lost light perception were classified as failures.

The approval by the local Ethical Committee from Pomeranian Medical University in Szczecin (Poland) was

Table 1. Demographic and preoperative data for patients treated with silicone or polypropylene Ahmed® glaucoma valves

Dempgraphic and preoperative data	Silicone plate (FP7) n = 27 (100%)	Polypropylene plate (S2) n = 23 (100%)	p-value
Age, years (mean ±SD) range	61.8 ±11.8 38–82	61.2 ±13.7 36–86	ns
Gender male/female	17 (62.0)/10 (38.0)	14 (60.0)/9 (40.0)	ns
Lens status phakic pseudophakic	 9 (33.0) 18 (67.0)	 8 (35.0) 15 (65.0)	ns
Previous surgery	23 (85.0)	20 (87.0)	ns
Number of procedures (mean ±SD) range	1.7 ±1.2 1–3	1.8 ±1.1 1–4	ns
Previous laser therapy	10 (38.0)	8 (35.0)	ns
Preoperative visual acuity (mean ±SD) range ≥0.2 0.1–0.15 0.05 CF HM	0.07 ±0.01 3 (11.0) 8 (30.0) 9 (33.0) 5 (19.0) 2 (7.0)	0.07 ±0.01 3 (13.0) 6 (26.0) 8 (35.0) 4 (17.0) 2 (9.0)	ns

CF – counting fingers; HM – hand movements; ns – nonsignificant statistically.

obtained for this prospective study, and all 50 patients signed informed consent before surgery.

Statistical analysis

Preoperative and postoperative data was compared between FP7 and S2 Ahmed® glaucoma valve groups. The Mann-Whitney U test was used to compare continuous variables between the 2 groups. The Wilcoxon signed-rank test was used to compare continuous parameters within the groups. The χ^2 test and Fisher's exact test were used for categorical variables. Success rates in both groups were compared using the Kaplan-Meier survival curves and long-rank test. The Cox proportional hazard regression model was used for the analysis of risk. The p-values < 0.05 were considered statistically significant.

Results

Both groups were comparable with respect to age, gender, lens status, previous surgery, the number of procedures, laser therapy and preoperative visual acuity. The preoperative data for the 2 groups of patients treated with silicone or polypropylene Ahmed® glaucoma valves is summarized in Table 1.

The following mean intraocular pressure values were detected in patients treated with silicone (FP7 group) and polypropylene (S2 group) plates, respectively: 48.63 ±11.34 mm Hg and 45.59 ±14,14 mm Hg preoperatively (p = 0.72); 11.2 ±6.45 mm Hg and 12.8 ±5.9 mm Hg at day 1 (p = 0.65); 15.4 ±9.1 mm Hg and 16.6 ±5.6 mm Hg at 3 months (p = 0.064); 14.2 ±4.6 mm Hg and 15.3 ±5.1 mm Hg at 6 months (p = 0.068); 13.6 ±3.3 mm Hg and 15.3 ±3.5 mm Hg at 12 months (p = 0.062); and 13.8 ±3.3 mm Hg and 15.7 ±4.2 mm Hg at 24 months (p = 0.061). The data is presented in Fig. 1.

The mean preoperative IOPs did not differ significantly between polypropylene and silicone valves (p = 0.72). The mean IOP in silicone valve group (FP7) was lower at 3, 6 and 12 months after surgery in comparison to propylene group (S2), but this difference was not statistically significant (p = 0.06). In both groups, on postoperative day 1 initial reduction of IOP was obtained. Then, the mean IOP gradually increased to a peak at 1 month, and then decreased and stabilized between 6 and 24 months in both groups.

The mean number of antiglaucomatous drops which were necessary to normalize IOP in both groups at all follow-up visits is shown in Fig. 2. The average drop number of medication was reduced from 3.74 ±0.52 and 3.32 ±0.59 before surgery to 0.92 ±1.3 and 1.27 ±1.3 at 2 years after treatment in patients with silicone and polypropylene plates, respectively. There were no statistically significant differences in the mean number of antiglaucomatous medications at all time points between the groups. However, a tendency for greater reduction of the mean of antiglaucomatous medications was detected with the implantation of a silicone valve (FP7) during 2 years of observation time (Fig. 2).

At 24 months, significant reduction of IOP was achieved in both silicone (FP7) and polypropylene (S2) group, although there were no significant differences between the groups (Table 2). Significant and similar reduction of the mean number of antiglaucomatous medication drops was obtained in both groups (Table 2). The number of patients who had a hypertensive phase within 3 months postoperatively was 8 (29%) in silicone group (FP7) and 14 (60%) in polypropylene group (S2) (p < 0.006) (Table 2).

A slight, similar improvement of the mean BCDVA was achieved in both groups 2 years after the surgery (FP7 – from 0.07 to 0.14; S2 – from 0.07 to 0.12; p < 0.34).

Table 2. Preoperative status and postoperative outcome at 24 months, a postoperative follow-up visit in patients treated with silicone or polypropylene Ahmed® valves

Preoperative status and postoperative outcome	Silicone plate (FP7) n (%)	Polypropylene plate (S2) n (%)	p-value
IOP, mm Hg (mean ±SD) preoperative postoperative	 48.63 ±11.34 13.81 ±3.3	 45.59 ±14.14 14.72 ±4.2	 0.72 0.07
Glaucoma medications (mean ±SD) preoperative postoperative	 3.74 ±0.52 0.92 ±1.3	 3.32 ±0.59 1.27 ±1.3	 0.61 0.18
Postoperative visual acuity (mean ±SD)	0.14 ±0.07	0.12 ±0.06	0.34
Surgical outcome by criterion 1 (IOP 6–21 mm Hg) success failure	 26 (96.0) 1 (4.0)	 19 (82.0) 4 (17.0)	0.007
Surgical outcome by criterion 2 (↓IOP > 30%) success failure	 26 (96.0) 1 (4.0)	 20 (86.0) 3 (13.0)	0.06

Table 3. Postoperative complications after the implantation of silicone or polypropylene Ahmed® glaucoma valves

Postoperative complications	Silicone plate (FP7) n = 27 (100%)	Polypropylene plate (S2) n = 23 (100%)	p-value
Number of eyes with complications	12 (44.0)	16 (69.0)	0.061
Choroidal effusion	1 (3.7)	2 (8.7)	0.923
Hypotony early (within 3 months) late (after 3 months)	 1 (3.7) 0	 1 (4.3) 0	 1.000 1.000
Shallow chamber	0	1 (4.3)	0.641
Tube obstruction	1 (3.7)	1 (4.3)	1.000
Corneal edema	0	1 (4.3)	0.641
Hyphema	5 (18.0)	7 (30.4)	0.063
Tenon's cyst	5 (18.0)	8 (34.7)	0.042

BCDVA was stable in most patients in both groups. Worsening of visual acuity (VA) of more than 2 lines of Snellen chart was observed in 5 patients (18%) in FP7 group and in 4 patients (17%) in S2 group ($p < 0.69$). No patient lost light perception.

In FP7 and S2 plate groups, the success rates were 96% (26 out of 27 eyes) and 82% (19 out of 23 eyes) as defined by criterion 1 ($p = 0.007$), and 96% (26 out of 27 eyes) and 86% (20 out of 23 eyes) as defined by criterion 2 ($p = 0.06$), respectively (Table 2).

The Kaplan-Meier analysis demonstrated a difference in the probability of success as defined by criterion 1 in adequate IOP control between FP7 and S2 groups (Fig. 3). The probability of success at 24 months of observation was 66.7% for silicone and 27.3% for polypropylene valve groups. The difference between the Kaplan-Meier survival curves was statistically significant (log-rank test = –2.7; $p = 0.007$). The use of a silicone valve presented a significantly lower risk of failure in terms of achieving adequate IOP and increased the chance for achieving adequate IOP in postoperative time by 35% (hazard ratio = 0.35).

Postoperative complications after the implantation of silicone or polypropylene Ahmed® glaucoma valves are presented in Table 3. The occurrence of valve encapsulation with the formation of Tenon's cyst was the only statistically significant difference in the number of complications between the 2 valve groups. The number of encapsulation and Tenon's cyst formation cases was lower in silicone valve group (18.0% vs 34.7%). The complication was successfully managed by surgically releasing the adhesions around the valve. The device tube obstruction that occurred in 2 cases (1 in each studied group) was managed surgically by flushing the device with balanced salt solution (BSS). No other surgical eye procedures were performed in the patients during the follow-up period.

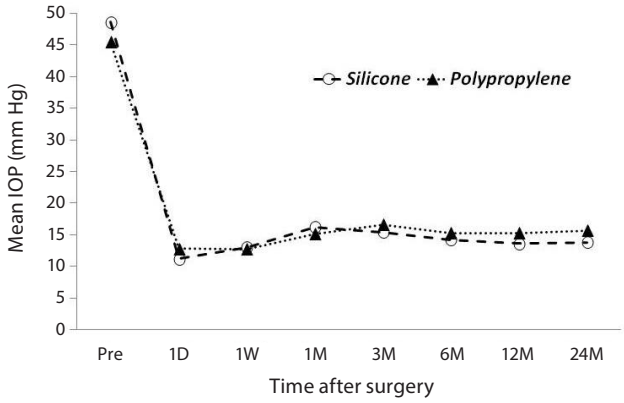

Fig. 1. Graph demonstrating the mean intraocular pressures (IOPs) before and after the implantation of silicone or polypropylene Ahmed® glaucoma valves

D – day; M – month(s); Pre – before surgery; W – week.

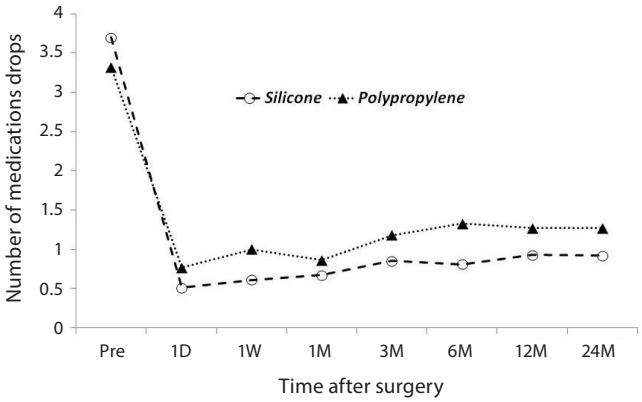

Fig. 2. The mean number of glaucoma medication drops after the implantation of silicone or polypropylene Ahmed® glaucoma valves

D – day; M – month(s); Pre – before surgery; W – week.

Discussion

Neovascular glaucoma, a type of secondary glaucoma, is caused by retinal hypoxia, which results in the release of a vascular endothelial growth factor (VEGF) into the vitreous and anterior chamber. The subsequent growth of a fibrovascular membrane covering the trabecular meshwork and sometimes peripheral anterior synechiae causes an increase of IOP through secondary open-angle or secondary closed-angle mechanisms.[7] Adequate treatment should be primarily directed toward treating retinal ischemia by the use of pan-photocoagulation, cryotherapy and/or anti-VEGF agents. Surgical lowering of IOP is, however, essential in most cases.[8]

Conventional trabeculectomy for the treatment of neovascular glaucoma is often complicated by intraoperative intraocular bleeding and the postoperative progression of the fibrovascular membrane, leading to a limited rate of success.[9,10]

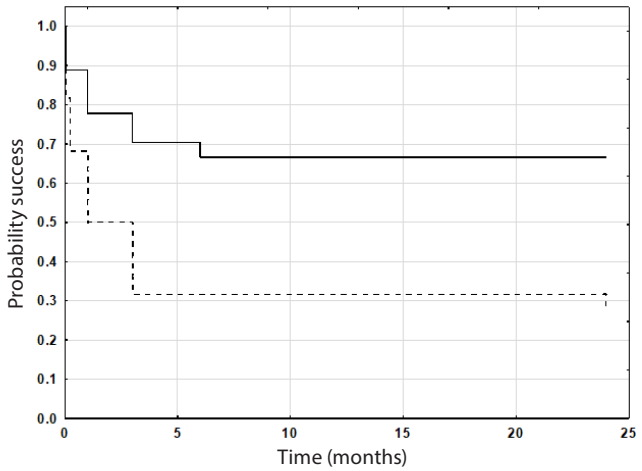

Fig. 3. The Kaplan-Meier survival curves show the probability of success as defined by criterion for silicone (FP7) and polypropylene (S2) valves

Probability of success at 24 months of observation was 66.7% and 27.3% for silicone and polypropylene valve group, respectively. The difference between the survival curves was statistically significant ($p = 0.007$).

Aqueous drainage implants may have higher success rates, but the results may vary not only due to different implant designs but also the valve material.[11,12]

In the present study, differences in clinical outcomes in treating neovascular refractory glaucoma using 2 types of Ahmed® glaucoma valves with similar dimensions but different materials were found.

The mean IOP did not differ significantly between polypropylene and silicone valve groups at any of the study milestone points (i.e., preoperatively, at day 1, and 1, 3, 6, 12, 24 months after surgery). However, the mean IOP in a group of patients could not be considered as a good statistical indicator of proper IOP control in an individual patient. Thus, the success of the implantation surgery was defined by the 2 criteria described above. Criterion 1 (IOP in the range of 6–21 mm Hg, with or without the use of additional antiglaucomatous drops) was met in 96.0% of patients with a silicone valve vs 82% of patients with a polypropylene valve ($p = 0.007$). Criterion 2 (IOP reduction of at least 30% relative to preoperative values) was met in 96% of patients with a silicone valve and 86% of patients with a polypropylene valve ($p = 0.06$). Thus, statistically, patients with neovascular glaucoma have a slightly better chance of achieving an acceptable level of IOP when silicone valves are used.

The results are consistent with the results of the study performed by Ishida et al., which showed an even bigger difference between silicone and propylene valves in achieving the same defined criterions of success: 82.4% vs 56.7% for criterion 1 and 78.3% vs 68.5% for criterion 2.[4]

The study, however, included a more heterogeneous group of refractory glaucoma patients, including patients with significant conjunctival scarring or inflammation. Also, the study of Mackenzie et al. showed greater success in achieving >30% IOP reduction at 2-year follow-up in patients with silicone valves: 82% vs 72%.[5] Both studies included quite a heterogeneous group of patients. Our study is, to our knowledge, the first study comparing 2 different valve materials in patients suffering only from diabetic neovascular glaucoma. We did not include any other type of refractory glaucoma patients to have a more homogenous group of patients.

The lower occurrence of valve encapsulation with the formation of Tenon's cyst in silicone valve group was the only statistically significant difference in the complication number between the 2 valve groups. We believe that this is the reason of the higher success rates in the silicone valve group.

Secondary scar formation around the valve is considered to be the most important reason for glaucoma surgery failure.[13] Ayyala et al. in an animal experimental study showed that polypropylene is more inflammatory than silicone.[14]

In addition to valve material, other factors may influence the success rate after implant glaucoma surgery. Greater surface of the end-plate of the valve may be associated with lower IOP, still with the upper limit of the surface area beyond which the valve implantation will stimulate a greater inflammatory response.[15–17] Some studies, however, do not show such an association.[18,19] The valve construction also may contribute to the final results. Some studies indicate that Ahmed® valves have a higher rate of encapsulation when compared to double-plate Molteno or Baerveldt implants.[20] However, Ahmed® valves were shown to be associated with fewer early postoperative complications, especially those related to hypotony.[21] Patients with Ahmed® implants are also at a lower risk of vision loss or the need of secondary glaucoma surgery.[22] In our study, none of the patients experienced vision loss.

To conclude, our results provide clinical evidence that different materials may influence the visual outcome after Ahmed® valve implantation in neovascular glaucoma patients, most likely because of the differences in biocompatibility of the materials.

The obtained results confirm the effectiveness of the valve mechanism in both devices, though the use of a silicone valve in patients with neovascular glaucoma increases the chance for achieving adequate IOP in postoperative time by 35%.

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Methodological exploration of bone marrow stem cell therapy in acute myocardial infarction – how to achieve greater benefit on cardiac outcomes: A systematic review and meta-analysis

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

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Abstract

Background. Clinical trials of intracoronary injection of bone marrow-derived stem cells (BMCs) in patients with acute myocardial infarction (AMI) have revealed promising but variable and modest results. One of the reasons underlying this situation may be the unstandardized preparation of BMCs.

Objectives. The aim of this study was to explore whether methodological differences affect the prognosis of acute myocardial infarction patients who received BMCs transplantation.

Material and methods. MEDLINE was searched for randomized controlled trials providing AMI patients with intracoronary BMCs injection or a standard therapy. Changes in cardiac parameters and clinical outcomes were analyzed. Subgroup analyses were conducted according to different methodologies for cell preparation, including supplement for serum or plasma, use of heparin and cell washout.

Results. Non-use of serum or plasma in the cell suspension is associated with more reduction in infarct size (IS) and a lower risk of all-cause mortality. Heparin usage could diminish the benefit in reducing IS. All-cause mortality rose significantly without the cell washout procedure when heparin was used.

Conclusions. Methodological differences in BMCs preparation as well as the use of heparin and serum/plasma impact on the prognosis of AMI patients.

Key words: acute myocardial infarction, bone marrow-derived stem cells transplantation, cell preparation, serum or plasma, heparin

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Introduction

Clinical trials of intracoronary injection of bone marrow-derived stem cells (BMCs) in patients with acute myocardial infarction (AMI) have revealed promising results. However, improved left ventricular ejection fraction (LVEF) and left ventricular remodeling were observed in most but not all studies. Moreover, the extent of improvement was variable between the trials and the benefit of cell therapy with BMCs was modest. One reason underlying the variable and modest improvement of cardiac function by cell therapy may be related to the unstandardized preparation of BMCs. Serum or plasma, which in some trials was used to provide a suitable physiological environment for BMCs, was reported to induce clotting of the cell product. Similarly heparin, used to prevent coagulation during cell preparation in many clinical trials, has recently been shown to affect the survival and functionality of BMCs. Therefore, the objective of this systematic review and meta-analysis is to explore whether the usage of serum or plasma, heparin and steps of washout during cell preparation may have influenced the results of previous trials.

Table 1. Overall analyses and the subgroup analysis examining the effect of serum/plasma addition, heparin use and the cell washout step after heparin use on cardiac parameters

Statistics and results	Total effect	Serum/plasma	Non-serum/plasma	Heparin	Non-heprarin	Cell washout	Non cell-washout
EF							
No. of RCTs	23	8	15	13	10	6	7
Difference in mean	1.50 [0.50, 2.49]	2.07 [0.89, 3.26]	1.30 [–0.13, 2.73]	1.83 [0.72, 2.95]	1.01 [–0.87, 2.88]	1.24 [–0.77, 3.24]	2.54 [1.44, 3.65]
p-value	0.003	0.0006	0.07	0.001	0.29	0.23	<0.00001
Subgroup p-value	–	0.41		0.46		0.26	
IS							
No. of RCTs	10	4	6	7	3	3	4
Difference in mean	–1.28 [–2.59, 0.02]	0.36 [–1.25, 1.98]	–2.03 [–3.45, –0.61]	–0.75 [–2.23, 0.74]	–4.37 [–7.20, –1.54]	–0.65 [–3.53, 2.24]	–0.84 [–2.62, 0.95]
p-value	0.05	0.66	0.005	0.32	0.003	0.66	0.36
Subgroup p-value	–	0.03		0.03		0.91	
LVESV							
No. of RCTs	19	8	11	13	6	6	7
Difference in mean	–3.3 [–5.44, –1.25]	–4.95 [–7.75, –2.14]	–2.25 [–5.29, 0.80]	–4.25 [–6.32, –2.17]	–0.93 [–5.98, 4.11]	–4.39 [–7.66, –1.12]	–3.78 [–6.74, –0.83]
p-value	0.002	0.0005	0.15	<0.0001	0.72	0.009	0.01
Subgroup p-value	–	0.2		0.23		0.79	
LVEDV							
No. of RCTs	20	8	12	13	7	6	7
Difference in mean	–2.75 [–5.47, –0.03]	–2.51 [–6.52, 1.50]	–3.14 [–6.91, 0.63]	–3.40 [–6.92, 0.12]	–0.70 [–4.95, 3.55]	–4.78 [–9.23, –0.33]	–2.93 [–8.46, 2.61]
p-value	0.05	0.22	0.1	0.06	0.75	0.04	0.3
Subgroup p-value	–	0.82		0.34		0.61	

Methods

Search strategy

We searched MEDLINE for studies of BMCs transplantation in patients with acute myocardial infarction by November 2015 using the following terms: "randomized controlled trials"; "cell therapy"; "stem cells"; "progenitor cells"; "precursor cells"; "bone marrow cells"; "mononuclear cells"; "cell transplantation"; "coronary artery disease"; "myocardial infarction"; "acute myocardial infarction"; and "myocardial ischemia".

Study selection

Since this was a study for exploring the methodology of cell transplantation, we prepared relatively strict criteria to avoid disturbance caused by different study design. Clinical trials were included if they 1) were randomized controlled clinical trials; 2) were conducted on patients with acute myocardial infarction who accepted primary percutaneous coronary intervention (PCI); 3) were using BMCs for cell transplantation; 4) were conducting cell therapy by injecting BMCs into the infarct-related coronary artery; 5) were providing a standard therapy for patients in both control and intervention group; 6) provided a detailed description of cell preparation; and if (7) the ratio of loss to follow-up was < 20%. Only articles published

in English were included. We used mean and standard deviation for statistical analysis, so studies reporting data as median and range were not included.

Data extraction

Two investigators independently screened all the titles and abstracts to include studies that met our inclusion criteria, and extracted the data from the papers. When there were disagreements, a third investigator was involved. Inclusion criteria or data would be reviewed and discussed until final decisions were made.

The cardiac outcome measures included changes in LVEF, infarct size (IS), left ventricular end-systolic volume (LVESV), and left ventricular end-diastolic volume (LVEDV). The clinical outcome measures from follow-ups included cardiac mortality, all-cause mortality, reinfarction, target-vessel restenosis, all-vessel restenosis,

Table 2. Overall analyses and the subgroup analysis examining the effect of serum/plasma addition, heparin use and the cell washout step after heparin use on clinical outcomes

Statistics and results	Total effect	Supplement		Heparin usage		Cell washout	
		serum/plasma	non-serum/plasma	heparin	non-heparin	cell washout	non-cell washout
Cardiac death							
Peto OR	0.41 [0.13, 1.27]	0.81 [0.11, 5.95]	0.39 [0.13, 1.17]	0.50 [0.12, 2.04]	0.29 [0.04, 1.91]	0.24 [0.03, 1.78]	1.01 [0.14, 7.22]
p-value	0.12	–	–	–	–	–	–
Subgroup p-value	–	0.42		0.65		0,.31	
All-cause mortality							
Peto OR	1.01 [0.49, 2.08]	3.01 [0.97, 9.31]	0.47 [0.18, 1.21]	1.35 [0.60, 3.03]	0.33 [0.07, 1.64]	0.47 [0.13, 1.68]	2.73 [0.96, 7.78]
p-value	0.98	–	–	–	–	–	–
Subgroup p-value	–	0.01		0.13		0.04	
Reinfarction							
Peto OR	0.49 [0.22, 1.09]	0.24 [0.08, 0.71]	1.03 [0.30, 3.58]	0.45 [0.18, 1.08]	0.76 [0.12, 4.87]	0.72 [0.22, 2.33]	0.23 [0.06, 0.90]
p-value	0,.06	–	–	–	–	–	–
Subgroup p-value	–	0.09		0.61		0.22	
Target-vessel restenosis							
Peto OR	0.96 [0.66, 1.40]	0.91 [0.56, 1.50]	1.03 [0.58, 1.84]	1.00 [0.67, 1.50]	0.77 [0.33, 1.82]	1.13 [0.64, 1.99]	0.88 [0.48, 1.61]
p-value	0.2	–	–	–	–	–	–
Subgroup p-value	–	0.75		0.62		0.55	
All-vessel restenosis							
Peto OR	0.96 [0.71, 1.31]	0.81 [0.54, 1.23]	1.17 [0.75, 1.81]	0.97 [0.70, 1.35]	0.92 [0.42, 2.02]	1.14 [0.71, 1.83]	0.84 [0.54, 1.33]
p-value	0.82	–	–	–	–	–	–
Subgroup p-value	–	0.24		0.91		0.38	
Stent thrombosis							
Peto OR	0.65 [0.22, 1.89]	0.58 [0.17, 1.90]	1.06 [0.09, 12.07]	0.65 [0.20, 2.05]	0.66 [0.04, 11.84]	0.99 [0.20, 5.01]	0.42 [0.08, 2.15]
p-value	0.43	–	–	–	–	–	–
Subgroup p-value		0.66		0.99		0.46	
Heart failure							
Peto OR	0.72 [0.39, 1.33]	0.64 [0.19, 2.15]	0.75 [0.37, 1.53]	0.64 [0.33, 1.24]	1.42 [0.29, 6.80]	0.41 [0.16, 1.09]	0.95 [0.38, 2.40]
p-value	0.3	–	–	–	–	–	–
Subgroup p-value	–	0.82		0.36		0.22	
VT/syncope/ICD implantation							
Peto OR	0.79 [0.41, 1.51]	0.76 [0.26, 2.20]	0.80 [0.35, 1.84]	0.76 [0.35, 1.66]	0.85 [0.26, 2.82]	0.76 [0.27, 2.11]	0.76 [0.23, 2.56]
p-value	0.47	–	–	–	–	–	–
Subgroup p-value	–	0.94		0.88		1	
Stroke							
Peto OR	0.65 [0.18, 2.36]	0.49 [0.08, 2.96]	0.88 [0.14, 5.56]	0.78 [0.15, 3.99]	0.49 [0.06, 3.94]	1.92 [0.20, 18.59]	0.29 [0.03, 3.11]
p-value	0.51	–	–	–	–	–	–
Subgroup p-value	–	0.66		0.73		0.26	

stent thrombosis, heart failure, ventricular arrhythmia/syncope/ICD implantation, and stroke. Data with the longest duration of follow-up were extracted as outcome measures. IS was defined as the ratio of infarct region to left ventricle. Left ventricular volumes were estimated from LV volume indexes when appropriate. Multiple imaging modalities were used in some studies. When available, data from MRI or SPECT was preferred for analyses to data from echocardiography or left ventricular angiography.

Data processing was done using the methods described in the Cochrane handbook when needed. For studies with more than one intervention arms, with different doses of BMCs or different time for intervention, outcome measures data was combined. For studies that did not report changes in outcome measures between baseline and follow-up, the mean differences and standard deviations were calculated using data from a reference study with presented mean and standard deviation for baseline and final measures as well as for changes from the baseline.

Data analysis

Statistical analyses were performed with Cochrane RevMan v. 5.2.11 (Cochrane, London, UK), and the results were expressed as weighted mean differences for continuous outcomes with 95% confidence intervals (CIs). Data was pooled by a random effects model. Because of low incidence of clinical cardiovascular events during follow-up and low heterogeneity, Peto OR was applied to obtain an overall estimate of the odds ratio for clinical outcomes, assuming fixed effects. Heterogeneity was analyzed with the I^2 statistic, and was defined as low (25–50%), intermediate (50–75%), or high (>75%). The p-values <0.05 (2-sided) were considered as statistically significant. Funnel plots were constructed to explore publication bias.

Quality assessment

The criteria established by Juni et al. were used to assess the quality of studies included, which were all randomized controlled clinical trials.[1]

Subgroup analysis for methodological exploration

To determine whether one particular procedure of cell preparation may impact the outcomes, subgroup analyses were conducted based on 1) the supplement of serum or plasma to the cell suspension or not; 2) the use of heparin in the cell suspension or not ('use' was defined as mixing BMCs with heparin during cell preparation); 3) cell washout or not after mixing with heparin during cell preparation.

Results

Search results

The initial search retrieved 1,126 reports with our search strategy. The majority of reports were excluded

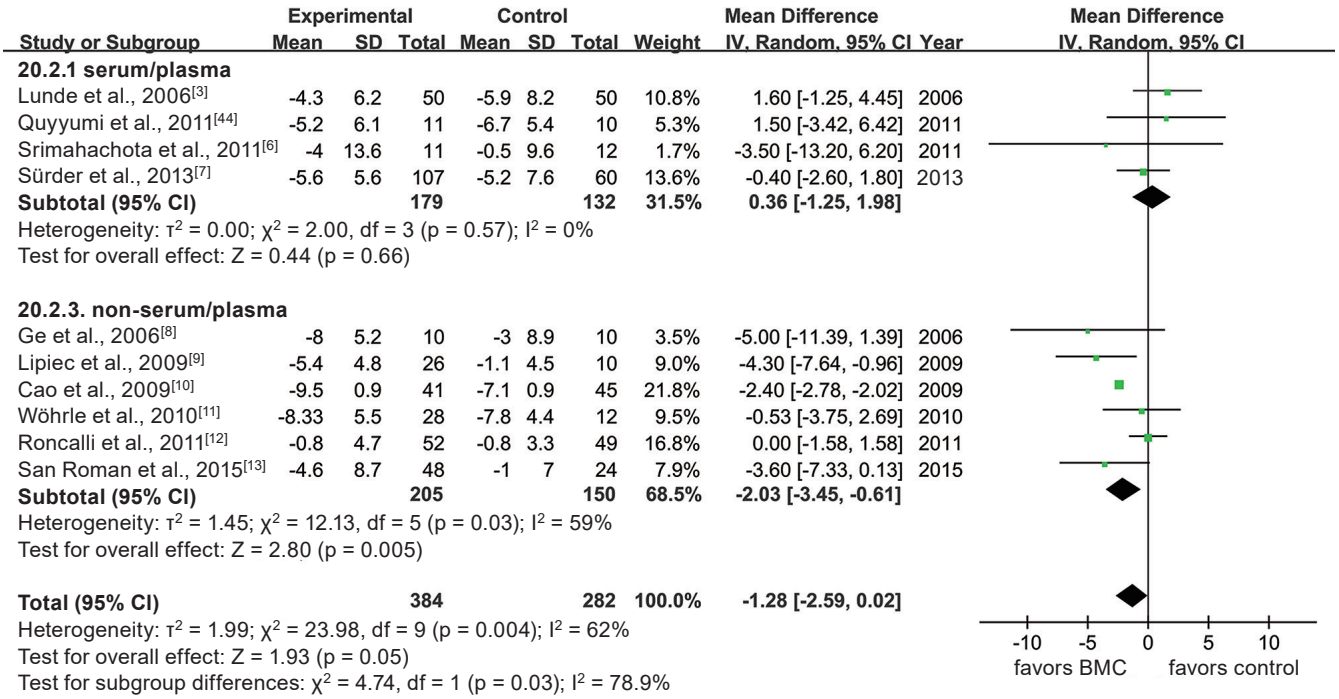


Fig. 1. Forest plot of the subgroup analysis of unadjusted difference in mean change in IS (with 95% CIs) among the trials using serum/plasma as a supplement for cell suspension or other supplements during BMCs preparation (the outcome was statistically in favor of non-use of serum/plasma for cell preparation)[3–13]

due to other types of study subjects (like chronic heart failure or granulocyte-colony stimulating factor treatment), wrong language, because they were reviews, or were regarded irrelevant on the basis of title and abstract. Full texts of 69 reports were thoroughly analyzed, and 36 reports were further excluded as being not randomized controlled trials, unmatched cell therapy approaches (like intramyocardial injection), a substudy, multiple publications, or without relevant outcomes. Two studies were excluded after full text analysis on account of an incomplete description of cell collection and preparation. One study was omitted because of a high percentage of loss to follow-up. Another study was excluded from this meta-analysis, because every patient in the cell therapy group received BMCs transplantation (or placebo) twice. The remaining 29 reports (24 clinical trials and 1,728 patients) were eventually included in this meta-analysis. All studies reported LVEF before and after cell transplantation in patients from both the cell therapy and control group, with or without other cardiac parameters and clinical outcomes.

Study characteristics

The median follow-up duration was 13 months (range: 3–61 months) and the median sample size was 72 patients (range: 10–204 patients). Only 7 (29.2%) studies included patients with acute anterior myocardial infarction, and the other studies were nonselective about the infarction segment. Based on the mean range of time from the onset of symptoms to PCI, the majority of patients underwent primary PCI in 24 h, while 3 studies did not give this data in their reports. Most patients cell therapy arm received cell transplantation within 7 days after AMI. Several imaging modalities were involved in these studies, including

Study or Subgroup	Experimental Events	Experimental Total	Control Events	Control Total	Weight	Peto Odds Ratio Peto, Fixed, 95% CI	Year
21.3.1 serum/plasma							
Schächinger et al., 2006[14]	2	101	0	103	6.8%	7.61 [0.47, 122.56]	2006
Janssens et al., 2006[15]	0	33	0	34		Not estimable	2006
Lunde et al., 2006[3]	1	50	1	50	6.7%	1.00 [0.06, 16.21]	2006
Huikuri et al., 2008[16]	0	40	1	40	3.4%	0.14 [0.00, 6.82]	2008
Grajek et al., 2010[17]	1	31	0	14	2.9%	4.27 [0.06, 294.50]	2010
Srimahachota et al., 2011[6]	0	11	0	12		Not estimable	2011
Quyyumi et al., 2011[5]	1	16	0	15	3.4%	6.94 [0.14, 350.54]	2011
Sürder et al., 2013[7]	6	133	0	67	17.7%	4.68 [0.84, 26.04]	2013
Subtotal (95% CI)		**415**		**335**	**41.0%**	**3.01 [0.97, 9.31]**	
Total events	11		2				
Heterogeneity: $\chi^2 = 3.89$, df = 5 (p = 0.57); $I^2 = 0\%$							
Test for overall effect: Z = 1.91 (p = 0.06)							
21.3.2. others							
Wollert et al., 2004[18]	2	30	2	30	12.9%	1.00 [0.13, 7.48]	2004
Go et al., 2006[8]	0	10	0	10		Not estimable	2006
Cao et al., 2009[10]	0	41	1	45	3.4%	0.15 [0.00, 7.49]	2009
Plewka et al., 2009[21]	2	40	2	20	11.5%	0.45 [0.05, 3.83]	2009
Nogueira et al., 2009[22]	0	14	0	6		Not estimable	2009
Silva et al., 2009[23]	0	14	0	6		Not estimable	2009
Wöhrle et al., 2010[11]	1	29	1	13	5.7%	0.40 [0.02, 8.34]	2010
Traverse et al., 2010[24]	0	30	0	10		Not estimable	2010
Colombo et al., 2011[25]	0	5	0	5		Not estimable	2011
Hirsch et al., 2011[26]	1	69	2	65	10.1%	0.48 [0.05, 4.68]	2011
Roncalli et al., 2011[12]	1	52	0	49	3.4%	6.97 [0.14, 352.12]	2011
Traverse et al., 2010[28]	0	58	2	29	6.0%	0.05 [0.00, 0.92]	2011
Traverse et al., 2011[29]	1	79	0	41	3.1%	4.57 [0.07, 284.74]	2012
San Roman et al., 2015[13]	0	48	1	24	3.0%	0.05 [0.00, 3.18]	2015
Subtotal (95% CI)		**519**		**353**	**59.0%**	**0.47 [0.18, 1.21]**	
Total events	8		11				
Heterogeneity: $\chi^2 = 7.27$, df = 8 (p = 0.51); $I^2 = 59\%$							
Test for overall effect: Z = 1.56 (p = 0.12)							
Total (95% CI)		**934**		**688**	**100.0%**	**1.01 [0.49, 2.08]**	
Total events	19		13				
Heterogeneity: $\chi^2 = 17.23$, df = 14 (p = 0.24); $I^2 = 19\%$							
Test for overall effect: Z = 0.03 (p = 0.98)							
Test for subgroup differences: $\chi^2 = 6.08$, df = 1 (p = 0.01); $I^2 = 83.5\%$							

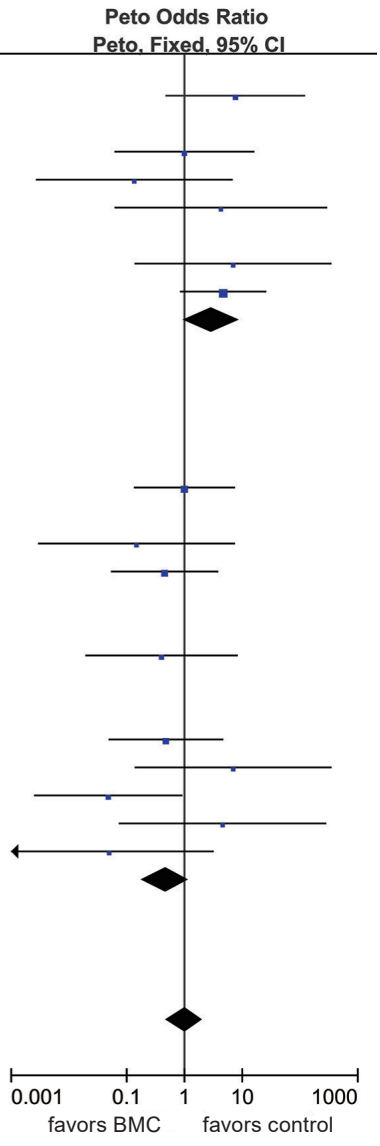


Fig. 2. Forest plot of the subgroup analysis of Peto OR in all-cause mortality among the trials using serum/plasma as a supplement for cell suspension or other supplements during BMCs preparation (the outcome was statistically in favor of non-use of serum/plasma for cell preparation)[14–30]

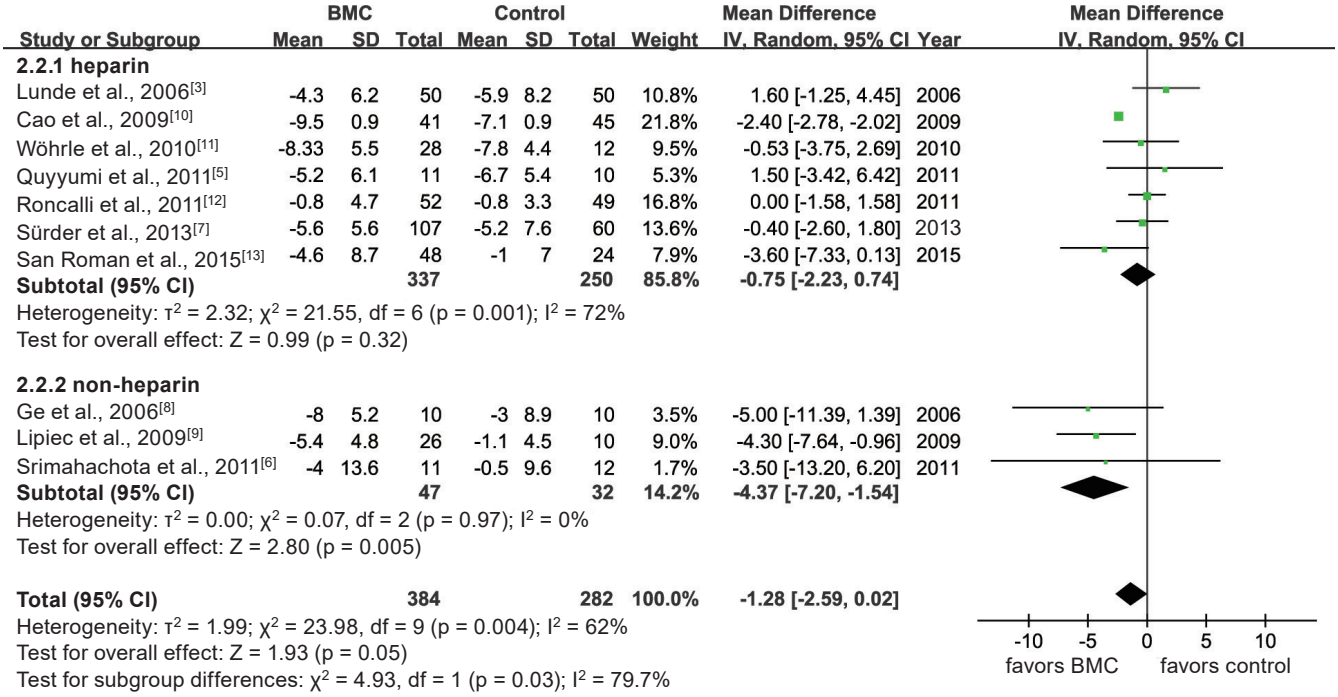


Fig. 3. Forest plot of the subgroup analysis of unadjusted difference in mean change in IS (with 95% CIs) among the trials using heparin or not during BMCs preparation (the outcome was statistically in favor of non-use of heparin for cell preparation)

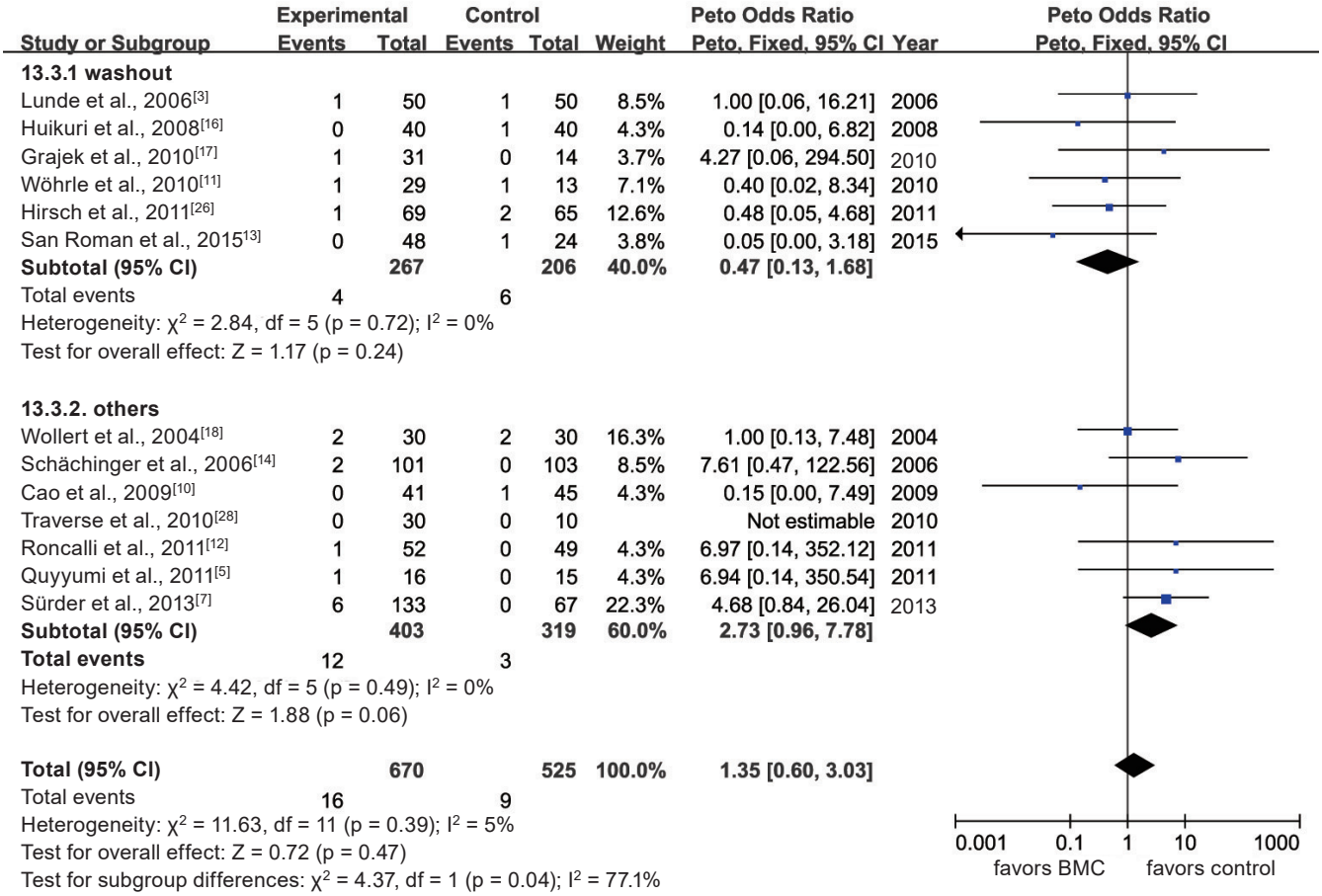


Fig. 4. Forest plot of the subgroup analysis of Peto OR in all-cause mortality among all the trials performing the cell washout step or not after heparin use during BMCs preparation (the outcome was statistically in favor of non-use of heparin for cell preparation)

transthoracic echocardiography, SPECT, cardiac MRI and left ventricular angiography. MRI was used for conducting measurements in 13 (54.2%) studies (Supplemental Table A).

Study quality

All studies were assessed using the Juni criteria (Supplemental Table B). All studies undertook adequate allocation and 79% of them provided detailed information about the method of randomization. Seven (29.2%) studies were double-blinded and 2 (8.3%) studies were single-blinded. Control patients did not undergo a sham biopsy and the infusion of cells in the other studies. Patient follow-up was completed in all studies. The percentage of patients lost to follow-up was acceptable.

Sensitivity analysis

The heterogeneity varied tremendously from low to high. The sensitivity analysis excluding 1 study at a time confirmed the results concerning all analyses in terms of direction and magnitude of statistical significance. The statistical heterogeneity(I^2) for LVEF vanished dramatically from 93% to 27%, indicating low heterogeneity among the majority of the studies included, except for the study by Wang et al.[2] Considering LVEF as the primary endpoint for almost all studies, this study was excluded in the following statistical analyses. After the exclusion, the heterogeneity for most cardiac parameters and all clinical outcomes was low.

Cardiac parameters and clinical outcomes

Only data with the longest duration of follow-up was referred to, although several follow-ups might have been conducted. Overall, BMCs transplantation to infarct-related region through the coronary artery was associated with statistically significant modification in all cardiac parameters (Table 1).

Twenty-three out of 24 studies conducted a follow-up and provided a clinical outcome in the reports. However, Peto OR results indicated no improvement in clinical outcomes in the cell therapy arm compared with the control arm (Table 2).

Subgroup analysis

Supplement of cell suspension

Many kinds of supplements were added to the final cell suspension such as serum, plasma, human serum albumin, phosphate-buffered saline, cell culture medium, etc. Whether serum or plasma was added was the only concern in this systematic review. Clinical trials which used serum/plasma in the BMC suspension were compared with those that did not (Table 1). No subgroup difference was found in the LVEF, LVESV and LVEDV (Supplemental Fig. 1–3). However, statistic results indicated that cell suspension without serum/plasma tended to diminish the IS (-2.03%, p = 0.005), while the subgroup with the serum/plasma addition showed no impact on IS change (0.36%; p = 0.66). The difference between the 2 subgroups was statistically significant (p = 0.03) (Fig. 1).

Although there were no significant differences in clinical outcomes, when all clinical trials included were considered, the analyses of subgroup differences showed that serum/plasma addition might increase all-cause mortality (Peto OR: 3.01; p = 0.06), which was significantly higher compared to non-serum/plasma subgroup (Peto OR: 0.47; p = 0.01) (Fig. 2). No other subgroup differences were found to be significant in the other clinical outcomes (Table 2).

Anticoagulant usage

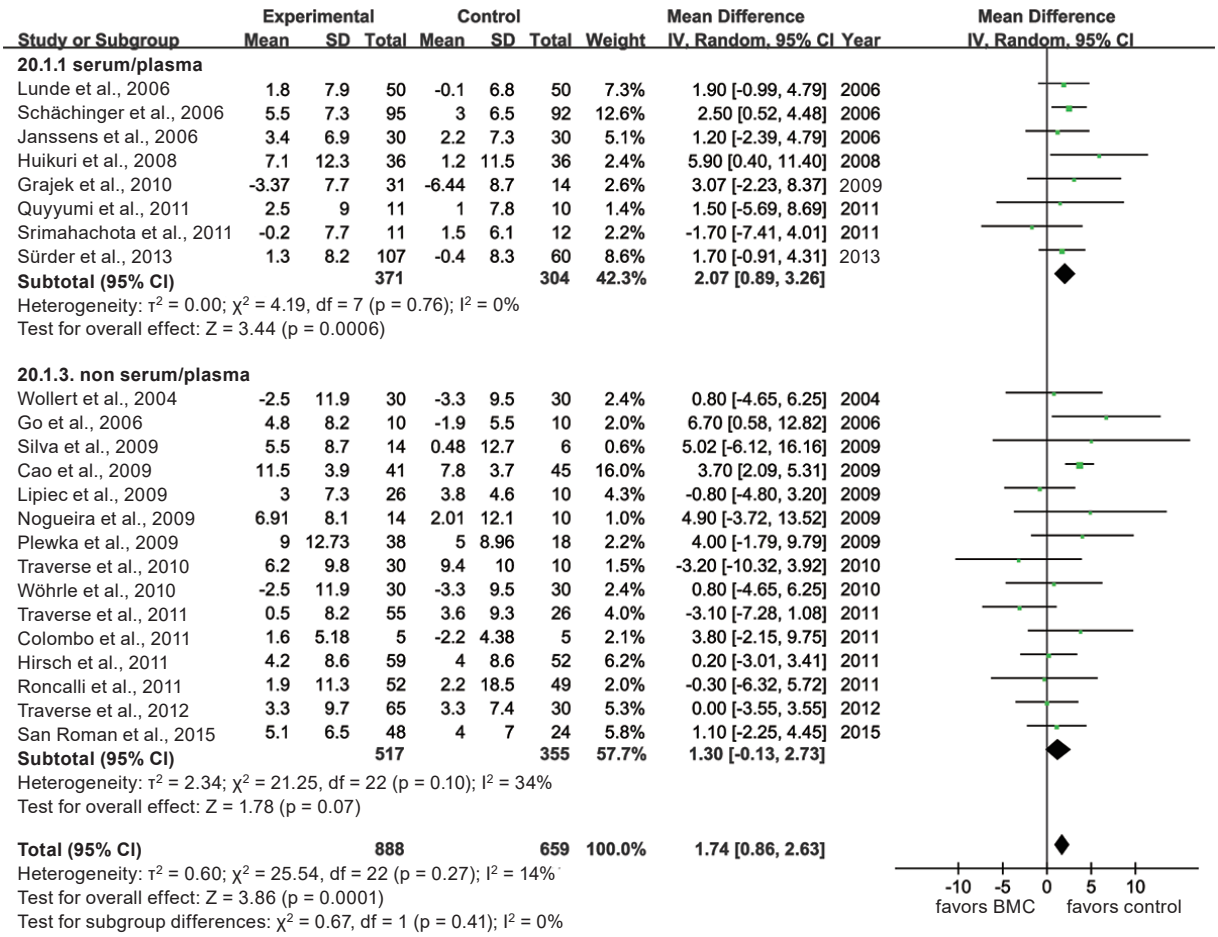
Thirteen out of 23 studies used heparin during cell preparation to prevent coagulation. The remaining studies used no anticoagulant during the procedure. Anticoagulant other than heparin was not found to be used in any study. When use of heparin and non-use of heparin was compared between the subgroups, IS change in the non-heparin subgroup was much greater than in the heparin subgroup (non-heparin vs heparin: -4.37% vs -0.75%; p = 0.03) (Fig. 3). No significant subgroup difference was found in LVEF, LVESV and LVEDV (Supplemental Fig. 4–6). Clinical outcomes were also compared between the 2 subgroups and no subgroup difference was found, either.

Cell washout

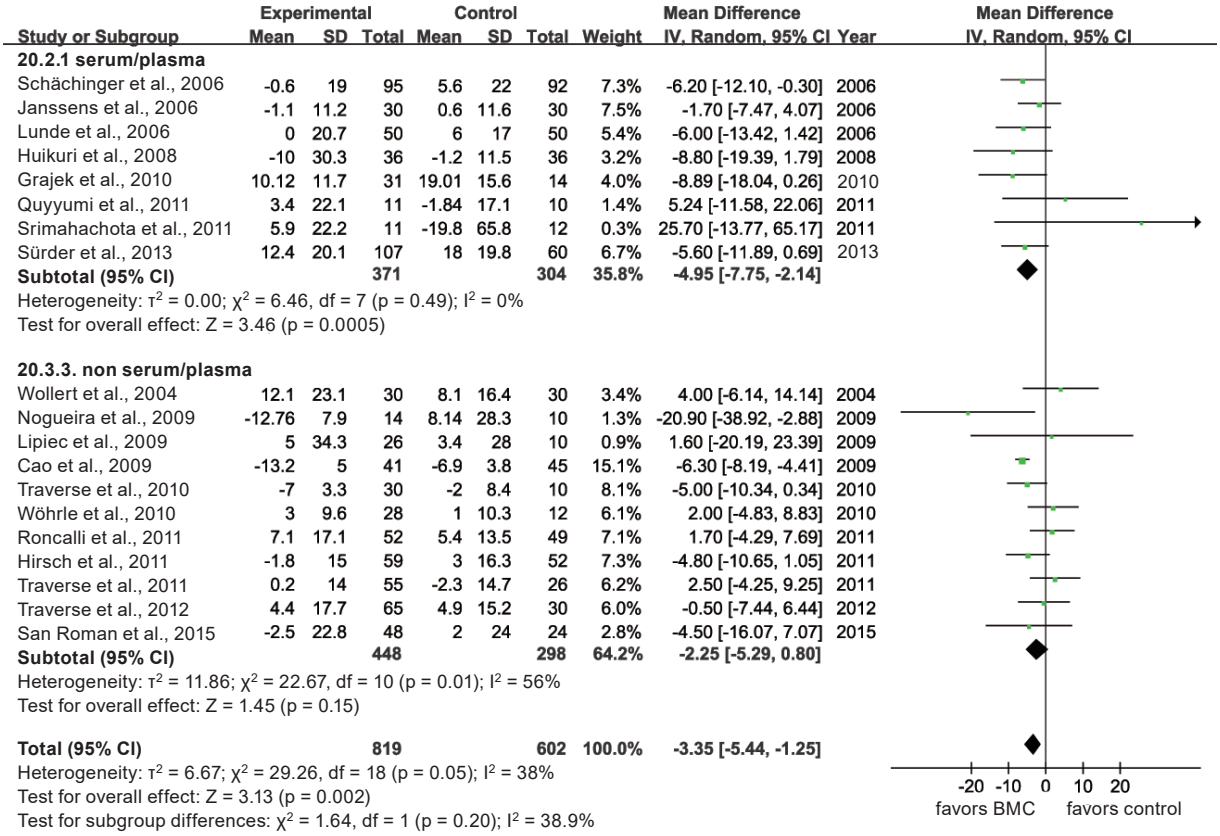
Thirteen studies added heparin into BMCs for coagulation, and in 6 of them, BMCs were further washed out before the final cell suspension for infection was obtained. In the case of studies with heparin usage, all cardiac parameters were compared between the studies with the cell washout application and those without. No subgroup difference was found in the cardiac parameters (Supplemental Fig. 7–10). With regard to clinical outcomes, all-cause mortality was increased in the non-washout subgroup (Peto OR: 2.73; p = 0.06) and the intra-subgroup differences were significant (Peto OR: 2.73 vs 0.47; p = 0.04) (Fig. 4).

Discussion

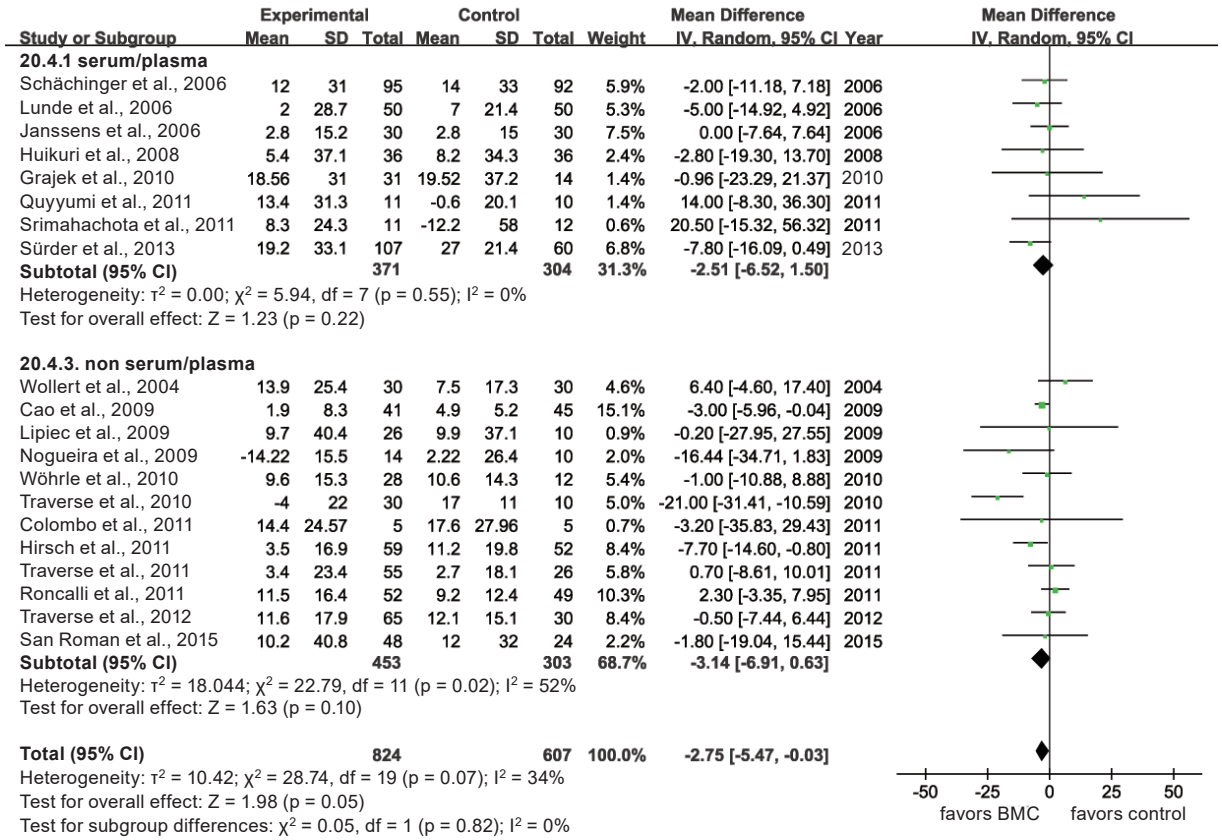
As far as we know, this is the first systematic review to focus on the impact of cell preparation on the efficacy and safety of intracoronary autologous BMCs transfer in patients with AMI. Firstly, this meta-analysis confirmed that, as compared with standard medical treatment, intracoronary BMCs therapy after AMI was associated with a significant increase in LVEF as well



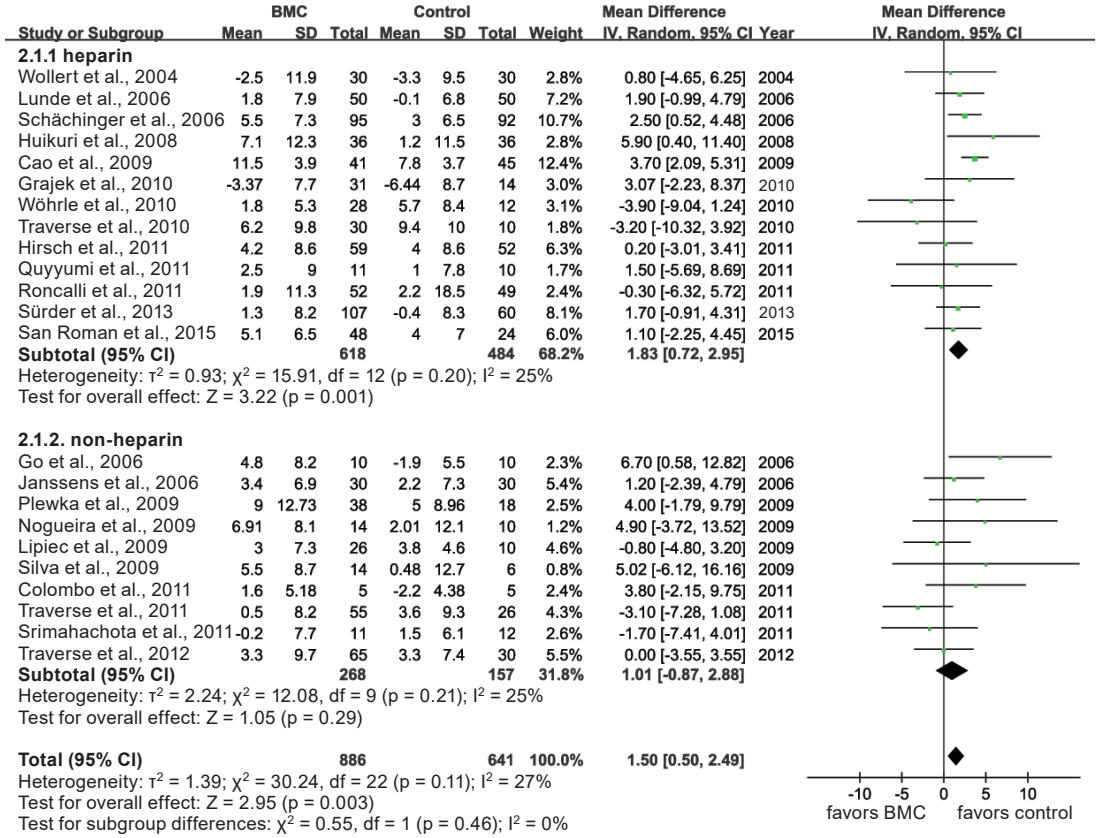
Supplemental Fig. 1.



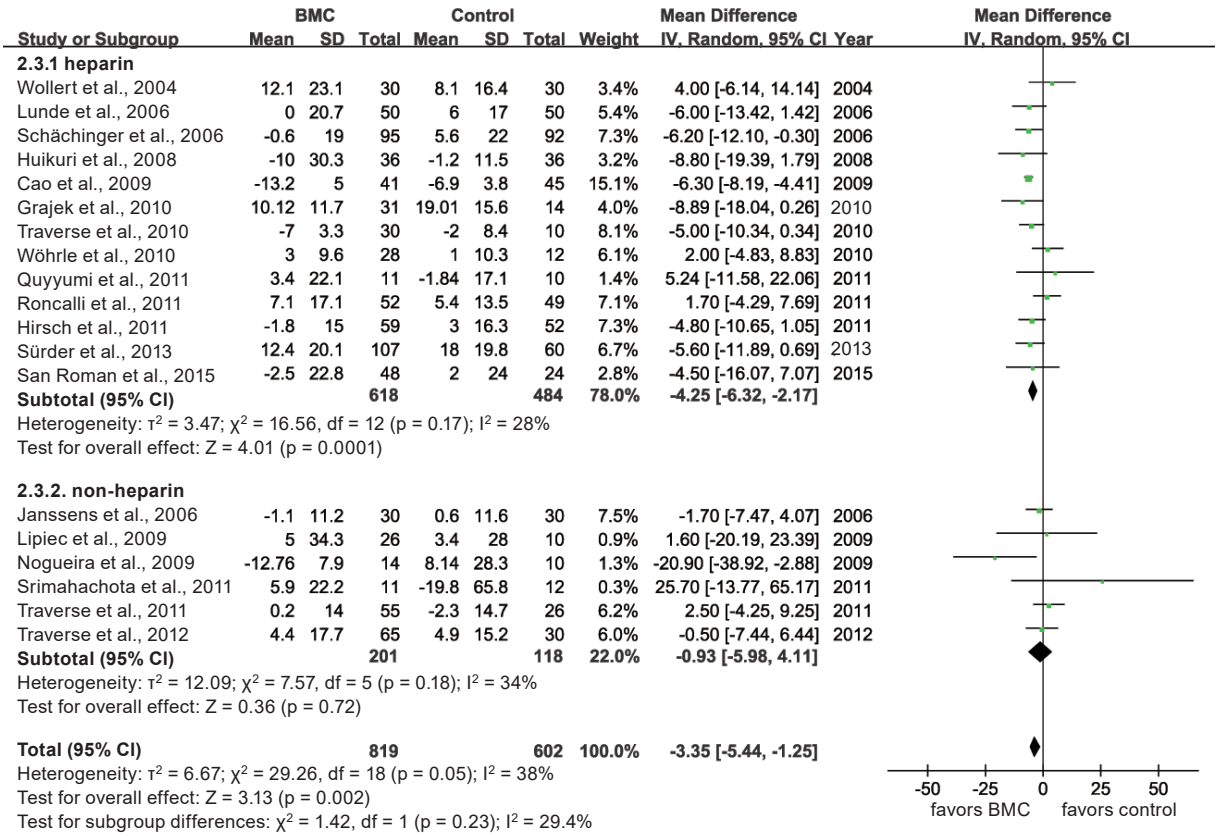
Supplemental Fig. 2.



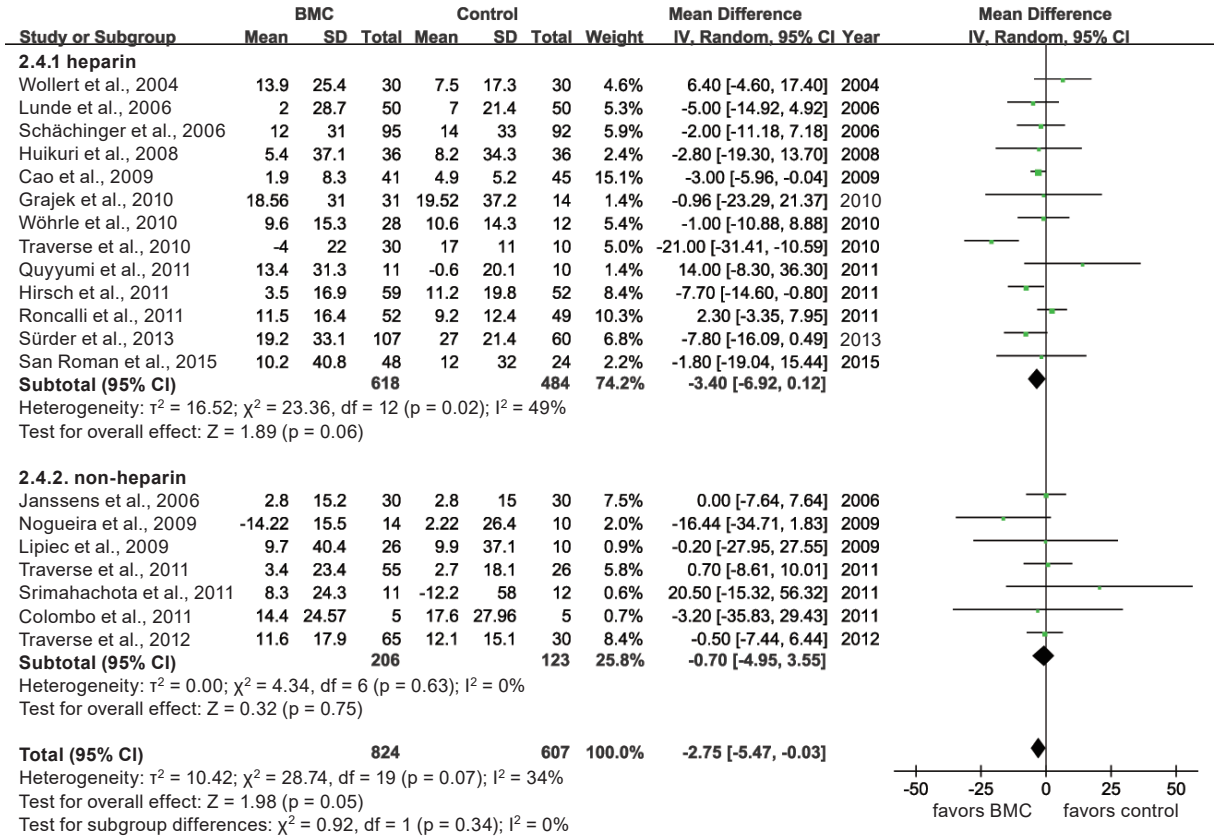
Supplemental Fig. 3.



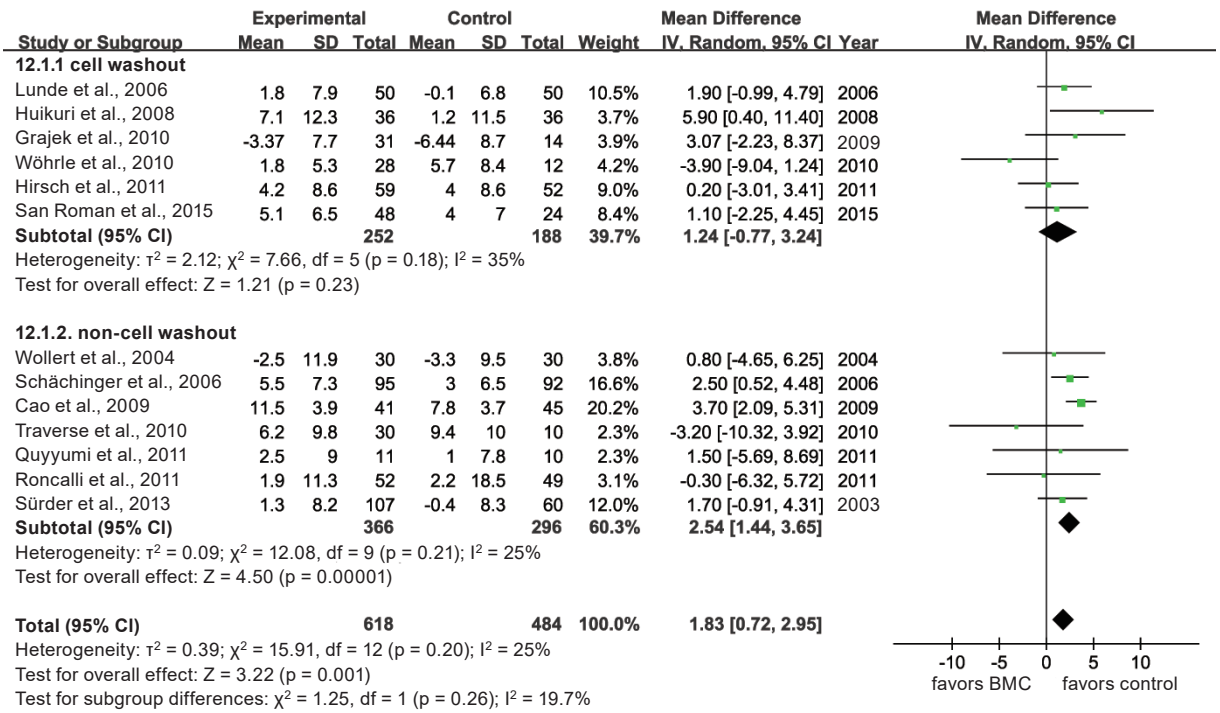
Supplemental Fig. 4.



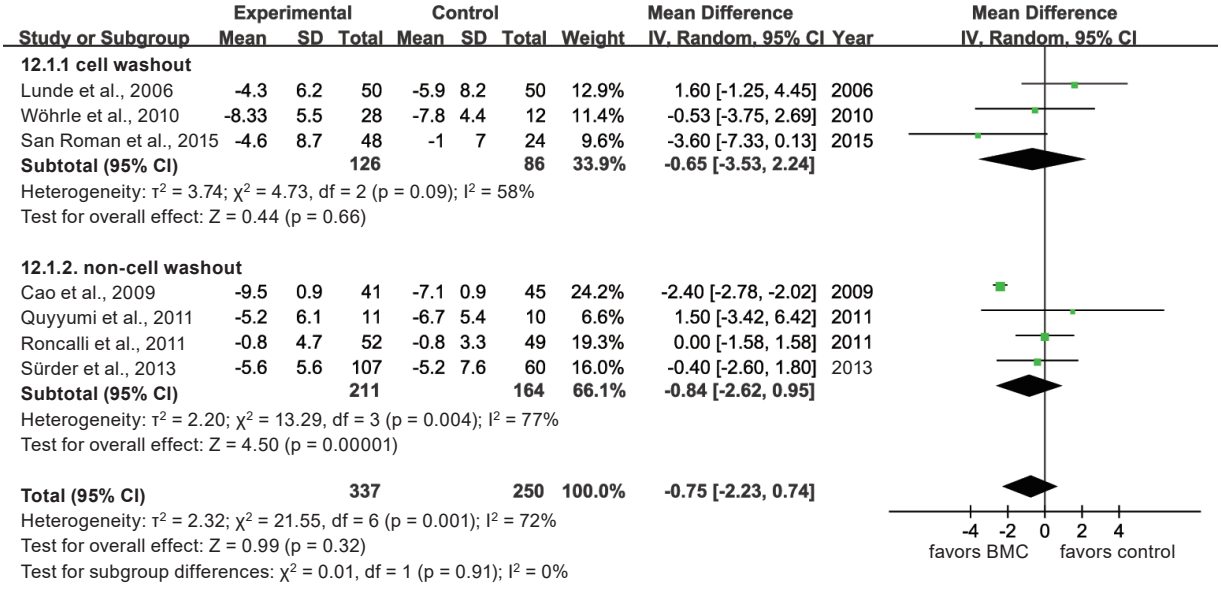
Supplemental Fig. 5.



Supplemental Fig. 6.



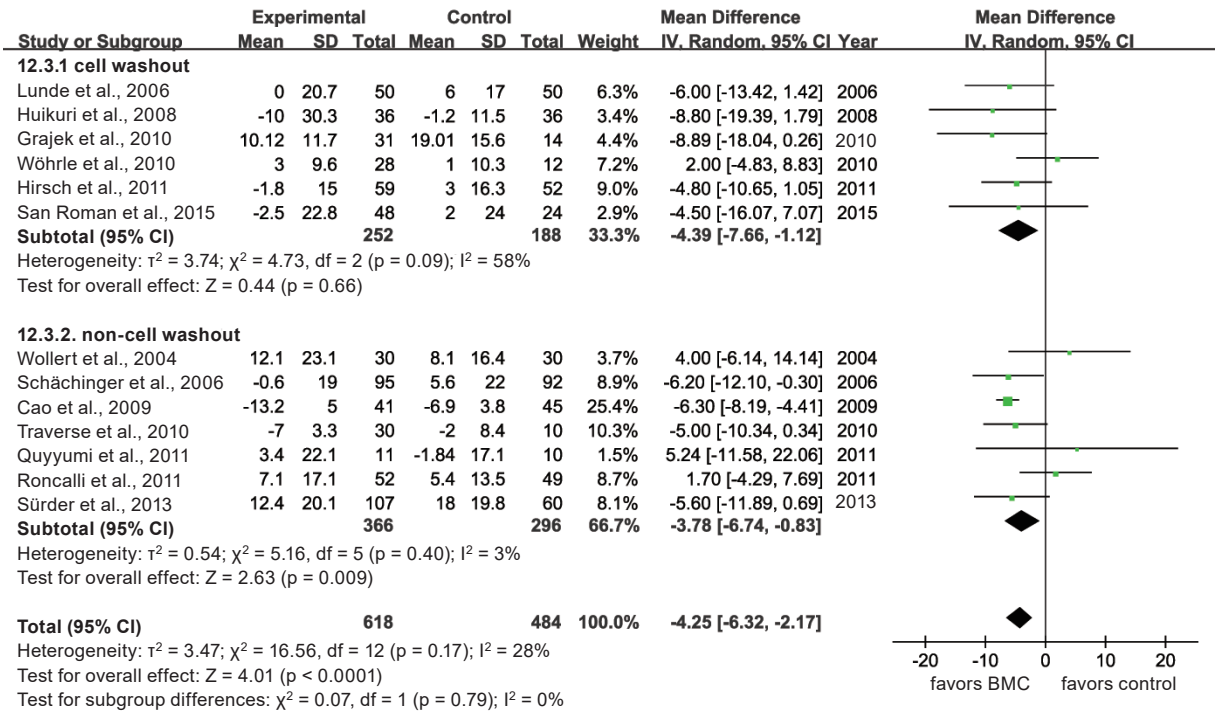
Supplemental Fig. 7.



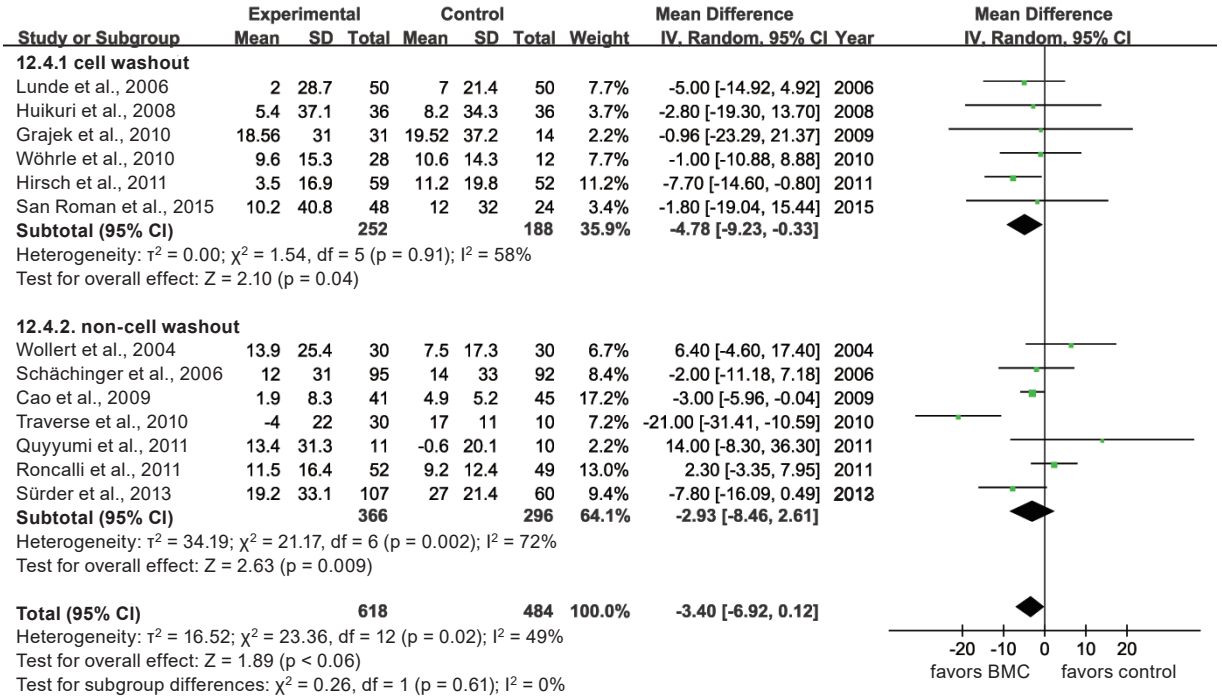
Supplemental Fig. 8.

as a significant decrease in IS, LVESV and LVEDV, which indicated an improvement in left ventricular function and modification in remodeling. Meanwhile, subgroup analysis was performed to determine the effect of serum/plasma in intracoronary BMCs therapy. Unfortunately, less benefits in IS and higher all-cause mortality was observed in patients treated with serum/plasma suspended BMCs. Moreover, further subgroup analysis suggested that a more significant decrease of IS was observed in patients treated with non-heparinized intracoronary-admitted BMCs. In the studies using heparin for anticoagulation, all-cause mortality was reported to rise dramatically without the cell washout procedure, although cell washout could not impact the cardiac parameters.

To date, most of the available clinical trials of intracoronary BMCs transplantation result in significant yet modest improvements in LVEF, IS, LVESV and LVEDV, which was confirmed by the present study and other meta-analyses.[7,31] Further studies indicated that the modest improvement of cardiac function after BMCs transplantation involves low cell survival and impaired cell



Supplemental Fig. 9.



Supplemental Fig. 10.

function.[32] Providing near-physiological conditions, and autologous plasma or serum containing growth factors and cytokines gives better results in terms of cell survival and preservation of cellular activities, and thereby it was applied in some clinical trials.[14,33,34] Therefore, we performed a subgroup analysis based on the cell suspension solution; however, the results showed a greater decrease in IS and a lower risk of all-cause mortality with the non-use of plasma or serum in the final BMC suspension. A recent study indicated that residual clotting activity due to insufficient coagulation during the preparation of autologous serum induced spontaneous clotting of the final cell product, and thus could cause microcirculatory obstructions and subsequent tissue damage

with intracoronary application.[35] Notably, delayed clotting of the cell product was observed only in autologous serum from patients with anti-thrombotic therapy, but not from healthy donors or from patients without anti-thrombotic therapy.[35] In most clinical trials, the patients who received cell therapy were patients with AMI, routinely provided with anti-thrombotic therapy according to the current guidelines. In the presence of anti-thrombotic drugs, autologous serum was not sufficiently coagulated and induced spontaneous clotting of the final cell product. Therefore, when autologous serum is considered for cell suspension solution, especially for patients with anti-thrombotic therapy, serum preparation should be optimized in order to avoid subsequent clotting of the cell product. Utmost care has to be taken if autologous components are used.

Given that the intracoronary application of stem cells reduced the blood flow or even occluded arteries, probably by causing cell clotting, in some trials, anticoagulant agents like heparin were used during cell preparation with the intention to improve the therapeutic benefits of cell therapy in patients with AMI.[15,26,35] However, recently it has been indicated that the addition of heparin to stem cell suspension solution might interfere with the SDF-1/CXCR4 axis, thereby resulting in impaired migration and homing of BMCs. Hence, heparin was not recommended by the authors as an ideal anticoagulant.[36] There are earlier meta-analyses investigating the effect of heparin during cell preparation on LVEF and LVESV, but they arrived at conflict results. Jeevanantham et al. found that heparin in the final BMC suspension brought a greater improvement in LVEF and LVESV.[37] Contrarily, de Jong et al. suggested that the use of heparin during cell preparation did not seem to affect the therapy outcome.[31] The inconsistent results might be explained by the authors' different inclusion criteria regarding the cell type, the spectrum of disease and the route of injection. In this study, we focused on the therapeutic effect of BMCs in patients with AMI through an intracoronary infusion of BMCs. A subgroup analysis suggested that a more significant decrease of IS was observed in patients treated with non-heparinized intracoronary-admitted BMCs. In the studies using heparin for anticoagulation, cell washout significantly lowered all-cause mortality, although this procedure could not impact the cardiac parameters. Since cell washout attenuates heparin and therefore diminishes its effect, it is reasonable to imply that heparin use for cell preparation might be useless or even harmful for the implantation of BMC in AMI patients, probably due to its interference with the SDF-1/CXCR4 axis.[36] Therefore, if anticoagulant agents are required to reduce potential cell clotting, we could deduce that the application of a new anticoagulant, like Bivalirudin, which does not interfere with the SDF-1/CXCR4 axis, should be of greater benefit for AMI patients undergoing cell therapy.

Conclusions and future perspective

Methodological differences in adult BMCs transplantation therapy have an impact on the cardiac parameters of patients suffering from an acute myocardial infarction. Non-use of serum or plasma in the cell suspension is associated with better performance in reducing the infarct area and lowering the risk of all-cause mortality. Meanwhile, heparin usage could diminish the benefit of infarct size reduction from BMCs therapy in AMI patients. In studies using heparin for anticoagulation, all-cause mortality rose dramatically without the cell washout procedure, although cell washout could not impact the cardiac parameters.

Further studies are needed to define the optimal components of cell suspension solution required for the maintenance of cell survival and activity. Instead of autologous medium supplements, the use of a combination of cytokines such as VEGF, IGF-1, HGF, SDF-1, PDGF and GCSF, which are known to improve cell activities, may be a preferred option. However, since our understanding and elaboration of the large amount of bioactive components within the serum are far from sufficient, further studies appear to be warranted to determine the optimal cocktail of factors that are required to maintain cell survival and bioactivities. Meanwhile, if new anticoagulant agents, such as Bivalirudin, Rivaroxiban, Apixaban, are required to reduce potential cell clotting, further systematic studies are needed to evaluate their effect on cell functions and long-term outcomes.

Supplemental Table A. Summary of all studies included

Source	Sample size n	Mean follow-up duration (months)	Baseline LVEF	Location of MI	Cell type	Cells transplanted n (*10^6)	Imaging modalities	Time from BMC aspiration to injection	Time from PCI/MI to transplantation	Heparin usage	Cell washout after heparin addition	Supplement
Wollert et al., 2004 [18]	60	61	nr	multiple	BMMNC	2460 ±940	MRI	6–8 h	4.8 ±1.3 days	yes	no	saline
Schächinger et al., 2006 [14]	204	4	nr	multiple	BMMNC	236 ±174	LVG	the same or next day	4.3 ±1.3 days	yes	no	X-VIVO™ 10 Medium, autologous serum
Lunde et al., 2006 [3]	100	36	nr	anterior	BMMNC	68 (median)	MRI (IS); Echo (EF, vol., WMSI)	the same or next day	6 days (median)	yes	yes	saline, 20% autologous plasma
Janssens et al., 2006 [15]	67	4	nr	multiple	BMMNC	172 ±72	MRI	4–6 h	1–2 days	no	no	saline, 5% autologous serum
Ge et al., 2006 [8]	20	6	nr	multiple	BMMNC	36.3 ±20.25	Echo (LVEF, vol.); SPECT (IS)	immediately after PCI	<3 hs	no	no	saline
Huikuri et al., 2008 [16]	80	6	nr	multiple	BMMNC	402 ±196	Echo (LVEF, vol.); LVA (LVEF, vol.)	within 3 h	the same day	yes	yes	saline, autologous serum
Lipiec et al., 2009 [9]	36	6	≤40%	anterior	CD34+, CD133+	CD34+ 3.36 ±1.87; CD133+ 0.33 ±0.17	SPECT	2–3 h	3–10 days	no	no	saline
Grajek et al., 2010 [17]	45	12	nr	anterior	BMMNC	2340 ±1200	Echo	the next day	5–6 days	yes	yes	X-VIVO™ 15 Medium, 2% heat-inactivated autologous plasma
Cao et al., 2009 [10]	86	48	nr	anterior	BMMNC	500 ±120	Echo (LVEF, vol., WMSI); SPECT (IS)	nr	7 days	yes	no	saline
Silva et al., 2009 [23]	20	6	nr	multiple	BMMNC	100.0	SPECT	8.5 ±1.44 h	5.5 ±1.3 days	no	no	saline, 5% human serum albumin
Plewka et al., 2009 [21]	60	6	<40%	anterior	BMMNC	144 ± 49	Echo	within 2 h	7 ±2 days	no	no	saline
Nogueira et al., 2009 [22]	20	6	nr	multiple	BMMNC	100.0	Echo	8.5 ±1.44 h	5.5 ±1.28 days	no	no	saline, 5% human serum albumin

Supplemental Table A. Summary of all studies included (cont.)

Source	Sample size n	Mean follow-up duration (months)	Baseline LVEF	Location of MI	Cell type	Cells transplanted n (*10^6)	Imaging modalities	Time from BMC aspiration to injection	Time from PCI/MI to transplantation	Heparin usage	Cell washout after heparin addition	Supplement
Wöhrle et al., 2010 [11]	42	6	nr	multiple	BMMNC	381 ±130	MRI	6.1 h (median)	6.3 ±0.8 days	yes	yes	saline, 6% human serum albumin
Traverse et al., 2010 [24]	40	6	<50%	anterior	BMMNC	100.0	MRI	within 8 h	3–10 days	yes	no	5% human serum albumin
Colombo et al., 2011 [25]	10	12	≤45%	anterior	CD133+	5.9 (median)	Echo (LVEF, vol., WMSI); PET (IS)	nr	10–14 days	no	no	saline, 10% human serum albumin
Hirsch, et al., 2011 [26]	134	4	nr	multiple	BMMNC	296 ±164	MRI	the same day	2.4–5.1 hours	yes	yes	saline, 4% human serum albumin
Quyyumi et al., 2011 [5]	31	6	≤50%	multiple	CD34+	5, 10, 15	MRI	24–48 h	8.3days	yes	no	PBS, 40% autologous human serum
Roncalli, et al., 2011 [12]	101	3	≤45%	multiple	BMMNC	98.3 ±8.7	MRI	5 h 28 min ±1h 30min	9.3±1.7days	yes	no	4% human albumin
Srimahachota et al., 2011 [6]	23	6	<50%	multiple	BMMNC	420.0 ±221.0 × 10	MRI	the same day	57.2±122.8 days	no	no	saline, 2% autologous serum
Traverse et al., 2011 [28]	87	6	≤45%	multiple	BMMNC	147 ±17	MRI	within 12 h	14–21days (median:17.4 days)	no	no	saline, 5% human serum albumin
Sürder et al., 2013 [7]	194	4	<45%	multiple	BMMNC	150.49 ±32.34 (median)	MRI	nr	5-7days or 3-4weeks	yes	no	X-VIVO™ 10 Medium, autologous serum
Traverse et al., 2012 [29]	120	12	≤45%	multiple	BMMNC	146.4 ±18.2	MRI	8.3 h	3.3 or 7.5 days	no	no	saline, 6% human serum albumin
Wang et al., 2014 [2]	58	6	nr	multiple	BMSC	200	LVG	7 days	15 days	yes	yes	LGDMEM + 20% (v/v) FBS
San Roman et al, 2015 [13]	90	12	nr	multiple	BMMNC	83 or 560	MRI	21(16–24) h	190 hours	yes	yes	PBS

MI – myocardial infarction; PCI – percutaneous coronary intervention; BMMNC – bone marrow mononuclear cells; BMSC – bone marrow mesenchymal stem cells; Echo – echocardiography; LVG – left ventriculography; vol. – volume; PBS – phosphate-buffered saline; FBS – fetal bovine serum; nr – not reported.

Supplemental Table B. Quality assesment of the studies included in the meta-analysis according to Juni criteria

Source	Was allocation adequate?	Was an adequate method of randomization described?	Were the groups similar at the start of the study?	Were patients/ caregivers blinded for intervention?	Was outcome ascertained blinded?	What percentage was lost in the follow up? (%)	Were all patients analyzed in the group to which they were assigned?
Wollert et al., 2004 [18]	Y	Y	Y	Y	Y	0	Y
Schächinger et al., 2006 [14]	Y	Y	Y	Y	Y	0	Y
Lunde et al., 2006 [3]	Y	Y	Y	N	Y	3	Y
Janssens et al., 2006 [15]	Y	Y	Y	Y	Y	10.4	Y
Ge et al., 2006 [8]	Y	Y	Y	Y	Y	0	Y
Huikuri et al., 2008 [16]	Y	Y	Y	Y	Y	3.7	Y
Lipiec et al., 2009 [9]	Y	Y	Y	N	Y	5.6	N
Grajek et al., 2009 [17]	Y	Y	Y	N	Y	0	N
Cao et al., 2009 [10]	Y	Y	Y	nr	Y	0	Y
Silva et al., 2009 [23]	Y	Y	Y	N	Y	0	Y
Plewka et al., 2009 [21]	Y	N	Y	N	Y	0	Y
Nogueira et al., 2009 [22]	Y	Y	Y	N	Y	0	Y
Wöhrle et al., 2010 [11]	Y	Y	Y	Y	Y	0	Y
Traverse et al., 2010 [24]	Y	Y	Y	Y	Y	0	Y
Colombo et al., 2011 [25]	Y	Y	Y	N	Y	0	Y
Hirsch et al., 2011 [26]	Y	Y	Y	N	Y	0	Y
Quyyumi et al., 2011 [5]	Y	N	Y	N	Y	0	N
Roncalli et al., 2011 [12]	Y	Y	Y	N	Y	4	Y
Srimahachota et al., 2011 [6]	Y	N	Y	N	Y	0	Y
Traverse et al., 2011 [28]	Y	N	Y	N	Y	1.1	Y
Sürder et al., 2012 [7]	Y	Y	Y	N	Y	0	Y
Traverse et al., 2012 [29]	Y	N	Y	Y	Y	10	Y
Wang et al., 2014 [2]	Y	Y	Y	Y	nr	0	Y
San Roman et al., 2015 [13]	Y	Y	Y	Y	Y	0	N

Y – yes; N – no; nr –not reported.

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Computerized planimetry evaluation of hyperbaric oxygen therapy in the treatment of diabetic foot

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

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Abstract

Background. Diabetic foot ulcer is one of the major complications of diabetes mellitus in adults.

Objectives. The aim of the study was to conduct a planimetry evaluation of the effectiveness of hyperbaric oxygen therapy (HBOT) in the treatment of patients with vascular disorders caused by diabetic foot.

Material and methods. The study included 94 patients, 30 females (32%) and 64 males (68%), aged 33–76 years, with diabetes lasting 1.5–32 years, who underwent HBOT due to diabetic foot. All patients from that group underwent vascular procedures prior to HBOT. In qualifying patients for hyperbaric oxygen therapy, transcutaneous oximetry method was applied (30–60 exposures in hyperbaric oxygen at pressure of 2.5 ATA). Progress in wound healing was evaluated by computerized planimetry system IRIS 4.

Results. In 26 patients the wounds were completely closed and in 37 patients the topical state was significantly improved – the wound surface decreased by 34% in average. During the treatment, in 11 patients amputation of fingers and metatarsal necrotic bones was performed, while in 9 patients amputation was prevented.

Conclusions. A planimetry evaluation showed that the application of HBOT in the treatment of diabetic foot enhances foot ulcer healing, reduces tissue damage, contributes to the reduction of complications related to soft tissue and bone infections.

Key words: diabetic foot, hyperbaric oxygen therapy, computerized planimetry

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Introduction

According to estimated values, diabetes mellitus affects 5% of the world's population, and the number is systematically growing. The estimated number of diabetic patients in Poland ranges between 1.5 and 2 million.[1] One of the most serious complications of diabetes is diabetic foot. Diabetic foot is a complex of acute and persistent symptoms occurring in patients suffering from long-lasting not compensated diabetes mellitus, related to damages in foot vascular and nervous system, as well as a tendency to delayed wound healing, infection or gangrene of the foot. Two main risk factors that cause diabetic foot ulcer are diabetic neuropathy and micro as well as macroangiopathy.[2]

Diabetic foot ulcer is one of the major complications of diabetes mellitus. It occurs in 15% of all patients with diabetes and precedes 84% of all lower leg amputations.[3] The probability of lower extremity amputation in diabetic patients is approximately 25 times higher than in patients with a vascular risk. Every 5 out of 6 amputations due to the lower extremity vascular dysfunction is performed in diabetic patients.[4]

Complications connected with diabetic foot, such as difficulties in healing wounds, or non-healing ulcers, wet and dry gangrene of soft tissue as well as foot osteitis, could lead to the necessity of amputating fingers, metatarsal necrotic bones or even the whole foot.[4,5] Nowadays, the combined treatment of diabetic foot is recommended including conservative treatment, revascularization of lesion area (stents, bypasses), surgical treatment including wound debridement (necrotic tissue demarcation, soft tissue incision, purulence cistern drainage, often resections within foot skeletal system, and amputations as well), empiric antibiotic therapy, and subsequently targeted therapy and hyperbaric oxygen therapy (HBOT). Hyperbaric oxygen therapy was defined as a medication by Gottlieb in 1977.[6] Diabetic foot syndrome is accepted as an indication for therapy in hyperbaric chamber by European Committee of Hyperbaric Medicine (ECHM) and Undersea and Hyperbaric Medical Society (UHMS) as well.[7,8]

Material and methods

The study comprised a group of 94 patients with vascular disorders caused by the diabetic foot ulcers, among them 30 female (32%) and 64 (68%) male, in the age between 33 and 76 years (mean age 42 years) who underwent HBOT at the Oxygen Therapy Ward of the Center for Burns Treatment in Siemianowice Śląskie between June 2012 and December 2015. Diabetes mellitus history of patients reported in medical records was between 1.5 and 32 years before starting hyperbaric oxygen therapy.

Depending on the pathogenic background of diabetic foot syndrome, the patients were divided into 2 groups: ischemic diabetic foot syndrome (50 patients) and neurogenic diabetic foot syndrome (44 patients), without any significant differences between them regarding sex and age of the patients, as well as the time of diabetes mellitus history.

In 9 patients with ischemic diabetic foot syndrome (9.6% of the whole group), blood flow disturbances of significant grade in lower extremity arteries with the symptoms of peripheral ischemia, diagnosed by Doppler ultrasonography and angio-CT, were observed. They were caused by advanced atherosclerosis. Among those patients, 5 (5.3% of the whole group) were subjected to vascular procedures (bypass – 2 and stent – 3) prior to HBO therapy.

Oxygen hyperbaric therapy procedure

After qualification basing on transcutaneous oxymetry examination, HBOT was carried out in a multiplace hyperbaric chamber once a day, 5 times a week. The treatment protocol consisted of 60 min lasting periods of inhalation of 100% oxygen at a pressure of 2.5 ATA, interspersed with 2 "air breaks" lasting 5 min, after each 30 min of inhalation. A cycle of HBOT consisted of 30 to 60 procedures, depending on the healing results.

Evaluation of healing process

The course of healing was evaluated by computerized planimetry (IRIS-4 system, manufactured by Medi.Com, Wrocław, Poland). This method is based on the wound's digital picture evaluation with a determination of the parameters listed below: wound perimeter (O), wound surface (S), distance between maximally outlaying points on wound edge (d), wound circularity (near-circle level of wound shape with values 0–1, where 1 means circle) (C). Pictures of the wound were taken every 5 to 7 days of therapy. From 3 to 7 measurements (mean 4) were performed for 1 patient.

Statistical analysis

Statistical analysis has been performed with the use of STATISTICA v. 6.0 software (StatSoft Inc., Tulsa, USA). A χ^2 test has been used to determine the significance of differences of non-parametric variables in both groups, while for the analysis of differences between the values of ulcer surface before and after treatment in each group, as well as between both groups, an analysis of variance with the subsequent Mann Whitney U test has been carried out. In all analyses, the significance level was set at $p < 0.05$.

Results

The results of HBOT of skin ulcer in patients from both groups are presented in Table 1. As a result of the treatment, ulcer healing was completed without any amputations in 26 (27.7%) patients (14 patients with ischemic foot

syndrome and 12 with neurogenic foot syndrome). Topical status of wound was significantly improved (wound was cleaned out from necrotic tissue and decontaminated) in 37 (39.4%) patients (20 patients with ischemic foot syndrome and 17 with neurogenic foot syndrome) who completed the whole cycle of the HBOT. In the group of patients with topical status improvement, a planimetric examination demonstrated a decrease of wound surface by 34% on average, compared with the results of initial planimetric examination before the beginning of HBOT. A total of 10 patients (10.6%) (5 patients with ischemic foot syndrome and 5 with neurogenic foot syndrome) discontinued HBOT after 1 to 19 exposures due to the deterioration of health status (laryngological complications – 6 patients, exacerbation of chronic circulatory insufficiency symptoms – 3 patients, claustrophobia – 1 patient), while 5 (5.3%) patients (2 patients with ischemic foot syndrome and 3 with neurogenic foot syndrome) discontinued therapy arbitrarily, without giving any reason. In those patients, a planimetric examination showed an improvement of topical status, resulting in a decrease of ulcer surface by 5% to 32% (mean 17.7%). In 14 (14.9%) patients (8 patients with ischemic foot syndrome and 6 with neurogenic foot syndrome) no topical improvement of ulcer surface was observed. In 2 (2.1%) patients (1 patient with ischemic foot syndrome and 1 with neurogenic foot syndrome) the topical status of the wound became exacerbated – the ulcer surface increased by 9% on average, compared with the results of planimetric examination before the beginning of HBOT. The statistical analysis performed with use of χ^2 test confirmed that the results of treatment in both groups did not differ significantly ($\chi^2 = 0.197$, $\chi^2_\alpha = 7.815$; $p > 0.05$).

In 11 (11.6%) patients (7 patients with ischemic foot syndrome and 4 with neurogenic foot syndrome) due to the lack of healing during HBOT cycle, amputations were performed of fingers and metatarsal necrotic bones, while in 9 (9.6%) patients (6 patients with ischemic foot syndrome and 3 with neurogenic foot syndrome) amputations which were initially planned at the beginning of the treatment were prevented by hyperbaric oxygen therapy.

The final comparison of the average ulcer surface (mean value ±SD) before and after HBOT in patients with ischemic and neurogenic foot syndrome is presented in Table 2. The statistical analysis confirmed a significant decrease in the average ulcer surface after HBOT both in patients with ischemic foot syndrome ($p = 0.004$) and in patients with neurogenic diabetic foot syndrome ($p < 0.05$). The average relative changes in ulcer surface values observed after the end of HBOT in both groups (45.3% in ischemic type and 41.8% in neurogenic type of diabetic foot syndrome, respectively) did not differ significantly ($p > 0.05$).

The obtained results proved that HBOT is an efficient method of treatment of topical lesions in both types of diabetic foot syndrome.

In Fig. 1–4, examples of topical status improvement estimated by computerized planimetry of ulcer dimensions in 3 patients with a diabetic foot ulcer exposed to combined treatment, including HBOT were presented.

Discussion

The results of the present study confirm findings reported in previous randomized, both double-blind and unblinded trials that HBOT enhances foot ulcer healing in patients with a diabetic foot.[9–12] Our healing rate following HBOT estimated immediately after completing hyperbaric oxygen cycle is in agreement with the aforementioned studies.

The incidence of previous vascular intervention at the time of randomization in our patients (9.6%) was much lower than in studies conducted by other authors: 38% and >50%, and this could explain the better results in long-term follow up obtained in those trials.[12,13]

Our findings are in agreement with some studies which have shown the beneficial effect of HBOT in preventing amputations.[10,11,13]

Due to Henry's law, the application of hyperbaric oxygen therapy causes a 15-fold increase in oxygen pressure in blood plasma (under therapeutic pressure of 2.5 ATA) with concomitant 100% oxygen saturation of hemoglobin. Therefore, during HBO compared to normobaric conditions, pO_2 gradient between the wound's edge and the center increases significantly.

Computerized planimetry enables an objective assessment of the healing process of wounds, based on the evaluation of wound surface,

Table 1. Therapeutic effect of HBOT in patients with skin ulcer in the course of both types of diabetic foot syndrome

Type of diabetic foot syndrome	Complete healing	Partial healing	No effect	Topical exacerbation	Total
Ischemic	14	27	8	1	50
Neurogenic	12	25	6	1	44
Total	26	52	14	2	94

Table 2. Comparison of the average ulcer surface (mean value ±SD) before and after HBOT in patients with ischemic and neurogenic foot syndrome, with statistical analysis

Type of diabetic foot syndrome	Ulcer surface before HBOT [mm^2]	Ulcer surface after HBOT [mm^2]	p-value
Ischemic	522.4 ±108.7	285.8 ±90.4	*p < 0.001
Neurogenic	509.3 ±99.6	296.2 ±82.8	*p < 0.001
Statistical significance	#p = 0.540	#p = 0.562	

* before and after treatment in particular groups; # comparison between both groups.

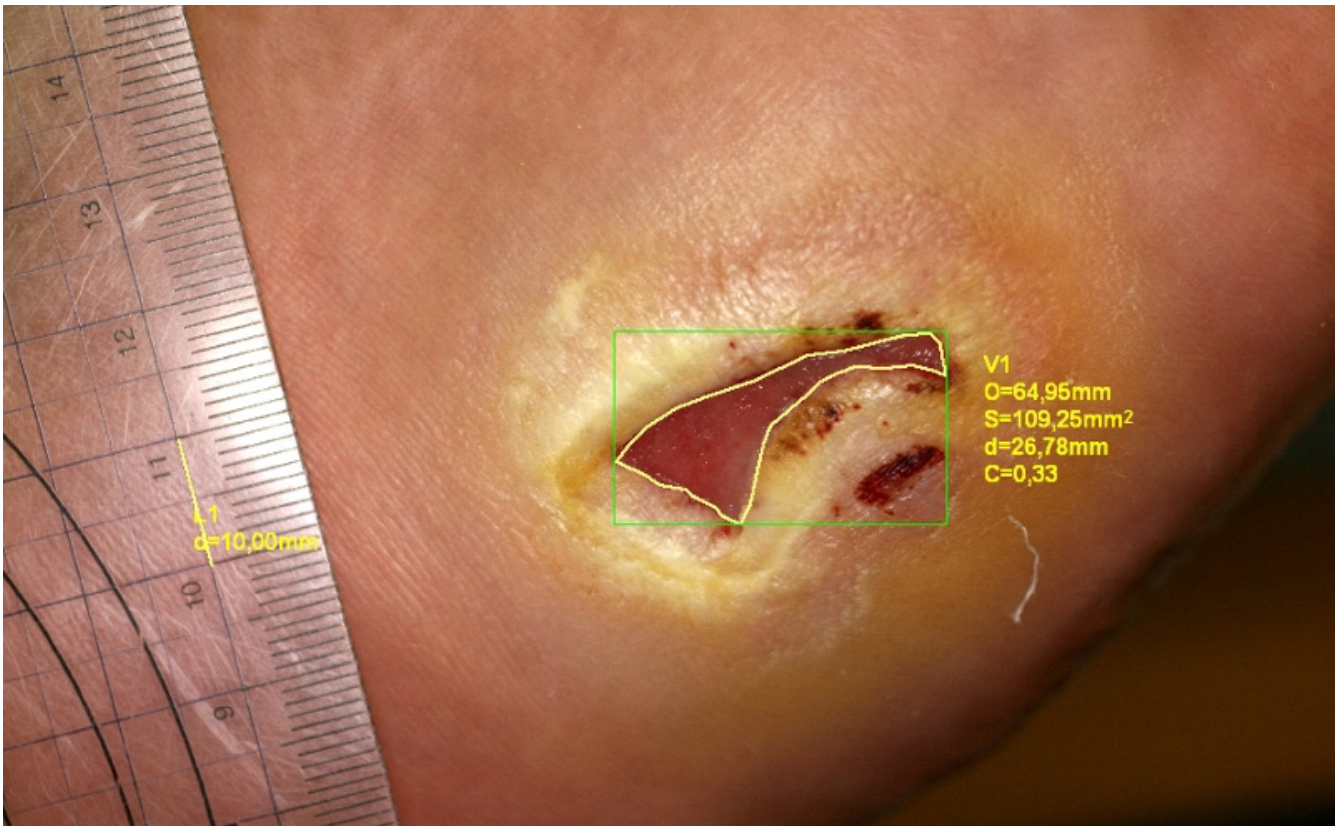

Fig. 1. Patient with diabetic foot ulcer (6 months) – the topical state before the beginning of combined treatment including HBOT with evaluation of wound dimensions by means of computerized planimetry; O – wound perimeter; S – wound surface; d – distance between maximally outlaying points on wound edge; C – wound circularity (near-circle level of wound shape with values 0–1, where 1 means circle)

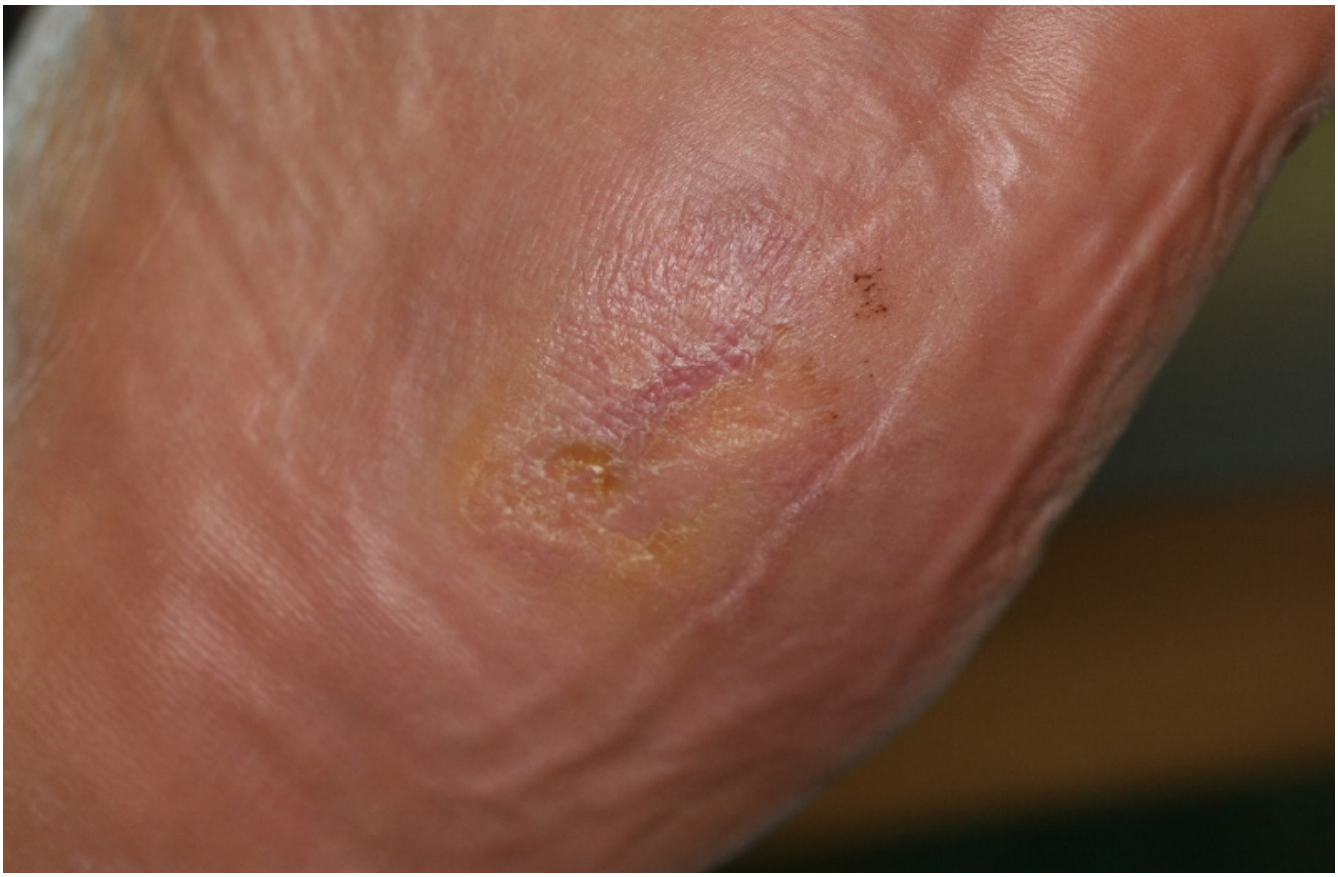

Fig. 2. The topical state after 36 procedures of combined treatment including HBOT – visible complete healing of the wound

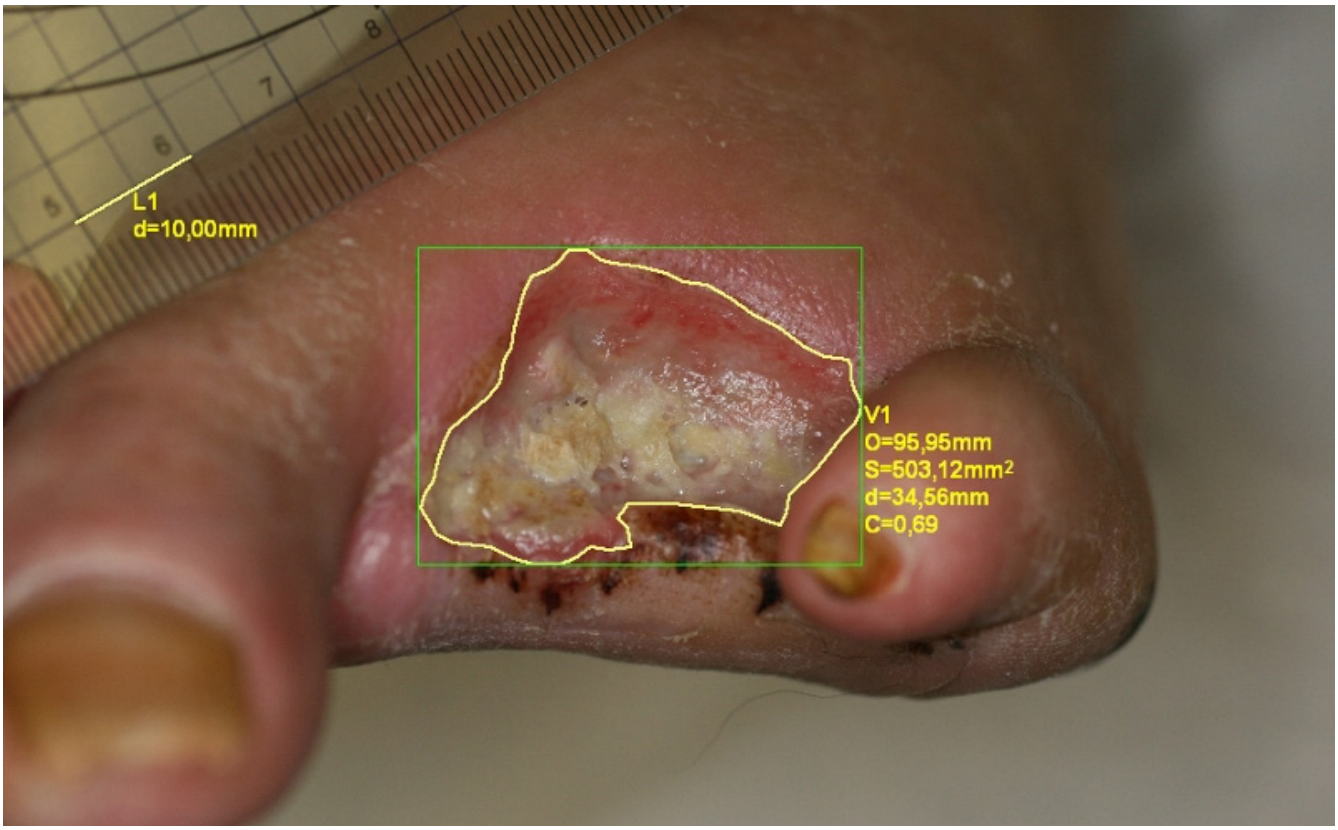

Fig. 3. Patient with diabetic foot ulcer (2 years) after amputation of right foot fingers II–IV – the topical state before the beginning of combined treatment including HBOT with evaluation wound dimensions by means of computerized planimetry; O – wound perimeter; S – wound surface; d – distance between maximally outlaying points on wound edge; C – wound circularity (near-circle level of wound shape with values 0–1, where 1 means circle)

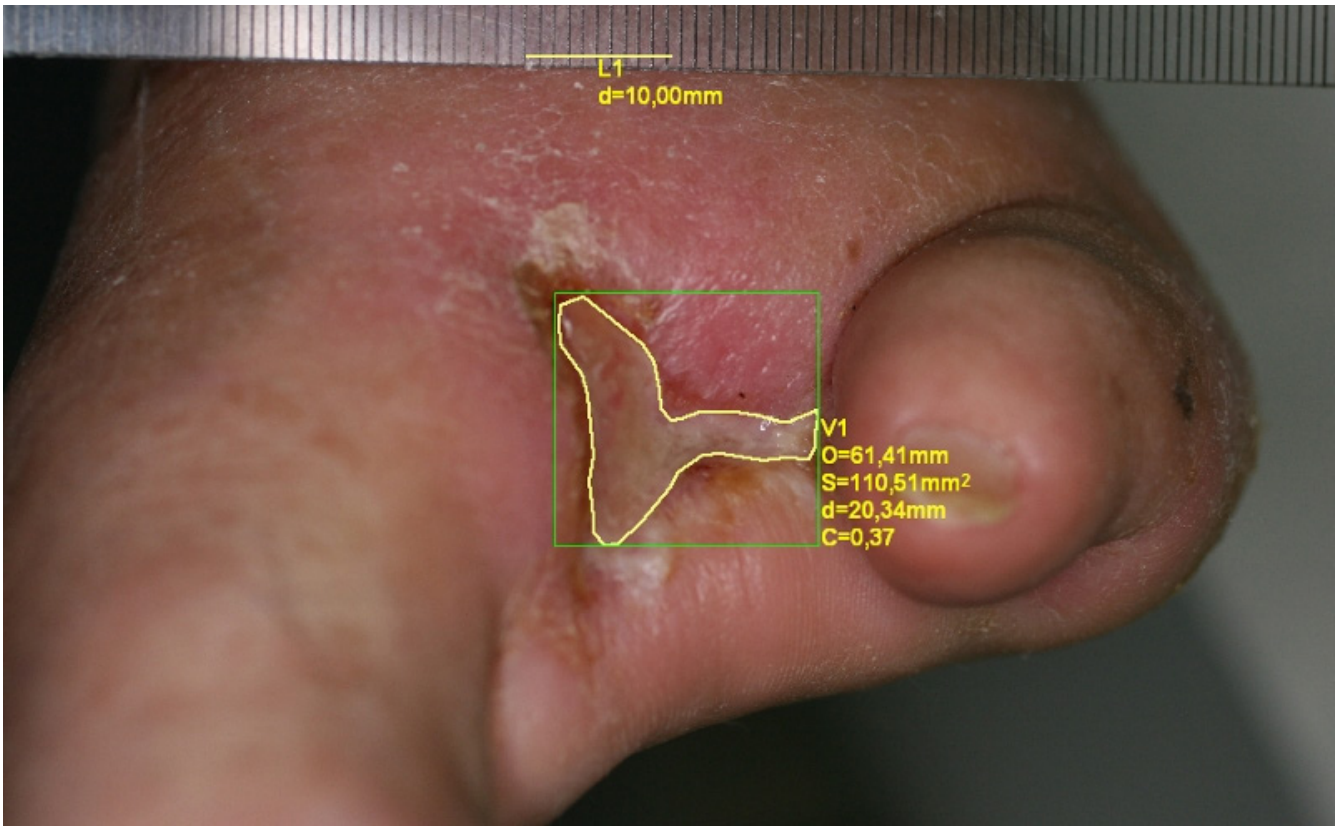

Fig. 4. The topical state after 45 procedures of combined treatment including HBOT with evaluation wound dimensions by means of computerized planimetry; O – wound perimeter; S – wound surface; d – distance between maximally outlaying points on wound edge; C – wound circularity (near-circle level of wound shape with values 0–1, where 1 means circle); visible distinct decrease of wound dimensions

wound periphery and changes of wound size (percentage of surface compared with previous measurement).[14] Planimetry is based on precise contouring of wound edges, using its digital image.

Monoplanar wounds of diabetic foot patients were covered by the measurement system IRIS-4. Ring-shape and multiplanar wounds cannot be objectively evaluated by this method. Wound photographing and evaluation by IRIS 4 program enabled us to determine the precise dimension and surface of tissue defects. The implementation of cyclic, timed documentation using high resolution digital imaging and data formulating in the IRIS system, demonstrated a gradual decrease of its surface, even in a small range, and objectified our clinical observation, allowing us to evaluate even discrete lesions in a wound.

Introducing a complex treatment of diabetic foot ulcer into clinical practice, combined with hyperbaric oxygen, offers an opportunity for a quick recovery and an active life, and reduces a budget load due to long-term therapy.[15] But one must remember that oxygen hyperbaric therapy cannot be considered as treatment of choice, as it is only an adjuvant method for basic conventional therapy of diabetic foot patients.

Conclusions

The application of HBOT in the treatment of diabetic foot enhances foot ulcer healing confirmed by computerized planimetry evaluation, both in the case of ischemic and neurogenic type of diabetic foot syndrome, it reduces tissue damage, contributes to the reduction of complications related to soft tissue and bone infections, and therefore enables idiopathic closure of wound.

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IL-6, *IL-1β*, and *TNF-α* only in combination influence the osteoporotic phenotype in Crohn's patients via bone formation and bone resorption

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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 the article

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Abstract

Background. Crohn´s disease (CD) is associated with a higher prevalence of osteoporosis. The pathogenesis of bone affliction remains controversial, especially if inflammatory cytokines or glucocorticoid therapy are the main contributors. In postmenopausal osteoporosis, bone resorption is induced by *IL-6*, *IL-1β* and *TNF-α*. In contrast, in children with CD, *IL-6* exclusively decreased bone formation without affecting bone resorption.

Objectives. The objective of this study was to further clarify the pathophysiology of bone affliction in adult patients with CD with the use of an osteoblast and osteoclast cell model.

Material and methods. Inflammatory cytokines *IL-6*, *IL-1β*, and *TNF-α* were measured in adult CD patients' serum. Mean values of these cytokines were applied with or without dexamethasone to the human cell line SCP-1 (osteoblastic cell model). Also, the effect of cytokines on primary human osteoclast differentiation and activity was determined.

Results. The combined cytokine application increased the receptor activator of NF-κB ligand/osteoprotegerin (*RANKL/OPG*) ratio 2-fold after 2 and 14 days. Additional application of dexamethasone to SCP-1 cells further increased the *RANKL/OPG* ratio 3-fold, but decreased *IL-6* and *IL-1β* expression to 10% and 50%, respectively. *TNF-α* expression was maximally suppressed to 16% by dexamethasone in the presence of cytokines. In osteoclasts, the combined cytokine treatment decreased expression of characteristic genes to approx. 30%, while increasing osteoclast resorption activity to 148%. In addition, a cytokine stimulated osteoblast cell culture-generated supernatant stimulated osteoclast resorption activity by 170%.

Conclusions. Our results suggest that *IL-6*, *IL-1β*, and *TNF-α* only in combination induced osteoclast-stimulating activity represented by the *RANKL/OPG* ratio in osteoblasts. Dexamethasone further increased this effect in osteoblasts, while decreasing cytokine expression. The results in osteoclasts support a direct and osteoblast-mediated effect on bone resorption. Our in vitro results differentiate for the first time the effect of cytokines on bone turnover as measured in adult CD patients from the additional dexamethasone effect on osteoblasts as part of the pathophysiology of osteoporosis.

Key words: cytokines, osteoprotegerin, Crohn's disease, bone remodeling, receptor activator of NF-κB ligand

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Introduction

Patients with Crohn's disease (CD) may develop secondary osteoporosis with fractures in the course of the disease.[1–4] The pathophysiology of osteoporosis in CD has not been clearly established, but, aside from steroid therapy, the disease itself appears to be a key factor.[5] Cytokines, such as interleukin 1 beta (*IL-1β*), interleukin-6 (*IL-6*), and tumor necrosis factor alpha (*TNF-α*), have been implicated in the pathogenesis of CD.[6–8] These cytokines are key regulators of bone turnover, and are involved in postmenopausal osteoporosis, as well as osteoporosis in rheumatic diseases.[9–12] Bone turnover is characterized by balanced bone formation by osteoblasts and the degradation of bone by osteoclasts. The most important factor for bone turnover is the ratio of the receptor activator for NF-κB ligand (*RANKL*) and osteoprotegerin (*OPG*), factors synthesized by osteoblasts and osteocytes, but acting only on osteoclasts.[13]

Inflammatory cytokines play a major role in shifting the *RANKL/OPG* balance toward excessive *RANKL*, which induces osteoclast activity and greater bone degradation.[14] We know from studies of postmenopausal osteoporosis that *IL1β*, *IL-6*, and *TNF-α* are associated with increased bone resorption in in vivo models.[15] Additionally, in patients with CD, inflammatory cytokines are correlated with markers of osteoclast activity.[16] However, in an in vitro organ culture model of bone formation, serum from children with CD exclusively influenced bone formation without inducing changes in bone resorption, suggesting that the inflammation from CD in children affects osteoblasts and bone formation, but not osteoclasts.[17] These findings were surprising, because *IL-6* is an osteoclast-activating factor in other inflammatory diseases such as rheumatic disorders.[18] In addition, it has been suggested that other cytokines, especially *TNF-α*, are the most relevant cytokines in CD.[19]

We now hypothesize that multiple factors in serum, but not *IL-6* or *TNF-α* alone, are primarily responsible for bone disease in adult CD patients, and that these factors are more highly expressed in patients with an acute phase of CD. In addition, we determined the effects of these cytokines with and without glucocorticoid on bone formation and/or bone resorption by monitoring the *RANKL/OPG* ratio using an osteoblast cell culture model.

We have also investigated the direct influence of the combined cytokine treatment on bone resorption using primary human osteoclasts.

Material and methods

Serum sampling in patients with acute Crohn's disease

Patients with acute CD were recruited either at the emergency clinic or at our Clinic of Gastroenterology and Gastrointestinal Oncology (UMG, Germany) for chronic inflammatory bowel diseases, and the diagnosis of CD was based on endoscopic, histological, or radiological findings. "Acute" disease was identified using the Crohn's disease activity index (CDAI), in which a score greater than 150 is defined as active disease.[20] Patients were excluded if they had been treated with any steroid or immunosuppressive compound during the previous 3 months. The use of calcium and vitamin D supplements, or estrogen prior to the study was allowed. Upon inclusion in the study, routine blood analysis was performed. An additional 50 mL of blood was drawn, placed directly on ice, and centrifuged within minutes, and the resulting serum was batched and stored at –70°C. Sera obtained from healthy, age- and gender-matched controls were treated under identical conditions. This study was approved by the Ethics Committee of Göttingen University Medical Center, and informed consent was signed by all subjects. The characteristics of these patients as well as detailed inclusion and exclusion criteria had been published previously.[21]

Cytokine production

The serum levels of the cytokines were determined using commercially available enzyme-linked immunosorbent assay (ELISA) kits according to the manufacturer's specifications (Table 1). *IL-6* (R&D Systems, Minneapolis, USA; 0.447–9.96 pg/mL, minimal detectable level 0.016–0.110 pg/mL), *IL-1β* (R&D systems, Minneapolis,

Table 1. Primer sequences used in the present study for real-time polymerase chain reaction (PCR)

Gene	Primer sequence
β-actin	Fwd:5′-ctggaacggtgaaggtgacg-3′ Rev:5′-agtcctcggccacattgtga-3′
OPG	Fwd:5′-ctcaggcacttgaggctttc-3′ Rev:5′-tgcaagcagtaataagggaaaa-3′
RANKL	Fwd:5′-agctcagccttttgctcatc-3′ Rev:5′-tggtgcttcctcctttcatc-3′
IL-6	Fwd:5′-tggctgaaaaagatggatgct-3′ Rev:5′-aactccaaaagaccagtgatgatt-3′
IL-1β	Fwd5′-aatttgagtctgcccagttccc-3′ Rev:5′-agtcagttatatcctggccgcc-3′
TNF-α	Fwd:5′-cccaggcagtcagatcatcttc-3′ Rev:5′-agctgcccctcagcttga-3′
Cathepsin K	Fwd:5′-tgaggcttctcttggtgtccatac-3′ Rev:5′-aaagggtgtcattactgcggg-3′
RANK	Fwd:5′-ggtgcagcctctaactcctg-3′ Rev:5′-ttgagaccaggctgggtaac-3′
TRAP	Fwd:5′-gaccaccttggcaatgtctctg-3′ Rev:5′-tggctgaggaagtcatctgagttg-3′
VNTR	Fwd:5′-actcaagcaaaagggagcaat-3′ Rev:5′-agattcatcccgcagatacgc-3′

OPG – osteoprotegerin; *RANKL* – receptor activator of NF-κB ligand; *IL-1β* – interleukin-1 beta; *IL-6* – interleukin-6; *TNF-α* – tumor necrosis factor alpha; *RANK* – receptor activator of NF-κB; *TRAP* – tartrate–resistant acid phosphatase; *VNTR* – vitronectin receptor.

USA; <1.996 pg/mL, minimal detectable level <0.1 pg/mL), and *TNF-α* (DPC Biermann GmbH, Bad Nauheim, Germany) reference values from the manufacturer were <8.1 pg/mL. Intra-assay precision was 2.6–3.6%, and inter-assay precision was 4–6.5%. The minimal detectable level was 0.1 pg/mL.

Experimental procedures

Cell culture

Disposable cell culture products were purchased from Nunc (Roskilde, Denmark). Fetal calf serum (FCS) was obtained from Lonza (Köln, Germany), cell culture medium and the medium supplements (antibiotics and glutamine) were obtained from GIBCO-BRL (Eggenstein, Germany). All reagents were purchased from Sigma Chemical Co. (Munich, Germany), unless otherwise stated.

Treatment of SCP-1 cells with cytokines

To simulate the conditions occurring during the acute phase of CD, a combination of the cytokines, rather than the single cytokines alone at the detected levels, was applied to an osteoblast cell model. To avoid the variability of primary human cell culture, an immortalized mesenchymal cell line (SCP-1; a single-cell-picked clone of hTERT immortalized human mesenchymal stem cells) was used as an osteoblast model.[22] This SCP-1 cell line can function as a progenitor cell but differentiates into an osteoblast phenotype when appropriately stimulated.[22] Osteogenic stimulation resulted in increased expression of alkaline phosphatase (*ALPL1*; 3.5-fold) and osteocalcin (*OC*; 6.6-fold). Adipogenic stimulation increased the expression of adipocyte protein 2 (*aP2*; 82-fold) and peroxisome proliferator activated receptor gamma (*PPARγ*; 8.5-fold) (data not shown). The cells were split 1:5 weekly and cultured under standard conditions (10% FCS in MEM alpha). For these experiments, the cells were trypsinized and plated on 6-well dishes at a density of 5×10^4 cells/mL.

For short-duration stimulation in bovine serum albumin (BSA) (dose response experiments), 10% FCS medium was changed to 0.1% BSA after reaching confluence, and the cytokines were applied as individual agents at various concentrations (*IL-1β*, 0.01–100 ng/mL; *TNF-α*, 0.01–100 ng/mL; or *IL-6*, 0.01–100 ng/mL) or as a combination (10 pg/mL *IL-6*, 1 pg/mL *IL-1β*, and 5 pg/mL *TNF-α*) to the cell medium without osteogenic differentiation. After incubation for 2 days, total RNA was isolated using a Qiagen RNeasy Mini Kit, and the RNA purity was confirmed based on the 260/280 ratio.

For SCP-1 cell experiments using 1% human serum from a healthy blood donor (Department of Transfusion Medicine), 10% FCS was exchanged for 1% human serum after 4 days of culture. After reaching confluence, the cells were supplemented with 10 mM β-glycerophosphate (bGP) and 10 µM ascorbic acid 2-phosphate (ascP) (osteogenic differentiation) and stimulated with the cytokines either individually or in combination (see above) to evaluate the effect of the cytokines on the osteoblasts.

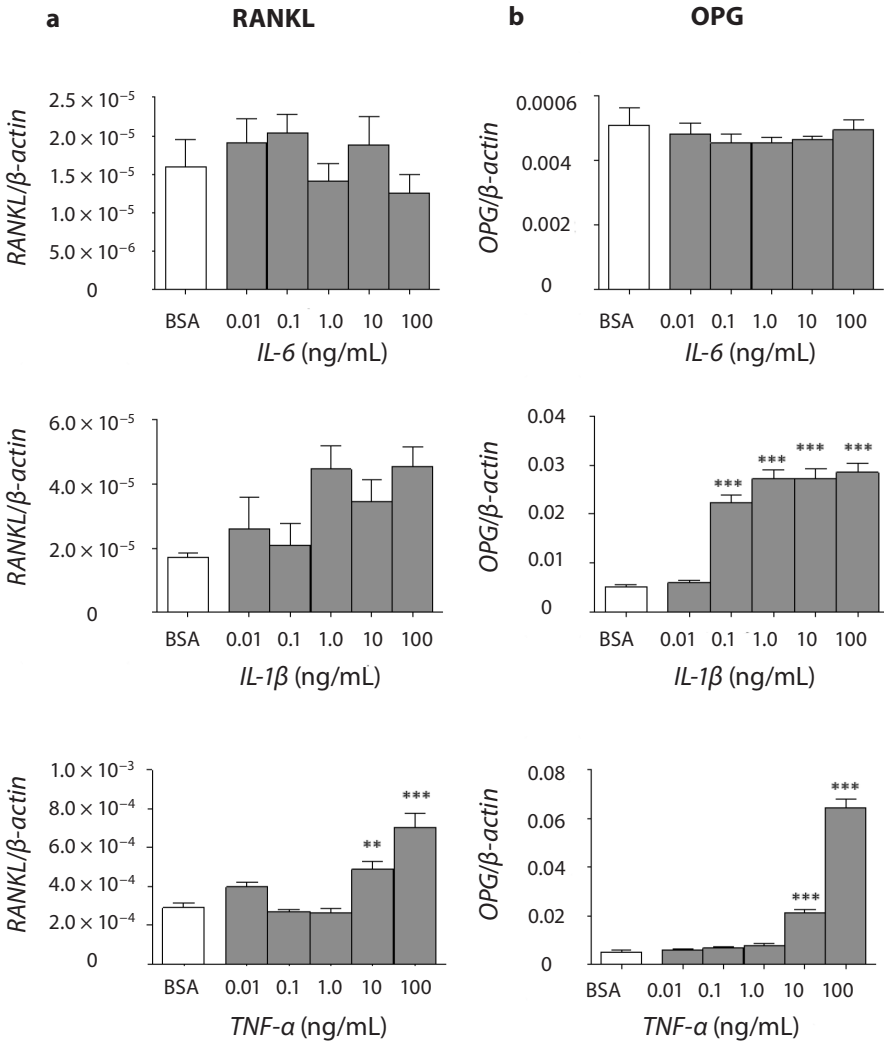

Fig.1. Gene expression of *RANKL* (A) and *OPG* (B) in SCP-1 cells after 48 h in 0.1% bovine serum albumin (BSA) and in the presence of various cytokine concentrations: *IL-6* (0.01–100 ng/mL), *IL-1β* (0.01–100 ng/mL), or *TNF-α* (0.01–100 ng/mL). *β-actin* was used as the housekeeping gene (n = 4). Significance is indicated as follows: ***p < 0.001; **p < 0.01; *p < 0.05 (Dunnett's test, treatments vs BSA)

Furthermore, experiments were also performed with or without dexamethasone (10 µM) to assess the dexamethasone effect in the presence of cytokines. The *RANKL/OPG* ratio and the self-induction of cytokines in osteoblasts were investigated to imitate glucocorticoid effects during the acute phase in CD. Based on the experimental design, treatment with cytokines, bGP, ascP, and dexamethasone was performed every 3–4 days.

Treatment of osteoclasts with cytokines

For osteoclast culture, monocytes obtained from healthy individuals were isolated using the magnetic-activated cell sorting (MACS) cell separation method (CD14) and cultured with Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% FCS, glutamine, and penicillin/streptomycin. Differentiation into mature osteoclasts

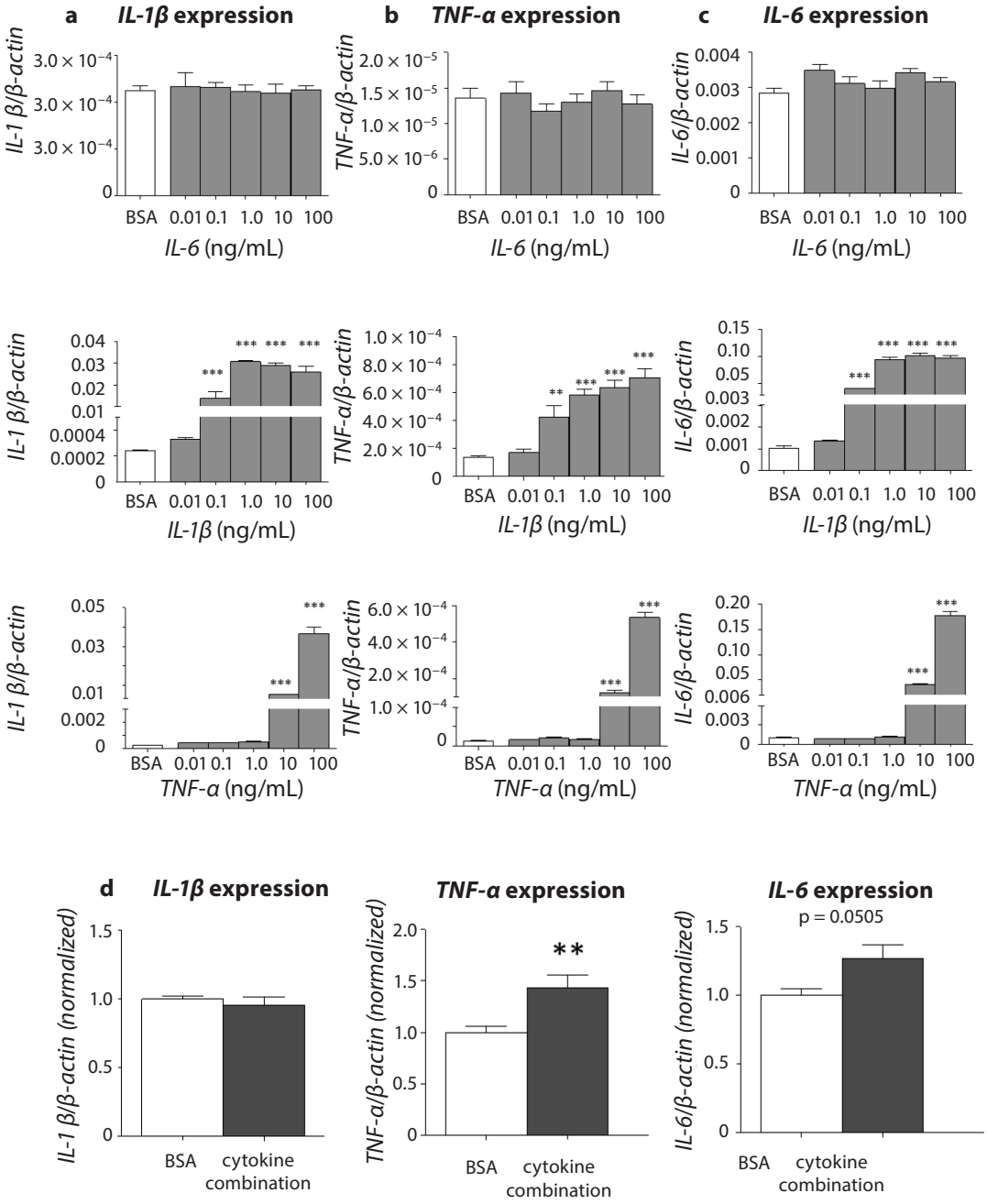

Fig. 2. Gene expression of *IL-1β* (A), *TNF-α* (B), and *IL-6* (C) in SCP-1 cells after 48 h in 0.1% bovine serum albumin (BSA) in the presence of various cytokine concentrations: *IL-6* (0.01–100 ng/mL), *IL-1β* (0.01–100 ng/mL), or *TNF-α* (0.01–100 ng/mL). D – Normalized gene expression of *IL-1β*, *TNF-α*, and *IL-6* in the absence or presence of the combined cytokines (10 pg/mL *IL-6*; 1 pg/mL *IL-1β*; 5 pg/mL *TNF-α*) from 3 independent experiments. *β-actin* was used as the housekeeping gene. P-values were calculated using Dunnett's test (A, B, and C) (n = 4) or unpaired t-test (D) (n = 12). Significance is indicated as follows: ***p < 0.001; **p < 0.01; *p < 0.05 (treatments vs BSA)

was achieved via the addition of *RANKL* (100 ng/mL) and macrophage colony-stimulating factor (M-CSF) (25 ng/mL) to the cells in 6-well plates for 17 days, and differentiation was monitored using toluidine blue (Sigma-Aldrich, St. Louis, USA) and tartrate-resistant acid phosphatase (TRAP) staining (Sigma-Aldrich). On days 9 and 13, the cells were treated with the combined cytokines (*IL-1β*, *IL-6*, and *TNF-α*) for 48 h. After 17 days, the total RNA from the osteoclasts was isolated. In 3 independent experiments, the expression of the markers was determined by real-time polymerase chain reaction (PCR), and β-actin (*ACTB*) was used as the housekeeping gene. The osteoclasts expressed a characteristic protein profile and resorbed osteoblast-derived matrix in a bone resorption assay.[23]

Bone resorption activity was determined as follows. Briefly, isolated monocytes (1×10^4 cells/mL) were plated onto a 24-well plate coated with a dense layer of SAOS-2-derived extracellular matrix (provided from Dr. Hempel) in the presence of 100 ng/mL *RANKL* and 25 ng/mL M-CSF. After 9 and 13 days, osteoclasts were stimulated for 48 h with either the control medium, the cytokine combination, an SCP-1 cell culture supernatant, or an "incubated" cytokine combination (incubated without cells for 48 h at 37°C in SCP-1 medium to be used as supernatant control). SCP-1 cells were treated with the cytokine combination for 48 h for the culture supernatant (Fig. 2d). To determine the pit area (resorptive activity), the osteoclasts were removed after 17 days by treating the coated plate with 5% sodium hypochlorite for 5 min and washing with water. The pits were photographed and analyzed using image analyzing software (Image J). Experiments were performed with n = 4.

RNA isolation and complementary DNA synthesis

Total RNA was isolated after treatment with cytokines and/or dexamethasone, depending on the individual experiment, using a Qiagen RNeasy Mini Kit, and the RNA purity was confirmed based on the 260/280 ratio. For each sample, 400 ng of RNA was reverse transcribed into cDNA using M-MLV reverse transcriptase, as previously described.[24] The real-time-PCR (RT-PCR) reactions were performed using a Peq-Lab Primus 96 thermal cycler in a total volume of 40 µL, as previously described.[25]

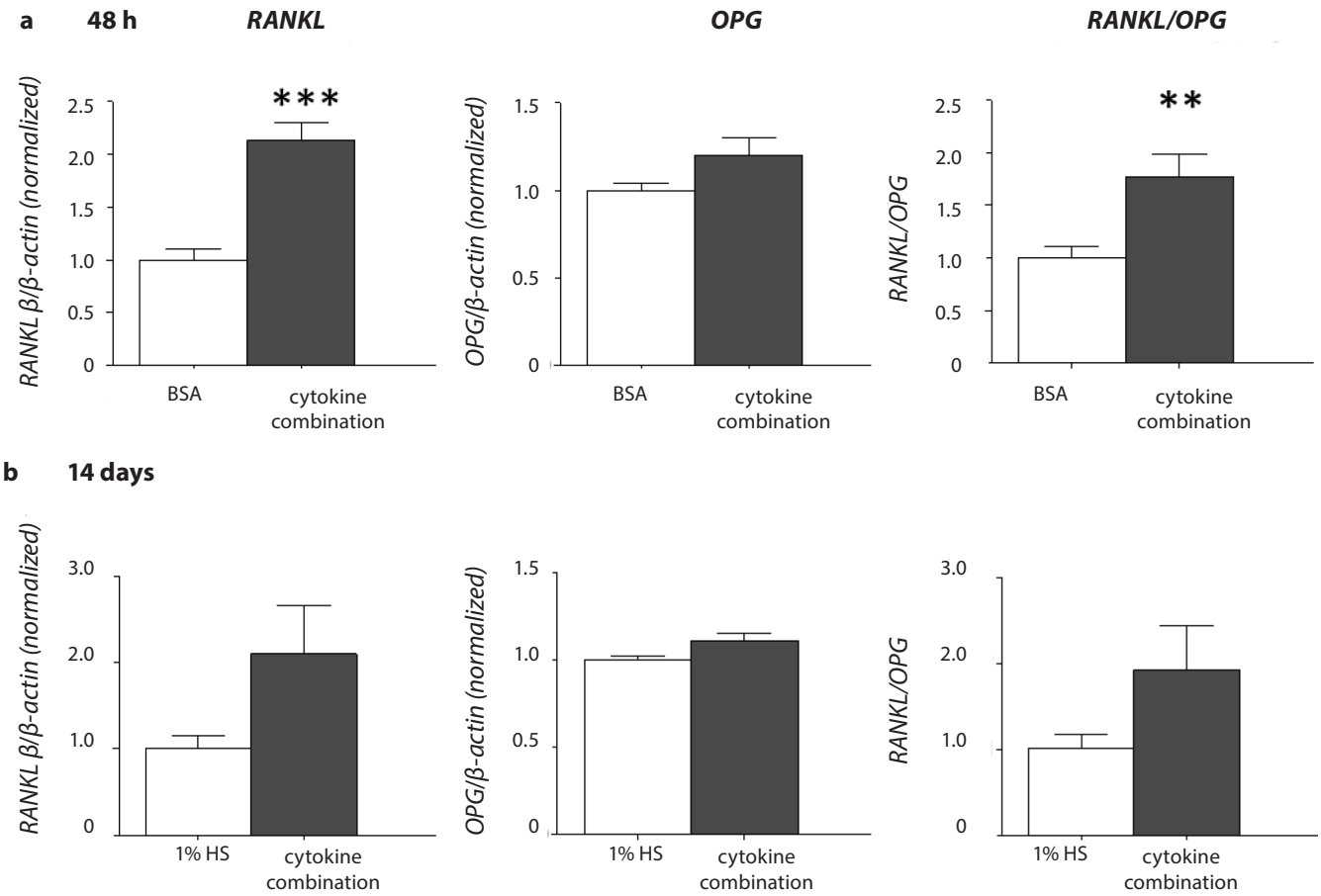

Fig. 3. Normalized gene expression of *RANKL* and *OPG* and the *RANKL/OPG* ratio in SCP-1 cells after 48 h in 0.1% bovine serum albumin (BSA) (A) or after 14 days in 1% human serum (B) with osteogenic stimulation (bGP, ascP) in the absence or presence of the combined cytokines (10 pg/mL *IL-6*; 1 pg/mL *IL1β*; 5 pg/mL *TNF-α*). The data were obtained from 3 (A; n = 12) or 2 (B; n = 8) independent experiments. *β-actin* was used as the housekeeping gene. Significance is indicated as follows: ***p < 0.001; **p < 0.01; *p < 0.05 (unpaired t-test)

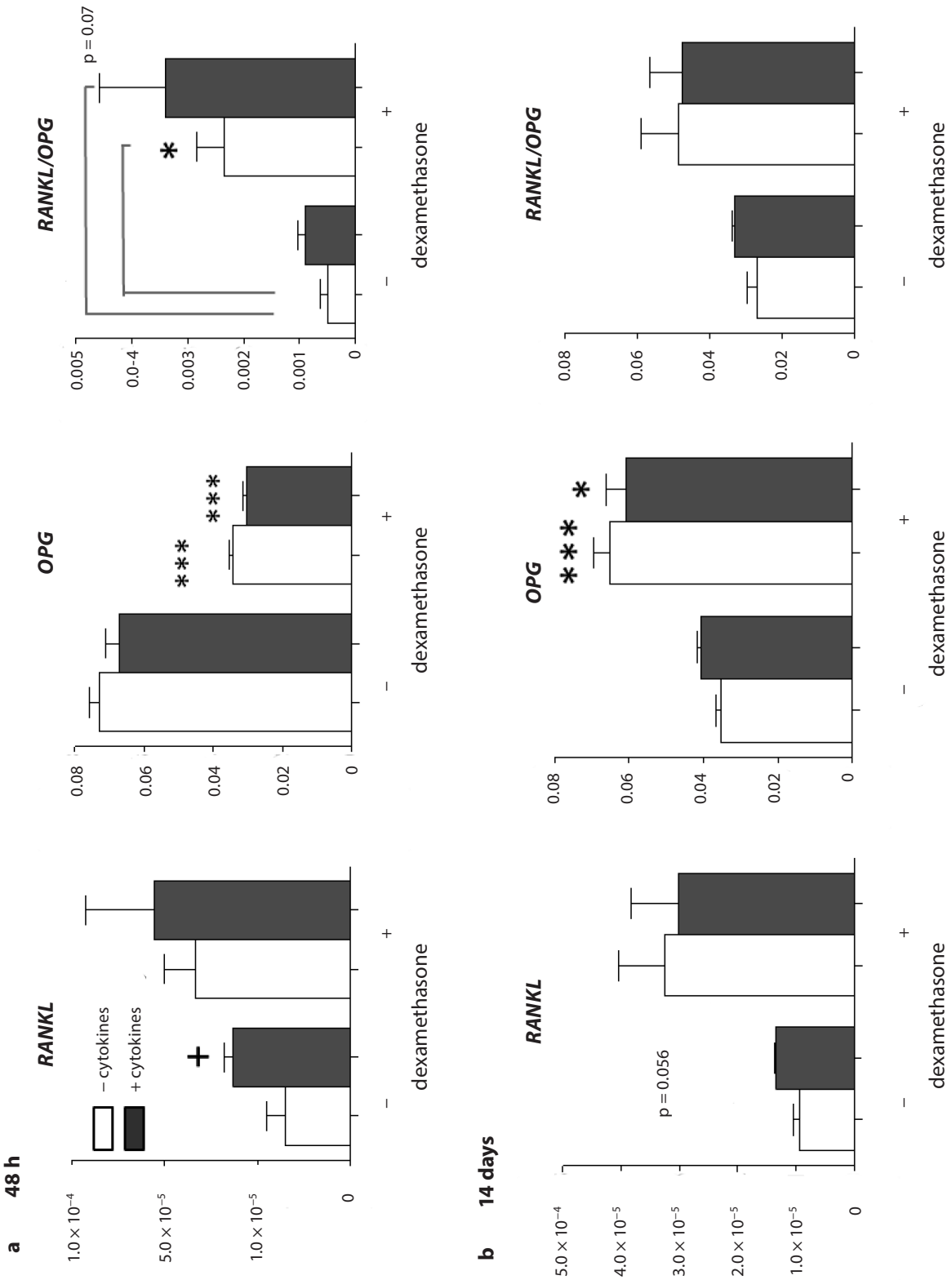

Fig. 4. Influence of dexamethasone on the gene expression of *RANKL* and *OPG* and the *RANKL/OPG* ratio in SCP-1 cells after 48 h in 0.1% bovine serum albumin (BSA) (A) or after 14 days in 1% human serum (B) and with osteogenic stimulation (bGP, ascP) in the absence (clear) or presence (gray) of the combined cytokines (10 pg/mL *IL-6*; 1 pg/mL *IL1β*; 5 pg/mL *TNF-α*). Dexamethasone was added at a concentration of 10 µM at the beginning of the experiment and every time the medium was changed (4 times in 14 days). *β-actin* was used as the housekeeping gene. Significance is indicated as follows: for the samples with or without dexamethasone, ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$; and for the samples in the presence or absence of cytokines, +, $p < 0.05$ (unpaired t-test)

RT-PCR analysis of complementary DNA (cDNA) was performed at 60–95°C for 40 cycles using the ABI Prism StepOnePlus sequence detection system (Applied Biosystems, Darmstadt, Germany) according to the manufacturer's instructions. The SYBR Green Reaction Master Mix (ABI Prism; Applied Biosystems) and the primers listed in Table 1 were used. All primers were synthesized by Invitrogen. Based on our experience from previous experiments, β-actin (*ACTB*) mRNA was used as an internal control in each RNA sample, because the expression of this gene is constant under different experimental conditions.

The results were normalized to *ACTB*, and the fold change in expression was calculated based on the threshold cycle (Ct) values ($2^{-\Delta Ct}$ method).[26]

Statistical analysis

The data was analyzed using Prism GraphPad 4 software (San Diego, USA). All the data is presented as means ±SEM (standard error of the mean). Statistical significance was calculated using the Mann-Whitney U test for clinical patient data, unpaired t-test (cytokine combination) or one-way analysis of variance ANOVA, followed by Dunnett's test (dose response experiments). The significance was set at $p < 0.05$.

Ethical considerations

All the patients provided informed consent, and the study was approved by the Ethics Committee of Göttingen University Medical Center (11/5/00). The study was conducted in accordance with good clinical practice and the Declaration of Helsinki.

Results

Serum parameters

The cytokines were significantly increased in sera from CD patients compared with those from healthy, age- and gender-matched controls (Table 2), i.e., *IL-6* (~100-fold), *IL-1β* (~10-fold), and *TNF-α* (~10-fold). Due to the small number of carefully chosen CD patients (n = 7), results must be judged carefully.

Treatment of SCP-1 cells with cytokines

To determine whether single cytokines influence bone turnover in SCP-1 cells, we applied cytokines for 2 days (dissolved in 0.1% BSA) and analyzed receptor activator of NF-κB ligand (*RANKL*) and osteoprotegerin (*OPG*), as shown in Fig. 1. During the short incubation, the cells remained vital and healthy. The expression of *RANKL* and *OPG* was not influenced by treatment with the lowest concentrations of *IL-6*, *IL-1β*, or *TNF-α* (0.01 ng/mL) comparable to acute phase in CD patients. Higher concentrations of *TNF-α* increased *RANKL* expression (2.4-fold, 10 and 100 ng/mL) and *OPG* expression (3–12.5-fold; 10 and 100 ng/mL). The expression of *OPG* was also induced by higher concentrations of *IL-1β* beginning at 0.1 ng/mL (4.4-fold) (Fig. 1).

The potential self-induction of exogenous applied cytokines in SCP-1 cells was examined by stimulation with each of the individual cytokines at increasing concentrations (Fig. 2a, 2b, 2c), or with the combined cytokines (Fig. 2d). As shown in Fig. 2a, 2b, and 2c, none of the cytokines was able to self-induce its own expression or the expression of the other cytokines in the lowest concentration adapted to the concentration found in CD patients.

Regarding higher concentrations, *IL-6* had no influence on the expression of itself or the other cytokines, even at the highest concentrations. *IL-1β* did induce its own expression and expression of *IL-6* and *TNF-α* beginning at 0.1 ng/mL. This influence accumulated with increasing *IL-1β* concentration (up to 100-fold). *TNF-α* induced its own expression and the expression of the remaining 2 cytokines only at the second highest and the highest concentrations (up to 50-fold).

In Fig. 2d the results of 3 independent experiments for the application of a combination of cytokines is presented. The concentration of cytokines in the combined treatment was chosen based on the measured cytokine concentrations in the sera of CD patients: 10 pg/mL *IL-6*; 1 pg/mL *IL-1β*; 5 pg/mL *TNF-α* (Table 1).

After 48 h, the cytokine combination showed no influence on the expression of *IL-1β*, a tendency towards increased *IL-6* expression (to 120%), and a significant increase in *TNF-α* expression to 140% compared with the control.

Based on the fact that the single cytokines at relevant concentrations had no effect on SCP-1 cells, we preceded with the experiments on SCP-1 cells with the cytokine combination.

To determine whether *RANKL* and/or *OPG* expression were affected, we treated undifferentiated SCP-1 cells with the combined cytokines for 2 days in 0.1% BSA solution

Table 2. Cytokine levels in CD patients during active disease and in healthy controls

Parameter	CD-patients (n = 7)	Healthy controls (n = 16)	p-value (Mann Whitney U)
IL-6 (0.447–9.96 pg/mL)	11.28 ±11.66	0.89 ±0.59	0.012
IL-1β (<1.996 pg/mL)	0.62 ±0.99	0.09 ±0.08	0.012
TNF-α (< 8.1 pg/mL)	2.12 ±2.02	0.2781 ±1.07	0.0055

CD – Crohn's disease; *IL-1β* – interleukin-1 beta; *IL-6* – interleukin-6; *TNF-α* – tumor necrosis factor alpha.

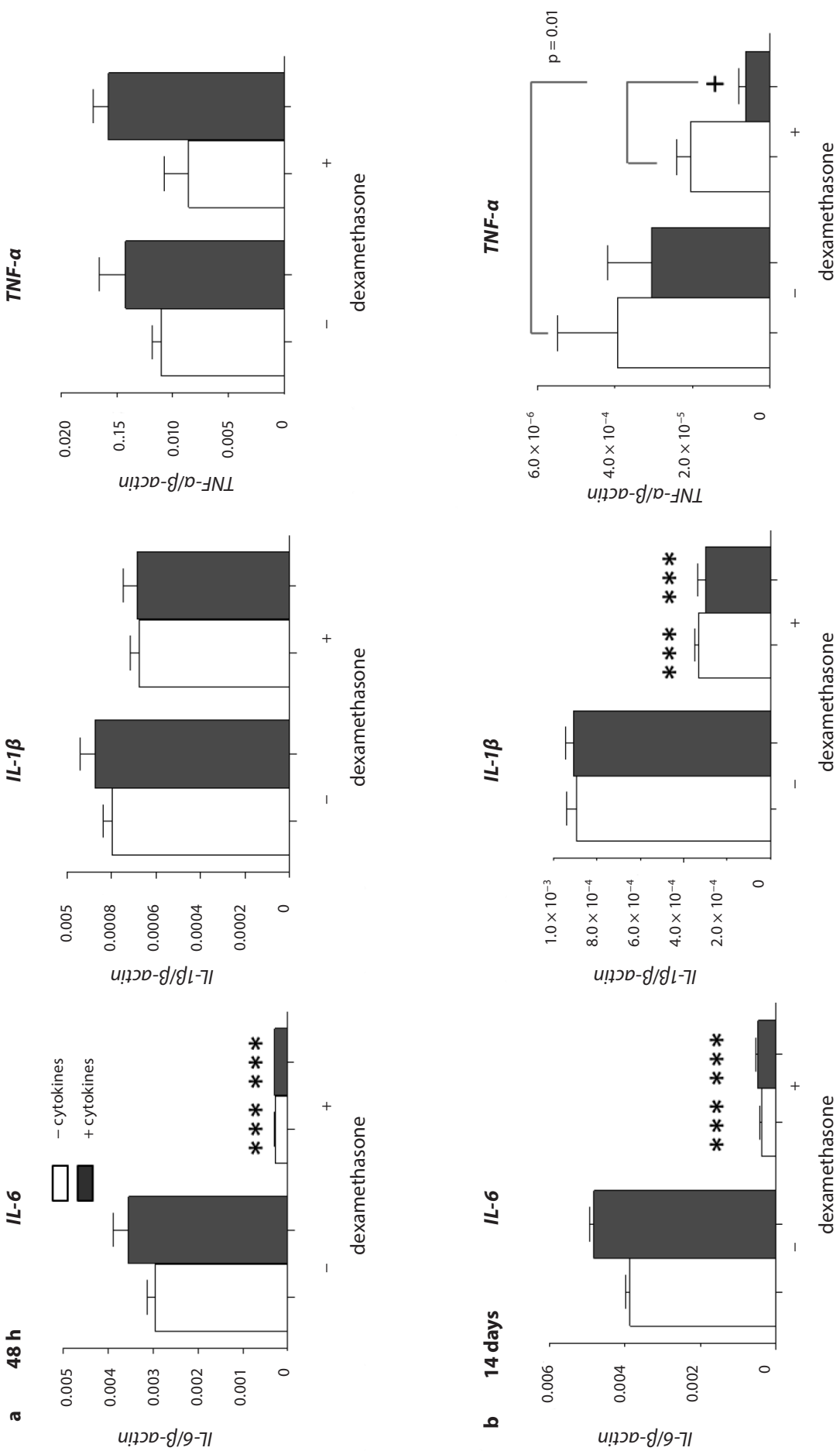

Fig. 5. Influence of dexamethasone on the gene expression of *IL-6*, *IL-1β*, and *TNF-α* in SCP-1 cells after 48 h in 0.1% bovine serum albumin (BSA) (A) or after 14 days in 1% human serum (B), and with osteogenic stimulation (bGP, ascP) in the absence (clear) or presence (gray) of the combined cytokines (10 pg/mL *IL-6*; 1 pg/mL *IL-1β*; 5 pg/mL *TNF-α*). Dexamethasone (10 µM) was added at the beginning of the experiment and every time the medium was changed (4 times in 14 days). *β-actin* was used as the housekeeping gene. Significance is indicated as follows: for samples with or without dexamethasone, *** $p < 0.001$; ** $p < 0.01$; * $p < 0.05$; and, for the samples in the presence or absence of cytokines, +, $p < 0.05$ (unpaired t-test)

(identical to that used for the treatment of the individual cytokines in Fig. 1), or for 14 days in 1% human serum obtained from a healthy donor under osteogenic stimulation.[6,27] Fig. 3 shows that the combined cytokine treatment increased *RANKL* expression by 2-fold after both the short-duration incubation in BSA ($p < 0.001$) (Fig. 3a) and the 14-day incubation in 1% human serum ($p = 0.1$) (Fig. 3b). *OPG* expression was not significantly affected by the combined cytokine treatment under these experimental conditions (Fig. 3b).

These findings indicate a clear influence of the combined cytokine treatment on the expression of *RANKL*, while *OPG* appeared to be unaffected. Therefore, the combined cytokine treatment shifted the *RANKL/OPG* ratio toward *RANKL* (Fig. 3).

Influence of dexamethasone on SCP-1 cells in the presence or absence of cytokines

CD patients are regularly treated with glucocorticoids during the acute phase of the disease. Therefore, we applied a dose-adapted concentration of dexamethasone to SCP-1 cells in either 0.1% BSA for 48 h (Fig. 4a) or in 1% human serum for 14 days (Fig. 4b) in the absence (white bars) or presence (black bars) of the combined cytokines. The data is shown in Fig. 4. The induction of the *RANKL/OPG* ratio by the cytokine combination was detectable at both time points. The ratio further increased by addition of dexamethasone for 48 h (right panel); this effect was independent of the cytokine combination ($p = 0.026$; 48 h in the presence of dexamethasone, absence of cytokines). The strongest induction (7-fold) occurred with dexamethasone and cytokines in combination ($p = 0.07$). The effect of dexamethasone on the *RANKL/OPG* ratio was caused by both an increase in *RANKL* expression and a decrease in *OPG* expression after 48 h.

After 14 days, the effect on *OPG* reversed, showing an increase in the presence of dexamethasone and, therefore, attenuated the effect on the *RANKL/OPG* ratio.

The effect of dexamethasone on cytokine expression is shown in Fig. 5. Dexamethasone significantly suppressed

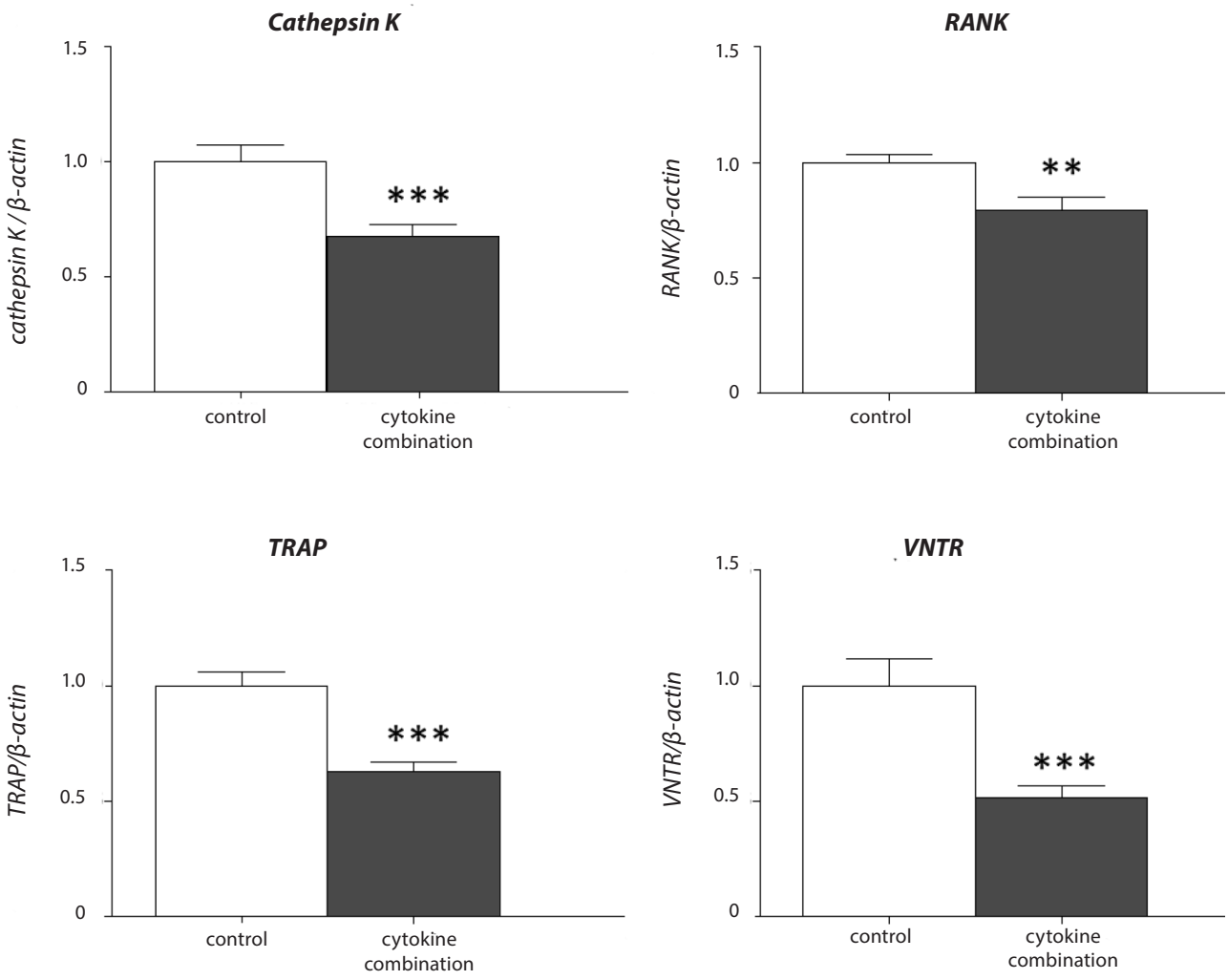

Fig. 6. Gene expression of *cathepsin K*, *RANK*, *TRAP*, and *VNTR* in osteoclast primary cultures after 17 days with or without (48 h bovine serum albumin (BSA)) the addition of the combined cytokines (at days 9 and 13 for 48 h) at the concentrations: 10 pg/mL *IL-6*; 1 pg/mL *IL-1β*; 5 pg/mL *TNF-α*). *β-actin* was used as the housekeeping gene. Probability values were calculated via unpaired t-tests

IL-6 expression to less than 10% at both time points, regardless of the presence of the cytokine combination (Fig. 5a and 5b, left panel). *IL-1β* expression was not influenced by dexamethasone after 48 h, but decreased to approx. 50% after 14 days (Fig. 5b, middle panel). *TNF-α* expression was not affected by dexamethasone after 48 h, but decreased to 20% under those conditions after 14 days. Notably, *TNF-α* was the only cytokine in which an additional effect evoked by the cytokine combination in the presence of dexamethasone was observed (p = 0.02) (Fig. 5b, right panel). This resulted in a decreased *TNF-α* expression to 16% in the presence of dexamethasone and cytokines of basal expression (p = 0.10).

Effect of cytokine combination on osteoclasts

Osteoclasts were incubated with the cytokine combination from day 6 to day 17 in the culture. The gene expression levels of the osteoclast markers *cathepsin K*, receptor activator of NF-κB (*RANK*), tartrate-resistant acid phosphatase (*TRAP*), and vitronectin receptor (*VNTR*) are presented in Fig. 6. The expression of these osteoclastic marker genes was significantly reduced (*cathepsin K* to 70%; *RANK* to 80%; *TRAP* to 64%, and *VNTR* to 53%, respectively) by treatment with the combined cytokines (Fig. 6), indicating a reduction in osteoclast function.

Osteoclast resorption assay

Osteoclast function was further analyzed by a bone resorption assay (Fig. 7). When comparing the freshly applied cytokine combination, bone resorption increased in relation to the control medium (p = 0.12; ns). In addition, we applied supernatant from the SCP-1 experiments for 48 h as conditioned medium, showing the same effect as the freshly applied cytokine combination.

As a direct control for the supernatant approach, cytokines were incubated in control medium without SCP-1 cells for 48 h and supplied to the osteoclasts. Under these conditions, a significantly lower osteoclast activity was observed (p = 0.017) compared with the stimulation with the 48-h supernatant. These results indicate an effect on the conditioned SCP-1 supernatant that is independent of the cytokines.

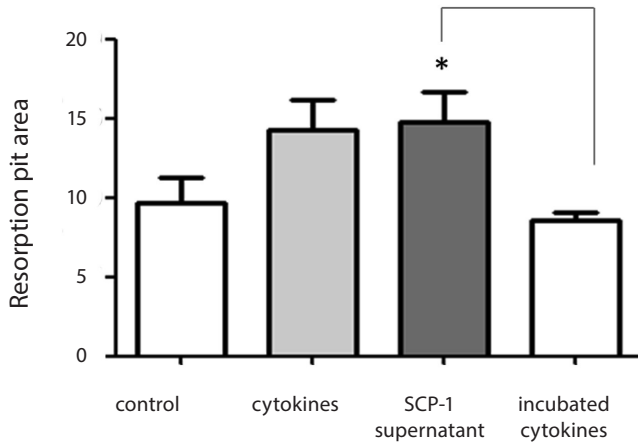

Fig. 7. Osteoclast resorption assay analyzing the pit area, indicating osteoclast activity after 17 days in culture. Osteoclasts were differentially stimulated at days 9 and 13 for 48 h with either control medium, cytokine combination (10 pg/mL *IL-6*; 1 pg/mL *IL-1β*; 5 pg/mL *TNF-α*), 48 h SCP-1 cell supernatant (*), cytokines incubated for 48 h in medium without cells. Every approach was performed 4-fold (n = 4). * SCP-1 cells were treated with cytokine combination for 48 h (Fig. 2d)

Discussion

The pathogenesis of osteoporosis in CD patients is multifactorial, but both the disease itself and the administration of glucocorticoids are thought to be among the primary contributors. We, therefore, analyzed sera from patients with active CD that had been glucocorticoid-free (steroid-free) for at least 1 month prior to analysis. Concentrations of cytokines *IL-1β*, *IL-6*, and *TNF-α* were measured.[21] Based on studies of pediatric patients with CD, all bone markers had returned to normal 1 month after the withdrawal of glucocorticoid treatment.[28–30]

As expected, we detected increased levels of *IL-6*, *IL-1β*, and *TNF-α* in sera from CD patients in the acute phase of the disease compared with age- and gender-matched controls. The data has to be interpreted carefully due to the small number of patients, but similar concentrations were measured in adult and pediatric CD patients.[6,16] Therefore, these cytokines possibly represent major pathogenic factors that influence the bone metabolism.[31]

To replicate the conditions occurring during the acute phase of CD, the osteoblast cell models were subjected to cytokines *IL-6*, *IL-1β*, and *TNF-α*, individually and in combination, at the detected levels.

In the following experiments of bone formation, the individual treatment of SCP-1 cells with *IL-6* did not influence the expression of *RANKL* or *OPG*. It has been suggested that *IL-6* is the main effector of bone resorption in children with CD.[6] In our system, none of the individually applied cytokines affected *RANKL* or *OPG* expression at the concentrations measured in CD patients. However, when applied in combination, the cytokines did influence the *RANKL/OPG* ratio toward bone resorption.

To verify the potential of self-induction, we investigated cytokine expression induced by the application of *IL-1β*, *IL-6*, and *TNF-α*, and produced dose–response curves. These experiments, applying the individual cytokines, showed a clear increase in *IL-6* expression via the single application of either *IL-1β* or *TNF-α*, but only at higher concentrations than those measured in the CD sera. An increase in *IL-6* after the application of *IL-1β* or *TNF-α* at higher concentrations has already been described, although that was in murine cells via the Stat3 pathway.[18] The cytokine levels as measured in CD patients

had no effect on *IL-6* expression when applied individually, and no self-induction of *IL-6* was observed. Therefore, our results using adult CD serum and SCP-1 cells are clearly different from those obtained by the organ culture model using serum from children with CD.[6] The slight increase in *TNF-α* and *IL-6* expression following the combined cytokine treatment gave no clear indication for a feedforward signaling cascade, because *IL-6* did not influence the expression of the other cytokines, and the effect of *TNF-α* on the expression of the other cytokines was only observed at considerable concentrations.

Therefore, treatment of osteoblasts with the combined cytokines applied in the concentrations found in adult CD patients produced results that contrasted absolutely with those observed using single cytokines, and partly with those observed in children with CD. The effects of cytokines on murine and human mesenchymal cells appear to be similar at high concentrations.[18]

To imitate the effects of glucocorticoid in the acute phase of CD, we performed experiments in the presence of dexamethasone. The effect of the combined cytokine treatment on the *RANKL/OPG* ratio was further increased when dexamethasone was added, regardless of the presence or absence of the cytokines. Dexamethasone reduced *OPG* transcript expression and protein secretion, and slightly increased *RANKL* gene expression in ST2 osteoblasts.[32] Our findings might be interpreted as being parallel to the clinical state in CD patients, indicating that an increased level of all 3 cytokines in combination shifts the *RANKL/OPG* ratio toward bone resorption, and that the additional application of dexamethasone further increases the *RANKL/OPG* ratio.

Dexamethasone strongly decreased *IL-6* expression, and the detectable original cytokine effect was small relative to the substantial dexamethasone effect. Therefore, in our model, *IL-6* stimulation in the presence of dexamethasone may have only minor effects on the bone. Other researchers have reported a similar inhibitory effect of glucocorticoids on the expression of *IL-6*.[33–35] Thus, the application of glucocorticoids to cultured cells exceeds the cytokine effect on the *RANKL/OPG* ratio, possibly mimicking their administration as a medication to CD patients.

Dexamethasone also reduced the expression of *IL-1β* independently of the presence of cytokines; this decrease was not as dominant as the *IL-6* decrease, but it was significant. *TNF-α* expression was decreased by the addition of dexamethasone, but, in contrast to the other 2 cytokines, the effect was further modified by the combined cytokine treatment. This supports the special role of *TNF-α* a in CD.[19,36,37]

The expression levels of *IL-6*, *IL1β*, and *TNF-α* were strongly reduced by dexamethasone. Moreover, dexamethasone shifted the *RANKL/OPG* ratio toward *RANKL*, independently of the presence or absence of cytokines. These findings demonstrate that the effect of dexamethasone on SCP-1 cells is not mediated by the reduced expression of cytokines in osteoblasts, but by the direct effect of dexamethasone on the expression of *RANKL* and *OPG*.

In addition to the treatment of osteoblasts with cytokines, we treated primary osteoclasts with the combined cytokines *IL-6*, *IL-1β*, and *TNF-α*. This treatment decreased all the examined markers of osteoclast function in cultures from 3 different donors. Assuming a decrease in osteoclastic function, this would pathophysiologically be in contrast to the effect observed in osteoblasts, where cytokines increase the *RANKL/OPG* ratio and, therefore, induce bone resorption via osteoclasts. The investigation of the effect of cytokines on osteoclasts as described in the literature mainly focuses on the differentiation between monocytes and mature osteoclasts, and not on the effects on mature osteoclasts, as in our experiments.[38] Nevertheless, it has been shown that *TNF-α* as well as *IL-1β* can have inhibitory effects on osteoclastic function.[39–41] However, we would have expected an increase in osteoclast number or function, because the development of monocytes or pre-osteoclasts into osteoclasts is strongly regulated by cytokines via *IL-6* and *RANKL*.[18,42] We, therefore, investigated osteoclast activity, using a bone resorption assay. The cytokine combination induced bone resorption activity. The conditioned cytokine medium from SCP-1 cells also increased osteoclast resorption, suggesting that factors produced by the osteoblasts and secreted into the medium stimulate increased osteoclasts resorption. The appropriate control of the conditioned cytokine medium clearly induced less osteoclast activity, suggesting an effect independent of "incubated" cytokines.

In the present study, we demonstrated that the combination of the 3 cytokines *IL-6*, *IL-1β*, and *TNF-α* increased the *RANKL/OPG* ratio in an osteoblast cell model, suggesting an indirect effect of cytokines on bone turnover via *RANKL/OPG*. The addition of dexamethasone to the osteoblast system caused a marked decrease in cytokine expression, especially that of *IL-6*, as well as a shift in favor of the *RANKL/OPG* ratio. In osteoclasts, treatment with the combined cytokines resulted in a decrease in osteoclast characteristic gene expression; however, bone resorption activity increased with the cytokine combination. In addition, a similar increase of osteoclast resorption activity by the conditioned medium suggested an additional influence of the SCP-1 supernatant independently of a direct *IL-6*, *IL-1β*, or *TNF-α* effect.

In conclusion, our in vitro results explain why in CD patients the increased cytokine levels together with glucocorticoid therapy will be deleterious to bone over an extended period, and may result in osteoporosis. Our results are the next step toward understanding osteoporosis in adult CD patients.

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Clinical and immunological characteristics of Polish patients with systemic lupus erythematosus

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Abstract

Background. Systemic lupus erythematosus (SLE) is a chronic autoimmune disease with varied clinical manifestations, which creates difficulties and delays in establishing a diagnosis.

Objectives. The aim of this study was to evaluate the prevalence and nature of the clinical symptoms of SLE, both at the onset of the disease and in its further course. An attempt to assess the immunological characteristics of the patients and to analyze autoantibodies variability over time was also made.

Material and methods. This retrospective study included 71 Caucasian patients, 63 women and 8 men, meeting the criteria for diagnosis of SLE according to ACR.

Results. The ratio of women to men was approximately 7.9:1. The average age of the onset of SLE was 31.5 years. The average time from the onset of symptoms to diagnosis was 5 years. The most common first manifestation of SLE were joint and muscles symptoms – 71.8%, skin lesions – 69.0%, fever – 57.7%. The main symptoms in the further course of the disease were neurological disorders – 69.0%, joint and muscle changes – 67.7%, and general symptoms – 59.2%. There was an increase in the incidence of renal involvement and neurological symptoms throughout the disease course. The most commonly detected antibodies were anti-dsDNA – 47.9%, anti-Ro/SSA – 40.8%, anti-nucleosomal antibodies – 29.6%, and lupus anticoagulant – 22.5%. A panel of antibodies typically did not change.

Conclusions. There is no typical clinical picture of SLE, the population suffering from this disease is very various. Therefore, early and accurate diagnosis can be a big challenge for any clinician, which justifies the need for this type of study to better characterize the disease.

Key words: systemic lupus erythematosus, course of systemic lupus erythematosus, onset of systemic lupus erythematosus

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Introduction

Systemic lupus erythematosus (SLE) is a chronic inflammatory autoimmune disease. It involves many organs and systems, mainly the skin, joints, kidneys, and the central nervous system.[1] In the course of the disease, general symptoms such as fever, weight loss, and fatigue are commonly seen. The first manifestations of SLE most often occur in young adults.[2]

To establish the diagnosis of SLE, patients have to fulfill at least 4 classification criteria developed by the American College of Rheumatology (ACR).[3,4] Based on the binomial coefficient, there are 330 combinations of symptoms that can be used to determine the diagnosis of SLE.

Due to this heterogeneous picture of the disease, SLE diagnosis is often delayed in relation to the appearance of the first symptoms. The occurrence of periods of exacerbation and remission is characteristic, and the course of the disease may take different forms, from mild to severe and even to life threatening.[5,6]

The aim of this study was to assess the incidence, clinical symptoms, dominant signs which indicate SLE, and the dominant signs in the course of the disease. Particular attention was paid to the delay in establishing the diagnosis of SLE in relation to the onset of symptoms, as well as to the average age of onset.

An attempt to assess the immunological characteristics of the patients was also made. Both the incidence of various autoantibodies at the time of establishing diagnosis and their variability over time were tested. The association between the presence of specific antibodies and the involvement of various systems, as well as the severity of the disease were examined.

Material and methods

The study records of 71 Caucasian patients, including 63 women (89%) and 8 men (11%) admitted to the Department of Rheumatology and Internal Medicine of the University Hospital in Wrocław in 2009–2011, were reviewed and included in a retrospective analysis. All patients fulfilled the criteria for the classification of SLE given by the ACR. Data was obtained from the patients' records and questionnaires on the basis of clinical symptoms and laboratory results. General symptoms (fever – more than 38°C after the exclusion of infection, weight loss, fatigue), skin lesions, mucosal changes, joint and muscle ailments, sensitivity to the sunlight, hair loss, lymphadenopathy, inflammation of the serous membranes, renal involvement (proteinuria, hematuria, pyuria), neuropsychiatric disorders (e.g., headaches, mood disorders, cognitive disorders), hematological symptoms (anemia, leukopenia, thrombocytopenia), sicca syndrome, and antiphospholipid syndrome were taken into account.

The presence of antinuclear antibodies, antiphospholipid antibodies, lupus anticoagulant was recorded. Antinuclear (ANA) and anti-double stranded DNA (anti-dsDNA) antibodies were routinely detected by indirect immunofluorescence on HEp-2 cells and *Crithidia luciliae* substrate, respectively. Antiextractable nuclear antigens (anti-Ro/SSA, anti-La/SSB, anti-Sm, and anti-RNP) antibodies were detected by qualitative enzyme-linked immunosorbent assays (ELISA), while the lupus anticoagulant (LA) was detected according to the guidelines of the International Society of Thrombosis and Hemostasis.

Specific symptoms and antibodies present in patients at the moment of diagnosis were monitored and compared at a later time in the course of the disease. The dominant frequency of symptoms and their correlation with the results of immunological studies were also considered.

The data is presented as mean values, standard deviations and percentages.

Results

In this study, the ratio of women to men was approximately 7.9:1, which correlates with the European SLE population.[5] The average age when the first symptoms appeared was 31.5 years (SD 11.8). The percentage of people whose onset began before the age of 14 years was 6%, and those with the first symptoms over the age of 50 years was 8%. The mean age of the establishing the diagnosis of SLE was 36.5 years (SD 13.94); therefore, the delay in diagnosis was 5 years (SD 5.24).

In the study group, joint and muscle symptoms dominated at diagnosis. They were present in 51 of 71 patients, which constituted 71.8%. The second most common signs were skin lesions – 69.0%. General symptoms were present in 57.7% of patients, including the dominant fever. Photosensitivity was observed in 52.1% of patients.

The average time between the appearance of the first symptoms and the conducted study examination was about 9 years, which allowed us to assess the subsequent course of the disease, in which neurological and psychiatric symptoms were dominant (occurring in 69.0%). The most frequent were headaches, mood disorders (depression), cognitive disorders, and cerebrovascular disease. Changes in joints and muscles were also important, as reported by 67.7% of the study group. General symptoms were present in 59.2% of patients, and the most commonly observed symptom was fatigue.

The prevalence of all symptoms evaluated in this study, both at the beginning and in the later stages of the disease, are included in Table 1 and Fig. 1 (A, B).

General symptoms

At least 1 of the general symptoms occurred in 62.0% of people in the early stage of disease and in 59.2% in the later course of SLE. Initially, the dominant general symptom was fever, which was observed in 47.9%

of patients. Fatigue (36.6%) and weight loss (26.8%) occurred less often. In the later stages of the disease, fever occurred less frequently (38.0%), and the most common symptom was fatigue (47.9%) and weight loss (19.7%).

Musculocutaneous symptoms

In our sample, the incidence of skin lesions at the disease onset was 69.0%, decreasing thereafter to 47.9%. Hypersensitivity to sunlight was observed in 52.1% of patients at the onset of the disease, and in 28.2% in its later course.

Table 1. Prevalence of all symptoms evaluated in this study, both at the beginning and in the later stages of SLE

The incidence of selected symptoms				
symptom	the initial period of the disease		the later period of the disease	
	number of patients	% (N = 71)	number of patients	% (N = 71)
Joint and muscle changes	51	71.8	48	67.6
Skin changes	49	69.0	34	47.9
Neuropsychiatric symptoms	42	59.2	51	71.8
Sunlight sensitivity	37	52.1	20	28.2
Fever	34	47.9	27	38.0
Fatigue	26	36.6	34	47.9
Anemia	24	33.8	30	42.3
Leucopenia	23	32.4	24	33.8
Alopecia	21	29.6	20	28.2
Weight loss	19	26.8	14	19.7
Headache	17	23.9	22	31.0
Proteinuria	16	22.5	32	45.1
Serositis	16	22.5	15	21.1
Sicca syndrome	14	19.7	15	21.1
Thrombocytopenia	13	18.3	9	12.7
Lymphadenopathy	11	15.5	6	8.5
Antiphospholipid syndrome	9	12.7	7	9.9
Hematuria	7	9.9	18	25.4

The presence of alopecia during the study was also pointed out, which was reported in 29.6% of patients at the time of diagnosis, dropping slightly to 28.2% as the disease progressed.

Erosions in the mouth were mainly manifested from the mucosal changes. Such changes were reported in 22.5% of patients at the time of appearance of the first symptoms, and in 19.7% at the later stages of the disease.

Symptoms of joints and muscles

The high prevalence of these symptoms was observed. About 71.8% of patients reported non-characteristic arthritis at the beginning of the disease, and 67.7% at a later stage, which makes it the most common first symptom and the second common ailment at the course of the disease.

Renal involvement

Proteinuria, hematuria and pyuria were the clinical symptoms evaluated in the study. In the initial stage of disease, at least 1 of these symptoms occurred in 23.9% of patients. Proteinuria occurred in nearly all patients (in 22.5% of patients and in 94.1% of patients with symptoms of renal involvement), whereas hematuria and pyuria occurred less often (in 9.9% and 5.6% of all patients, respectively). In the later period, symptoms of kidney disease were reported in 52.1% of patients. The incidence of proteinuria (45.1%) and hematuria (25.4%) was doubled. The incidence of pyuria (15.5%) was tripled.

Neurological and psychiatric symptoms

In the initial stage of the disease, neurological symptoms were found in 40.8% of patients. At a later course of the disease, 69.0% of the patients

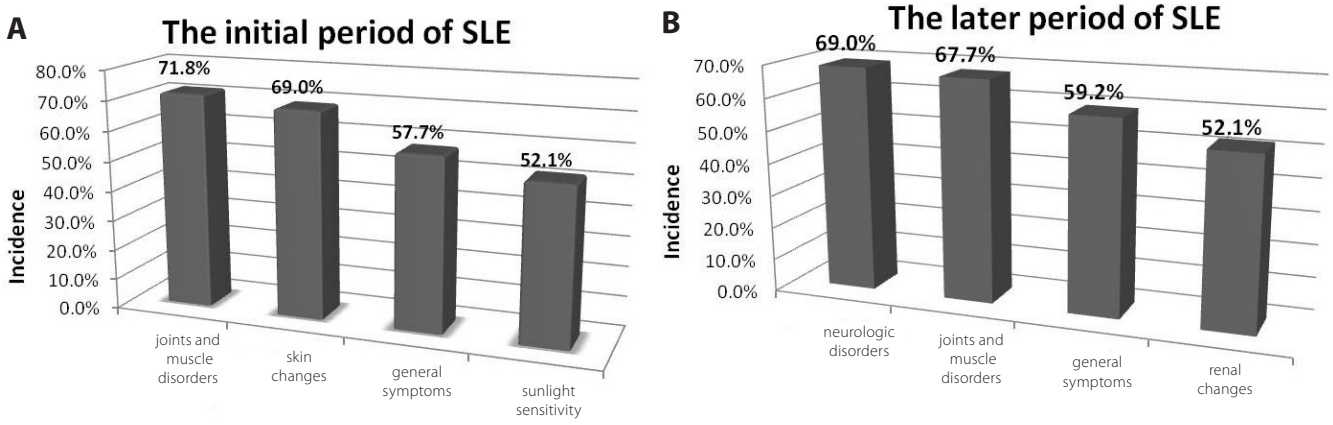

Fig. 1. The most frequent symptoms, both at the beginning and in the later stages of the disease

complained of at least 1 of the disorders of the nervous system. The most commonly reported disorders were headache (23.9% at the beginning and 31.0% in the advanced stage), and affective disorders (9.9% at diagnosis and 28.2% in the later course of the disease). Cognitive impairment in an advanced stage of SLE was manifested in 16.9% of patients. Symptoms of cerebrovascular disease, including stroke, were experienced by 9.9% of patients.

Hematological changes

In more than half of the patients (53.5%), hematological changes (anemia, leucopenia, thrombocytopenia) were observed at the beginning of the disease. The percentage of patients who suffer from these symptoms remained the same in the later course of SLE, but with an increased number of patients who experienced at least 2 of these 3 symptoms. Leucopenia (32.4%) and anemia (33.8%) occurred initially with similar frequency, while in the course of the disease anemia (42.3%) was significantly more frequently demonstrated than leucopenia (33.8%). Thrombocytopenia was initially diagnosed in 18.3% of patients, and in the further course of SLE incidence fell about 30% and amounted to 12.7%.

Sjörgen's syndrome and inflammation of the serous membranes

In the study, these symptoms were present in 22.5% of patients at the time of diagnosis. A similar incidence of occurrence (21.1%) was noted in the further course of the disease. Sicca syndrome accompanying SLE was reported in 19.7% of patients initially and in a similar number of patients (22.5%) in the further course of the disease.

Immunological profile of patients

ANA positive result was observed in the initial period in 87% of patients, and later in 94% of them. The full panel of antibodies was not determined for each patient in the initial stage of the disease. However, the available data shows that the most common antibodies in SLE patients in this period were anti-double stranded DNA (anti-dsDNA) and anti-Sjögren's-syndrome-related antigen A (anti-SSA autoantibodies). Similarly, in the later stages of the disease, anti-dsDNA (47.9% of patients) and anti-SSA (40.8%) were most common, followed by antinucleosomal antibodies (29.6%), and lupus anticoagulant (22.5%). Anti-ribosomal P protein was checked in 39 patients. All patients with a positive test result (6/39, 15.4%) had neurological symptoms. However, 21.2% of patients with negative test result also had neurological symptoms.

In 40 patients, data was available from both the onset of the disease as well as during its duration; an antibody panel in these patients mostly did not change.

Discussion

Numerous studies showing the incidence of SLE in the world has been previously established. Petri et al. and Alarcon et al. studied the American population, Wang et al. investigated the Asian population, and Cervera et al. covered European population in a large cohort study Euro-lupus.[5,7–9] Clinical manifestation of SLE varies considerably depending on the geographical region and, therefore, it was decided that this study should be performed based on the Polish population. Data was collected in 1 region of Poland, the Lower Silesia.

The presented results are similar to those shown in the study of the Euro-Lupus. Despite the significant difference in the number of patients in groups, similar basic symptoms of SLE were observed in both cases. These include changes in joints and muscles, as well as skin.

However, a considerably lower number of documented changes in kidney disease symptoms, such as proteinuria, hematuria and pyuria were found at the beginning of the disease. Nevertheless, analyzing the incidence of nephropathy in the further course of SLE, its presence was observed in 50% of patients (Euro-Lupus 27.9%). Similar results were included in the research on the US population (55.6%). In the Asian population, nephropathy was present in 74% of patients. This shows that the variability in the incidence of particular symptoms differs significantly depending on the geographical region and needs to be studied in particular groups to determine the natural history of SLE.

Clinical manifestation of symptoms also varies with the duration of illness. Initial dominance of presentation of skin symptoms is later replaced by symptoms of the nervous system, such as headaches, mood disorders, cognitive disorders, and vascular changes. The incidence of depression drastically increased. This indicates the need for more frequent neurological, psychological and mental health consultations throughout the course of the disease.

SLE is a disease appearing mainly in young adults. In the present study, the vast majority (86%) of the first symptoms of SLE appeared at the age of 15–49 years, but 6% were observed at a younger age, and 8% in the elderly. In the group of 50+ years of age, female dominance is not as strongly pronounced (only 67%), while in the youngest group it is equal to 100% (in the whole study population 89%). In neither the younger nor the older age group was there a significant difference in the incidence of individual symptoms of SLE in comparison to the general population, as suggested by other studies.[5,10–13]

The observed time from the appearance of the first symptoms of the disease to the time when 4 of the ACR criteria were met was 5 years, and it is significantly longer compared to a European study (2 years).[5] This difference may be due to the milder initial course of the disease in the Polish population, or to other methods of collecting information (Euro-Lupus is a prospective study; the current study collected data retrospectively).

Although headaches are the most frequently reported neuropsychiatric symptoms, they have a low specificity for SLE and are classified as minor syndromes. Studies also demonstrated that headaches are not more common in people suffering from lupus compared to the population of healthy people, and characteristic features of the pain were not observed.[14,15]

Depression may also result from causes not directly related to the SLE disease process. It can be due to steroid therapy, among others, and likely depends on the predisposition of the individual. The appearance of seizures and vascular lesions in the brain (stroke, transient ischemic attack – TIA) are related largely to the presence of antiphospholipid antibodies, which are responsible for the occurrence of prothrombotic state.[16,17]

In the study population, the most common symptoms at presentation were changes in muscle and joint system, skin, general symptoms, and sensitivity to UV light. As the disease progresses, the percentage incidence of neurological symptoms and renal involvement are increased, while syndromes from joint and muscle system and the skin continue to be an important concern. The average time from onset of symptoms to diagnosis was 5 years. Profile of autoantibodies did not change during the course of the disease with anti-dsDNA and anti-SSA as the most common autoantibodies.

SLE as a systemic disease is characterized by the presence of many symptoms associated with the activity of various organs. Most of them are non-specific, and even those characteristics of lupus are present only in some patients. There is no typical clinical picture of SLE; therefore, the patient population suffering from this disease is very various. Differences in the course of the disease are also evident due to the geographic area. Therefore, early diagnosis can be a big challenge for any clinician, which justifies the need for this type of study to better characterize the disease.

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Heart infarct as the major cause of death of hematological patients as identified by autopsy

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

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Abstract

Background. Despite progress in diagnostic procedures, clinical diagnosis is not always confirmed by an autopsy. An autopsy is a valuable tool in evaluating diagnostic accuracy.

Objectives. The aim of the study was to compare clinical diagnoses of immediate causes of death with autopsy findings in patients with hematological malignancies or aplastic anemia.

Material and methods. In this study, the results of 154 autopsies (1993–2004) of patients with hematological diseases were reviewed and compared with clinical data. The most probable causes of death in the case of particular hematological diseases as well as the discordances between clinical and autopsy diagnoses and their relation to the clinical characteristic were identified in the studied cohort, which primarily included patients whose death at that particular time was not explained by the clinical course, and in 50% of cases was sudden.

Results. Although various combined infections have been found to be responsible for the largest number of deaths (26.6%), the most common single cause was myocardial infarction (29 patients, 18.8%). The discordance between clinical and post-mortem diagnoses of immediate causes of death was found in 55 patients (35.7%; 95% CI 28.2–42.8%), with 50.9% of cases considered class I discrepancies according to Goldman's criteria. The myocardial infarction was found to be clinically undiagnosed in 69% of cases. In 41% of cases, it was a class I discrepant diagnosis.

Conclusions. This data suggests that hematological patients require special attention and probably preventive measures concerning coronary heart disease, particularly during the initiation of antineoplastic therapy.

Key words: hematology, malignancies, cause of death, discordance, autopsy

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Introduction

In the past few decades, great progress has been made in the management of hematological malignancies. However, the mortality rates in these diseases are still high and the immediate causes of death are not always clear when only clinical data is taken into consideration. It is essential to elucidate the precise causes of mortality in hematological malignancies and aplastic anemia in order to properly focus the research efforts and to improve the management and treatment.

Previous studies in different medical specialties have shown that clinical diagnoses of causes of death are characterized by a high discordance rate (0–29%) with autopsy findings.[1–6] It could be even higher in specific groups with particularly severe or rare diseases, e.g., in cancer patients.[1,7,8] This suggests the post-mortem examination to be the best tool to verify the clinical diagnosis.[9–12]

In this study, we have compared clinically made diagnoses of immediate causes of death with autopsy findings in 154 patients with hematological malignancies or aplastic anemia. We intended to identify the most probable causes of death in different hematological diseases, the most frequently missed diagnoses and the discordance rate between clinical and anatomopathological investigation.

Material and methods

Patients

The study was performed in the Department of Hematology, Oncology and Internal Diseases at Medical University of Warsaw, Poland. After reviewing the reports of 1,179 cases of patients who died in the years 1993–2004 in this Department, it was found that 181 autopsies (15.6%) were performed, and from that group 27 cases (14.9%) were excluded because of incomplete records of either the clinical course of disease (2 cases), autopsy (23 cases) or both (2 cases). The remaining 154 cases (13.1% of all deaths) composed the study population, which was characterized by a median age of 50.9 years (±17.1), even sex distribution – 77 males (50%) and 77 females (50%), and clinical diagnosis and the stage of the disease as shown in Table 1.

The distribution of primary hematological diseases in the entire reviewed group of 1,179 cases is displayed in Table 1, together with the distribution of diagnoses in patients who underwent an autopsy examination.

Autopsy

Patients were selected for the autopsy in this Department on the basis of relatively uniform criteria, defined as follows:

- any death within 24 h of admission;
- death unexplained by the clinical situation of the patient, including sudden death;
- death while participating in a clinical trial;
- death after any form of hematopoietic stem cell transplantation.

For the purpose of this study, patients were categorized into early or advanced primary disease at the time of death. Early disease, for the purpose of this study, is the disease that should not be by itself an immediate cause of death and involves a patient who still has available treatment options. Advanced disease, for the purpose of this study, is the end-stage, progressive disease, not responding to treatment, which is most likely the immediate cause of death or results in such a deterioration of performance status that could make a patient vulnerable to any secondary cause of death.

In Poland, the law requires an autopsy for all patients who have died in hospital, but when there is no doubt regarding the cause of death, the Chief of the Department can evade this requirement upon written request from the patient's family. Routinely, autopsies are bypassed in the case of almost all patients with advanced diseases. Bypassing an autopsy is not permitted in the case of patients dying within 24 h of admission as it is legally assumed that the hospital did not have enough time to precisely identify the clinical problems of the patient. An autopsy is performed not earlier than 12 h after death, and in this hospital it is done usually on the next day, except for patients dying on a Friday and Saturday. Their autopsies are performed on a Monday.

A complete autopsy includes a gross examination of the viscera of the cranium, thorax, abdomen and pelvis, and a collection of tissue specimens that are used for subsequent microscopic evaluations, including special immunohistochemistry staining if necessary. Moreover, special attention to the requests of clinicians is given, who may suggest a collection of a larger than routine number of specimens for microscopic analysis from selected locations.

Data analyzed

The medical documentation was analyzed with attention to the patient's sex, age, hematological diagnosis and its prognosis, treatment given, time from diagnosis, length of the last hospitalization, comorbidities, suggested cause of death, presence of the *Aspergillus* spp. infection.

In each case, all the clinical data and autopsy records were evaluated by at least 2 physicians. All doubtful autopsy cases were reviewed by a pathologist other than the one who performed a given autopsy.

The patients were assigned to 3 age groups (18–42; 43–62; >62 years). Specific treatment for the disorders of the last period of life, chemotherapy, radiotherapy and bone marrow or peripheral blood stem cell transplantation were also taken into consideration. Time from diagnosis meant the time from precise diagnosis of hematological disease until death. The mode of dying was an indication whether the process of dying was rapid (hemorrhage, myocardial infarction, arrhythmia, pulmonary embolism or septic shock) or prolonged (others). Special attention

Table 1. Basic data on the evaluated population of hematological patients

Diagnosis	Total number of patients deceased	Number of autopsied patients (%)	Number of autopsied patients deceased in the early stage of primary disease n (%)	Number of discrepancies between clinical and autopsy diagnoses n (%)
Acute myelogenous leukemia (AML)	269	47 (30.5)	26 (33.8)	12 (21.8)
Chronic myelogenous leukemia (CML)	90	10 (6.5)	5 (6.5)	1 (1.8)
Other myeloproliferative diseases	30	3 (1.9)	2 (2.6)	2 (3.6)
Myelodysplastic syndrome (MDS)	47	5 (3.2)	4 (5.2)	3 (5.5)
Acute lymphoblastic leukemia (ALL)	79	15 (9.7)	5 (6.5)	7 (12.7)
Non-Hodgkin's lymphoma (NHL)	217	36 (23.4)	13 (16.9)	12 (21.8)
Hodgkin's disease (HD)	30	6 (3.9)	0 (0.0)	3 (5.5)
Chronic lymphocytic leukemia (CLL)	118	9 (5.8)	5 (6.5)	5 (9.1)
Multiple myeloma (MM)	215	13 (8.4)	8 (10.4)	5 (9.1)
Aplastic anemia (AA)	24	4 (2.6)	4 (5.2)	3 (5.5)
Other	60	6 (3.9)	5 (6.5)	2 (3.6)
Total	1179	154	77	55

Table 2. Goldman's criteria for major class missed diagnosis[17,39]

Goldman's class	Description
Class I	major misdiagnoses that could have direct impact on management
Class II	major misdiagnoses that would probably not have changed the management because of: • lack of appropriate therapy, • refusal of further evaluation or treatment, • cardiac arrest appearance without response to resuscitation, • application of appropriate treatment without diagnosis.

was given to the *Aspergillus* spp. infection because of its known high incidence in hematological patients.[13]

Discordance evaluation

The most probable immediate cause of death was defined on the basis of an autopsy study and compared with clinical diagnosis. As a result, the clinically suspected cause of death was classified as concordant or discordant with autopsy findings and categorized according to Goldman's criteria (Table 2).[5] An experienced clinical hematologist reviewed the doubtful cases. Minor misdiagnoses, not contributing to the immediate cause of death, were not considered. The group of 5 patients whose immediate cause of death was not identified even after an autopsy was excluded from further analysis.

The association between the presence of major class discrepancies and the clinical characteristics of the patients was evaluated. The characteristics comprised of the following factors: age (18–42 years; 43–62 years; >62 years), time from the diagnosis of primary hematological disease (<1 month; >1 month), stage of primary disease (advanced or early), duration of the last hospitalization (<8 days; 8–30 days; >30 days), mode of dying (rapid, prolonged), treatment with bone marrow transplantation (BMT) or peripheral blood stem cell transplantation (PBSCT), and presence of the *Aspergillus* spp. infection.

Statistical analysis

The data was processed using statistical analysis system (SAS) and Microsoft Excel software. Simple proportions were reported with their 95% confidence intervals (95% CI). The association between the clinical characteristics and discrepancy rates was analyzed using the χ^2 test. A p-value of 0.05 or less was considered to indicate statistical significance.

Results

The entire group of 154 autopsies consisted of 11 (7.1%) autopsies performed because patients died within 24 h of admission, 142 (92.2%) autopsies performed because of sudden or unexplained death, 8 (5.2%) autopsies performed on patients participating in clinical trials, and 22 (14.3%) autopsies performed on patients dying after earlier hematopoietic transplantation. Twenty-six (16.9%) autopsies were performed because of more than 1 reason.

The median time from the diagnosis of primary hematological disease to death was 494 days. Seventy-seven patients (50.0%; 95% CI 41.9–58.2%) died in advanced stages of their diseases. The median length of the last hospitalization was 26.5 days (±34.9). The mode of dying was rapid in 95 patients (61.7%; 95% CI 53.5–69.4 %) and did not

differ significantly between hematological malignancies. Nineteen patients (12.3 %; 95% CI 7.6–18.6%) had an active *Aspergillus* spp. infection at the time of death.

Immediate causes of death

Clinical diagnoses of death of patients who were forwarded for autopsy evaluation are listed in Table 3. The most frequent diagnoses of the immediate causes of death as determined by the autopsies of the evaluated patients are shown in Table 4. As it can be observed, the most frequent autopsy-identified cause of death was myocardial infarction. In almost each hematological malignancy, myocardial infarction (ischemia) was the most frequent pathological diagnosis of the immediate cause of death (Table 5). Also considering the age groups, myocardial infarction appeared to be the most common cause of death, characterized by a frequency rate increasing with age (Table 6).

Table 3. Clinical diagnoses of the immediate cause of death in hematological patients

Major clinical diagnosis	n (%)	Number of discordant diagnoses
CNS bleeding	15 (9.7)	6
Disseminated hematological disease	15 (9.7)	7
Pneumonia (bacterial)	13 (8.4)	1
Septic shock	12 (7.8)	6
Pulmonary embolism	11 (7.1)	4
Respiratory failure (undefined)	11 (7.1)	7
Myocardial infarction (ischemia)	10 (6.5)	2
Pulmonary edema	2 (1.3)	0
Gastrointestinal bleeding	8 (5.2)	2
Infection without septic shock	8 (5.2)	2
Adult respiratory distress syndrome	6 (3.9)	3
DIC	6 (3.9)	0
Pulmonary bleeding	6 (3.9)	2
Cardiopulmonary failure (undefined)	6 (3.9)	5
Cardiac failure (undefined)	4 (2.6)	4
Asystole	4 (2.6)	0
Peritoneal bleeding	3 (1.9)	0
Paralytic ileus	2 (1.3)	1
Hepatic failure	2 (1.3)	1
Intestinal perforation	1 (0.6)	0
Aspergillosis of CNS	1 (0.6)	0
Pulmonary aspergillosis	1 (0.6)	0
Acute renal failure	1 (0.6)	1
Encephalitis	1 (0.6)	1
Undefined	5 (3.2)	0
Total	154 (100)	55

CNS – central nervous system; DIC – disseminated intravascular coagulation; major clinical diagnosis – clinical diagnosis that served to estimate the discordance with autopsy diagnosis.

With regard to the patients with early stages of primary diseases, the most frequent immediate causes of death were myocardial infarction (15; 19.5%), bacterial pneumonia 9 (11.7%), and pulmonary embolism (7; 9.1%).

Considering the etiology, post-mortem diagnoses of the immediate causes of death were assigned to 9 categories (Table 7). In 5 patients (3.2%; 95% CI 0.4–5.6%), the exact cause of death was not identified, even after an autopsy.

The most frequent immediate cause of death in 22 patients with prior bone marrow or peripheral blood stem cell transplantation was aspergillosis (27.3%), mainly in the pulmonary location (4 out of 6 patients).

Discordances between clinical and anatomopathological investigation

The discordance between clinical and autopsy diagnoses of the immediate cause of death was found in 55 patients (35.7%; 95% CI 28.2–42.8%). The discrepancies were considered class I in 28 cases (18.2%) and class II in 27 cases (17.5%). The details of the discordant cases (class I) are displayed in Table 8. Myocardial infarction was the most frequent class I discrepant diagnosis (42.9%). Pulmonary aspergillosis, myocardial infarction and gastrointestinal bleeding were the most frequently clinically undiagnosed causes of death (Fig. 1). A more precise analysis of patients with myocardial infarction showed that 6 of them (20.7%) had a previously diagnosed cardiac disease. The remaining 23 patients with myocardial infarction had a 65.2% discrepancy rate. The major discrepancy in the diagnosis of myocardial infarction regarded patients in whom

Table 4. Autopsy diagnoses of hematological patients

Diagnosis	n (%)
Myocardial infarction (ischemia)	29 (18.8)
Pneumonia (bacterial)	17 (11.0)
CNS bleeding	11 (7.1)
Disseminated hematological disease	10 (6.5)
DIC	10 (6.5)
Pulmonary edema	9 (5.8)
Pulmonary embolism	9 (5.8)
Gastrointestinal bleeding	9 (5.8)
Infection without septic shock	8 (5.2)
DAD	7 (4.5)
Septic shock	7 (4.5)
Pulmonary aspergillosis	7 (4.5)
Pulmonary bleeding	5 (3.2)
Peritoneal bleeding	3 (1.9)
Unknown	5 (3.2)
Others	8 (5.2)

CNS – central nervous system; DAD – diffuse alveolar damage; DIC – disseminated intravascular coagulation.

clinical diagnoses included central nervous system (CNS) hemorrhage (5; 25.0%), septic shock (3; 15.0%) and disseminated hematological disease (3; 15.0%).

There were no significant differences in the discrepancy rate between patients with different primary hematological diseases (Table 1).

The discrepancy rate in patients with the *Aspergillus* spp. infection was 42.1%; however, in the group with the pulmonary location, it was 71.4%.

No significant association was found between a major class discordant diagnosis and age, diagnosis time and prognosis of hematological disease, duration of the last hospitalization, mode of dying, prior transplantations (BMT or PBSCT), presence of the *Aspergillus* spp. infection (Table 9).

Discussion

To the authors' best knowledge, this study concerns the largest series of autopsies of patients with hematological malignancies and aplastic anemia, and the only one that is focused on an actual, immediate cause of death.

The analysis concerned a group of patients with a fully diagnosed hematological disease. All of them were diagnosed, examined and treated in a highly specialized hematological unit, which assures the uniformity of patient care and decreases the risk of misdiagnosis.[1,14]

The study regarded only the patients with the post-mortem examination because it seems to be the best tool to verify clinical diagnosis.[1,5,8–10,14–16]

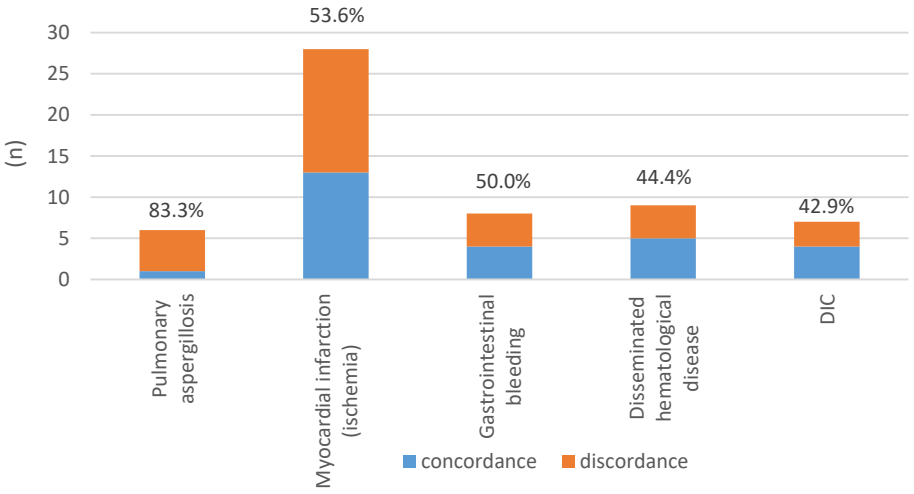

Fig. 1. The percentage of discrepancies between clinical and post-mortem diagnosis of cause of death (n = number of patients)

Table 5. The most frequent autopsy diagnoses of the immediate cause of death in various blood disorders

Hematological disease	The most frequent cause of death	n (%)
Acute myeloblastic leukemia	myocardial infarction (ischemia)	9 (19.1)
Non-Hodgkin's lymphoma	myocardial infarction (ischemia)	8 (25.0)
Multiple myeloma	myocardial infarction (ischemia) or pulmonary embolism	2 (16.7)
Acute lymphoblastic leukemia	CNS bleeding or respiratory tract bleeding	2 (13.3)
Chronic myeloid leukemia	pulmonary aspergillosis	3 (33.3)
Chronic lymphocytic leukemia	myocardial infarction (ischemia)	3 (33.3)
Hodgkin's lymphoma	myocardial infarction (ischemia)	2 (33.3)

CNS – central nervous system

Table 6. The most frequent causes of death by age

<43 years	43–62 years	>62 years
myocardial infarction (ischemia) 11.5%	myocardial infarction (ischemia) 19.6%	myocardial infarction (ischemia) 25.5%
pneumonia (bacterial) 11.5%	pulmonary edema 9.8%	pneumonia (bacterial) 13.7%
pulmonary aspergillosis 7.7%	disseminated hematological disease 7.8%	gastrointestinal bleeding 9.8%

However, the 15.6% autopsy rate and the limitation to the preselected group of patients may result in the lack of representativeness for the entire population of hematological patients and can lead to an overestimation of discrepancies. On the other hand, the clinical characteristics of the patients including age, sex, underlying hematological disease and its prognosis, length of patient care, length of the last hospitalization, suggest that the studied population was very diverse. The differences in the autopsy rate between primary hematological diseases probably result from an acute course of leukemia with a high frequency of death unexplained by the clinical situation of a patient.

The post-mortem study allowed us to identify the immediate cause of death in 96.8% of cases. Only close collaboration of pathologists and clinicians can make an autopsy study such an effective tool in defining the immediate cause of death.[7,14,17]

Various combined infections were the most frequent causes of death (26.6% of cases), especially in younger patients (<43 years; 36.5%). Such data is in accordance with the studies conducted by Goldman et al. (24%) and Gerrain et al. (23.5%) in cancer patients, still our values are lower than those reported by Nosari et al. (63%) in hematological patients, Manci et al.(60%) in sickle cell disease patients and Provencio et al.(43%) in patients with Hodgkin's lymphoma.[5,7,8,17,18] A more detailed analysis of infectious causes of death revealed that bacterial pneumonia, pulmonary aspergillosis and septic shock were the most frequent causes. Additionally, it was found that 24 out of 41 infectious cases (58.5%) involved the respiratory tract.

Table 7. Post-mortem diagnosis assignment to the autopsy categories

Autopsy categories	n (%)
Infectious	41 (26.6)
Myocardial infarction (ischemia)	29 (18.8)
Hemorrhages	28 (18.2)
Pulmonary edema / DAD	17 (11.0)
Disseminated neoplastic disease	10 (6.5)
DIC	10 (6.5)
Pulmonary embolism	9 (5.8)
Other	5 (3.2)
Unknown	5 (3.2)

DAD – difffuse alveolar damage; DIC – disseminated introvascular coagulation.

Table 8. Patients with class I discrepancy according to Goldman's classification

Diagnosis	Age (years)	Clinical cause of death	Post-mortem diagnosis	Prognosis
PMF	51	cardiac failure, undefined	pulmonary embolism	P
AA	54	respiratory failure	myocardial infarction (ischemia)	P
NHL	48	cachexy	pulmonary Aspergillosis	N
CLL	61	respiratory failure	myocardial infarction (ischemia)	P
CML	47	congestive heart failure	pulmonary Aspergillosis	N
AML	25	ARDS, gastrointestinal bleeding	pneumonia	P
ALL	18	CNS bleeding	septic shock	N
AML	36	cachexy	pulmonary Aspergillosis	P
NHL	46	gastrointestinal bleeding	myocardial infarction (ischemia)	P
AML	42	CNS bleeding, intestinal paralysis	myocardial infarction (ischemia)	N
MM	42	CNS bleeding	myocardial infarction (ischemia)	P
HD	70	respiratory failure, septic shock	myocardial infarction (ischemia)	N
HD	28	pneumonia, renal failure	disseminated hematological disease	P
CLL	65	pneumonia	pulmonary embolism	P
AML	76	pulmonary embolism	myocardial infarction (ischemia)	P
HD	42	cachexia	myocardial infarction (ischemia)	N
HD	28	respiratory failure, hepatic failure	pulmonary embolism	N
PMF	59	cachexy	myocarditis	N
NHL	62	stroke, CNS bleeding	myocardial infarction (ischemia)	P
ALL	67	respiratory failure (cerebral cause)	myocardial infarction (ischemia)	N
AML	38	cardiopulmonary failure, pneumonia	gastrointestinal bleeding	N
AML	68	CNS bleeding	myocardial infarction (ischemia)	P
8AML	53	respiratory failure, undefined cause	pneumonia	P
AML	32	cachexy	myocardial infarction (ischemia)	N
ALL	39	septic shock	gastrointestinal bleeding	N
NHL	34	cachexy	myocardial infarction (ischemia)	N

PMF – primary myelofibrosis; P – positive prognosis; N – negative prognosis.

The most frequent cause of death, when considering single pathology, was myocardial infarction (18.8% of cases). Furthermore, myocardial infarction was observed to be the most frequent immediate cause of death in all age groups (<43 years; 43–62 years; >62 years) and almost in each hematological malignancy. There are many factors that predispose hematological patients to heart failure: age, anemia, cardiotoxicity of chemotherapeutics, overload with fluids, increased body temperature, to name a few. Moreover, some symptoms of cardiac insufficiency may be masked by therapy or other complications, e.g., pain, tachypnea. The major discrepancy in the diagnosis of myocardial infarct considered CNS bleedings. The discrepancy is probably due to the unspecific symptoms that mask heart failure, and the diagnosis of CNS hemorrhage seems to be a safe guess for the doctor. The majority of patients who died of myocardial infarction (22/29; 75.9%) were exposed to chemotherapeutic agents of known cardiotoxicity, usually anthracyclines or radiotherapy.[12,19–22,23] To the authors' best knowledge, this is the first study reporting myocardial infarction as the most frequent cause of death.

The underlying hematological disease was found to be the immediate cause of death in 10 patients (6.5%). Such an observation differs from that reported by Provencio et al. in patients with Hodgkin's lymphoma (37%), but is similar to the observation of Gerrain et al. in cancer patients (11.8%).[7,8]

The discordance rate between clinical and post-mortem diagnoses of the immediate cause of death was 35.7%. This may seem high, but an autopsy was ordered primarily in cases where clinical diagnosis was doubtful or the cause of death was at least partially unexplained to the ordering physician. Previously conducted studies in hematological and oncological units showed a higher incidence of discrepancies.[7,8] A systematic review performed by Shojania et al., who evaluated 53 studies of autopsy series concerning patients with various disorders, other than hematological, revealed a range of major errors from 4.1% to 49.8%.[11] Our data is within these limits.

In this study, management would have been modified (class I discrepancies) in 18.2% of cases. It is apparently more frequently than in other studies of patients without neoplasia but less frequently than in the studies of oncological patients.[1,5,7,11,23,24] A higher rate of class I discrepancies reported by Xavier et al. in hematological patients (31.3%) may be due to the treatment in an unspecialized unit.[14] All the data might confirm that a highly complex course of malignant disorders might result from the elevated rates of discrepancies. The evaluation of the prognosis was performed in order to assess if the modification of management in class-I-discrepant patients could have influenced the survival. It was shown that 39.3% of class-I-discrepant patients were in the early stages of their primary diseases. This may suggest that an appropriate modification of management focused on early diagnosis and treatment of heart infarct may decrease the mortality rate.

An unexpected and previously not reported observation was that myocardial infarct or extensive myocardial ischemia was undiagnosed in 69% of cases. The clinically suspected causes of death in these patients were hemorrhages, especially cerebral, septic shock and disseminated hematological disease. This suggests more intensive attention to the cardiac problems in hematological patients.

Infection was misdiagnosed in 18 out of 41 cases (43.9%). This finding is in accordance with various studies.[5,7,16,25,26] However, the number of undiagnosed infections in some studies is higher.[18,27] A more detailed analysis showed that 42.1% of the *Aspergillus* spp. infections and 71.4% of pulmonary aspergillosis were overlooked. Donhuijsen et al. reported a high and increasing incidence of mycoses in hematological patients, which positively correlates with the present study.[13] As previously suggested by other authors, infectious processes are likely to remain undetected and their symptoms can mimic tumor progression.

Pulmonary embolism was observed as the most frequent missed diagnosis in various studies.[7,25,28] This was not confirmed by our study (33.3% of discrepant cases). The difference may result from the limitation of our group to hematological patients and previously reported decreasing incidence of misdiagnosed pulmonary embolism.[5]

Xavier et al. reported hematological disease to be the most commonly discordant diagnosis in hematological patients.[14] The present study was performed in a specialized hematological unit, which probably limited the rate of misdiagnoses.

Another purpose of this study was to assess the occurrence of discrepancies in accordance with clinical characteristics. No clinical feature showed a statistically significant

Table 9. Occurrence of discrepancy per clinical characteristic

Factor	Number of patients	Number of discrepancies n (%)	p-value
Age (years)			
18–42	44	19 (43.2)	0.47
43–62	42	14 (33.3)	
>64	43	13 (30.2)	0.94
Prognosis			
negative	60	27 (45.0)	0.06
positive	69	19 (27.5)	
Time of hospitalization			
≤7 days	30	9 (30.0)	0.60
8–30 days	65	24 (36.9)	0.91
>30 days	34	13 (38.2)	0.88
Diagnosis of hematological disease			
<1 month	25	6 (24.0)	0.21
>1 month	100	40 (40.0)	
<8 months	63	22 (34.9)	0.80
≥8 months	62	24 (38.7)	
Transplantation	19	8 (42.1)	0.77
Aspergillosis	14	6 (42.9)	0.82
Rapid death	79	22 (27.9)	0.03
Long-term death	50	24 (48.0)	

relation to major or class I discrepancies. Underrepresented studies regarding the association of a clinical characteristic with major class discrepancies reported various data, of which only care in a specialized unit was independently and inversely related to the occurrence of class I discrepancy.[2,14,29,30] In the present study, all the patients were hospitalized in a specialized unit, which excludes the possibility of such a relation.

The underlying hematological disease by itself rarely leads to death, while the complications of therapy and specific disorders appearing in its course in the majority of cases are responsible for the patient's death. An autopsy study together with a clinical investigation providing the information about the immediate cause of death focus the attention of clinical physicians on frequently misdiagnosed, life-threatening pathologies. It seems essential to perform comprehensive analyses of the results of post-mortem examinations to make the autopsy findings available to a wider group of physicians in charge of the patients, which might result in a decrease of discrepancies in the future.

Conclusions

This data suggests that hematological patients require special attention and preventive measures concerning coronary heart disease, particularly during the initiation of antineoplastic therapy.

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The relationship between QDASH scale and clinical, electrophysiological findings in carpal tunnel syndrome

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Abstract

Background. Carpal tunnel syndrome (CTS) occurs as a result of compression of the median nerve at the wrist. The Quick Disabilities of the Arm, Shoulder, and Hand (QuickDASH) questionnaire is a self-administered region-specific outcome instrument which measures symptom severity and functional status.

Objectives. The aim of this study was to evaluate the clinical and electrophysiological relationship with QDASH scale in CTS.

Material and methods. The study included 99 females and 22 males in total out of 121 idiopathic CTS patients with the mean age of 47.9 ±9.5 years. Patients were divided clinically and electrophysiologically into 2 groups as severe and mild based on modified criteria defined by Italian CTS working group. Pain severity was evaluated by visual analog scale (VAS). Patients were evaluated functionally by QDASH scale and the relationship between clinical and electrophysiological effect intensity (degree) was examined.

Results. QDASH scores were found significantly high in female patients, in patients with long disease duration (6 years and more), patients with clinically severe symptoms, and the ones with positive phalen test in both hands. Statistically significant positive relationship was found between QDASH scores, disease duration and clinical severity. However, no relationship was found between electrophysiological severity and QDASH. A mild and positive correlation was observed among disease duration, clinical severity and VAS. A small and positive correlation was detected between VAS and QDASH.

Conclusions. Although electrophysiological findings were prioritized in the follow-up and treatment strategies of CTS patients, clinical and patient-oriented assessment scales should be dealt together. Despite the electrophysiological findings, we believe that individual differences are effective in clinical and functional capacity. We conclude that since QDASH scale has a simple applicability in a short time, it can be used for assessing the symptom severity and disability of patients with CTS.

Key words: carpal tunnel syndrome, visual analog scale, electrophysiology, Quick Disabilities of the Arm, Shoulder, and Hand

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Introduction

Carpal tunnel syndrome (CTS) occurs as a result of compression of the median nerve at the wrist, and this constitutes about 90% of all entrapment neuropathies. CTS prevalence varies between 2% and 3% in the general population.[1] It is seen 3 times more often in women than in men. It is most commonly observed in women between the ages of 40 and 60 years.[2]

The most common symptoms are pain and paresthesia in the hand. Paresthesia is evident in the first 3 fingers and the radial side of the 4th finger. The symptoms get worse at night and patients try to reduce the physical disturbance by shaking their hands.[1] Sensory symptoms are also felt in areas other than the median nerve innervation and the whole hand is affected.[3] In addition, pain and paresthesia may spread to the forearm and shoulder; this phenomenon has been associated with central sensitization.[4,5] Some of the patients feel that their hand is swollen, even if it is not, and their complaints are evident in the morning. As the CTS progresses, paresthesia starts to occur also in the morning. In a further period, muscle weakness in the thenar area and atrophy occur. The thumb cannot do abduction and opposition. In a small number of cases, there may be findings of autonomic sympathetic nerve involvement in median nerve distribution area.[6] Continuous wrist extension and flexion, and repetitive hand movements aggravate the symptoms.

Paresthesia occurs in median nerve innervation area with the percussion of median nerve at the wrist (Tinel's sign) or passive wrist flexion (Phalen's maneuver). Although radiological methods such as ultrasound and magnetic resonance imaging (MRI) are used for diagnosis, electrophysiological studies are the gold standard.[7]

The Disabilities of the Arm, Shoulder and Hand (DASH) scale was developed by Hudak et al. in 1996 to evaluate the symptoms and physical functions of the patients with pathology of the upper extremities, and it was found to be reliable in evaluating CTS results.[8] The Quick Disabilities of Arm, Shoulder and Hand (QDASH) scale is a shortened and improved version of DASH, and the validity and reliability of Turkish version was shown in patients with CTS.[9,10] In this study, we aim to assess the patients with CTS by QDASH (functionally) and to examine the relationship between clinical and electrophysiological findings.

Material and methods

In this study, 121 patients with idiopathic CTS were evaluated according to the CTS diagnostic criteria of the American Academy of Neurology (AAN).[11] Patients with neurological, endocrinological, orthopedic, rheumatologic and upper extremity surgery history were excluded. The demographic data of the patients is shown in Table 1. Patients were examined in 2 groups as mild and severe (Table 2, 3) according to clinical and electrophysiological findings

Table 1. Demographic characteristics

Characteristics of patients	n = 121	%
Age		
mean ±SD	47.9 ±9.5	–
Gender		
female	99	81.8
male	22	18.2
Occupation		
worker	42	34.7
officer	7	5.8
housewife	72	59.5
Hand dominance		
right	114	94.2
left	7	5.8
Education		
illiterate	10	8.2
primary school	66	54.5
secondary school	16	13.2
high school	19	15.7
university	10	8.4
Duration of disease		
<1 year	55	45.5
2–5 years	47	38.8
6–9 years	9	7.4
≥10 years	10	8.3

Table 2. Clinical classification in CTS

Severity		Clinical history and objective findings
Mild	0	asymptomatic
	1	only night paresthesia
	2	night and day paresthesia
Severe	3	sensory loss
	4	in the median innervated thenar muscles atrophy and/or weakness
	5	paralysis in the median innervated muscles

Table 3. Electrophysiological classification of CTS according to median nerve conduction study findings

Severity		Electrophysiological findings
Mild	0	negative CTS: normal findings
	1	minimal CTS: in the palm–wrist segment abnormal sensory conduction study or abnormality in 4th f. comparative tests
	2	mild CTS: abnormal sensory transduction in finger (1, 2, 3)–wrist segment
Severe	3	moderate CTS: abnormal sensory transduction in finger (1, 2, 3)–wrist segment and prolonged motor distal latency
	4	severe CTS: absence of SNAP in the finger (1, 2, 3)–wrist segment prolonged motor distal latency
	5	extreme CTS: absence of CMAP and SNAP

CTS – carpal tunnel syndrome; SNAP – sensory nerve action potential; CMAP – compound muscle action potential; finger (1, 2, 3) – thumb, index and middle finger.

on the basis of modified criteria that were defined by Italian CTS working group.[12–14] Pain severity was evaluated by Visual Analogue Scale (VAS). Patients were examined functionally by QDASH scale and the relationship between clinical and electrophysiological response severity was examined. A neurological examination of the patients, electrophysiological examination and QDASH disability scale were conducted by 2 neurologists. Additionally, patients of treatments were recorded according to the disease duration. There were 55 patients whose duration of disease was >1 year. Out of that group, 22 patients were treated conservatively and the other 23 patients were not treated because they were newly diagnosed with CTS. The group of 47 patients whose duration of the disease was 2–5 years was only conservatively treated, e.g., by teaching patients how to use their hands in daily activities and how to use a splint. Patients whose duration of disease was 6–9 years and over 10 years were treated with medical, physical and kinesio therapies, and they also underwent surgery.

The study was carried out in accordance with the Declaration of Helsinki. Patients were informed about the details of the study. The study was approved by the local ethics committee of Dıskapı Yıldırım Beyazıt Training and Research Hospital (19/24–2015).

QDASH is a shortened version of the DASH scale that was developed to measure the physical symptoms and function of patients with upper extremity pathology. The questionnaire consists of 2 scales; one of them is the disability and symptoms scale with 11 items, and also an optional scale related to working, sport and playing instruments. There are 5 answer choices to each question. One point is given for the mildest symptom or functional status, 5 points for the most severe symptom or functional status. A score is calculated by the following formula: total points of n/n) – 1 × 25 (where n is the total number of questions) and if there is more than 1 unanswered questions, the QDASH score cannot be calculated. The score varies from 0 to 100. Our patients did not answer the questions in the optional scale because of the sociocultural situation, which is why the scale was not evaluated.

Electrophysiological examination was done by Dantec-Keypoint (Alpine Biomed ApS., Skovlunde, Denmark) using surface electrodes and the temperature of extremity was kept above 31°C. The median sensorial conduction velocity in the 2nd finger-wrist segment and ulnar nerve sensorial conduction velocity in 5th finger-wrist segment were studied antidromically. For median nerve sensorial conduction, velocity ≥50 ms for sensory nerve action potential (SNAP) and amplitude ≥20 μv were considered normal. For ulnar nerve sensory conduction, velocity ≥50 ms, SNAP amplitude ≥17 μv were considered normal. Also, it was interpreted in favor of CTS that in the 4th finger-wrist, segment median and ulnar sensory peak latency difference was longer than 0.5 ms.[15]

The median nerve motor conduction velocity was recorded from the abductor pollicis brevis muscle. It was stimulated in 8 cm proximally from the active electrode and the antecubital fossa. Motor distal latency <4.2 ms, conduction velocity ≥50 ms and over, and median nerve compound muscle action potential (CMAP) amplitude ≥4 mV were considered normal. Ulnar nerve was stimulated in 7 cm proximally from the active electrode, under and over the elbow and recorded from abductor digiti minimi muscle. For motor conduction velocity ≥49 ms was considered normal. The ulnar nerve CMAP ≥6 mV was considered normal.[15]

Table 4. The distribution of QDASH scores according to clinical and electrophysiological data

Characteristics of patient	QDASH	p-value
Gender		
female	52.5 ±20.1	0.0001*
male	32.7 ±19.5	
Duration of disease		
<1 year	43.3 ±22.4	
2–5 years	50.4 ±20.6	0.01*
6–9 years	64.3 ±7.2	
≥10 years	58.7 ±16.1	
Phalen right		
positive	54.5 ±19.4	0.0001*
negative	39.3 ±21.1	
Phalen left		
positive	57.0 ±19.9	0.0001*
negative	41.1 ±19.8	
Tinel right		
positive	52.4 ±20.1	0.01
negative	43.0 ±22.0	
Tinel left		
positive	52.1 ±21.7	0.10
negative	45.8 ±20.5	
Clinical severity		
mild	45.9 ±20.6	0.0001*
severe	71.9 ±7.8	
EMG severity		
mild	45.4 ±18.3	
severe	50.4 ±22.7	0.05
right severe – left mild	60.0 ±15.1	
left severe – right mild	32.7 ±14.0	

* $p < 0.5$ is statistically significant.

Statistical analysis

All statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS), v. 15.0 for Windows (SSPS; Chicago, USA). Descriptive values were stated as "number" and "percent". According to the data distribution, variables were defined as mean ±standard deviation or median (min–max). At the end of the evaluation, categorical data was compared using χ^2 test. According to the data distribution, to compare paired numerical data, the Mann-Whitney U-test or the Student's t-test were used. For the comparison of more than 2 pieces of data, the Kruskal-Wallis or the one-way ANOVA test were used. The relationship between the variables was tested by Pearson's or Spearman's correlation analysis, according to the distribution of the parameters. The significance level was set at $p < 0.05$.

Results

This study included 99 females and 22 male patients (the mean age was 47.9 ±9.5 years). CTS was found on 1 side in 24 patients, bilaterally in 97 patients. In total, CTS was detected electrophysiologically in 218 hands. QDASH values were found significantly high (Table 4) in female patients ($p = 0.0001$), in patients whose duration of disease is >6 years ($p = 0.01$), patients with severe clinical symptoms ($p < 0.0001$), and patients with positive Phalen's test in both hands ($p = 0.0001$).

In female patients ($p = 0.02$), in patients whose duration of the disease is >6 years ($p = 0.02$) and in patients with positive Phalen's test on the right hand ($p = 0.01$), with clinical ($p = 0.02$) and severe EMG findings ($p = 0.01$), VAS results were found significantly high.

In a correlation analysis, a statistically significant positive relationship was found between QDASH results, duration of the disease ($r = 0.30$; $p = 0.001$), and clinical severity ($r = 0.43$; $p = 0.0001$). Statistically significant positive mild relationship was found between VAS results, duration of the disease ($r = 0.28$; $p = 0.002$) and clinical severity ($r = 0.23$; $p = 0.01$). Statistically significant positive moderate relationship was found between severe clinical symptoms and EMG severity ($r = 0.27$; $p = 0.002$).

Discussion

In this study, we examined the relationship between QDASH scale and clinical electrophysiological findings in CTS. As stated in the literature, CTS was found more often in female patients and in the dominant hand (about 94.4% of the patients were right-handed).[1,2,15] Although CTS is usually bilateral, both clinically and electrodiagnostically, especially in idiopathic cases, the dominant hand may be affected more severely. In patients with a positive bilateral Phalen's test and a positive Tinel's test on the right, QDASH scores were high. The sensitivity of the Phalen's test ranged from 67 to 83% and its specificity from 40 to 98%. The sensitivity of the Tinel's test ranged from 48 to 73%, and its specificity from 30 to 94%.[16]

QDASH results were high in patients whose duration of the disease was >6 years and in patients with severe clinic symptoms. Also, in a correlation analysis, we found a positive correlation between disease duration and clinical severity with QDASH results. However, no relationship between the electrophysiological level and QDASH results was found.

Itsubo et al. have applied preoperative and postoperative QDASH disability scale to patients with CTS but they did not find any relationship between electrophysiological findings. They reported a correlation with QDASH results and clinical severity in patients who were evaluated postoperatively – in this study, similar results were obtained.[17] In addition, in various studies, it was stated that there was no relationship between scales that were used to evaluate symptom severity and functional status in CTS and electrophysiological findings.[17–19] Chan et al. did not find any relationship between symptom severity and functional status with electrophysiological findings in patients with mild CTS. They reported that all of the tests were required to be applied in evaluation of CTS.[18] Longstaff et al. did not find any correlation between symptom severity and electrophysiological findings in patients with CTS who underwent a preoperative and postoperative scan retrospectively.[19] De Campos et al. reported that in CTS, paresthesia, disability and nocturnal symptoms were not associated with electrophysiological findings.[20]

Electromyogram (EMG) is the most sensitive test for the diagnosis of CTS. In the literature, it is reported that the sensitivity of the median nerve studies ranges from 49 to 84%, its specificity from 95 to 99%.[6] However, as stated above, in the studies, no correlation was found between sensory symptom severity and electrodiagnostic data in CTS. This is mainly because in electrophysiological studies, thick myelinated Aβ fibers are evaluated. However, pain and paresthesia symptoms are related to the thin myelinated Aδ fibers, neuropathic pain is located in the unmyelinated C fibers. Thin and thick myelinated fibers are affected at different stages of CTS. Thin fibers are affected in the early stages of the illness. Another reason for the lack of correlation between sensory symptom severity and electrodiagnostic findings is the central sensitization and plasticity that is triggered by abnormal median nerve imputes.[21] In clinical studies, laser-evoked potentials, sympathetic skin responses and quantitative sensory tests show that thin fibers were affected in CTS. However, no relationship was detected with electrophysiological data.[21] Skin biopsy is the gold standard for demonstrating the affection of thin fibers. Recently, Schmind et al. found a reduction in the density of intra-epidermal fibers in CTS histopathologically, and it has been reported that there was no correlation between this result and electrophysiological findings.[22]

In other studies, it is suggested that in CTS symptom pathogenesis C-fibres are not effective but Aδ fibers play an important role, and in CTS a correlation was reported between spontaneous pain intensity and Aδ fiber function.[21,23] In our study, we found a mild correlation in the duration of the disease, clinical symptom severity and QDASH values with VAS scores that evaluated pain severity. Similarly, Koldas et al. reported moderate correlation between VAS and QDASH in their CTS QDASH Turkish version reliability and validity study.[10] İmaeda et al. found a correlation between VAS and QDASH in the validity and reliability study of QDASH scale.[24] Recently, Yücel et al. reported that QDASH was well correlated with pain and paresthesia, and they suggested that QDASH questionnaire appears to be more practical for carpal tunnel release patients.[25] Although VAS values were found high in the patients with severe electrophysiological findings, we did not find a correlation between electrophysiological severity level and VAS.

As a result, we found that in patients with CTS, QDASH values were related with clinical severity and the duration of the disease. Although we found a positive correlation between clinical severity level and EMG results, there was no corelation between QDASH results and EMG results. These findings are consistent with the inconsistencies between symptoms in CTS and electrophysiological data that was previously reported in the literature. However, in the clinical follow-up and treatment strategies of CTS patients, clinical, electrophysiological findings and patient-centered assessment scales should be considered together. Despite the electrophysiological findings, we think that individual differences are effective, while their clinical and functional capacities are being evaluated. We are of the opinion that QDASH scale can be applied in a short time and can easily be used for patients with CTS to evaluate the symptom severity and disability.

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Oxidative stress in colonic adenocarcinoma: An impact on the body's antioxidative status and oxidative protein damage

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

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Abstract

Background. Thus far, the pathogenesis of these intestinal tumors has not been fully explained. However, the analysis of risk factors and research regarding their formation that have continued for 3 decades have allowed us to demonstrate a significant role of oxidative stress in the processes leading to the development of cancer in the large intestine as well as in some other organs.

Objectives. The aim of the study was to examine the level of anti-oxidative status and the degree of oxidative protein damage in patients with varying severity of colonic adenocarcinoma (CAC) in relation to healthy individuals.

Material and methods. The study involved 4 groups (A–D) of patients with increasing severity of CAC stages according to Dukes' classification and a control group of healthy volunteers. Total antioxidant capacity (TAC) of blood plasma, as well as carbonyl (C=O) group contents in blood plasma proteins as a product of their oxidative damage, were estimated in all participants. Both parameters were determined by spectrophotometric methods using commercial kit to test TAC and 2,4-dinitrophenylhydrazine to assay the contents of C=O groups.

Results. In each of the studied groups, A–D, a statistically significant reduction in the TAC values was noted relative to the control group, which progressed with increased severity of CAC stages: 1.783 mmol/L vs 1.191 mmol/L (group A), 1.07 mmol/L (group B), 0.931 mmol/L (group C), and 0.899 mmol/L (group D). At the same time, significantly increased contents of protein C=O groups were observed compared to the controls, also progressive in the course of growing CAC severity: 0.496 nmol/mg protein vs 0.57 nmol/mg protein (group A), 0.689 nmol/mg protein (group B), 0.804 nmol/mg protein (group C), and 1.054 nmol/mg protein (group D).

Conclusions. The CAC-related oxidative stress considerably reduces the systemic anti-oxidative status and increases the protein damage; both those changes become worse in parallel with the progression of this cancer.

Key words: oxidative stress, total antioxidant capacity, colonic adenocarcinoma, oxidative protein damage

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Introduction

Malignant tumors of the large intestine are one of the main subjects of research on neoplasms, as they are on the 3rd top position in respect to morbidity and mortality rates, preceded only by breast cancer in women and lung cancer in men.[1] This applies especially to their histologically dominating form (>95%), that is colonic adenocarcinoma (CAC), in 62% of cases located in the distal section of large intestine: sigmoid and rectum.[2] Thus far, the pathogenesis of these intestinal tumors has not been fully explained. However, the analysis of risk factors and research on hypotheses of their formation that have continued for 3 decades have allowed us to demonstrate the significant role of the oxidative stress in the processes leading to the development of cancer in the large intestine as well as in some other organs.

Oxidative stress is the shifting of the body's oxido-reductive balance towards oxidative reaction, resulting from disorders of oxygen metabolism.[3] Its main cause is the excessive production of reactive oxygen species (ROS), which are toxic to cells. It has been documented that oxidative stress participates in the formation and development of numerous diseases, including cancer. This is due to the fact that exposure of cells to excessive amounts of ROS causes damage to the structural cellular macromolecules: lipids, proteins, nucleic acids and carbohydrates, and this in turn leads to pathogenic dysfunctions of cells.[4]

The body possesses, however, an anti-oxidative defense system, which protects it against ROS. It includes some specialized enzymes, non-enzymatic antioxidants (e.g., glutathione, vitamins C, E and many others), as well as some microelements.[5] In diseases with the intensive production of ROS, that is with the intense oxidative stress, the defense system becomes significantly weaker due to the accelerated depletion of its elements. In consequence, the body is much more susceptible to the detrimental influence of ROS.

Determining the degree of intensity of oxidative stress in the related diseases is, therefore, clinically valuable, as it aids in the rational diagnosis as well as control over the progress in the treatment. For this purpose, it is possible to assay either concentrations of the most important elements of the body's anti-oxidative defense system or the products of reactions between ROS and the components of the cells.

However, the assay of even only the most important antioxidants is difficult and laborious, and in total simply unfeasible. Instead of this, while undertaking the below described research, it was decided to assay the total anti-oxidative capacity (TAC). This is an acknowledged measure of the oxido-reductive balance, well reflecting the anti-oxidative properties of complex biological systems existing in the human body, e.g., blood plasma.[6] In order to simultaneously evaluate the destructive influence of ROS, it was decided to include in the research also the oxidative damage of proteins. This parameter was not as well tested as similar damages of lipids or DNA, even though proteins dominate among the elements of the human body with normal BMI (in obese subjects lipids are dominant and obesity is a risk factor for colon cancer). Therefore, the results of the respective tests relating to the proteins have special cognitive value. It is possible to assay 3 markers of damage of these fundamental macromolecules: the content of amino ($-NH_2$), thiol (–SH) or carbonyl (C=O) groups. Because of the fact that the latter seems to be the best marker of oxidative stress, it was decided that it would be applied in our research reported in this paper.[7]

The aim of this work was, therefore, to assess the status of the anti-oxidative defense system (based on the TAC assays), as well as the oxidative damage of proteins (based on the assay of C=O groups) in patients at various stages of CAC severity. At the same time, an attempt has been made to determine if there is any correlation between these 2 parameters.

Material and methods

Patients

The study group comprised 102 patients (42 women and 60 men; mean age for the entire group was 64.4 ±10.9 years), who underwent surgery on the premises of our department due to adenocarcinoma located in their rectum or sigmoid. This group was divided into 4 subgroups: A, B, C, and D, depending on the degree of the severity of the above-mentioned cancer according to Dukes' classification (Table 1). All the patients had normal BMI (there were no subjects with obesity).

The control group consisted of 20 healthy volunteers (8 women, 12 men; mean age for the entire group was 61.2 ±13.9 years) with the negative family history of CAC. All participants had not been smoking tobacco or taking any vitamins or microelement supplements for at least 6 months before the start of the research.

The clinical diagnosis was performed in accordance with the following pattern: a) physical examination and taking medical history; b) examination of large intestine (rectoscopy and/or colonoscopy with collection of samples

Table 1. Classification of participating patients with colonic adenocarcinoma (CAC) into subgroups according to Dukes' classification

Division data	Degree of clinical severity of CAC according to Dukes' classification			
Subgroup symbols	A	B	C	D
Number of patients	34	17	30	21
Women:men	15:19	6:11	12:18	8:13
Mean age [years]	63.1 ±12.8	65.9 ±9.4	65.9 ±9.0	62.6 ±10.2

for histopathological assessment; transanal ultrasound examination); c) imaging examination in search of distant metastases (ultrasound examination and/or CT scan of abdominal cavity, chest X-ray scan). Based on the above diagnostic tests, all subjects were preliminarily qualified for surgical treatment. In most cases surgical resection of the tumor was performed.

Tissue samples obtained during the surgeries were subject to histopathological assessment. The results constituted the basis for classifying patients into particular groups with respect to the degree of severity of the disease. But, in some patients, the extent of intrasurgically detected neoplastic proliferation was a cause of disqualification from surgical treatment (cases recognized as a non surgical).

Blood and plasma sampling

The analyzed material was blood (5.0 mL from each patient) drawn on an empty stomach from the basilic vein by means of Vacuette (heparinised tube) system. The material was temporarily stored in a refrigerator (4°C), and within no later than 2 h from sample collection centrifuged for 10 min in a MPW 360 centrifuge (Mechanika Precyzyjna, Warszawa, Poland) at 650 × g. Separated portions of plasma were moved to Eppendorf test tubes and stored at −80°C until the material was collected from all participants.

Biochemical determinations

The described further assays were carried out immediately after defrosting the collected samples. Spectrophotometric measurements were performed by means of a Lambda 14 P (Perkin Elmer, Überlingen, Germany) apparatus.

The Total Antioxidant Status Kit (Randox, Crumlin, United Kingdom), based on Miller et al. method, was used to assay TAC in blood plasma.[7] In accordance with the assay procedure, a synthetic water-soluble analogue of vitamin E (Trolox catalogue No. 238813-1G, Sigma-Aldrich, St. Louis, USA) was used as a standard. After performing the steps specified by the producer of the kit and carrying out the spectrophotometric measurements at 600 nm, the resultant values were expressed in terms of mmol/L Trolox equivalents.

The content of C=O groups in plasma proteins was assayed in accordance with Levine et al. method.[8] Proteins were precipitated from the analyzed samples with 10% trichloroacetic acid and the separated sediments were incubated in 37°C (1 h) with 0.2% solution of 2,4-dinitrophenylhydrazine in 2M HCl and dissolved in guanidine hydrochloride. After carrying out the spectrophotometric measurements at 370 nm and performing the steps specified by the recalculation method, the resultant values were expressed in terms of nmol/mg protein.

Ethics

The study was conducted in accordance with the Declaration of Helsinki and with principles of the Good Clinical Practice. The study protocol was approved by the Bioethical Commission of the Medical University of Lodz, Poland (decision No. RNN/586/07/KB). All participants agreed to take part in the tests voluntarily and gave their consent in writing.

Statistical analysis

The results were statistically analyzed by means of STATISTICA v. 6.0 software (StatSoft, Tulsa, USA). Results obtained in different groups of patients were compared to those of healthy individuals as well as between groups. Compliance of parameters with normal distribution was assessed using the Shapiro-Wilk test. Most results did not present normal distribution (analysis of variance ANOVA, Kruskal-Wallis test by rank); therefore, variables were compared between groups using the nonparametric Dunn-Bonferroni method. The level of statistical significance for the results of all tests was assumed to be $p < 0.05$. Evaluation of correlations between the results of TAC and C=O groups assays was calculated using Spearman's rank correlation coefficient r_s. Based on the obtained analytical data, a trend (regression) line was established between the sets of both types of the assayed parameters.

Results

Results of TAC assays performed in the plasma of patients with CAC at various stages of clinical severity (according to Dukes' classification), as well as in people from the control group (healthy volunteers) are graphically demonstrated in Fig. 1a.

It was found that in all stages of severity of the tumor, the values of plasma TAC were statistically significantly lower (1.783 mmol/L in the control group vs 1.191 mmol/L in group A, 1.07 mmol/L in group B, 0.931 mmol/L in group C, and 0.899 mmol/L in group D; $p < 0.05$).

At the same time, it was observed that the degree of decrease in the results for patients with CAC was becoming deeper with the progression of the disease. Differences between subgroups A and B, as well as B and C were statistically significant, while the difference between subgroups C and D did not show this significance.

The results of assays of C=O groups content in plasma proteins of patients with CAC at all stages of severity of the disease in accordance with Dukes' classification, as compared to the control group, are presented in Fig. 1b.

Statistically, significantly ($p < 0.05$) higher values of C=O groups in plasma proteins were observed at all severity stages of the cancer in comparison with the control group. It was found that the above mentioned values of the results

were increasing in patients with CAC to an extent which reflected the advancement of the disease (0.496 nmol/mg protein vs 0.57 nmol/mg protein in group A, 0.689 nmol/mg protein in group B, 0.804 nmol/mg protein in group C, and 1.054 nmol/mg protein in group D. The differences between the results obtained in all subgroups of participating patients were statistically significant.

Observations on the decrease in plasma TAC values progressing with the stages of severity of CAC, as well as the increase in C=O groups content in plasma proteins prompted us to analyze the mutual correlation between alternations of these 2 parameters. As a result of the performed analysis, a negative correlation was noted between values of plasma TAC and the content of C=O groups in plasma proteins (r_s = -0.566; $p < 0.05$) (Fig. 2).

Discussion

For many years now, TAC assays in clinically collected material (usually blood plasma) have had an acknowledged position in studies with patients suffering from diseases with oxidative stress, including cancer. This is due to the fact that they provide a comprehensive view of the status of the body's anti-oxidative defense system. Our own assays of TAC revealed a weakening of this system in patients with CAC. As it has been mentioned in the Results section, values of TAC in subsequent stages of CAC severity (according to Dukes' classification) were statistically significantly lower in comparison with the control group. The results of this study have been fully confirmed by the data presented in the references.

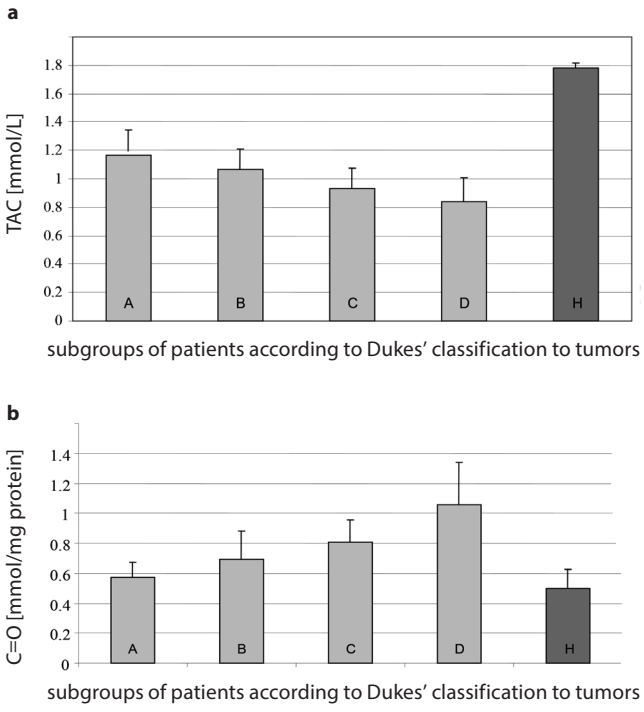

Fig. 1. Mean values of assays

a – total antioxidant capacity (TAC); b – contents of carbonyl (C=O) groups, performed in patients with various degrees of severity of the tumor. Mean values of both parameters for all subgroups were significantly different at $p < 0.05$ vs control group H (healthy).

Brodzki et al. noted decreased values of TAC in animals with malignant perianal tumors.[9] The same observations were made by Gupta et al. in patients with head and neck squamous cell carcinoma. These findings show that oxidative stress is elevated in cancer patients as evidenced by elevated levels of lipid peroxidation products – malondialdehyde (MDA).[10] In the study conducted by Subramanyam et al., the decreased levels of glutathione peroxidase activity and total antioxidant capacity, and increased malondialdehyde, glutathion reductase, were observed in cervical cancer patients when compared to healthy controls.[11] Decreased levels of TAC in patients with lung cancer, correlating with oxidative damages of proteins, were acknowledged by Erhola et al.[12] Similar observations were made by Hietanen et al. with regard to patients suffering from breast or prostate cancer, as well as colorectal carcinoma (CC), which was especially significant compared to the results of the research described herein.[13] Equally significant for the comparison of the obtained results were the studies carried out by Czeczot et al., who noted the decrease in TAC levels with the advancement of CC severity.[14] The weakening of the body's antioxidative status in accordance with the severity of cases was also observed by Saygili et al., but this research was restricted to stages B and C according to Dukes' classification (patients at stages A and D were not included in the research).[15] However, in that case, the authors did not assay TAC values, but only the chosen antioxidants: concentrations of glutathione and activities of dependent enzymes. Results of a study conducted by Kocot et al. indicated that in stages I, II and III of colorectal cancer, the total antioxidant status value was increased in tumor tissue as compared to healthy tissue and markedly decreased in stage IV in contrast to the other stages of the disease.[16]

The references also describe studies on assays of the destructive influence of ROS on the macroparticles of cells in clinical material, which are equally important for the research on carcinogenesis. As it was already pointed out, the most commonly analyzed were the products of lipid peroxidation, generally MDA and oxidative modification of DNA, especially 8-OHdG (8-hydroxy-2′-deoxyguanosine).[17,18] Much less common were assays of products of the adverse influence of ROS on proteins, even though proteins in various forms (including enzymes) are the main component of the body. As a result, the correlations between oxidative damages of proteins and the process of carcinogenesis have not been sufficiently explained. Stadtman et al. suggested, for example, that the abovementioned damages may be in vivo more biologically significant than lipid damages but, unfortunately, there are too few studies documenting this.[19]

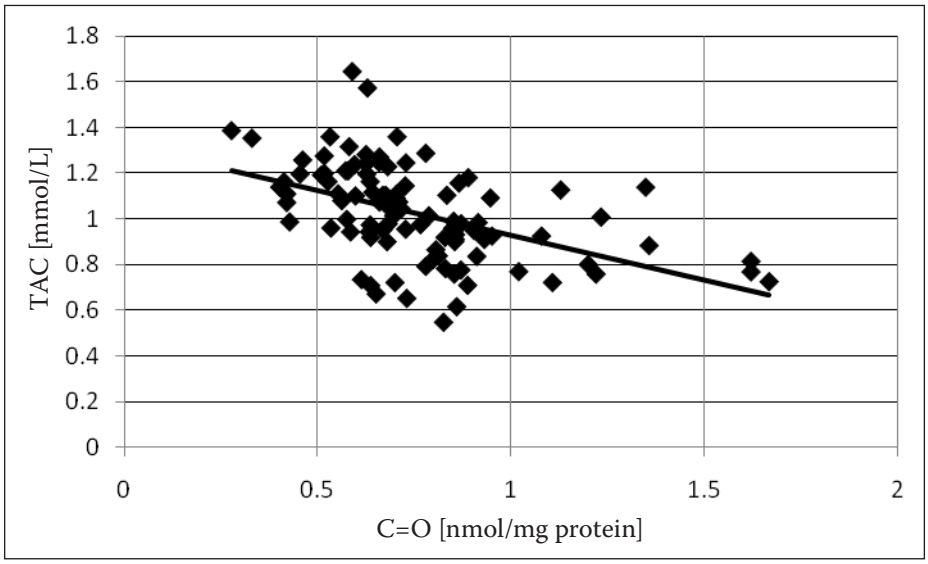

Fig. 2. Correlation between assayed values of total antioxidant capacity (TAC) and contents of carbonyl (C=O) groups: Correlation coefficient rs and trend line

An important opinion on the oxidative damages of proteins was expressed by us at the beginning of this paper. It was said that for the evaluation of the effects of ROS influence on proteins, the most reliable are the assays of contents of C=O groups. These groups are components of aldehydes or ketones, which are formed as a result of ROS oxidation of proteinaceous amino acid residues with free groups $-NH_2$, $-CONH_2$ or $-OH$, as well as during the splitting of polypeptide chains.[20] Formation of these carbonyl derivatives of proteins takes place during a reaction with ROS earlier than in the case of other products, which supports the opinion on the reliability of C=O group assays quoted above and convinced us to include them in the study.

Results of our assays of C=O group contents in plasma proteins, as presented in the Results section, were significantly higher in patients with CAC than in the control group. Moreover, the increase of this parameter was observed in each subsequent degree of CAC severity (according to Dukes' classification). Other researchers obtained analogous data with regard to other tumors.

Renke et al. noted that contents of C=O groups in plasma proteins of children with several types of malignant tumors were twice as high as in healthy children.[21] Yilmaz et al. observed significantly elevated values of this parameter in patients with bladder cancer and noted its additional increase in the invasive form, although the differences were not statistically significant.[22] The most important data for comparison with our results was obtained by Chang et al., who observed elevated contents of C=O groups in plasma proteins in CC patients, with a simultaneous decrease of activities of antioxidative enzymes and contents of vitamins C and E.[23] The same authors noted that patients with CC had a lower concentration of MDA in their plasma than healthy people, which indicates a greater sensitivity of assays of protein C=O groups for the evaluation of oxidative damages caused by ROS. Avinash et al. observed a significant increase in advanced oxidation protein products (AOPP), percent hemolysis (that indirectly indicates the degree of membrane damage secondary to lipid peroxidation), and a highly significant increase in globulin in colorectal carcinoma. Results of this experiment demonstrated oxidative stress, decreased antioxidant status, and secondary inflammatory response in colorectal carcinoma.[24]

The results of our study demonstrate the existence of the increased oxidative stress in patients with CAC. It seems, however, that it is an effect of the increased severity of the neoplasm rather than its cause. Such a conclusion seems to be confirmed by Erhola et al., who observed an improvement of competence markers of the body's anti-oxidative defense system after resection of the tumor.[12] According to these authors, this was due to the elimination of the oxidative stress caused by the tumor. A similar opinion was presented by Hamada et al., who studied mice with breast cancer, as well as by Lusini et al., who examined patients with kidney cancer.[25,26] These conclusions appear to be justifiable by the complexity of mechanisms regulating the processes of pro- and anti-oxidative balance.[27]

There are 2 interesting theories attempting to explain the shift of the balance in the prooxidative direction in patients with advanced tumors. The first is based on cancer-related nutritional disorders which are significant for these stages of tumors and which result in vomiting, lack of appetite or even anorexia. They prevent sufficient supply of anti-oxidative nutrients, indispensible for the elimination of excessive amounts of ROS. This effectively leads to oxidative stress.[28] The other theory assumes that the developing tumor progressively stimulates the body's immune system by increasing the production of pro-inflammatory cytokines and intensifies the inflammatory reaction. The increased activation of leukocytes intensifies the production of ROS and, as a result, causes oxidative stress.[29] This theory is confirmed by the increase in inflammatory markers (concentrations of CRP and IL-6 in plasma), correlating with the severity of the cancer, while the connection between chronic inflammation and oxidative stress is recognized as one of the "release mechanisms" of carcinogenesis.[28]

The conclusion from the first theory is of major importance for the research, i.e., an insufficient supply of nutrient antioxidants, including anti-oxidative vitamins, is weakening the cells' shield against ROS, which may lead, among others, to the initiation and stimulation of carcinogenesis.

Therefore, the opinions arising from epidemiological studies become obvious – a diet rich in these vitamins lowers the risk of developing various cancers, including CAC.[30,31] These substances prevent the transformation of cells and the proliferation and invasion of the existing tumors. The systemic effect of their use is the strengthening of the anti-oxidative defense system (that is why the study included exclusively subjects who did not take any anti-oxidative supplements).[31] This is a rational argument for systematic consumption of fruit and vegetables rich in the abovementioned vitamins as a preventative strategy against CAC, as well as for supplementing people from the groups of increased risk with relevant preparations as a form of chemoprevention, or as an adjuvant therapy for patients with CAC.

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Expression of $VEGF_{165}b$, VEGFR1, VEGFR2 and CD34 in benign and malignant tumors of parotid glands

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Abstract

Background. Vascular endothelial growth factor (VEGF) is an angiogenic factor and could be involved in the pathogenesis of salivary gland tumors. VEGF exerts its biological function by binding to its receptors, VEGFR1 and VEGFR2. An alternative splice variant of VEGF (VEGFxxxb) is an anti-angiogenic factor. Binding $VEGF_{165}b$ with VEGFR2 results in an impaired angiogenic response. The imbalance of VEGFxxx and VEGFxxxb isoforms can underpin pathological angiogenesis.

Objectives. The purpose of this study was to evaluate and compare the expression of $VEGF_{165}b$, VEGFR1, VEGFR2, and CD34 in benign and malignant parotid gland tumors and to explore the possible correlations between their expression and clinicopathological features of tumors.

Material and methods. The study was performed on archived paraffin-embedded tissue samples derived from 70 patients with benign and malignant parotid gland tumors (25 with malignant tumors, 23 with pleomorphic adenoma and 22 with Warthin's tumor). Immunohistochemical staining of selected tissue sections was performed using monoclonal antibodies. Immunohistochemical staining of selected molecules was used for evaluation of their expression in tissue sections.

Results. There were no statistically significant differences in the expression of the selected proteins localized in the tumor and surgical margin taken from the same patient. Expression of VEGFR2 correlated with $VEGF_{165}b$ in mixed tumors. There was a statistically significant difference in the expression of VEGFR1 in malignant tumors between females and males, and between the expression of VEGFR1 and the score of T classification in malignant tumors.

Conclusions. $VEGF_{165}b$ cannot be treated as a prognostic factor. VEGF receptors correlated with selected clinicopathological data of malignant tumors, indicating their possible role as a prognostic marker. The balance of VEGF isoforms have a limited influence on the development of parotid glands tumors. The correlation between $VEGF_{165}b$ and VEGFR2 in mixed tumors suggests the existence of an additional antiangiogenic pathway in poorly vascularized mixed tumors.

Key words: endothelial growth factor, angiogenesis, salivary gland tumors, CD34, Warthin's tumor

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Introduction

Malignant salivary gland tumors are highly invasive and frequently result in distant metastasis. The ability of cancer cells to invade stroma and then to metastasize to both the regional lymph nodes and distant tissue is a complex, multistage process. Tumor growth is regulated by the intensity of angiogenesis. Moreover, newly formed vessels have an impact on the ability of tumor cells to form metastases. Specific angiogenic molecules produced by tumor cells stimulate the surrounding capillary endothelial cells resulting in angiogenesis into tumor tissues. Of these, the vascular endothelial growth factor (VEGF) is the most potent factor in the neovascularization of several neoplasms of salivary glands, especially adenoid cystic carcinomas (ACC) and mucoepidermoid carcinomas.[1–6] VEGF is a selective mitogen for vascular endothelial cells, which promotes angiogenesis and induces vascular permeability and therefore its inhibition could be integrated into treatment strategies.[3] Several studies have evaluated whether VEGF immunohistochemical expression may be a prognostic factor for salivary gland carcinomas, but the results are conflicting or inconclusive. In our opinion, the possible source of these conflicting results may be the antagonistic antiangiogenic and proangiogenic properties of VEGF.

VEGF belongs to the platelet-derived growth factor (PDGF) superfamily and consists of glycoproteins with different structures and functions. It functions as a general tumor proangiogenic factor, but there are also different isoforms of VEGF with antiangiogenic properties. Two families of VEGF proteins are formed by an alternative splice-acceptor-site to give to 2 distinctive C-terminal sequences differing in their angiogenic properties. VEGF exerts its biological function by binding to its receptors: VEGFR1, VEGFR2 and VEGFR3. These 2 isoforms bind to VEGFR2 with the same affinity, but the binding of $VEGF_{165}b$ results in an insufficient activation of VEGFR2 and an impaired angiogenic response. The imbalance of $VEGF_{165}$ (proangiogenic) and $VEGF_{165}b$ (antiangiogenic) isoforms can underpin pathological angiogenesis. Primarily, the influence of $VEGF_{165}$ and $VEGF_{165}b$ on the cancerogenesis of colorectal, renal and breast cancers has been examined.[7–13] The imbalance of VEGF isoforms in the pathogenesis of salivary gland tumors has not yet been examined.

Another very important issue is the distribution of these isoforms and their receptors in intratumoral and peritumoral tissues. Their different distribution in tumor and stromal cells may have a crucial role for tumor invasiveness.[7] Although angiogenesis is difficult to measure directly in human tumors, there is increasing evidence that MVD may be an indirect marker of angiogenesis. One of the most common antibodies used for microvessel staining is CD34, which has been used in immunohistochemical studies to evaluate the intra- and peritumoral changes induced by VEGF.[14,15] Despite the progress made in recent years in understanding the role of VEGF in salivary gland tumors, more data is needed to better elucidate the relationship between the expression of VEGF isoforms and tumor prognosis. To address this need, we determined the prognostic potential of these markers.

The purpose of this study was to evaluate and compare the immunohistochemical expression of $VEGF_{165}b$, VEGFR1, VEGFR2, and CD34 in benign and malignant parotid gland tumors and in surgical margins from the same patients taken as a control group, and to explore the possible correlations between expression of these peptides and the clinicopathological features of tumors.

Material and methods

Study group

The study was performed on archived paraffin-embedded tissue samples derived from 70 patients with benign and malignant parotid gland tumors (25 with malignant tumor, 23 with pleomorphic adenoma, and 22 with Warthin's tumor), which were collected in the Department of Oncologic Pathology, Poznan University of Medical Sciences and in the Department of Pathomorphology, Collegium Medicum in Bydgoszcz, Nicolaus Copernicus University in Toruń, after surgical treatment of the primary tumor. Exclusion criteria included the presence of lymphoma, previous parotid gland surgery, and other parotid gland pathologies. Clinical examinations included ultrasound and, in selected cases, computed tomography (CT) or magnetic resonance imaging (MRI) to confirm the diagnosis. All tumors were described by location and size. Additionally, malignant tumors were evaluated for stage (7^{th} edition of the American Joint Committee on Cancer TNM staging), level of differentiation, regional lymph node metastasis status, and grade.[16] Tumor diagnosis was performed by pre- and postoperative histopathological examinations. The tumor diagnosis was performed independently by 2 pathologists. Disease-free tumor resection margins, located at least 2 cm from the tumor, were used as controls.

The protocol for this study was approved by the Bioethics Committee of Poznan University of Medical Sciences, Poland (No. 744/11). All patients provided signed written informed consent.

Tissue microarray paraffin blocks

Tissue microarray paraffin blocks (TMA) paraffin blocks were prepared for the evaluation of selected protein expression in the same location in the primary tissue sections. The primary paraffin embedded tissue sections (donor blocks) were re-embedded into paraffin-wax tissue blocks. Two tissue cylinders (2 mm in diameter) from each

case were selected from the "donor blocks", punched from marked regions, and subsequently placed into the recipient TMA blocks using 3DHISTECH TMA Master v. 1.14 (3DHISTECH Ltd, Budapest, Hungary).

Immunohistochemistry

The paraffin blocks were cut into 4 μm tissue sections using a rotary microtome (Accu-Cut®SMRTM200, Sakura, Japan). To establish immunohistochemical procedures, a series of positive control reactions were performed by determining the presence of antigens (The Human Protein Atlas).[17] The negative control reactions were performed on additional tissue sections during proper immunohistochemical staining, by substituting the primary antibody for a solution of 1% bovine serum albumin (BSA) in phosphate-buffered saline. Immunohistochemical staining was performed according to protocols described in detail elsewhere.[2] Immunohistochemical staining of selected tissue sections was performed using the monoclonal antibodies listed in Table 1. Epitopes were unmasked by Epitope Retrieval Solution high-pH (Dako Denmark A/S, Glostrup, Denmark) and then the slides were incubated overnight (16 h) with a primary antibody at 4°C. The antibody complex was detected with EnVisionFlex Anti-Mouse/Rabbit HRP Labeled Polymer (Dako Denmark A/S, Glostrup, Denmark). Antigens were localized according to the presence of a brown reaction product, using DAB as a chromogen. Finally, the sections were counterstained with hematoxylin, dehydrated in increasing grades of ethyl alcohol (80%, 90%, 96%, and 99.8%) and mounted with Shandon Consul Mount (Thermo Fisher Scientific, Waltham, USA).

The results were analyzed using a light microscope (ECLIPSE E800; Nikon Instruments Europe, Amsterdam, the Netherlands), and the level of expression was estimated using morphometric principles. To assess the level of protein expression, we used the modified Remmele-Stegner scale (Index Remmele-Stegner IRS – immunoreactive score), according to the intensity of expression and the number of cells/tissue area positively expressed in our previous publications.[2,18] Expression of $VEGF_{165}b$, VEGFR1 and VEGFR2 was localized in the cytoplasm and graded as follows: (–) negative; (+) weak positive staining; (++) moderate positive staining; and (+++) strong positive staining. Expression of CD34 was assessed as a positive (>5 blood vessels in the field of view) or negative (<5 blood vessels in the field of view) staining.

Statistical analysis

The calculations were carried out with the use of Microsoft Excel 2010 and STATISTICA v. 10 software (StatSoft Inc., Tulsa, USA). The distributions of continuous variables obtained at each step of data processing were evaluated for normality using the Shapiro-Wilk test. The age of each group is expressed as a median with an interquartile range (IQR). Categorical variables resulting from immunohistochemistry are presented in contingency tables and they were tested depending on the number of cases using the χ^2 test or two-tailed Fisher's exact test. For paired nominal data, the on-line McNemar's test with continuity correction was used.[19] Probability value <0.05 was considered statistically significant.

Results

There was a statistically significant difference in the expression of VEGFR1 in malignant tumors between females and males (Table 2). A statistically significant difference in the expression of VEGFR1 and a score of T classification in malignant tumors was found (Table 2). A statistically significant correlation between the expression of $VEGF_{165}b$ and VEGFR2 in pleomorphic adenomas was found (Table 3). There were no statistically significant differences in the expression of the selected proteins localized in the tumor and the surgical margin taken from the same patient (Table 4).

Discussion

Although it is clear that there is a relationship between the expression of VEGF and the development of salivary gland tumors, the exact nature of these relationships remains to be fully explained. In the present study, we analyzed the tissue expression of $VEGF_{165}b$, VEGFR1, VEGFR2, and CD34 in pleomorphic adenomas, Warthin's tumors and malignant tumors of the parotid glands to determine their potential value as prognostic and differentiating markers for these tumors. We also assessed expression of $VEGF_{165}b$, VEGFR1, VEGFR2, and CD34 to check for correlations in tumorous and non-tumorous tissues, finding no difference between expression of these molecules in these different tissues. VEGF receptors correlated with selected

Table 1. Primary antibodies used in immunohistochemistry (IHC)

Specificity	Animal	Type	Clone	Source	Catalog No.
$VEGF_{165}b$	mouse	monoclonal	7F17	Acris antibodies GmBH, Herford, Germany	DM3615P
VEGFR1	mouse	monoclonal	MM0001–7G96	Novus Biologicals, Abingdon, UK	NB110–60964
VEGFR2	rabbit	monoclonal	SP123	Acris antibodies GmBH, Herford, Germany	AM2104PU-M
CD34	mouse	monoclonal	QBend/10	BioGenex, Fremont, USA	AM236–5M

clinicopathological data of malignant tumors, indicating their possible role as a prognostic marker of salivary tumors. Taken together, these findings suggest that the balance of proangiogenic and antiangiogenic VEGF isoforms seems to have a limited influence on the development of parotid gland tumors, and therefore further research is required. $VEGF_{165}b$ cannot be treated as a prognostic and differentiating factor. The correlation between $VEGF_{165}b$ and VEGFR2 in mixed tumors suggests the existence of an additional antiangiogenic pathway in poorly vascularized mixed tumors.

There were some reports of correlations between the clinicopathological factors of salivary gland tumors and VEGF. According to Lim et al., increased VEGF expression correlated with lymph node metastasis, clinical stage, perineural invasion, vascular invasion, recurrence, and survival.[20] Similar results were obtained by Lequerica-Fernández et al., who found a relationship between VEGF expression and neck node disease, clinical stage, survival and local control of the tumor.[6] In our study, antiangiogenic $VEGF_{165}b$ did not correlate with better prognosis and a less severe clinical stage in malignant tumors. $VEGF_{165}b$ expression in intratumoral and peritumoral tissues was insufficient for the inhibition of tumor growth and for its invasiveness, or it was limited by $VEGF_{165}$ or other undetermined angiogenic factors.

The regulation of VEGF alternative splicing is unknown. VEGF gene expression is transcriptionally regulated by a diversity of factors including hypoxia, growth factors,

Table 2. Clinical characteristics of patients with malignant parotid gland cancers, pleomorphic adenoma and Warthin's tumor, and the association of selected parameters with the distribution of $VEGF_{165}b$, VEGFR1, VEGFR2 assessed as negative (–) for no or weak intensity of expression (0 and 1) and positive (+) for moderate and strong intensity of expression (2 and 3), and CD34 expression assessed as negative (–) or positive (+) by immunohistochemistry

Parameter, n		Protein											
		$VEGF_{165}b$			VEGFR1			VEGFR2			CD34		
		–	+	p-value	–	+	p-value	–	+	p-value	–	+	p-value
Malignant tumors	n = 25[#]	3	22	–	21	4	–	8	17	–	0	25	–
Age (years), 66 (26)*													
≤60	11	0	11	0.2300[a]	9	2	1.000[a]	5	6	0.3892[a]	0	11	1.000[a]
>60	14	3	11		12	2		3	11		0	14	
Gender, n													
males	16	3	13	0.2800[a]	16	0	0.0100[a]	5	11	1.000[a]	0	16	1.000[a]
females	9	0	9		5	4		3	6		0	9	
Location, n													
left side	10	1	9	1.000[a]	10	0	0.1245[a]	4	6	0.6668[a]	0	10	1.000[a]
right side	15	2	13		11	4		4	11		0	15	
Invasion, n													
deep/superficial	12	2	10	0.5930[a]	12	0	0.0957[a]	4	8	1.000[a]	0	12	1.000[a]
superficial	13	1	12		9	4		4	9		0	13	
Facial palsy, n													
yes	4	1	3	0.4217[a]	3	1	0.5269[a]	1	3	1.000[a]	0	4	1.000[a]
no	21	2	19		18	3		7	14		0	21	
Grade, n													
G1	4	1	3	–[b]	2	2	0.1288[b]	1	3	0.2802[b]	0	4	–
G2	11	2	9		10	1		2	9		0	11	
G3	10	0	10		9	1		5	5		0	10	
T classification, n													
T1	9	1	8	–[b]	6	3	–[b]	3	6	–[b]	0	9	–
T2	3	0	3		2	1		0	3		0	3	
T3	9	1	8		9	0		3	6		0	9	
T4	4	1	3		4	0		2	2		0	4	
N classification, n													
Nx	1	0	1	–[b]	1	0	–[b]	0	1	–[b]	0	1	–
N0	12	2	10		9	3		4	8		0	12	
N1	5	0	5		5	0		2	3		0	5	
N2	7	1	6		6	1		2	5		0	7	
M classification, n													
M0	22	3	19	1.000	18	4	1.000	7	15	1.000	0	22	–
M1	3	0	3		3	0		1	2		0	3	

* median (IQR); [#] *C. ductale* (n = 4); adenocarcinoma (n = 4); *C. planoepitheliale kera* (n = 4); *C. adenoides cysticum* (n = 3); *C. mucoepidermale* (n = 3); *C. myoepithelial* (n = 2); acinic cell carcinoma (n = 1); basal cell carcinoma (n = 1); *C. glandulae salivare* (n = 1); *C. neuroendocrine* (n = 1); *C. ex pleomorphum* (n = 1); TNM: T – size or direct extent of the primary tumor, N – degree of spread to regional lymph nodes, M – presence of distant metastasis; [a] two-tailed Fisher's exact test; [b] χ^2 for contingency tables.

Table 2. Clinical characteristics of patients with malignant parotid gland cancers, pleomorphic adenoma and Warthin's tumor, and the association of selected parameters with the distribution of $VEGF_{165}b$, VEGFR1, VEGFR2 assessed as negative (–) for no or weak intensity of expression (0 and 1) and positive (+) for moderate and strong intensity of expression (2 and 3), and CD34 expression assessed as negative (–) or positive (+) by immunohistochemistry (cont.)

Parameter, n		Protein											
		$VEGF_{165}b$			VEGFR1			VEGFR2			CD34		
		–	+	p-value	–	+	p-value	–	+	p-value	–	+	p-value
Pleomorphic adenoma	n = 23	10	13	–	20	3	–	12	11	–	0	23	–
Age (years), 44 (24)*													
≤60	16	9	7	0.0886	14	2	1.000	9	7	0.6668	0	16	1.000
>60	7	1	6		6	1		3	4		0	7	
Gender, n													
males	8	5	3	0.2213	8	0	0.5257	5	3	0.6668	0	8	1.000
females	15	5	10		12	3		7	8		0	15	
Location, n													
left side	19	8	11	1.000	17	2	0.4529	11	8	0.3168	0	19	1.000
right side	4	2	2		3	1		1	3		0	4	
Invasion, n													
deep/superficial	8	4	4	0.6850	7	1	1.000	4	4	1.000	0	8	1.000
superficial	15	6	9		13	2		8	7		0	15	
Warthin's tumor	n = 22	7	15	–	16	6	–	6	16	–	1	21	–
Age (years), 62 (5)*													
≤60	8	2	6	1.000	6	2	1.000	1	7	0.3512	1	7	0.3636
>60	14	5	9		10	4		5	9		0	14	
Gender													
males	15	5	10	1.000	11	4	1.000	3	12	0.3341	1	14	1.000
females	7	2	5		5	2		3	4		0	7	
Location													
left side	12	3	9	0.6517	9	3	1.000	4	8	0.6462	0	12	0.4545
right side	10	4	6		7	3		2	8		1	9	
Invasion													
deep/superficial	3	1	2	1.000	2	1	1.000	1	2	1.000	0	3	1.000
superficial	19	6	13		14	5		5	14		1	18	

* median (IQR); # *C. ductale* (n = 4); adenocarcinoma (n = 4); *C. planoepitheliale kera* (n = 4); *C. adenoides cysticum* (n = 3); *C. mucoepidermale* (n = 3); *C. myoepithelial* (n = 2); acinic cell carcinoma (n = 1); basal cell carcinoma (n = 1); *C. glandulae salivare* (n = 1); *C. neuroendocrine* (n = 1); *C. ex pleomorphum* (n = 1); TNM: T – size or direct extent of the primary tumor, N – degree of spread to regional lymph nodes, M – presence of distant metastasis; [a] two-tailed Fisher's exact test; [b] χ^2 for contingency tables.

such as insulin-like growth factor 1 (IGF-1), transforming growth factor β1 (TGF-β1), transforming growth factor α (TGF-α), oncogenes and tumor suppressor genes.[10] Ishibashi et al. postulated that hypoxia may be one of the factors inducing angiogenesis in salivary gland carcinomas by producing VEGF.[1] Distribution of these angiogenic factors in tumorous and non-tumorous tissues varies depending on the type of tissue and the local microenvironment. In our opinion, for a comprehensive assessment of the $VEGF_{165}b$ and $VEGF_{165}$ relationship and their distribution in tumor tissues, the influence of other possible splicing factors should be considered.

Among the molecules which were determined in our study, VEGFR1 expression correlated with the T score in malignant tumors. According to Younes et al., AEE788, a dual inhibitor of EGF and VEGF receptor tyrosine kinases, increased tumor and cell apoptosis, and decreased microvessel density, which correlated with a decrease in the incidence of vascular metastasis of salivary adenoid cystic carcinoma. This data showed that both receptors, EGFR and VEGFR, can be molecular targets for therapy of salivary ACC.[3] In the study by de Faria et al., VEGF and its receptor expression levels discriminated benign and malignant tumors of salivary glands but they cannot be treated as a predictor of metastasis from non-metastasizing tumors.[4] In our study, VEGFR1 was weakly expressed but correlated with the T score in malignant tumors. This result may suggest its important influence in tumor growth and invasiveness. VEGFR1 seems to be a more sensitive marker of tumor growth than VEGFR2. Although VEGFR2 was more strongly expressed in our study than VEGFR1, it did not correlate with clinical data for malignant tumors. In our opinion, these positive correlations reflected the increased levels of proangiogenic VEGF isoforms in malignant tumors. Expression of VEGFR2 resulted from both $VEGF_{165}$ and $VEGF_{165}b$ activity.

VEGFR1 expression in malignant tumors varied for different genders. This correlation was observed only in malignant tumors. In a previous study by Lim et al., the male gender was connected with shorter survival in salivary gland carcinomas.[20] Many studies have concentrated on the differences in survival in colon cancers according

to gender-dependent polymorphism of the *VEGF* gene.[21] Similar associations between gender, age and family history – dependent *VEGF* gene polymorphism and the increased risk of osteosarcoma – were determined in studies by Tie et al.[22] Patel et al., in contrast, did not observe any demographic and gender predispositions for Warthin's tumor compared to other benign salivary gland tumors. However, in the growth of benign tumors, VEGF and its isoforms seem to play a limited role.[23] Polymorphisms of *VEGF* and their receptors in salivary gland tumors have not been yet determined. Previously, only the influence of EGF, its receptor EGFR and their polymorphism in salivary gland carcinomas were explored. EGF and VEGF belong to the same family of growth factors and their functions overlap very often. In our view, a larger-scale study would be needed to explain the relation between gender and angiogenic factors in parotid tumors.

There were no statistically significant differences in the expression of molecules determined in the study and localized in the tumor and surgical margin taken from the same patient. Similar results were obtained by Tayama et al. In this study, VEGF was expressed on similar levels in both tumor and stromal cells. Tumor VEGF expression correlated with an advanced clinical stage, and VEGF expression in stromal cells was increased in the earlier clinical stage. Additionally, a splice variant revealed that tumor VEGF was mainly composed of $VEGF_{165}$ and that stromal VEGF included both $VEGF_{165}$ and $VEGF_{165}b$. The microvessel density tended to be lower in cases with higher $VEGF_{165}b$ mRNA levels.[7] Taken together, we suggest that the $VEGF_{165}b/VEGF_{165}$ ratio, especially in the surrounding stromal cells, can prove more important for tumor invasiveness or inhibition than the overexpression of these isoforms. Additionally, we did not explain what kinds of cells secrete $VEGF_{165}b$ and what factors induce $VEGF_{165}b$ expression. Malignant tumors of the salivary glands vary according to their histological structure and their sensitivity to VEGF-induced angiogenesis. Most studies have concentrated on salivary ACC. For comprehensive assessment of the role of VEGF in parotid gland tumors, a study conducted on a bigger and more homogenous group of malignant tumors is needed.

Table 3. Associations of $VEGF_{165}b$, VEGFR1, VEGFR2, and CD34 expression in malignant and benign parotid tumors

Tumor	Expression			$VEGF_{165}b$			VEGFR1			VEGFR2		
				–	+	p[a]	–	+	p	–	+	p
Malignant tumors	N			3	22	–	21	4	–	8	17	–
	VEGFR1	–	21	2	19	0.4217	–	–	–	–	–	–
		+	4	1	3							
	VEGFR2	–	8	1	7	1.000	7	1	1.000	–	–	–
		+	17	2	15		14	3				
	CD34	–	0	0	0	1.000	0	0	1.000	0	0	1.000
		+	25	3	22		21	4		8	17	
Pleomorphic adenoma	N			10	13	–	20	3	–	12	11	–
	VEGFR1	–	203	9	11	1.000	12	0	–	–	–	–
		+		1	2		8	3				
	VEGFR2	–	12	8	4	0.0361	0	0	0.0932	–	–	–
		+	11	2	9		20	3				
	CD34	–	0	0	0	1.000	16	6	1.000	0	0	1.000
		+	23	10	13					12	11	
Warthin's tumor	N			7	15	–	–	–	–	6	16	–
	VEGFR1	–	16	6	10	0.6158	–	–	–	–	–	–
		+	6	1	5							
	VEGFR2	–	6	3	3	0.3341	5	1	0.6341	–	–	–
		+	16	4	12		11	5				
	CD34	–	1	1	0	0.3182	1	0	1.000	0	1	1.000
		+	21	6	15		15	6		6	15	

[a] two-tailed Fisher's exact test; N – number $VEGF_{165}b$, VEGFR1, VEGFR2 assessed as negative (–) for no or weak intensity of expression (0 and 1) and positive (+) for moderate and strong intensity of expression (2 and 3), and CD34 expression assessed as negative (–) or positive (+) by immunohistochemistry.

Table 4 . Associations of $VEGF_{165}b$, VEGFR1, VEGFR2, and CD34 expression assessed as weak (–) or strong (+) between the tumor and surgical margins taken as a control in the groups studied

Parameter			Control											
			$VEGF_{165}b$			VEGFR1			VEGFR2			CD34		
			+	–	p*	+	–	p*	+	–	p*	+	–	p*
Tumor	malignant tumor	+	12	7	0.3588	0	3	0.6831	8	3	0.7237	20	2	.4795
		–	12	1		3	16		5	3		0	0	
	pleomorphic adenoma*	+	5	4	1.000	0	1	0.1306	4	3	0.3428	15	2	.4795
		–	3	4		6	10		7	2		0	0	
	Warthin's tumor	+	3	4	1.000	2	2	1.000	8	1	0.4795	9	2	1.000
		–	3	3		1	5		1	3		1	0	

* McNemar's test with the continuity correction for paired data.

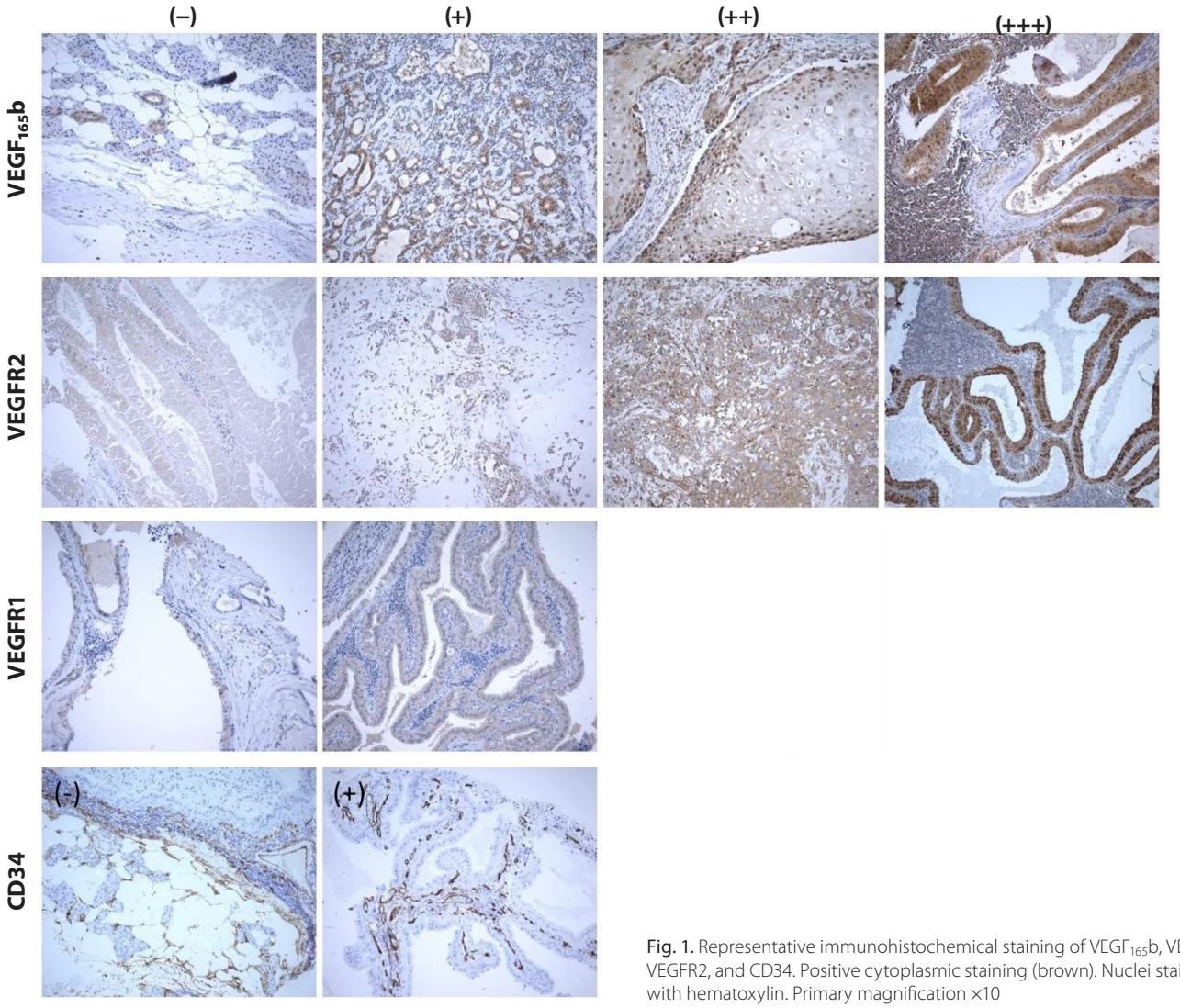

Fig. 1. Representative immunohistochemical staining of $VEGF_{165}b$, VEGFR1, VEGFR2, and CD34. Positive cytoplasmic staining (brown). Nuclei stained with hematoxylin. Primary magnification ×10

In our study, $VEGF_{165}b$ correlated with VEGFR2 in mixed tumors. This is a highly unexpected result, because the stroma of pleomorphic adenoma is always poor in terms of vascularity. This is a contradiction to the general idea of highly vascular neoplasm architectures on which antitumor therapeutic strategies have been devised. On the other hand, in the poorly vascularized tissues of mixed tumors, the hypoxic conditions promote higher VEGF expression. Swelam et al. demonstrated VEGF and its receptors in normal duct epithelial and myoepithelial cells as well as in tumor cells in ductal structures and in myxochondroid stromata. Additionally, they determined at least 4 proangiogenic VEGF isoforms, among which $VEGF_{121}$ was the most enhanced. They also observed higher HIF-1α levels in mixed tumors.[5] These previous results suggested that mixed tumor cells produce VEGF in several functional forms for their own proliferation or differentiation, and that VEGF expression is controlled by the hypoxic circumstances of poorly vascularized mixed tumors. Our results also revealed the existence of an antiangiogenic $VEGF_{165}b$/VEGFR2 pathway in mixed tumors. In our opinion, this pathway is strictly associated with the special histological structure or special conditions of a mixed tumor. However, we do not explain which mixed tumor cells might be a source of this antiangiogenic mechanism and whether it is primary or secondary to the proangiogenic $VEGF_{165}$, and which factors can foster its production.

The development of parotid gland tumors is not associated with deterioration in the balance between proangiogenic and antiangiogenic VEGF isoforms. $VEGF_{165}b$ levels did not correspond with a less severe clinical stage of parotid gland tumors. Furthermore, this cannot be treated as a prognostic or differentiating factor of malignant and benign parotid gland tumors. Without a comprehensive assessment of the $VEGF_{165}b/VEGF_{165}$ ratio in tumor and stromal tissues and the influence of their receptors, VEGFR1 and VEGFR2, its expression has limited importance. In our opinion, a more important issue is the ratio of proangiogenic/antiangiogenic VEGF isoforms in the tissue in question. VEGFR1 expression correlated with selected clinicopathological data of malignant tumors, indicating an additional field

for further research. VEGFR2 correlated with $VEGF_{165}b$ levels in mixed tumors. Its avascularity and complex histological structure with a high proportion of myxoid and chondroid contents seem to have a great affinity with antiangiogenic VEGF isoforms and requires further research.

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Imatinib in the treatment of chronic myeloid leukemia in children and adolescents is effective and well tolerated: Report of the Polish Pediatric Study Group for the Treatment of Leukemias and Lymphomas

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Abstract

Background. Chronic myeloid leukemia (CML) constitutes only 2–3% of all leukemias in pediatric patients. Philapelphia chromosome and BCR-ABL fusion are genetic hallmarks of CML, and their presence is crucial for targeted molecular therapy with tyrosine kinase inhibitors (TKIs), which replaced hematopoietic stem cell transplantation (HSCT) as a standard first-line therapy. The disease in pediatric population is rare, and despite molecular and clinical similarities to CML in adults, different approach is needed, due to the long lifetime expectancy and distinct developmental characteristics of affected children.

Objectives. The objective of this study is to evaluate treatment with imatinib in Polish pediatric patients with CML.

Material and methods. We analyzed the results of treatment with imatinib in 57 pediatric patients (June 2006 – January 2016) from 14 Polish pediatric hematology and oncology centers.

Results. In the study group, 40 patients continued imatinib (median follow-up: 23.4 months), while in 17 the treatment was terminated (median follow-up: 15.1 months) due to therapy failure. In the latter group, 13 patients underwent HSCT, while 4 switched to second-generation TKIs. The 5-year overall survival rate (OS) in the study group was 96%, and the 5-year event-free survival (EFS) was 81%.

Conclusions. Our results confirm that the introduction of TKI therapy has revolutionized the treatment of CML in the pediatric population by replacing the previous method of treatment with HSCT and allowing a high percentage of OS and EFS.

Key words: children, imatinib, adolescents, chronic myeloid leukemia

Introduction

Chronic myeloid leukemia (CML) is a myeloproliferative neoplasm typically diagnosed in the adult population and is relatively rare in children with the incidence of 0.6–1.2 million per year.[1] CML is a clonal disorder of hematopoietic progenitor cells resulting from the balanced translocation (9;22) called the Philadelphia chromosome (Ph) at the molecular level, resulting in the formation of a fusion gene *BCR-ABL*. The BCR-ABL protein encoded by the fusion gene exhibits tyrosine kinase activity and promotes uncontrolled proliferation of pluripotent stem cells in bone marrow (BM). CML has a 3-phase course: chronic phase (CML-CP); accelerated phase (CML-AP); and blast crisis phase (CML-BC). CML is most commonly diagnosed in the CML-CP and only in about 10% of cases in advanced phases: CML-AP or CML-BC.[2]

Before the implementation of imatinib, hydroxyurea +/- interferon alpha remained the first-line treatment of CML, followed by HSCT after achieving hematologic remission.[3] The identification of tyrosine kinase inhibitors (TKI) with BCR-ABL blocking ability revolutionized the CML therapy due to pharmacological control of leukemic clone. TKI-based therapy has proved to be very effective and quickly led to the withdrawal of HSCT as the first-line treatment. A first-generation TKI – imatinib was approved for the treatment of adult patients in 2001. Then, in 2003, it was approved, for the therapy in children. It should be emphasized that treatment with TKI is not a way to cure CML, as in most patients leukemia cells are still present. However, a unique feature of these drugs is a significant reduction of the risk of CML progression.[2] In the era of TKIs, the CML-CP can last beyond 20 years.

The recommended starting dose of imatinib for children is 260–300 mg/m^2 (max daily dose: 400 mg) in CP, 400 mg/m^2 (max daily dose: 600 mg) in AP, and 500 mg/m^2 (max daily dose: 800 mg) in the blastic phase.[3] In recent years, due to the development of the targeted therapy and the implementation of TKIs in pediatric patients, there has been significant progress in the treatment of CML, but the data is relatively limited due to low incidence of CML. One should remember that long-term side effects of TKI therapy may occur, because the drug has only been in use for approx. 15 years. Despite the excellent results of imatinib therapy, one should not forget about HSCT. It is the only method for obtaining definite cure of CML. HSCT is the first-line treatment in patients with CML who have become resistant to the first- and second-generation TKIs, or when serious side effects of the therapy occur and there is a matched donor available. In some cases, the preference of HSCT by the patient or his/her parents is also very important. It is worth noting that HSCT should be considered more frequently in patients diagnosed with CML before puberty due to growth impairment after TKI therapy. The transplant is in fact the only method that can lead to the cure of this disease.

Material and methods

We conducted a retrospective, nation-wide analysis of the imatinib therapy results in children and adolescents with CML in Poland. The study group consisted of 57 patients (M = 35, F = 22) from 14 Polish pediatric hematology and oncology centers treated in 2006–2016. The majority of patients (n = 54) were diagnosed in the chronic phase and only 3 patients in the accelerated phase. The diagnosis of CML was made according to the 4th edition of WHO classification of hematopoietic and lymphoid tissues, with mandatory molecular confirmation of BCR-ABL fusion. The first-line treatment with imatinib was performed accordingly to previous I-BFM recommendations. Patients and/or their legal guardians signed the appropriate consents.

The effectiveness of TKI therapy was evaluated on the basis of "milestones" of the therapy: complete hematologic remission (CHR); complete cytogenetic response (CCyR); and major molecular response (MMR). CHR was defined as leukocyte count <10 × 10^3/μL, <5% basophils and <450 × 10^3/μL platelets in peripheral blood, absence of myelocytes, promyelocytes and blasts in the peripheral blood, and non-palpable spleen during physical examination. Starting from the moment of CML diagnosis blood counts with blood smear should be performed every 15 days until achieving CHR, and then at least once every 3 months. Complete cytogenetic response (CCyR) was defined as the absence of Ph (+) cells in the bone marrow in classical cytogenetics or the FISH method, and evaluated within 12 months of the treatment.

Partial cytogenetic response (PCyR) was the presence of 1–35% Ph (+) cells in the bone marrow. Minor and minimal cytogenetic response was the presence of 36–65% or 66–95% Ph (+) cells in the bone marrow, respectively. Cytogenetic evaluation was performed after 3 and 6 months after the implementation of imatinib, then every 6 months until CCyR achievement, and every 12 months thereafter or in the case of treatment failure.

Molecular response was evaluated after 18 months of the treatment. Major molecular response (MMR) was defined as the BCR-ABL transcript level below 0.1% Molecular tests were performed every 3 months until MMR confirmation, and then not less than every 6 months. If there was an unsatisfactory response, testing for BCR-ABL kinase domain mutations was performed.

Statistical analysis

The statistical analysis was performed using STATISTICA v. 10.0. As events in the analysis of event-free survival (EFS) we considered the following: death of the patient; switch to second-generation tyrosine kinase inhibitors (2G TKIs) due to therapy failure or imatinib intolerance; proceeding with HSCT due to intolerance of imatinib and/or loss of cytogenetic or molecular response during imatinib therapy.

Results

Study group

The median age at CML diagnosis was 13.6 years (the youngest child was 1.2 year, the oldest 17.9 years). The most commonly reported symptoms at the diagnosis were: asthenia (n = 22); weight loss (n = 18); abdominal pain (n = 17); fever (n = 16); limb pain (n = 10); and hemorrhage (n = 7). Other reported symptoms included ecchymoses (n = 4); headache (n = 4); diplopia (n = 2); pallor (n = 2); cough (n = 2); priapism (n = 2); breast pain (n = 1); night sweats (n = 1); arthritis (n = 1); dyspnoea (n = 1); polydipsia (n = 1); hair loss (n = 1); and intramuscular hematoma (n = 1). The most frequent signs were splenomegaly (n = 43; median size of the spleen was 5.5 cm below the costal margin), and hepatomegaly (n = 34; median size of the liver was 3 cm below the costal margin). In 8 cases, the diagnosis was set on the basis of routine blood tests without other accompanying symptoms. Laboratory tests revealed significant hyperleukocytosis in the majority of patients (median 226.28 10^3/μL, range 7.17–810 10^3/μL) and thrombocytosis (median 471 10^3/μL, range 27.9–3444.7 10^3/μL). During the follow-up period, 2 patients died, both after transplantation. One of them died on day +307 after HSCT from central nervous system aspergillosis and multiorgan failure, while the other died on day +76 after HSCT from grade IV acute Graft vs Host Disease (aGvHD) and pulmonary hemorrhage. The 5-year OS in the study group was 96% (Fig. 1) and the 5-year EFS was 79% (Fig. 2).

Treatment with imatinib

All pediatric patients were qualified for first-line treatment with imatinib, according to CML-PAED II and I-BFM-CML protocol. Hydroxyurea was administered as pre-imatinib cytoreductive phase in 21 patients (median duration: 16 days). In 1 case, anagrelide was administered alternatively for the treatment of CML-associated thrombocytosis. The median time of imatinib implementation from the moment of diagnosis was 7 days (range 0–202 days). The median initial dose was 300 mg/m^2 (range 220–468 mg/m^2). The maximum dose was implemented in a single patient in CP, who had become resistant to the standard doses and experienced lack of CCyR and MMR in 12 and 18 months after the initiation of therapy, respectively. The dose of imatinib during therapy was modified in 23 cases. In 13 patients, the dose

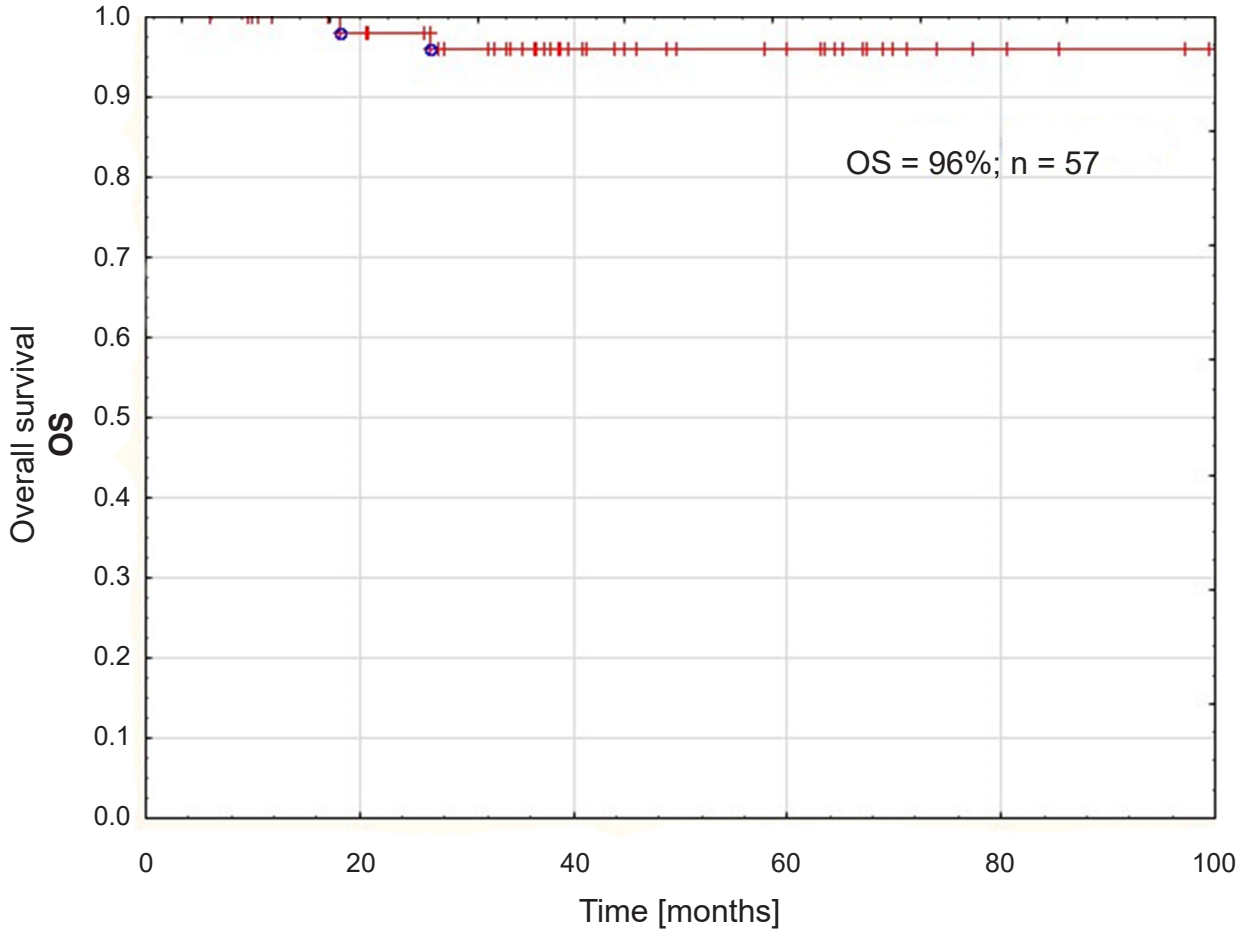

Fig. 1. 5-year overall survival (OS) in the study group (n = 57; alive = 55, dead = 2)

had to be increased from 300 mg/m^2 to 400 mg/m^2 due to resistance to imatinib therapy. In 10 patients, the imatinib dose was reduced due to toxicity (n = 8) or satisfactory response to a higher dose (n = 2) (median dose after reduction: 170 mg/m^2). Toxicity included myelotoxicity (WHO grade 4, n = 1); thrombocytopenia (n = 1); leukopenia (n = 5); and headache (n = 1). In 4 patients, 2G TKIs were implemented after imatinib: dasatinib in 3 cases, and nilotinib in 1 case. In 19 patients, due to unsatisfactory response to imatinib, testing for BCR-ABL kinase domain mutations was performed. In 3 cases, the T315I mutation was confirmed, and all these patients underwent HSCT afterwards. In 1 case, ponatinib achieved molecular remission.

Complete hematologic remission

Within 3 months complete chematologic remission (CHR) was achieved in 57 patients. In 2 patients, we observed hematologic relapse. One patient underwent HSCT afterwards, and the other was switched to 2G TKI-nilotinib and achieved another CHR.

Complete cytogenetic response

Within 12 months, complete cytogenetic response (CCyR) was evaluated in 45 out of 57 patients. In 4 cases, the follow-up period did not exceed 1 year, 4 patients underwent HSCT in less than 12 months since the start of the treatment, 2 patients were transferred to an adult ward before the evaluation, while in 2 patients cytogenetic response was not assessed. Within 12 months from the onset of therapy, CCyR was achieved in 31/45 patients (i.e., 69%). After 12 months, CCyR was observed in 9/45 cases (i.e., 20%). CCyR was not achieved in 5 cases (i.e., 11%). Out of the 5 patients who failed to achieve CCyR, 3 patients were switched to dasatinib, and 2 patients underwent HSCT afterwards.

Major molecular response

After 18 months, major molecular response (MMR) was evaluated in 46 out of 57 patients. In 5 cases, the follow-up period did not reach 1.5 year. Four patients underwent HSCT in less than 18 months since the start of the treatment,

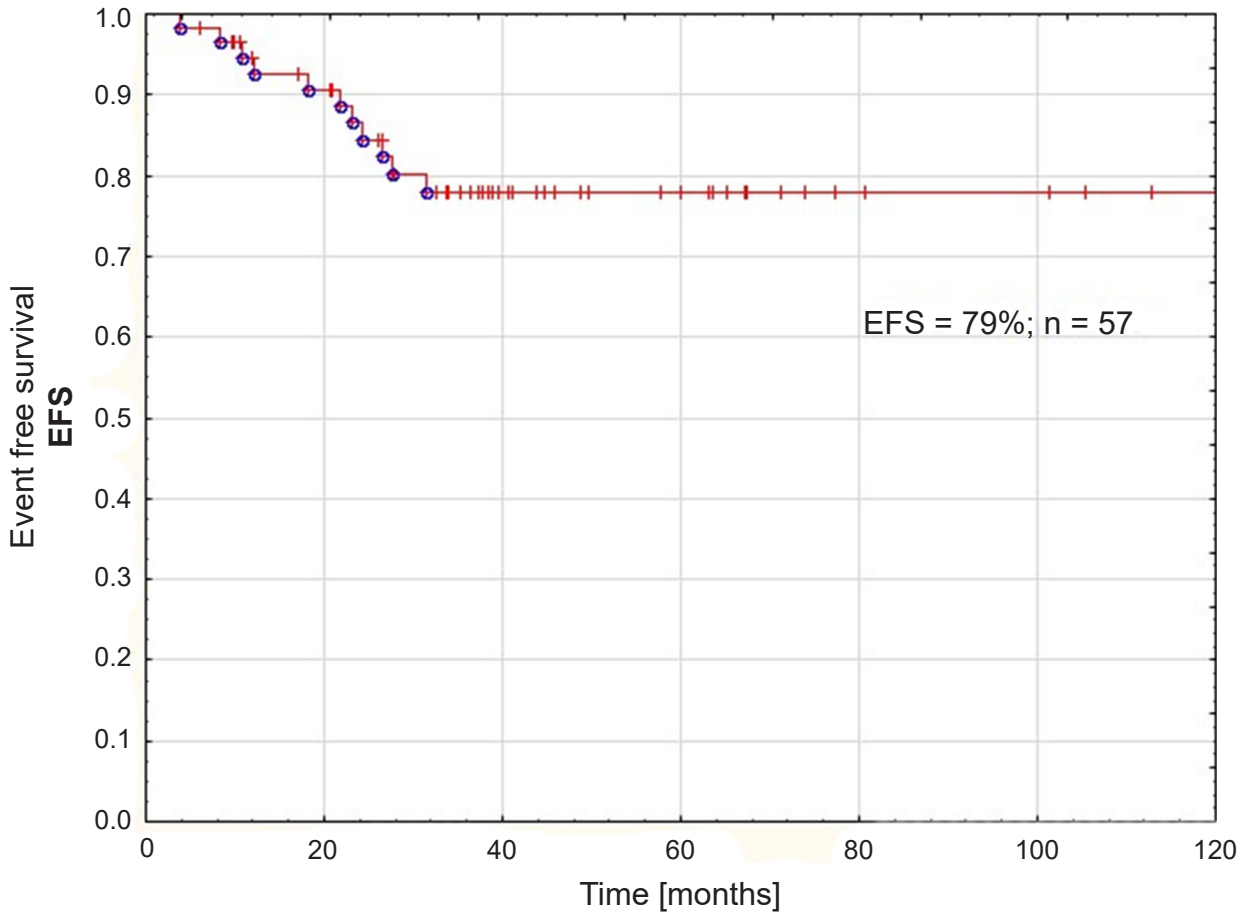

Fig. 2. 5-year event free survival (EFS) in the study group (n = 57)

event free = 45; events = 12 (death of the patient; switch to 2G TKIs due to therapy failure or imatinib intolerance; HSCT due to intolerance of imatinib and/or loss of cytogenetic or molecular response during imatinib therapy).

and 2 patients were transferred to an adult ward. MMR was achieved in 28/46 patients (i.e., 61%) after 18 months since the beginning of the treatment. MMR later than 18 months from therapy start was seen in 8/46 patients (i.e., 17%) with median time of 24 months. MMR was not achieved in 10/46 cases (i.e., 22%). Five of these patients underwent HSCT afterwards, and 2 were switched to 2G TKI-dasatinib. In 3 cases, the dose of imatinib was increased, with continuous decrease in the BCR-ABL level. Characteristics of the study group and the results of treatment are summarized in Table 1.

Table 1. Characteristics of the study group. Results of treatment with imatinib in 57 pediatric patients (June 2006 – January 2016) from 14 Polish pediatric hematology and oncology centres

Study group	Number of patients
Patients total	57
male	35
female	22
Phase at the diagnosis	
chronic phase	54
accelerated phase	3
Hematologic remission	
complete hematologic remission (CHR)	55
hematologic relapse	2
Cytogenetic response evaluable	45/57
CCyR within 12 months	31 (69%)
CCyR after 12 months	9 (20%)
failed to achieve CCyR	5 (11%)
switch to dasatinib	2
HSCT	3
Molecular response evaluable	46/57
MMR within 18 months	28 (61%)
MMR after 18 months	8 (17%)
failed to achieve	10 (22%)
HSCT	5
switch to dasatinib	2
increase of the imatinib dose	3

Hematopoietic stem cell transplantation

Hematopoietic stem cell transplantation (HSCT) was performed in 13 children (M = 9, F = 4). In 4 patients, the transplant was performed in the 1st CML-CP due to the local preferences of the center. In 3 patients, the reason for HSCT was the advanced phase of CML (CML-BC, n = 1; CML-AP, n = 2), and these children were transplanted in the 2nd CP (CML-CPII), after TKI treatment. Hematologic toxicity of imatinib was the reason for a transplant in 1 case. In 5 other cases, the loss of molecular and/or cytogenetic response were observed. The donors were either matched unrelated (MUD: n = 9) or sibling (MSD: n = 4). Median time from the diagnosis of CML to HSCT was 14.8 months (range 5.7–49 months). In 9 children, HSCT was performed in the 1st CP, and in 3 cases in the 2nd CP. In 1 case, a reduced-intensity conditioning regimen according to CML-SCT I-BFM study was introduced. So far, tests have confirmed 100% allogenic chimerism and non-detectable BCR-ABL (MR 4.5) in the patients' peripheral blood, but the follow-up period is too short (2 months) to confirm the complete success of transplantation.

Follow-up

Out of 57 patients enrolled in the treatment, 40 patients continue therapy with imatinib, while 17 completed the treatment. Thirty-five patients continue the therapy in pediatric centers (median follow-up: 33 months), and 3 patients in adult centers, with which we are in constant contact (median follow-up: 93 months), while 2 patients were lost to follow-up treatment after being transferred to adult centers (status at the last contact: continuation of the TKI therapy). Among patients who completed the treatment, 13 underwent HSCT (2 patients died due to complications of the post-transplant period), and 4 patients were switched to second-generation TKIs (dasatinib, n = 3; nilotinib, n = 1).

Discussion

Clinical data concerning the treatment of CML in pediatric population are limited because of its low incidence in children. It should be noted that for the purpose of our analysis we have gathered a relatively large group of patients (n = 57) compared with the available literature. The entire observation period amounted for 33 months in patients who continue the treatment in pediatric centers, and 93 months in patients who continue the treatment in adult centers.

Hematopoietic stem cell transplantation

Before the era of imatinib, HSCT was a standard first-line therapy. Among pediatric patients who underwent transplantation from MSD in the 1st chronic phase (CML-CP) in the years 1982–2004, EFS during the observation period of 3–5 years after HSCT ranged from 61 to 63%. OS ranged from 66 to 87%.[4,5] In transplant patients, MUD results were inferior, with EFS ranging from 27 to 55%, and OS from 45 to 65%.[4–7] The main cause of death in both groups was acute and chronic GvHD, more common in children after MUD-HSCT. In patients transplanted in the advanced phase (CML-AP or CML-BC) or the 2nd chronic phase (CML-CPII), the results were worse with EFS from 34 to 35%, and OS from 39 to 46%.[6] In our study group, only 13 patients underwent HSCT, which was mainly due to the good response to TKI therapy in most cases. OS among these children was 86%. We should remember that despite giving up the use of HSCT as a first-line therapy, it is still the only method by which we can completely eliminate leukemia cells and cure the disease.

Treatment with imatinib

One of the first trials with TKI in children was published in 2004 by the Children's Oncology Group and it presented promising results.[8] For the 2nd phase trial of this study, 31 pediatric patients who experienced failure of interferon therapy were qualified. In this group, all the children achieved CHR, and in 83% of them CCyR was observed. Another study covered 8 European countries and 30 patients were enrolled in it.[9] CHR was achieved in 80% cases, and CCyR in 60% of the patients enrolled in the trial in the CP and 29% of the children enrolled in the AP. In half of the children, MMR was reported. Similar results were published in France, in the 4th phase study conducted on a group of 40 patients.[10] The average duration of the follow-up period in this study was 16 months. CHR within 3 months was achieved in 86% of the patients, and within 6 months in 98% of the patients. CCyR was observed in 62% of the children within 1 year after the inclusion of imatinib therapy, in 34% of them MMR within 18 months after initiation of the treatment was confirmed. Similar results were achieved in a German clinical trial CML-PAED II published in year 2009.[11] Out of 42 enrolled patients, 40 achieved CHR within 3 months. In 26 out of 28 patients, CCyR was observed within 1 year after the start of the treatment, and in 17 out of 19, MMR after 18 months from the onset of the treatment. It should be emphasized that all patients from our study group are simultaneously registered in an international database I-CML-Ped Study, so far consisting of 351 children diagnosed with CML. This study, aimed at optimizing the treatment of CML in children, is still ongoing. Preliminary results presented at the 56th American Society of Hematology (ASH) Annual Meeting are promising and comparable with the results of treatment achieved in the Polish population.[12] Our study group consisted of 57 pediatric patients diagnosed in Poland with a median overall follow-up period of 31 months. CHR within 3 months after the implementation of the treatment with imatinib was documented in all patients. CCyR after 12 months of therapy was observed in 69% of patients, while MMR in 61% of patients after 18 months of treatment. These results correspond with the quoted literature, in particular with the results of Suttorp et al.'s analysis from 2009, and the results of treatment in the context of clinical trial I-CML-Ped Study.[11,12] Moreover, when comparing the proportion of patients achieving CCyR and MMR, our results are above promising. However, despite the excellent results of imatinib therapy, one should not forget about HSCT. It is worth noting that HSCT should be considered more frequently in patients diagnosed with CML before puberty due to the fact that long-term TKI intake may in the future be the cause of short stature among these patients.[13]

Side effects of imatinib

TKI are usually well tolerated; however, some side effects may occur. In most cases, they are classified as mild to moderate and occur mainly in patients in whom TKI therapy was introduced in advanced phases of CML. Non-hematological, relatively common side effects include nausea; vomiting; diarrhea; skin rash; swelling; limb pain; muscle spasms; bone and joint pain; headaches; weight gain; and an increase in liver enzymes.[4,9,10,14] In one of our patients, we documented severe headaches, which resolved after a temporary decrease in the dose of imatinib. Millot et al. described neutropenia grade 3 or 4 in 27% of the children receiving imatinib; thrombocytopenia grade 3 or 4 in 5%; and anemia grade 3 or 4 in 2.5% of the patients.[15] However, these cytopenias were treatable by temporary discontinuation of the therapy or the administration of granulocyte-colony stimulating factor (G-CSF) in some children with neutropenia. In 5 of our patients, we observed leukopenia, and in 1 thrombocytopenia, which also resolved after a temporary dose reduction of imatinib. Only in 1 case, myelosuppression as a side effect after imatinib implementation was a reason for HSCT. Despite the potential cardiotoxicity, hepatotoxicity, immune disorders and thyroid gland dysfunction observed in adults treated with imatinib, they have not been documented so far in children.[16] In the group of pediatric patients with CML, the aspect of TKI therapy impact on bone metabolism is very important. Imatinib impairs the differentiation and reduces the activity of osteoblasts and osteoclasts. This can result in growth retardation in children, particularly those who are starting the treatment in prepubertal age.[13,17,18] In our study group, we observed no impact of imatinib on calcium and phosphate metabolism. We also noticed no abnormalities in serum phosphate, calcium, parathyroid hormone (PTH) and vitamin D levels or tubular function disorders (phosphate absorption). However, we should keep in mind that particular attention should be paid in the group of the youngest patients chronically receiving TKI. They require regular and detailed clinical evaluation, and performing the panel of basic laboratory tests during each visit. Our results confirmed that the recommended daily dose of imatinib is well tolerated in pediatric patients, and severe side effects are relatively rare.

Second-generation tyrosine kinase inhibitors

2G TKIs – dasatinib and nilotinib – were registered for the treatment of adult patients with CML in 2006, and dasatinib only for the therapy of children in 2007. Nilotinib is not currently recommended for the treatment of pediatric patients. 2G TKIs are recommended when intolerance or resistance to imatinib occur.[19,20] They are more effective in the treatment of CML due to the linking of both active and inactive conformations of the BCR-ABL protein. In addition, they show greater activity in the case of mutations of *BCR-ABL* gene, associated with resistance to TKI therapy.[21] Unfortunately, there are an even more limited number of studies on the use of 2G TKIs

in children. The first reports are from 2011 as the results of the 1st phase clinical trial conducted by the Children's Oncology Group. Thirty-nine patients were enrolled for the treatment with dasatinib, including 9 with CML who were resistant to imatinib or who had had an adverse event after using it. In 8 patients, cytogenetic response was observed, in 3 CCyR, in 3 PCyR, in 1 minor, and in 1 minimal cytogenetic response. In 1 patient, the cytogenetic response was not possible to assess.[22] The 1st phase study CA 180–018 from 2013 was conducted on a group of 63 pediatric patients, 17 of whom were enrolled in the chronic phase of CML, and only 3 in the advanced phase. Among patients with CML-CP, CHR was achieved in 94%, CCyR in 82%, and MMR in 47%. Patients enrolled in the study in the advanced phase of CML achieved slightly worse results.[23] In our study group, dasatinib was administered in 3 patients, while nilotinib in 1 case. In all 4 cases, these drugs were implemented due to the failure of imatinib treatment. Patients continue the treatment with 2G TKIs with a satisfactory outcome, and no side effects have been documented. Clinical trials on the use of 2G TKIs are being carried out and are raising great hopes, especially in patients in whom imatinib has proven to be ineffective.

Discontinuation of the treatment

Observations in adult patients show that during the TKI therapy and after documenting the undetectable level of BCR- ABL over a period of 24 months or more, one can try to discontinue the treatment with imatinib.[24–26] In approx. 40% of these patients, one can confirm continuous MMR despite cessation of the therapy. In our study group, the treatment was discontinued in 1 patient after continuous 26 months of the undetectable level of BCR-ABL. The patient remained under constant control and, after 11 months of continuous MMR, molecular relapse was confirmed (level of BCR-ABL transcript 3%). Imatinib was reintroduced and the patient achieved another MMR within 3 months. The results of controlled trials evaluating the efficacy of TKI withdrawal in the groups of pediatric patients are promising, but they have not been published yet.[27] These results will be very important because of the potential side effects of long-term therapy with imatinib.

Conclusions

Our results confirm that the introduction of TKI therapy has revolutionized the treatment of CML in the pediatric population by replacing the previous method of treatment with HSCT and allowing a high percentage of OS (96%) and EFS (81%). Although the use of TKIs and 2G TKIs is not a cure for CML, and only reduces the risk of disease progression significantly, the results of ongoing clinical trial evaluating the safety of the treatment withdrawal after confirming a continuous 24-month undetectable level of BCR-ABL are promising. Despite the initial enthusiasm due to the excellent results of TKI therapy, there are more reports confirming that the use of imatinib is not devoid of serious side effects. However, in our study group, we observed in only 1 case myelotoxicity WHO grade 4, which was the reason for HSCT. We should keep in mind the fact that the goal of the therapy in pediatric patients should be rather to cure the disease than to suppress it, which can be achieved only by performing HSCT. In the context of very promising results of HSCT in pediatric patients with CML after reduced-intensity conditioning regimen, HSCT should be taken into consideration, especially in prepubertal children.

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Elevated serum concentrations of metalloproteinases (MMP-2, MMP-9) and their inhibitors (TIMP-1, TIMP-2) in patients with Graves' orbitopathy

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Abstract

Background. Graves' orbitopathy (GO), also known as thyroid-associated ophthalmopathy, is characterized by dramatic tissue reactivity. Both inflammation and tissue remodeling characterize the clinical course of GO. Some data has been found regarding the association of MMPs and TIMPs in GO.

Material and methods. Serum concentrations of MMP-9, MMP-2, TIMP-1, and TIMP-2 were determined by ELISA method.

Objectives. Forty-eight patients (34 females, 14 males, with median age 51.5 years) with GD and hyperthyroidism were enrolled in the study. In 28 patients, active, moderate-to-severe grade orbitopathy was diagnosed. The aim of this study was to assess the serum concentrations of MMP-2, MMP-9, TIMP-1, and TIMP-2 in patients with Graves' disease (GD), with and without GO, and their relationship with disease severity, as well as to evaluate how these concentrations change after successful treatment.

Results. Median serum concentrations of MMP-2 and MMP-9 were significantly higher in all patients with GD as well as in the subgroup with GO than in the control group. Median serum concentrations of TIMP-1 and TIMP-2 were significantly higher in all patients with GD than in controls. The same significant differences were observed in the subgroups with and without GO in comparison with controls. The GO subgroup showed a significant positive correlation between the MMP-9 concentration and the serum level of TSHRAb antibodies, and a clinical activity score ≥4 according to EUGOGO.

Conclusions. In our study we found that only MMP-9 differentiates the patients with and without GO, and may be used as a marker of the disease severity in patients with this manifestation of GD.

Key words: Graves' disease, matrix metalloproteinases, tissue inhibitors of metalloproteinases, orbithopathy

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Introduction

Graves' orbitopathy (GO), also known as thyroid-associated ophthalmopathy, is characterized by dramatic tissue reactivity. It is clinically proven in 10–45% of patients with Graves' diseases (GD).[1] The clinical course of GO is characterized by both inflammation and tissue remodeling. Inflammation dominates at the beginning of GO, followed by the remodeling of the orbital connective tissue, including the accumulation of extracellular matrix (ECM) macromolecules and fibrosis.[2,3] ECM metalloproteinases (MMPs) constitute a group of 28 zinc-dependent endopeptidases involved in the proliferation, migration, differentiation, angiogenesis, apoptosis, and host defense. Among MMPs, MMP-2 and MMP-9 are very important for collagen degradation. The concentration of MMPs is very low in the quiescent tissue, but their expression can be increased by inflammatory cytokines, hormones, growth factors, and cell interactions. A number of factors regulate these processes taking place in the extracellular matrix, including natural tissue inhibitors of metalloproteinase (TIMPs). Four TIMPs are known: TIMP-1,-2,-3, and -4. TIMP-1 and TIMP-3 can inhibit proteolytic activity of MMP-9, andTIMP-2,-3, and -4 bind and inactivate MMP-2.[4–7] Some data has been found regarding the association of MMPs and TIMPs in GO. It was found that glucocorticosteroid administration in patients with GO significantly decreased MMP-9 but not MMP-2 serum concentration, whereas TIMP levels have not been examined.[8] Therefore, the aim of this study was to assess the serum concentrations of MMP-2, MMP-9, TIMP-1, and TIMP-2 in patients with GD, with and without GO, and their relationship with disease severity, as well as to evaluate how these concentrations change after successful treatment.

Patients

Forty-eight patients (34 females, 14 males, with median age 51.5 years) diagnosed with GD and hyperthyroidism between October 2010 and October 2012 at the Department of Endocrinology, Diabetes and Isotope Treatment of Wroclaw Medical University (Poland) were enrolled in the study. GD diagnosis was determined using a clinical examination, laboratory results and thyroid gland ultrasonography.[3] Commercially available agents were used to determine thyroid-stimulating hormone (TSH), thyroxine (FT4), and triiodothyronine (FT3) levels. Serum concentration of thyroid-stimulating hormone receptor antibodies (TSHRAb) was analyzed by ELISA. Table 1 presents the patients' characteristics. All of the patients were established ophthalmologically evaluated. In 28 patients, active, moderate-to-severe grade orbitopathy was diagnosed. Severity and activity of GO were established based on the recommendations of the European Group on Graves' Orbitopathy (EUGOGO).[9] One or more of the following clinical signs were observed: lid retraction ≥2 mm, moderate-to-severe orbital soft tissue involvement, proptosis ≥3 mm, permanent or periodic diplopia. According to the EUGOGO, a clinical activity score of ≥4/7 indicates active GO.[10] Patients in the current study presented with conjunctival injection, edema of the eyelids, and chemosis. Additionally, patients with clinically active orbitopathy were tested with magnetic resonance imaging (MRI) and ultrasonographic examination of the orbital muscles. Normal thyroid function was achieved using a thyreostatic drug (thiamazole–Thyrozol) beginning with 20–60 mg per day and decreasing the dose based on clinical and biochemical parameters. Supportive medication was propranolol (20–40 mg per day). The duration of treatment varied from 12 to 18 months. After reaching normal thyroid function, patients with active GO were given methylprednisolone intravenously, 3 times per week over a period of 4 weeks. This regimen was followed by oral prednisone starting with 60 mg and gradually decreasing the dose during 6 weeks. In all patients, marker analysis was repeated after successful treatment. In the GO subgroup, full remission of ocular symptoms was achieved, and was confirmed clinically and by ultrasonography. In patients without GO, the measure of successful treatment was the normalization of hormonal parameters and the remission of the clinical symptoms of hyperthyroidism.[3] This clinical and laboratory remission was achieved in all patients. No infections were observed at the time of parameter measurements. The control group consisted of 19 persons without endocrine, neoplastic or inflammatory pathological changes, and matching the age and gender of the patient population.

Table 1. Clinical and biochemical data of patients with GD

Parameter	GD (all patients)	GO	GO-negative
Number of patients	48	28	20
Age [years], median	51.5	52.5	50.0
Gender [f/m]	34/14	20/8	14/6
FT3 [pmol/L], median (25Q-75Q)	8.0 (0.01–46.8)	8.2 (0.01–46.8)	7.3 (1.1–40.8)
FT4 [pmol/L], median (25Q-75Q)	18.6 (7.0–70.0)	19.9 (7.0–70.0)	17.4 (9.6–34.9)
TSH [U/L], median (25Q-75Q)	0.1 (0.01–13.0)	0.2 (0.01–13.0)	0.06 (0.01–3.0)
TSHRAb [U/L], median (25Q-75Q)	180.0 (7.0–1333.0)	186.5 (8.0–1234.0)	155.5 (7.0–1333.0)

f – females; m – males; FT3 – free triiodothyronine; FT4 – free thyroxine; TSH – thyroid-stimulating hormone (thyrotropin); TSHRAb – thyroid-stimulating hormone receptor antibodies; GD – Graves' disease; GO – Graves' orbitopathy.

Methods

Serum from whole peripheral blood was collected from patients at diagnosis and after successful treatment, during remission. All sera were kept in –70°C until

assessment. Serum concentrations of MMP-9, MMP-2, TIMP-1, and TIMP-2 were determined by ELISA (R&D Systems Inc., Minneapolis, USA), according to the manufacturer's protocol. All samples and standards were measured in duplicate. Minimum detectable concentrations were 0.16 ng/mL (MMP-2), 0.156 ng/mL (MMP-9), 0.08 ng/mL (TIMP-1), and 0.011 ng/mL (TIMP-2). Serum TSHRAb concentration was measured at the time of diagnosis by ELISA (Bio Vendor, Brno, Czech Republic) according to the manufacturer's protocol. Values <0.4 U/L were considered negative.

Statistical analysis

The results were analyzed by the Mann-Whitney U test for uncorrelated data, the paired sample test, and the Spearman's rank correlation test.

Results

Median serum concentrations of MMP-2 and MMP-9 were significantly higher in all patients with GD as well as in the subgroup with GO before treatment than in the control group. This observation was not found in GO-negative patients regarding MMP-2. Moreover, median MMP-9 concentration was significantly higher in patients with GO in comparison with GO-negative patients. Median serum concentrations of TIMP-1 and TIMP-2 were significantly higher in all patients with GD before treatment than in controls. The same differences were observed in the subgroups with GO and without GO in comparison with controls. The data is presented in Table 2.

Additionally, in the GO and GO-negative subgroups, median MMP-2, MMP-9, TIMP-1, and TIMP-2 concentrations significantly decreased after successful treatment. Moreover, the MMP-9/TIMP-1 ratio was significantly higher in all patients and in the groups with GO and without GO than in controls, but we did not observe ratio normalization after treatment. The data is presented in Table 3.

It is worth noting that the subgroup with GO showed a positive correlation between the MMP-9 concentration and the serum level of TSHRAb antibodies, and a clinical activity score ≥4 according to EUGOGO ($r = 0.265$, $p = 0.04$; $r = 0.8180$, $p = 0.0001$, respectively). The data is presented in Fig. 1 and 2.

Discussion

MMPs play a key role in ECM remodeling and are involved in a variety of processes including inflammation, migration, differentiation, angiogenesis, and fibrosis. Although the expression of some MMPs is considered to be constitutive (MMP-2) or inducible (MMP-9) in the quiescent tissue, many factors affect their synthesis. Therefore, the clinical significance of MMPs, in particular MMP-2 and MMP-9, has been revealed in many pathological conditions such as neoplasms, autoimmune diseases, and chronic inflammation.[4–6] Increased production of MMP-2 and MMP-9 appears to be a useful marker of several autoimmune disorders and neoplasms (e.g., multiple myeloma).[11–13] The involvement of MMPs in pathological processes includes matrix degradation and an imbalance between MMPs and their specific inhibitors, TIMPs. TIMPs play important roles in maintaining the delicate balance between ECM production and disposal. Changes in their concentrations are associated with tissue dysfunction. The participation of these metalloproteinases as well as their inhibitors, TIMP1 and TIMP9, in the pathogenesis of ocular manifestation of GD, i.e., Graves' orbitopathy (GO), has not been clearly established. Myśliwiec et al. reported that serum concentration of MMP-9 was significantly higher in GO patients compared with both GO-negative and normal individuals, and decreased after successful steroid treatment.[8] The MMP-2 concentration remained unchanged. In another report, the authors described an increase of the production of TIMP-1 in GO orbital fibroblasts after stimulation by interleukin 1β.[14] Moreover, Yoon et al. observed that quercetin, a flavonoid phytoestrogen, inhibited in vitro inflammation and

Table 2. Comparison of median [M (25Q-75Q)] concentrations of MMP-2, MMP-9, TIMP-1, TIMP-2, and the MMP-9/TIMP-1 ratio in the serum of GD, GO and GO-negative patients before treatment and controls

No.	Patients	MMP-2 [ng/mL]	MMP-9 [ng/mL]	TIMP-1 [ng/mL]	TIMP-2 [ng/mL]	MMP-9/TIMP-1
1	GD (n = 48)	181.5 (95–296)	860.0 (160.0–2640.0)	216.6 (122.1–321.6)	112.5 (85.0–150.0)	3.85 (0.7–16)
2	GO (n = 28)	186.0 (95–296)	1030.0 (210.0–2640.0)	223.34 (135.0–321.6)	115.0 (90.0–145.0)	4.80 (0.8–16.0)
3	GO-negative (n = 20)	171.0 (119–260)	700.0 (160.0–1230.0)	216.6 (122.1–290.7)	110.0 (85.0–150.0)	3.7 (0.7–5.6)
4	controls (n = 19)	149.9 (110.4–211.1)	248.3 (142.1–553.8)	128.6 (98.4–217.3)	92.1 (77.9–120.5)	2.1 (1.2–4.3)
p	1 vs 4 2 vs 4 3 vs 4 2 vs 3	0.005 0.003 ns ns	0.001 0.001 0.0001 0.005	0.001 0.001 0.0003 ns	0.001 0.001 0.0003 ns	0.004 0.005 0.003 ns

n – number of patients; GD – Graves' disease; GO – Graves' orbitopathy; ns – not significant; MMP-2 – metalloproteinase-2; MMP-9 – metalloproteinase-9; TIMP-1 – tissue inhibitor of metalloproteinase-1; TIMP-2 – tissue inhibitor of metalloproteinase-2.

accumulation of the extracellular cell matrix in GO orbital fibroblasts stimulated with pro-inflammatory cytokines.[15,16] The same authors demonstrated that quercetin inhibited MMP-2 and MMP-9 in orbital fibroblasts, which led to the prevention of chronic fibrosis in GO patients.[17] However, it is not clear whether the concentration of MMP-2 or MMP-9 allows for the differentiation between patients with and without an ocular manifestation. In particular, is it not clear which pathogenic mechanisms are involved in the development of ocular symptoms observed in some patients with GD. Therefore, the aim of the present study was to evaluate the serum concentrations of MMP-2 and MMP-9, and their inhibitors, TIMP-1 and TIMP-2, in patients with GD, with and without GO, both in the active phase and after successful treatment. We determined that serum concentrations of all cytokines tested, as well as the MMP-9/TIMP-1 ratio, were significantly higher in the whole group of GD patients, and also in the sub-groups with and without GO, than in control subjects. However, only the MMP9 serum concentration was significantly more elevated in the patients with GO than in persons without ocular manifestations of GD. It may be thus hypothesized, in line with the above quoted reports of Myśliwiec et al. and Yoon et al., that this enzyme is involved in a specific way in the pathogenesis of GO. The positive correlation we found between the MMP-9 concentration and TSHRAb and the disease activity according to the EUGOGO in the GO subgroup provides an additional argument in favor of a particular role played

Table 3. Comparison of median [M (25Q-75Q)] concentrations of MMP-2, MMP-9, TIMP-1, TIMP-2, and the MMP-9/TIMP-1 ratio in the serum of GD, GO and GO-negative patients, before and after treatment, and controls

No.	Patients	MMP-2 [ng/mL]	MMP-9 [ng/mL]	TIMP-1 [ng/mL]	TIMP-2 [ng/mL]	MMP-9/ TIMP-1
1	GD before treatment (n = 48)	181.5 (95–296)	860.0 (160.0–2640.0)	216.6 (122.1–321.6)	112.5 (85.0–150.0)	3.85 (0.7–16)
2	GD after treatment (n = 20)	133.0 (110.5–172.5)	331.1 (175.1–602.9)	101.7 (83.3–144.7)	92.4 (77.8–111.8)	2.9 (1.8–5.9)
3	GO-negative before treatment (n = 20)	171.0 (119–262.0)	700.0 (160.0–1230.0)	216.6 (122.1–290.7)	110.0 (85.0–150.0)	3.7 (0.7–5.6)
4	GO-negative after treatment (n = 10)	135.1 (111.5–171.6)	330.8 (173.2–599.9)	104.2 (83.5–146.6)	91.2 (75.8–111.8)	2.8 (1.7–5.6)
5	GO before treatment (n = 28)	186.0 (95–296)	1030.0 (210.0–2640.0)	223.34 (135.0–321.6)	115.0 (90.0–145.0)	4.80 (0.8–16.0)
6	GO after treatment (n = 10)	133.6 (110.4–173.5)	332.1 (175.1–602.9)	101.6 (83.4–144.6)	92.3 (77.8–111.8)	2.9 (1.7–5.8)
7	controls (n = 19)	149.9 (110.4–211.1)	248.3 (142.1–553.8)	128.6 (98.4–217.3)	92.1 (77.9–120.5)	2.1 (1.2–4.3)
p	1 vs 2	0.006	0.0003	0.0001	0.0002	ns
	3 vs 4	0.005	0.0004	0.0001	0.0002	ns
	5 vs 6	0.0005	0.0002	0.00001	0.0001	ns
	2 vs 7	ns	ns	0.01	ns	0.03
	4 vs 6	ns	ns	ns	ns	ns

n – number of patients; GD – Graves' disease; GO – Graves' orbitopathy; ns – not significant; MMP-2 – metalloproteinase-2; MMP-9 – metalloproteinase-9; TIMP-1 – tissue inhibitor of metalloproteinase-1; TIMP-2 – tissue inhibitor of metalloproteinase-2.

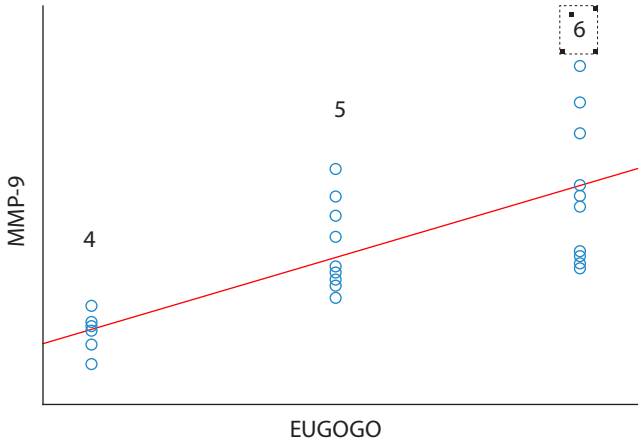

Fig. 1. Positive correlation between the MMP-9 concentration and clinical activity score ≥4 according to EUGOGO in patients with GO (r = 0.8180; p = 0.0001)

MMP-9 – metalloproteinase-9.

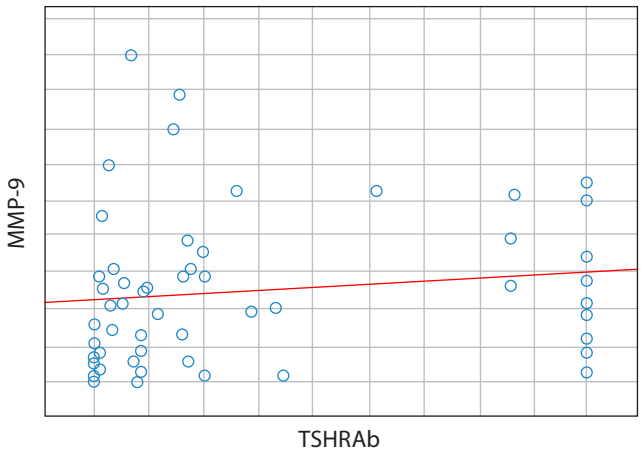

Fig. 2. Positive correlation between the MMP-9 concentration and the serum level of TSHRAb antibodies in patients with GO (r = 0.265; p = 0.04)

MMP-9 – metalloproteinase-9; TSHRAb – thyroid-stimulating hormone receptor antibodies.

by this enzyme in the pathogenesis of the ocular manifestation of GD, and also suggests the possibility of a relationship between its release to the blood and the activity of the disease. The significant increase of the MMP9/TIMP1 ratio we found in all subgroups of patients as compared to healthy controls deserves particular attention. It may indicate that, although the concomitant rise of both metalloproteinases and their inhibitors occurs in GD with and without GO, the increase in MMP-9 is more pronounced than the increase of its inhibitor TIMP1, and this finding strengthens the hypothesis of the role of MMP-9 in the development of GD with or without orbitopathy. Our findings showing that the serum concentration of all cytokines tested significantly decreased after successful treatment of all subgroups of patients suggest that these substances not only are involved in the pathogenesis of GD but also their production correlates with clinical activity of the disease. It is of interest that TIMP1 and the MMP9/TIMP1 ratio are the only parameters which remain significantly elevated in GD patients after successful treatment as compared to healthy subjects. Therefore, their usefulness as the most sensitive markers of the disease should be further evaluated.

Conclusions

To the best of our knowledge, our study is the first analysis of the combined determination of serum concentration of 2 metalloproteinases and their inhibitors in GD with and without orbitopathy, in active disease and after successful treatment. Although the serum concentrations of MMP-9, MMP-2, TIMP-1, and TIMP-2 were elevated in patients with GO and decreased after the treatment, we found that only MMP-9 differentiates the patients with and without GO, and may be used as a marker of disease severity in patients with this manifestation of GD.

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The effect of hemodialysis on intraocular pressure

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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 the article

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Abstract

Background. The effect of hemodialysis (HD) on intraocular pressure (IOP) has been investigated before, but there is a lack of consensus. Clinicians dealing with renal failure patients are interested in the potential negative effects of HD on IOP and the course of glaucoma.

Objectives. The aim of this study was to investigate the effects of HD on IOP in patients with end-stage renal disease.

Material and methods. This prospective study included 106 patients who were receiving outpatient hemodialysis. Patient history of systemic and ophthalmologic conditions was recorded. Serum osmolality (mOsm), blood urea nitrogen (BUN), blood glucose (BG), bicarbonate (BC), and hematocrit (Hct) levels at the start of HD (pre-HD), at the end of HD (end-HD), and 30 min after HD (post-HD) were measured. Systolic and diastolic blood pressures (SBP and DBP) and IOP were measured at pre-HD, 1-hour intervals during HD, end-HD, and post-HD.

Results. A significant decrease in mOsm and BUN and a significant increase in BG, BC, and Hct levels were observed at end-HD ($p < 0.05$). Mean IOP was 16.71 ±2.51 mm Hg at pre-HD, 15.52 ±3.18 mm Hg at end-HD, and 15.23 ±2.73 mm Hg at post-HD ($p = 0.001$; $F = 4.439$). Post-HD SBP and DBP were significantly lower than at pre-HD ($p < 0.001$). There was a positive correlation between the change in IOP and the change in mOsm and the change in BUN at end-HD ($r = 0.315$, $p = 0.004$; and $r = 0.279$, $p = 0.012$, respectively).

Conclusions. IOP decreased significantly during HD in this study. Additional research on the effects of the change in blood parameters and ocular perfusion pressure on IOP and optic nerve perfusion during HD is recommended.

Key words: glaucoma, hemodialysis, intraocular pressure

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Introduction

Glaucoma is an optic neuropathy with multifactorial etiology. While the etiological mechanism is not precisely known, elevated intraocular pressure (IOP) remains the focus of its treatment; however, some glaucoma patients continue to experience optic nerve damage despite decreased IOP.[1,2] Altered ocular blood flow was reported to accelerate the development and progression of the disease in such patients; as such, not only elevated IOP, but also medical conditions that negatively affect ocular blood flow will obviously cause ischemia and reperfusion damage in glaucoma patients and in individuals prone to glaucoma.[3–5]

Hemodialysis (HD) is an important component of the treatment of end-stage renal disease (ESRD). Some studies have reported the effects of HD on IOP, but the findings are inconsistent. It was reported that IOP increases in response to HD, whereas it was also reported that IOP decreases or does not change during or after HD.[6–11] The present study aimed to investigate the effect of HD on IOP in ESRD patients. To the best of our knowledge this is, to date, the largest prospective study on the effect of HD on IOP in ESRD patients.

Material and methods

Study design and the selection of study subjects

This prospective study included 106 patients (51 females and 55 males) who were receiving HD and met the inclusion criteria. The inclusion criteria were a negative history of glaucoma, and a healthy cornea. The exclusion criteria were IOP > 21 mm Hg at the beginning of HD, and optic nerve abnormalities suggesting glaucoma (cup-disc ratio >0.3; cup-disc asymmetry between the eyes). Refractive errors, uveitis, and diabetic retinopathy were not considered as exclusion criteria unless they caused high IOP. The study was conducted in accordance with the tenets of the Declaration of Helsinki, and the institutional review board approved the study protocol. All the patients provided written informed consent.

Measurements

A Braun Dialog Hemodialysis System (Braun, Melsungen, Germany) was used for HD treatment. This system is part of a completely new generation of dialysis systems aimed at delivering optimum care with highest efficiency. It uses the principles of spectroscopy to determine the reduction in the molar concentration of urinary excreted substances in the dialyzate drain, and it enables measurement in the used dialyzate. All patients underwent 4-hour HD sessions 3 days per week. Patient body weight, and history of diabetes mellitus, hypertension, asthma, renal diseases, eye diseases, and medications were recorded. Blood samples were collected at the start of HD (pre-HD), at the end of HD (end-HD), and 30 min after HD (post-HD) to measure serum osmolality (mOsm), blood urea nitrogen (BUN), blood glucose (BG), bicarbonate (BC), and hematocrit (Hct). Systolic and diastolic blood pressure (SBP and DBP) and IOP were measured at pre-HD, at 1-hour intervals during HD (1st hour, 2nd hour, and 3rd hour), at end-HD (4th hour), and at post-HD. Pre-HD blood pressure and IOP measurements were performed after 15 min of rest, before the HD needle was inserted. Blood pressure measurement using a sphygmomanometer was always performed before IOP measurement. IOP measurement was performed using an Icare Pro tonometer (Icare, Tiolat Oy, Helsinki, Finland), with patients in the supine position. The recorded IOP was the mean of 5 successive measurements.

Statistical analysis

Statistical analysis was performed using SPSS v. 17.0 for Windows (SPSS Inc., Chicago, USA). Descriptive data is expressed as an arithmetical mean ±SD. The independent samples t-test was used to compare the patients' right and left eyes. The paired samples t-test and one-way ANOVA for repeated measures were used to evaluate the changes in blood parameters, IOP, SBP and DBP, and the effect of systemic β-blockers on IOP change. Pearson correlation coefficient was used to determine correlations between the changes in blood parameters, IOP, SBP, and DBP. At the 95% CI, p-values <0.05 were accepted as statistically significant.

Table 1. Serum osmolality, blood urea nitrogen, blood glucose, biocarbonate, and hematocrit levels

Variables	Mean ±SD			p-value		
	pre-HD	end-HD	post-HD	pre-HD vs end-HD	pre-HD vs post-HD	end-HD vs post-HD
mOsm (mOsm/kg)	338.40 ±10.63	305.22 ±5.99	307.22 ±6.18	<0.001	<0.001	<0.001
BUN (mg/dL)	148.23 ±24.98	47.14 ±10.38	53.80 ±12.23	<0.001	<0.001	<0.001
BG (mg/dL)	128.22 ±64.29	136.72 ±53.39	135.73 ±54.35	0.01	0.013	1.000
BC (mEq)	21.74 ±8.33	27.60 ±2.55	26.95 ±2.23	<0.001	<0.001	<0.001
Hct (%)	31.42 ±3.91	33.78 ±4.18	33.88 ±4.12	<0.001	<0.001	0.319

mOsm – serum osmolality; BUN – blood urea nitrogen; BG – blood glucose; BC – bicarbonate; Hct – hematocrit; pre-HD – at the start of hemodialysis; end-HD – at the end of hemodialysis; post-HD – 30 min after hemodialysis.

Results

Mean body weight before HD was 70.22 kg (range: 42–103.5 kg) and mean age of the patients was 61.65 ±14.39 years (range: 24–91 years). In all, 21 (20.3%) patients had a history of diabetes mellitus, 80 (77.7%) had a history of hypertension, 89 (88.1%) had a history of renal disease (such as kidney stone, polycystic kidney disease, glomerulonephritis, etc.), and 18 (17%) had a history of diabetic retinopathy. Among the patients, 53 (50%) were using systemic β-blockers, 38 (35.8%) were using Ca-channel blockers, and 12 (11.3%) were using angiotensin converting enzyme inhibitors. None of the patients reported using any eye drops. Pre-HD, end-HD and post-HD mOsm, BUN, BG, BC, and Hct are shown in Table 1 and Fig. 1. The values of mOsm and BUN were significantly lower, and BG, BC, and Hct were significantly higher at end-HD and post-HD, as compared to pre-HD ($p < 0.05$). Moreover, mOsm and BUN were significantly lower at end-HD than at post-HD, and BC was significantly higher at end-HD than at post-HD ($p < 0.001$) (Table 1). There was no significant difference in IOP between right and left eyes; therefore, only right eye measurements were used for further analysis ($p > 0.05$).

IOP, SBP, and DBP measurements obtained at pre-HD, 1-hour intervals during HD, end-HD, and post-HD are shown in Table 2 and Fig. 2. As compared to pre-HD, IOP decreased significantly at the 2nd hour of HD ($p = 0.009$), end-HD ($p = 0.042$), and post-HD ($p = 0.001$). Additionally, IOP was lower at post-HD than at end-HD, but not significantly. Systemic β-blockers did not affect the changes in IOP ($p = 0.443$). SBP was significantly lower at the 2nd hour of HD ($p < 0.001$), and DBP was significantly lower at the 1st hour of HD ($p = 0.046$), as compared to pre-HD. Among the 106 patients, 105 had SBP ≥ 80 mm Hg and 104 had DBP ≥ 50 mm Hg during HD, at end-HD, and at post-HD. SBP and DBP increased from end-HD to post-HD, but not significantly ($p > 0.05$).

Among all the evaluated measurements, there was a positive correlation between the IOP change and the mOsm and BUN change at end-HD ($r = 0.315$, $p = 0.004$; $r = 0.279$, $p = 0.012$, respectively). In addition, there was a negative correlation between the change in DBP and the change in IOP between end-HD and post-HD ($r = -0.255$, $p = 0.036$).

Discussion

The potential effects of HD on IOP prompt clinicians who treat ESRD patients with glaucoma to considering the potential negative effects of HD on IOP and the course of glaucoma, because the deterioration of hydrodynamic parameters and blood constituent levels during dialysis are common in such patients. Altered ocular blood flow negatively affects the pathophysiology of glaucoma.[3–5] In a population-based study on adults in Malaysia, chronic kidney disease was associated with elevated IOP.[12] During HD, abrupt changes in blood parameters can affect the osmotic gradients between body compartments, leading to changes in IOP; thus, if HD adversely affects IOP in ESRD patients without glaucoma, those with glaucoma will not only have higher IOP, but the optic nerve may also be damaged due to hydrodynamic changes and altered ocular blood flow during HD.

The relationship between HD and IOP was first evaluated in 1964, yet there remains a lack of consensus about the effects of HD on IOP.[6] It is difficult to compare findings from various studies because of the differences in IOP measurement techniques, and the ocular and non-ocular parameters investigated. Many published studies reported changes in IOP based on measurements obtained using a Goldmann applanation tonometer.[7,9,11,13,14] Patients must be seated when using a Goldmann applanation tonometer. The reduction in blood volume that occurs during HD can cause hypotension and any movement during HD can cause blood pressure variation, all of which can indirectly affect IOP.[15] In the present study, all measurements were made with patients in the supine position, eliminating the need for mobilization.

Some earlier studies reported that IOP increases during HD due to a decrease in mOsm.[6,7] Accordingly, a rapid decrease in mOsm during HD results in an osmotic gradient and causes fluid shift from blood to the eyes, causing an increase in IOP. If there is obstruction in the outflow facility (such as anterior synechia or narrow angle), the increase in IOP is apparent, but if the outflow pathway for the aqueous humor is not obstructed, there is only a minimal increase in IOP. Sitprija et al. reported a mean rise in IOP of 5.9 mm Hg in patients undergoing HD.[6]

Table 2. Introvascular pressure, systolic blood pressure and diastolic blood pressure measurements during hemodialysis

Variables	Mean ±SD						p-value
	pre-HD	1st hour	2nd hour	3rd hour	end-HD	post-HD	
IOP (mm Hg)	16.71 ±2.51	15.78 ±3.12	15.42 ±3.28 [a]	15.78 ±3.53	15.52 ±3.18[a]	15.23 ±2.73[a]	0.001 (F = 4.439)
SBP (mm Hg)	132.01 ±22.29	129.64 ±21.84	123.44 ±19.23[a,b]	120.37 ±20.08[a]	116.02 ±20.23[a]	118.05 ±19.08[a]	<0.001 (F = 23.096)
DBP (mm Hg)	75.37 ±10.88	73.37 ±10.28[a,b]	72.03 ±9.85[a]	69.74 ±10.28[a]	68.71 ±10.67[a]	70.04 ±9.51[a]	<0.001 (F = 14.091)

p – represents significance for one-way ANOVA for repeated measures; IOP – intraocular pressure; SBP – systolic blood pressure; DBP – diastolic blood pressure; pre-HD – at the start of hemodialysis; end-HD – at the end of hemodialysis; post-HD – 30 min after hemodialysis; [a] a statistically significant change compared with pre-HD measurements; [b] a statistically significant change compared with the previous measurement.

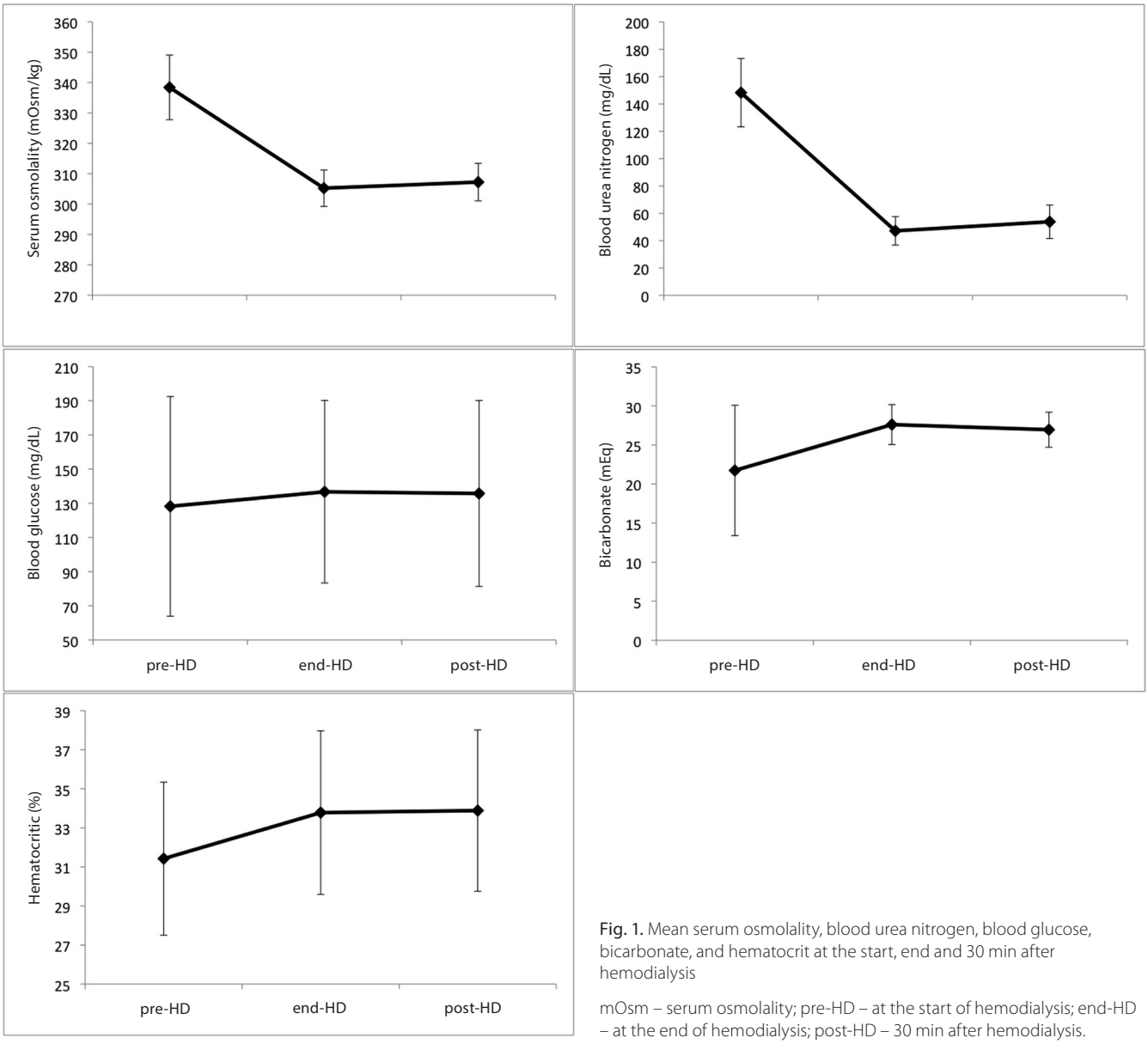

Fig. 1. Mean serum osmolality, blood urea nitrogen, blood glucose, bicarbonate, and hematocrit at the start, end and 30 min after hemodialysis

mOsm – serum osmolality; pre-HD – at the start of hemodialysis; end-HD – at the end of hemodialysis; post-HD – 30 min after hemodialysis.

When compared with the previously reported marked increases in IOP during HD, Leiba et al. reported that the slight increase in IOP (0.35 mm Hg) in their study was due to improved dialysis techniques.[7]

Some studies reported that there is no correlation between mOsm and IOP, while others reported no change in IOP during HD.[10,11,13,16] Tokuyama et al. observed a decrease in IOP (–1.8 mm Hg) and plasma osmolarity following HD, but not a significant correlation between IOP and plasma osmolarity; however, they did note a correlation between IOP and plasma colloid osmotic pressure ($r = -0.510$, $p = 0.0012$) and a change in body weight ($r = 0.534$, $p = 0.0006$).[9] According to Fauchald, plasma colloid osmotic pressure is critical to hydrodynamic changes during HD.[17] The removal of water during HD decreases plasma volume and the concentration of plasma proteins increase; this increase in colloid osmotic pressure draws water from the aqueous humor into plasma.[9,16]

In the present study there was a significant decrease in IOP during HD (from 16.71 ±2.51 at pre-HD to 15.52 ±3.18 at end-HD), which might have been due to the improvement in HD techniques since the time the earlier studies were performed. Slower HD may prevent abrupt changes in mOsm, and fluid shift to the eyes may be limited. Mean mOsm change was -8.30 ± 1.98 mOsm $\cdot$ kg^{-1} $\cdot$ h^{-1} in the present study. Sitprija et al. reported an increase in IOP when mean mOsm change was –11 mOsm $\cdot$ L^{-1} $\cdot$ h^{-1}.[6] During HD, an increase in colloid osmotic pressure draws water from the aqueous humor into plasma.[9,16] In the present study, the effect of elevated colloid osmotic pressure on IOP might have been greater than the effect of the decrease in mOsm on IOP, which is why HD led to lower IOP values. Despite the reported marked rise in IOP, in the present study there was a 1.2 mm Hg of IOP decrease during HD, which is also comparable with the study conducted by Tokuyama.[6,9] New dialysis techniques seem to prevent marked increases in IOP during HD.

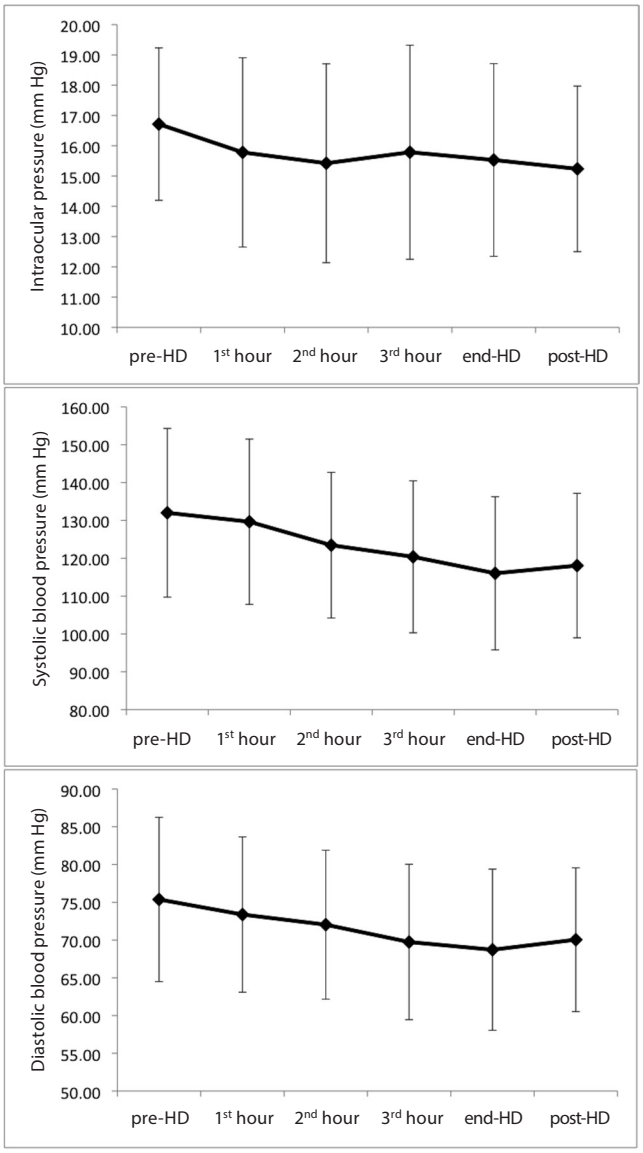

Fig. 2. Mean introvascular pressure, systolic blood pressure and diastolic blood pressure during hemodialysis

During HD, a decrease in urea in the extracellular environment precedes the intracellular decrease. The difference between compartments reaches equilibrium as urea exits cells and enters extracellular fluid. This causes an increase in the serum urea level, which is known as post-dialysis urea rebound (PDUR). According to Tovbin et al., PDUR was strongly correlated with intradialytic changes in IOP.[8] They subtracted the urea level 1 h after dialysis from that measured at the end of dialysis. In the present study, BUN was measured 30 min after HD (post-HD) and the increase in BUN at post-HD was not correlated with the IOP change from end-HD to post-HD, but the decrease in BUN during HD was positively correlated with the observed decrease in IOP during HD.

As mentioned above, altered ocular blood flow accelerates the development and progression of glaucoma. Modern HD devices, such as that used in the present study, make it possible to adjust the ultrafiltration rate according to the changes in blood pressure.[18] This biofeedback system prevents hypotension via the regulation of the ultrafiltration rate and facilitates the protection of the optic nerve from the hazardous effects of hypotension, which can offer protection against ischemia and reperfusion damage in glaucoma patients and individuals prone to glaucoma.[19]

Aqueous humor dynamics in a glaucomatous eye differs from the normal eye. This difference is mainly related to the outflow pathways. It is reported that the resistance of the trabecular meshwork is increased in glaucomatous eyes due to an increase in extracellular matrix thickness and the deposited cochlin protein and mucopolysaccharides.[20] The glycosaminoglycans in the extracellular matrix of the trabecular meshwork may generate osmotic forces and induce hydration of the trabecular meshwork. Therefore, the increased thickness in extracellular matrix in glaucomatous eyes may cause an increased resistance to aqueous outflow and eventual higher IOP. As mentioned above, the rapid decrease in mOsm with the earlier HD techniques causes a fluid shift from blood to the eyes. This would further increase IOP in glaucomatous eyes due to thicker extracellular matrix. Colloid osmotic pressure, which draws water from the aqueous humor into plasma, may counterbalance the effect of mOsm on IOP. Therefore, slower dialysis techniques seem to be beneficial for glaucomatous eyes by preventing rapid changes in serum and plasma constituents, and thereby preventing IOP rises.

ESRD patients often have chronic diseases that require the use of systemic medications. Little is known about the effects of systemic medications on IOP. A population-based study has investigated the association between the use of common systemic medications and IOP.[21] According to the results of that study, the use of systemic β-blockers and nitrates were independently associated with lower IOP. However, in the present study we did not find any effects of systemic β-blocker use on IOP decrease during HD.

The present study has some limitations. The anterior chamber was not evaluated, which would have provided some data on outflow facility; patients identified as having obstructed outflow pathways may have had findings during HD that differed from those of other patients. Additionally, plasma colloid osmotic pressure – possibly the primary mechanism responsible for decreasing IOP – was not measured. Another limitation of the present study is the lack of evaluation of central corneal thickness, as measured IOP has been proven to vary depending on central corneal thickness.[22,23]

In conclusion, the previously reported increase in IOP during HD was not observed in this study. In fact, IOP decreased significantly during HD in this study, which may be a favorable outcome for patients with ESRD and glaucoma. With continuing improvements in dialysis techniques and the advent of new devices, hydrodynamic changes during HD are controlled more effectively and the regulation of blood parameters and hypervolemia

is improved, positively affecting IOP. We think that the observed decrease in IOP during HD in the present study was associated with a change in colloid osmotic pressure, mOsm, ocular perfusion pressure, and the outflow facility in the patients' eyes. Based on the present findings, we think additional research on the above-mentioned changes and the effects of HD on optic nerve perfusion is warranted.

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Typing of *Enterococcus* spp. strains in 4 hospitals in the Małopolska region in Poland

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

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Abstract

Background. In the Małopolska province, the first case of vancomycin resistant enterococci (VRE) occurrence was an outbreak in 2001 caused by strains of the genus *E. faecium* carrying the *vanA* operon.

Objectives. The aim of this study is to determine the antimicrobial resistance and the occurrence of virulence determinants among *Enterococcus* spp. in patients hospitalized in the Małopolska region in 2015.

Material and methods. Antimicrobial susceptibility was determined by disc diffusion and the E test. The presence of aminoglycoside and glycopeptide resistance genes and virulence genes (*asa1*, *gelE*, *cylA*, *esp*, *hyl*) was investigated using multiplex polymerase chain reaction (PCR). Also, the presence of IS*16* was investigated. The activity of gelatinase, cytolysin (hemolysin), and DNase was tested.

Results. All *E. faecalis* were susceptible to ampicillin, vancomycin, teicoplanin, linezolid and tigecycline. All *E. faecium* strains were susceptible to quinupristin-dalfopristin. Resistance to ampicillin and vancomycin was detected among all *E. faecium* isolates from hospitals C and D. 87.32% of *E. faecium* presented high-level aminoglycoside-resistant (HLAR) phenotype, including 78.33% of strains from hospital C and 100% from hospital D. In hospital C (98.3%) and D (96%), resistance to ciprofloxacin, levofloxacin and norfloxacin was observed. Gene *esp* was detected in all *E. faecium* isolates and the majority of *E. faecium* isolates carried *hyl* (97%). In *E. faecalis*, different combinations of virulence genes were detected. All analyzed *E. faecium* strains showed the presence of IS*16* insertion element.

Conclusions. *E. faecalis* isolates were more susceptible to antimicrobials than *E. faecium*, which were largely multidrug-resistant. *E. faecalis* strains have diverse virulence factors. *E. faecium* showed a high percentage of *hyl* and *esp* genes and the presence of IS*16*.

Key words: virulence factors, healthcare-associated infections, *Enterococcus*, vancomycin resistant enterococci, antimicrobial susceptibility

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Introduction

Enterococci are now recognized as a significant cause of healthcare-associated infections worldwide.[1–3] The emergence of multidrug resistance (MDR), including high-level aminoglycoside-resistant (HLAR) enterococci and vancomycin-resistant enterococci (VRE), causes great difficulties in clinical antimicrobial therapy.[4] VRE infections have been associated with higher mortality, longer hospital length of stay, and higher costs compared with vancomycin-susceptible isolates.[5,6] The first vancomycin-resistant *E. faecium* (VRE*fm*) strains carrying the *vanA* operon and *vanB* operon were reported in Poland in 1996 and 1999, respectively.[7] In the Małopolska province, the first case of VRE occurrence was an outbreak caused by strains of the genus *E. faecium* carrying the *vanA* operon in 2001.[8,9]

The aim of this study is to determine the virulence factors and antibiotic resistance patterns of enterococcal isolates from 4 hospitals with different specialties from the Małopolska region.

Material and methods

Hospital settings

Pieces of information were obtained from hospitals from which enterococcal strains were isolated, regarding ward locations and body sites from which VRE and other enterococci were recovered. Material for the study was taken according to the following criteria: from patients with symptomatic infection as well as from patients admitted from other hospitals, Social Welfare Homes, Health Care Centers; patients who had previously been treated in another hospital and had been given broad-spectrum antibiotics; patients who had information about VRE colonization in their discharge card; the ones who were repeatedly hospitalized for 12 months.

Characteristic of *Enterococcus* strains

In this hospital-based study, a total of 154 *Enterococcus* strains isolated from 4 hospitals during the period from January 2015 through December 2015 were collected. Six of the samples were collected from hospital A, 13 samples were from B, 60 were from C, and 75 samples were acquired from hospital D. The enterococcal isolates were obtained from different specimens and were classified as colonization or infection. The study comprised consecutive, non-repetitive enterococci isolates.

All strains had already been identified with the species level using conventional biochemical tests and with the VITEK-2 COMPACT fully automated microbiological system in hospital laboratories. The identification was confirmed by species-specific multiplex polymerase chain reaction (PCR) as described by Jackson (*E. faecalis* – 360 bp, *E. faecium* – 215 bp and *E. avium* – 368 bp) using the DNA samples of each *Enterococcus* strain isolated by Genomic Mini (A&A Biotechnology, Gdynia, Poland).[10]

Antibiotic susceptibility testing

Susceptibility to antimicrobials was evaluated by the disc-diffusion method (Oxoid, Basingstoke, England) according to manufacturer's procedure. The minimum inhibitory concentrations (MICs) of ampicillin, ciprofloxacin, tigecycline and linezolid were evaluated by the E tests method (bioMérieux, Marcy l'Etoile, France) according to the manufacturer's procedure.

The presence of HLAR phenotype, HLGR (high-level gentamicin resistance) and HLSR (high-level streptomycin resistance) phenotypes were identified by the disk susceptibility tests with streptomycin and gentamicin. VRE phenotype was detected by teicoplanin and vancomycin. The HLAR and VRE phenotypes were confirmed by the E tests method. All tests carried out using both methods were done on freshly prepared Mueller Hinton II Agar (Biocorp, Warszawa, Poland). The interpretation was performed in accordance with the recommendations of the European Committe on Antimicrobial Susceptibility Testing (EUCAST) guidelines.[11]

Assay of gelatinase activity

The production of gelatinase in *Enterococcus* strains was detected with the method described by Strzelecki.[12] Gelatinase activity was observed as a transparent halo around the enterococcal colonies.

Detection of antimicrobial resistance genes

To detect genes responsible for HLAR phenotype in the genomes of all the strains resistant to gentamicin and/or streptomycin, multiplex PCR was applied according to the method of Vakulenko.[13] The results of VRE testing by the phenotype method were confirmed by investigating *vanA* (732 bp) and *vanB* (635 bp) operons using the multiplex PCR as described by Biendo.[14]

Table 1. Characteristics of the studied hospitals

Hospital	Body sites			Units		
	blood	rectal swab	others*	ICU	surgery	others**
A	5	0	1	2	0	4
B	0	0	13	2	7	4
C	1	49	10	25	15	20
D	2	55	18	57	7	11
Total	8	104	42	86	29	39

* pleural effusion, cerebrospinal fluid, urine, bronchial aspirates, surgical wound; ** Clinical Department of Interventional Cardiology, Department of Pulmonary Diseases, Department of Internal Medicine, Department of Orthopedic Trauma, Department of Neurology, Stroke Unit, Department of Urology; ICU – intensive care unit.

Detection of virulence factor genes

To detect the presence of genes encoding selected virulence factors (*asa1*, *gelE*, *cylA*, *esp*, *hyl* – 375bp, 213 bp, 688 bp, 510 bp, 276 bp, respectively) in the enterococcal DNA, multiplex PCR was applied according to the methods of Vankerckhoven.[15] The presence of quorum-sensing genes (*fsrA*, *fsrB*, *fsrC*) in the genomes of all the strains positive for the *gelE* gene was tested by multiplex PCR and was applied pursuant to Qin.[16] The product sizes for *fsrA*, *fsrB* and *fsrC* were 484 bp, 574 bp and 580 bp, respectively.

Assay of cytolysin (hemolysin) activity

Cytolysin activity in *Enterococcus* strains was detected by the method described by Kowalska-Krochmal.[17] The presence of a clear zone surrounding the studied strain colonies indicated the production of cytolysin.

Assay of DNase activity

DNase activity was detected on BD DNase Test Agar (Becton Dickinson, Oxford, England) described by Kowalska-Krochmal.[17] DNase positive organisms will be surrounded by clear zones of depolymerized DNA. Colonies of DNase negative organisms will not show any clearing around the colonies.

Detection of a specific mobile insertion element IS*16* by polymerase chain reaction

PCR was performed with primers encoding gene fragment of the insertion sequence IS*16*.[18] The product sizes were 547 bp, which is specific for IS*16* element.

Results

Hospital settings

Generally, over half of the strains were isolated from patients hospitalized in ICU (55.84%), of which the highest proportion of enterococci was isolated from patients in hospital D and accounted for, respectively, the highest percentage (76%) of strains in that hospital and among other hospitals.

In hospital A, no strain originated from a patient operated on, while in hospital B, it was the surgical unit that was the source of most strains (53.84%). In general, most enterococci were isolated from perianal swabs, which resulted from a high rate of isolation in hospitals C and D. In hospitals A and B, no enterococci were isolated from rectal swabs. Only in hospital A, the biggest proportion of enterococci was isolated from the blood. At the remaining facilities, enterococci were significantly more often isolated from materials other that blood, as there is no procedure for screening for VRE on admission (Table 1).

Distribution of *Enterococcus* species in various clinical specimens

The highest prevalence of *E. faecium* was demonstrated in rectal swabs (75%), followed by wounds (12.5%), lower respiratory tract specimens (5.8%), urine (3.7%), blood (2.2%), and others (0.8%). The highest prevalence of *E. faecalis* was detected in wounds (47%), followed by blood and lower respiratory tract specimens (23.5% each), and cerebrospinal fluid (6%). In our study, only one *E. avium* isolate was detected, and it was found in a postoperative wound swab.

Table 2. Prevalence of antimicrobial resistance in *Enterococcus* species isolated from hospitals A, B, C, and D (disk diffusion method)

Antibiotic	Hospital							
	A		B		C		D	
	S	R	S	R	S	R	S	R
Ampicillin	5 (83.3)	1 (16.7)	8 (61.5)	5 (38.5)	0 (0)	60 (100)	0 (0)	75 (100)
Ciprofloxacin	4 (66.7)	2 (33.3)	6 (46.2)	7 (53.8)	1 (1.7)	59 (98.3)	3 (4)	72 (96)
Levofloxacin	4 (66.7)	2 (33.3)	7 (53.8)	6 (46.2)	2 (1.7)	60 (98.3)	3 (4)	72 (96)
Norfloxacin	4 (66.7)	2 (33.3)	6 (46.2)	7 (53.8)	3 (1.7)	61 (98.3)	3 (4)	72 (96)
Gentamicin	3 (50)	3 (50)	6 (46.2)	7 (53.8)	3 (5)	57 (95)	0 (0)	75 (100)
Streptomycin	6 (100)	0 (0)	8 (61.5)	5 (38.5)	8 (13.3)	52 (86.7)	9 (12)	66 (88)
Vancomycin	6 (100)	0 (0)	12 (92.3)	1 (7.7)	0 (0)	60 (100)	0 (0)	75 (100)
Teicoplanin	6 (100)	0 (0)	12 (92.3)	2 (7.7)	15 (25)	45 (75)	70 (93.3)	5 (6.7)
Tigecycline	6 (100)	0 (0)	13 (100)	0 (0)	60 (100)	0 (0)	75 (100)	0 (0)
Linezolid	6 (100)	0 (0)	13 (100)	0 (0)	60 (100)	0 (0)	75 (100)	0 (0)
Quinupristin-dalfopristin	1*(100)	0 (0)	3*(100)	0 (0)	60*(100)	0 (0)	75*(100)	0 (0)

S – susceptible; R – resistant; n (%) – number (percentage) of strains; * quinupristin-dalfopristin was evaluated for *E. faecium* only.

Comparison of antimicrobial resistance between *E. faecium* and *E. faecalis* and antimicrobial resistance in *Enterococcus* species isolated from various hospitals

The E test method confirmed the resistance to antimicrobials shown by the disc diffusion method (vancomycin, teicoplanin, ampicillin, gentamicin, ciprofloxacin, linezolid, and tigecycline) (Tables 2, 3). A strain resistant to linezolid and tigecycline was not reported among the tested enterococcal strains. All *E. faecalis* were also susceptible to ampicillin, vancomycin and teicoplanin. HLAR, HLGR and HLSR phenotypes were present in 33.3%, 20% and 6.6% of *E. faecalis* strains, respectively. All enterococci from *E. faecium* species were also susceptible to quinupristin-dalfopristin. Among all *E. faecium* isolates from hospitals C and D, resistance to ampicillin and vancomycin was detected. A high percentage of *E. faecium* (87.32%) presented HLAR phenotype, including 78.33% of strains from hospital C and 100% from D. Furthermore, in hospital D, HLSR (1.67%) and HLGR (10%) were detected in *E. faecium*. Also, very high rates of resistance to ciprofloxacin and 2 other fluoroquinolones (levofloxacin and norfloxacin) were observed in hospitals C (98.3%) and D (96%). In our study, *E. faecalis* isolates were more susceptible to antimicrobials than *E. faecium*, which were largely multidrug-resistant. Detailed results are shown in Tables 2 and 3. In hospitals A and B, MDR was not reported among the tested *Enterococcus* strains. In hospitals C and D, 95% and 96%, respectively, were classified as MDR strains.

Prevalence of resistance genes

In the study group there were 136 gentamicin resistance strains containing the *aac(6')-Ie-aph(2")-Ia* gene, which encodes the bifunctional enzyme AAC(6')-APH(2"), and 6 strains encoding the *aph(2")-Id* gene that also mediates resistance to gentamicin. All 123 streptomycin resistance strains contained the *aph(3')-IIIa* gene. 115 *vanB* genes and only 21 *vanA* genes were detected in enterococci. PCR analysis confirmed the phenotypic analysis.

Detection of virulence factor genes

Esp genes were detected in all *E. faecium* isolates and the majority of *E. faecium* isolates carried *hyl* (97%), in contrast to *E. faecalis* strains, in which different combinations of *asa1*, *esp*, *gelE*, and *cylA* genes were detected (Fig. 1).

Phenotypic analysis of virulence factors

In hospitals A and B, where *E. faecalis* constituted the majority of the isolated strains, cytolysin as well as gelatinase were produced. 100% of the gene responsible for

Table 3. Comparison of in vitro activity of 7 antimicrobials against enterococci isolated from hospitals A, B, C, and D

Antimicrobials	Hospital															
	A				B				C				D			
	MIC range	MIC_{50}	MIC_{90}	% resistant strains	MIC range	MIC_{50}	MIC_{90}	% resistant strains	MIC range	MIC_{50}	MIC_{90}	% resistant strains	MIC range	MIC_{50}	MIC_{90}	% resistant strains
Vancomycin	0.5–2.0	0.75	1.5	0	0.5–>256	2	3	7.7	>256	256	256	100	>256	256	256	100
Teicoplanin	0.5	0.5	0.5	0	0.38–>256	0.5	0.95	7.7	0.25–>256	0.75	256	75	0.38–>256	0.5	0.75	93.3
Ampicillin	2.0–>256	2	129	16.7	0.26–>256	2	256	38.5	>256	>256	>256	100	>256	256	256	100
Gentamicin	32–192	128	192	50	8.0–>256	16	256	53.8	192–>256	256	256	95	192–>256	256	256	100
Ciprofloxacin	0.5–32	2	22	33.3	0.5–256	6	32	53.8	2–256	256	>256	98.3	2–256	256	>256	96
Linezolid	0.75–4.0	2	3	0	1.0–4.0	2	2	0	0.75–2.0	1.25	2	0	0.75–2.0	2	2	0
Tigecycline	0.125–0.5	0.25	0.5	0	0.125–0.75	0.38	0.7	0	0.016–0.5	0.25	0.5	0	0.032–0.5	0.125	0.5	0

MIC – minimum inhibitory concentration; $MIC_{50/90}$ – MICs at which 50% and 90% of the isolates were inhibited, respectively; MIC values are given in mg/L.

producing cytolysin underwent expression. The activity of gelatinase was detected only for 5 strains that had all 3 regulator genes (*fsrA*, *fsrB* and *fsrC*) from 9 enterococci containing the gene encoding gelatinase. No DNase activity was observed in any of the tested strains from all the hospitals (Table 4).

Detection of a specific mobile insertion element IS*16*

Molecular analysis of all analyzed *E. faecium* strains showed the presence of a gene fragment specific for IS*16* insertion element.

Discussion

In our study, the majority of patients were hospitalized in ICU, which is similar to other reports and seems to be an important risk factor for enterococci infections.[19,20]

Table 4. Distribution of cytolysin (hemolysin), gelatinase and DNase activity in the genus *Enterococcus* isolates from hospitals A, B, C, and D

Hospital	Virulence determinants		
	cytolysin	gelatinase	DNase
A	1	2	0
B	2	3	0
C	0	0	0
D	0	0	0
Total	3	5	0

Antimicrobial resistance

Our study confirms that *E. faecalis* is still a rare reservoir of acquired vancomycin resistance, and this trend is well-known in our country and in Europe.[2,21] The majority of *E. faecalis* strains were susceptible to ampicillin and other antimicrobials, and it seems that *E. faecalis* species is not a therapeutic problem in hospitals in Małopolska.

The major determinant of vancomycin resistance among Polish VRE*fm* is still the VanA gene cluster, which is also most prevalent in some European counties.[2,19,22] However, in our study, *E. faecium* VanA phenotype strains were in the minority, while VanB phenotype isolates were in the majority. This seems to be the characteristic feature for VRE*fm* from the Małopolska region, because in other provinces VanA phenotype is predominant.[8,23,24] In Małopolska, in recent years, the presence of both VanA and VanB phenotypes has been described.[25–27] All VREfm strains isolated in this study were MDR and this is a problem for the therapeutic management of patients. The most important problem with the multidrug resistance of the studied strains is that they are able to easily acquire (by plasmids and/or transposon transfer) new resistance traits between enterococci.

In our research, we isolated only 1 strain which belonged to the *E. avium* species and included the HLAR phenotype but was susceptible to other antimicrobials. Therefore, it seems that other enterococcal species are not a threat to the therapeutic situation in the Małopolska region nowadays. In our study, only a few *E. faecalis* strains and a large group of *E. faecium* strains, including all VRE*fm* isolates, were resistant to ampicillin. It is a very disturbing and

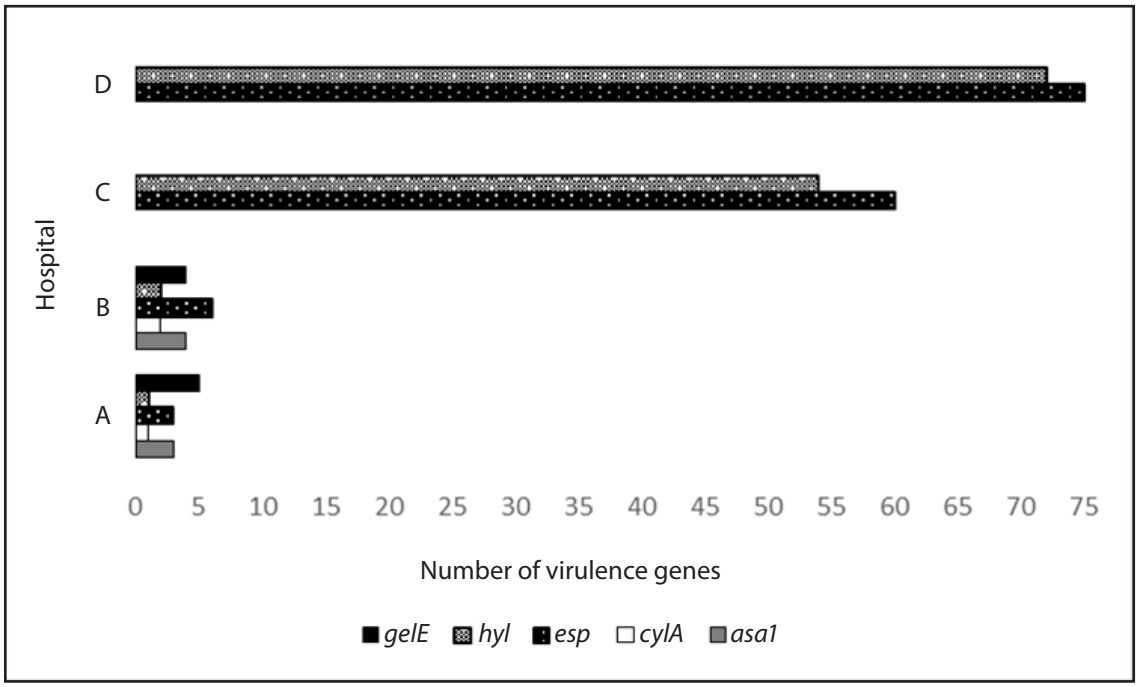

Fig. 1. Occurrence of virulence determinants *asa1*, *gelE*, *cylA*, *esp* and *hyl* in enterococci isolates from hospitals A, B, C, and D

dangerous situation, because β-lactam antibiotics are a vital group of drugs employed for the treatment of infections caused by enterococci. Ampicillin resistance also indicates resistance to amoxicillin, piperacillin and preparations combined with β-lactamase (ampicillin/sulbactam, amoxicillin/clavulanic acid, piperacillin/tazobactam).[28] Moreover, it is well known that ampicillin-resistant *E. faecium* are a precursor of multidrug-resistant strains, including VRE, and are widespread in Polish and European hospitals.[2,19] Enterococci are inherently resistant to low concentrations of aminoglycosides, which is associated with reduced permeability of these antibiotics through the cell wall.[29] In agreement with other studies from Poland, also ours, the spread of the *aac(6')-Ie-aph(2")-Ia* gene, which encodes the bifunctional enzyme AAC(6')-APH(2") and eliminates the synergistic effect between β-lactams or glycopeptide antibiotics and aminoglycosides, was responsible for high-level resistance to gentamicin among the majority of enterococci.[22,28] Thus, it precludes its therapeutic application in severe infections caused by enterococci, including bacteremia and endocarditis.[28,29] If the strains are resistant to high concentrations of gentamicin with simultaneous sensitivity to streptomycin (HLGR), streptomycin can be used in combination with β-lactams and glycopeptides. If the strains display HLSR phenotype, which means that they are resistant to high concentrations of streptomycin with simultaneous sensitivity to gentamicin, streptomycin therapy can be applied combined with β-lactams and glycopeptides, since the therapeutic effect is present. Detection of resistance to high concentrations of gentamicin and streptomycin at the same time (HLAR) means that there is no synergy of aminoglycosides with β-lactams and glycopeptides, which was found in a high degree in the strains examined by us.[28,29] For years, a tendency that has appeared among enterococci, also in Poland, regards their growing resistance to fluoroquinolones. In hospital A, this regularity is poorly marked (only 33.3% of resistant strains). In hospital B, it increases to approx. 50% of resistant strains, but it is best marked by a high resistance to ciprofloxacin, levofloxacin and norfloxacin in the enterococci investigated in hospitals C and D. This also proves that a very high percentage of resistant strains is common among the species *E. faecium*, which is also described in other studies originating in Poland.[19,28] Quinupristin-dalfopristin (Q/D) (with the exception of the *E. faecalis* species), linezolid and tigecycline have bacteriostatic activity against VRE, so they are recommended for the treatment of various infections caused by strains simultaneously resistant to several groups of antibiotics, such as ampicillin, glycopeptides, and a high concentration of aminoglycosides. Our data suggests that these antimicrobials may be effective therapeutic options for the treatment of serious infections caused by the studied enterococci strains. Unfortunately, Q/D treatment has failures and adverse effects, whereas acquired resistance to linezolid has been observed in enterococci, but this phenomenon is still rare and associated with the duration of previous linezolid therapy.[3,6]

Virulence factors

Enterococci displaying virulence factors are more likely to cause an infection than the strains devoid of them. There are factors that facilitate colonization and those that facilitate the infection of previously colonized tissue. The majority of VREfm strains from hospitals C and D both contained *esp* and hyl_{Efm} genes. Hyaluronidase (encoded by the *hyl* gene) contributes to the destruction of connective tissue and thus makes it easier for bacteria to spread in an infected organism.[15] The enterococcal surface protein Esp (encoded by *esp*) is associated with increased virulence, colonization and persistence in the urinary tract and biofilm formation.[15,18] In hospitals A and B, the situation was more diverse, and *gelE*, *asa1*, *esp*, hyl_{Efm}, and *cylA* genes in different combinations were detected in the enterococcal isolates. Gelatinase (encoded by *gelE*) has the ability to hydrolyze collagen, gelatin and small peptides.[15,18] Aggregation substance (encoded by *asa1*) facilitates the adhesion of enterococci to host cells, supports aggregation and facilitates the survival of bacteria in macrophages. Cytolysin has bacteriocin activity and the ability to lyse certain eukaryotic erythrocytes and gram-positive bacterial cells.[15,18] Among the studied enterococci, there were also strains which did not have virulence determinants. Moreover, like other researchers, we did not find DNase activity in either *E. faecalis*, *E. faecium* or *E. avium* isolates.[17] Therefore, it seems that this is not a virulence factor that occurs in enterococci.

The correlation between drug resistance and virulence genes

Nowadays, in European hospitals, the vancomycin-susceptible enterocci are replaced by high-level ampicillin and ciprofloxacin resistance, HLAR and VRE phenotype simultaneously, which are typical of hospital-acquired VREfm. The *E. faecium* strains examined in our study also shared other subpopulation characteristics of hospital-acquired strains, such as the presence of *esp*, *hyl* and IS*16*.[18,30] Phenotypic and molecular characterization of the *E. faecium* isolates tested coming from hospitals C and D corresponds to the characteristics of strains with high epidemic potential occurring in hospitals in Europe (Clonal Complex CC17). The specific mobile insertion element IS16 is highly specific for predicting hospital-associated strain types.[30] In our study, all strains belonging to the *E. faecium* species showed the presence of IS*16*, which is a similar result to other studies from Poland.[19,22,23,30]

Conclusions

The *E. faecalis* strains that appear in Małopolska hospitals are largely sensitive to antibiotics and have a variable amount of virulence factors. The *E. faecalis* species were

isolated less frequently than *E. faecium* from patients hospitalized in Małopolska hospitals. In contrast, the strains of the species *E. faecium* are a more uniform group with resistance to a number of significant therapeutic drug types, such as ampicillin, high concentrations of aminoglycosides, fluoroquinolones and glycopeptides (also more often displaying the VanB phenotype). VRE*fm* strains also have a high proportion of *hyl* and *esp* genes, which is characteristic of hospital strains of enterococci.

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Probiotics: Myths or facts about their role in allergy prevention

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Abstract

Background. The hygiene hypothesis proposed by Strachan in the 1980s clearly emphasized the role of microorganisms in atopy prevention.

Objectives. The study objective was to assess the preventive role of probiotics in patients with allergic rhinitis, bronchial asthma, atopic dermatitis, and/or food allergy.

Material and methods. The methods used in the study were the International Study of Asthma and Allergies in Childhood (ISAAC) questionnaires for 6–7- and 13–14-year-olds and the European Community Respiratory Health Survey II (ECRHS II) questionnaire targeted for the 20–44 age group. The study was conducted as part of the cross-sectional Epidemiology of Allergic Diseases in Poland study conducted in 9 Polish regions (8 urban: Warszawa, Lublin, Białystok, Gdańsk, Poznań, Wrocław, Katowice, Kraków, and the rural regions of Zamojski and Krasnostawski counties). The study material was a group of patients diagnosed with food allergy (n = 407), atopic dermatitis (n = 311), allergic rhinitis (n = 1.353), bronchial asthma (n = 505), and healthy volunteers (n = 2,403).

Results. Genetic factors play an important role in the allergy development. A family history positive for chronic skin disorders increased the risk of atopic dermatitis and food allergies (OR = 1.456, CI = 1.14–1.85, p = 0.002; and OR = 1.378, CI = 1.05–1.81, p = 0.02, respectively). The consumption of products containing live bacterial cultures showed no preventive effects in any of the evaluated disorders in early childhood. Conversely, over the age of 14 years, probiotic formulations exhibit health-promoting effects and may lower the risk of allergic diseases.

Conclusions. The use of probiotics in the Polish population showed no protective effect in the first years of life. The changes in dietary habits introduced during late adolescence demonstrated significantly greater preventive effects of live bacterial cultures against the development of allergic diseases.

Key words: probiotics, prevention, allergy

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The prevalence of allergic diseases poses a serious problem in the field of modern medicine and public health. Estimates suggest that nearly 40% of the general population suffers from some form of allergy.[1] Measures such as primary and secondary prophylaxis are crucial in allergy prevention and contribute to health improvement. Recent decades saw an increased interest in diet supplementation with live bacterial cultures (*Lactobacillus rhamnosus GG, Lactobacillus acidophilus, Lactobacillus reuteri*, and mixed cultures) which – apart from having protective gastrointestinal effects – modulate the immune system (the intestinal microflora regulates the Th1/Th2 ratio and stimulates IL10).[2,3]

The objective of this study was to assess the rates of probiotic use in the Polish population as well as to detect any probiotic-induced protection against allergic diseases, including atopic dermatitis (AD), food allergies (FA), allergic rhinitis (AR), and bronchial asthma (BA).

Material and methods

Study group

A total of 22,454 respondents took part in the questionnaire survey, of whom the quality verification process was passed by 18,617 people in the age ranges: 6–7 years (n = 4510; 24.2% of all the subjects); 13–14 years (n = 4721; 25.4%); and 20–44 years (n = 9386; 50.4%). There were 10,011 (53.8%) women and 8,606 (46.2%) men altogether in the survey. The percentage distribution of respondents in individual research centers in Poland was similar: Białystok – 3,411 (18.3%); Katowice – 2,434 (13.1%); Lublin – 2,422 (13.0%); Warszawa – 2,281 (12.3%); Zamość – 2,055 (11.0%); Gdańsk – 1,837 (9.9%); Kraków – 1,642 (8.8%); Wrocław – 1,317 (7.1%), and Poznań – 1,218 (6.5%). The selection of the group was based on purposive (nonprobability) sampling, and within the surveyed centers, the subjects were randomly selected with their personal identification number (PESEL) used as the sampling frame (the PESEL system is administered and maintained by the Ministry of the Interior and Administration). For the purposes of sampling in the research centers, the following 3 criteria were taken into account: geographical location, the number of inhabitants, the level of air pollution. From among the entire population from the 1st stage of the study, 30% (4,783) of all the respondents were qualified on an outpatient basis for further analysis. They were in 3 age ranges: children (6–7 years; 29.5%); adolescents (13–14 years; 28.0%); and adults (20–44 years; 22.7%). The study involved subjects who had been diagnosed with AR (n = 1,353), BA (n = 505), AD (n = 311) and/or FA (n = 407), and 2,403 healthy controls (HC) (Fig. 1). The diagnoses were verified based on the Allergic Rhinitis and its Impact on Asthma (ARIA) criteria, Global Initiative for Asthma (GINA) criteria, and according to Hanifina and Rajka. The patients underwent additional complementary skin prick tests (16 environmental allergens), a spirometry test, an assessment of specific IgE (sIgE) antibodies, and had their nasal patency measured by peak nasal inspiratory flow (PNIF).

Methods

The assessment tools used in the study were the European Community Respiratory Health Survey II (ECRHS II) and the International Study of Asthma and Allergies in Childhood (ISAAC); the latter had been adapted to European conditions. The project involved the use of the innovative Computer-Assisted Personal Interviewing (CAPI) technique and Personal Digital Assistant (PDA) equipment, which was employed to ensure quality control of the survey questionnaire. Furthermore, after the interview had been completed, all the data was transferred via GPRS to the central office and exported to the project database. This cross-sectional study was part of the project "Implementation of a System for the Prevention and Early Detection of Allergic Diseases in Poland" (No. 6 PO5 2005 C/06572), conducted on the residents of 8 large Polish cities (Gdańsk, Wrocław, Poznań, Katowice, Kraków, Lublin, Białystok, Warszawa) and rural areas of Zamojski and Krasnostawski counties. The study was approved by the Ethics Committee at Medical University of Warsaw (KB/206/2005).

Table 1. Coinheritance factors for allergic diseases

Food allergy (FA)	OR	CI	p-value
Genetic factors – mother*	1.850	1.44–2.38	1.64e-06
Genetic factors – father*	2.058	1.56–2.72	3.45e-07
Chronic skin disorders in the family**	1.456	1.14–1.85	0.00227
Atopic dermatitis (AD)			
Genetic factors – mother*	1.831	1.38–2.43	2.84e-05*
Genetic factors – father*	1.822	1.33–2.50	0.000192*
Genetic factors – paternal grandfather	1.896	1.16–3.09	0.010303
Chronic skin disorders in the family**	1.378	1.05–1.81	0.021048
Allergic rhinitis (AR)			
Genetic factors – mother*	1.277	1.07–1.53	0.00751
Genetic factors – father*	1.767	1.44–2.17	4.23e-08*
Genetic factors – siblings	1.300	1.10–1.53	0.00160
Bronchial asthma (BA)			
Genetic factors – father*	1.386	1.05–1.84	0.02338
Genetic factors – siblings	1.275	1.01–1.61	0.03968
Genetic factors – maternal grandparents*	1.480	1.04–2.10	0.02833
Genetic factors – paternal grandfather	1.946	1.28–2.95	0.00176

Asterisks indicate statistically significant results (Bonferroni-corrected due to a high number of tests); * declared responses according to the ECRHS/ ISAAC standardized questionnaire (Does anyone in the immediate family suffer from allergy?); ** declared responses according to the ECRHS/ ISAAC standardized questionnaire (Is there any history of skin disease in your family?).

Statistical analysis

The odds ratio (OR) for individual risk factors was calculated based on the appropriate logistic regression models. The Wald test was used to determine statistical significance. The 95% confidence intervals (CI) for the OR were also provided. Due to a large number of factors (28 factors from Table 1 and 6 factors from Table 2, stratified by 4 diseases), a total of 136 tests were conducted. Consequently, the standard significance level of 0.05 was divided by the number of tests, which yielded a significance level of 3.7e-4. The results marked with an asterisk (*) in Tables 1 and 2 represent findings that are significant according to this new significance level (with the Bonferonni correction).

Results

Genetic factors (the risk factor analysis included 28 variables; Table 1) significantly increased the risk of allergic diseases, with a higher risk of allergy in children with a family history of paternal atopy. Interestingly, chronic hereditary skin disorders tended to increase the risk of FA and AD. FA manifestation rates were 5-fold (OR = 5.45; p < 2e-16; 95% CI 4.127809–7.185846), AO manifestations were 2-fold (OR = 2.32; p = 2e-8; 95% CI 1.73–3.11), and AR manifestations were 2-fold (OR = 1.85; p = 2.7e-7; 95% CI 1.47–2.35) higher in patients with AD.

The use of probiotics was declared by 80% of patients diagnosed with FA, 79% of patients with AD, 71% of patients with AR, and 78% patients with BA. The most commonly used probiotic products were Lakcid (*Lactobacillus rhamnosus*), Lacidofil (*Lactobacillus rhamnosus, Lactobacillus helveticus*) and Trilac (*Lactobacillus*). The frequency distribution of probiotic use was nearly identical between the groups, with slightly higher rates in the AD and FA groups, with the difference being nonsignificant. Probiotic products used for the purpose of allergy prevention did not exhibit any prophylactic effects in subjects under 14 years of age (1 exception was a case of AR, in which preventive effects were observed from the age of 1 year) (Table 2). Supplements such as kefir (*Lactobacillus kefiri, Leuconostac*) and yogurts (*Streptococcus thermophilus, Lactobacillus*), administered several times a week in late adolescence (above the age of 14 years), showed health-promoting effects in allergic diseases.

Discussion

The effect of environmental changes as well as outdoor and indoor factors play a key role in allergic disease development. The hygiene hypothesis proposed by Strachan

Table 2. Products containing live bacterial

Food allergy (FA)	OR	CI	p-value
Products containing live bacterial cultures	1.902	1.46–2.46	1.27e-06*
Products containing live bacterial cultures, used independently of antibiotic therapy	1.703	1.18–2.44	0.00386
Products containing live bacterial cultures, used until the age of 1 year	1.835	1.17–2.87	0.008012
Products containing live bacterial cultures, used at the age of 3–7 years	1.336	1.01–1.75	0.036345
Products containing live bacterial cultures, used at the age of over 14 years	0.490	0.33–0.71	0.000232*
Yogurts consumed 3 times a week or more at the age of over 14 years	0.311	0.19–0.49	5.14e-07*
Atopic dermatitis (AD)			
Products containing *Lactobacilli*	2.061	1.52– 2.78	2.69e-06*
Products containing live bacterial cultures, used independently of antibiotic therapy	1.610	1.07–2.40	0.0202
Products containing live bacterial cultures, used at the age of over 14 years	0.378	0.23–0.59	3.22e-05*
Kefirs consumed 3 times a week or more at the age of over 14 years	0.389	0.18–0.81	0.0124
Yogurts consumed 3 times a week or more at the age of over 14 years	0.381	0.23–0.60	4.83e-05*
Allergic rhinitis (AR)			
Products containing live bacterial cultures	1.165	1.00–1.34	0.041
Products containing live bacterial cultures, used until the age of 1 year	1.477	1.02–2.13	0.0371
Products containing live bacterial cultures, used at the age of 1–3 years	0.751	0.58– 0.96	0.0277
Yogurts consumed 3 times a week or more at the age of 3–7 years	0.725	0.56–0.92	0.00929
Bronchial asthma (BA)			
Products containing live bacterial cultures	1.850	1.45–2.34	4.09e-07*
Products containing live bacterial cultures, used in combination with antibiotic therapy	0.576	1.12–2.81	0.0141
Products containing live bacterial cultures, used at the age of over 14 years	0.673	0.49–0.91	0.0104
Kefirs consumed 3 times a week or more at the age of 7–14 years	0.399	0.22–0.71	0.00201

Asterisks indicate statistically significant results (Bonferroni-corrected due to a high number of tests).

in the 1980s clearly emphasized the role of microorganisms in atopy prevention.[4] Excessive hygiene or genetic factors lead to disturbances in the intestinal microbiota, which begins forming during the first days of life in a breastfed neonate and reaches its mature composition at the age of 2 years.[5] Due to the disturbances the activity of lymphocytes Th1 is limited. A study by Bjorksten et al. on the relationship between allergic diseases and the composition of intestinal microflora showed considerably lower levels of *Lactobacillus* and *Bacteroides* in the group with a history of allergies as compared to the control group. This clearly indicates the necessity to use probiotic supplementation apart from an elimination diet.[6]

As mentioned before, live bacterial cultures play a distinct role in maintaining good health via their effects on the gastrointestinal tract (the stomach, intestines). Although studies on the health-promoting influence of probiotics showed good therapeutic and preventive effects in animal models, the treatment of allergic diseases in humans showed only moderate effects (most pronounced in atopic eczema).[7,8] Moreover, the World Allergy Organization and McMaster University Guidelines for Allergic Disease Prevention (GLAD-P) panel of experts clearly indicated a lack of robust evidence confirming the effectiveness of probiotics in a group of pregnant or breastfeeding women, or infants at a high risk of atopy.[9]

Fig. 1. Study group characteristics

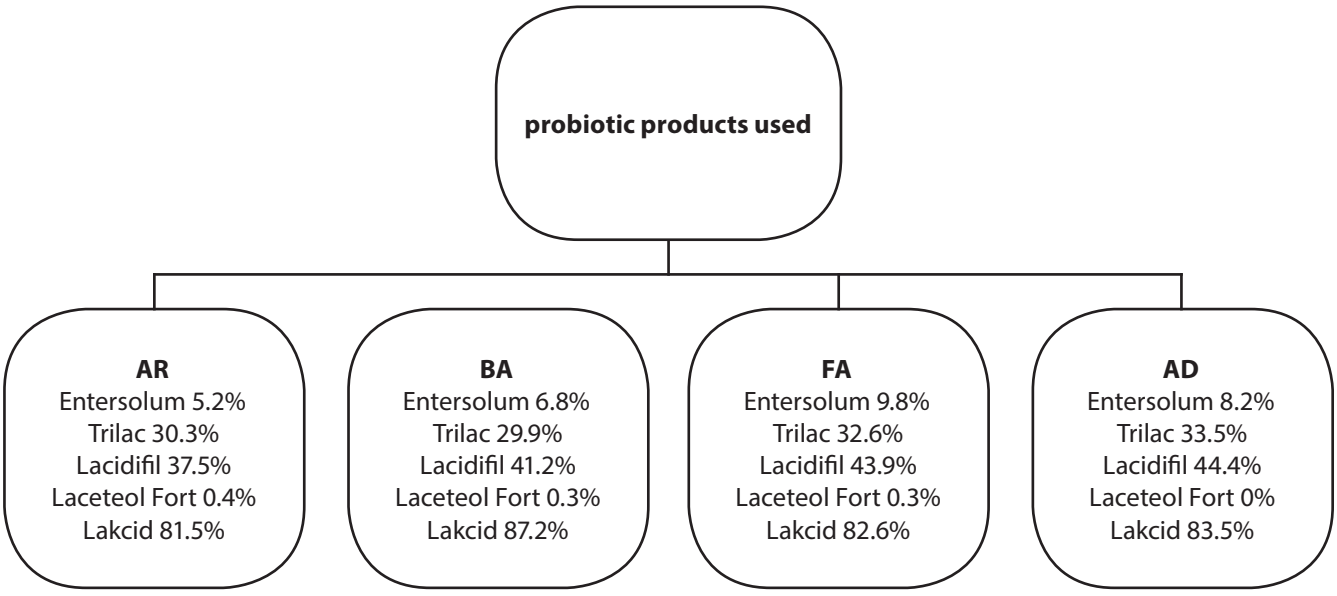

Fig. 2. Probiotic products used by the study population

Despite the fact that a meta-analysis (n = 2,403) by Cuello-Garcia et al., based on the Cochrane Central Register of Controlled Trials, MEDLINE and Embase, documented a preventive effect of probiotics on atopic eczema in a group of women using products containing live bacterial cultures (OR = 0.71; 95% CI 0.60–0.84), in breastfeeding women (OR = 0.57; 95% CI 0.47–0.69) and in neonates (OR = 0.80; 95% CI 0.68–0.94), it did not indicate the necessity of supplementation due to a number of confounding factors.[10] A similar study by Boyle et al. evaluated the effects of *Lactobacillus rhamnosus* (LGG®) supplementation from 36 Hbd until birth as a factor significantly reducing the development of AD in infants; likewise, the use of *Lactobacillus casei DN114001* in neonates for 6 weeks minimized the risk of AD in a group of children under 6 months of age.[11]

The GUSTO cohort study with 3 assessment time points (at 6 months of age, 6–12 months of age, and at consecutive years after the age of 12 months) showed a significantly high risk of developing AD associated with antibiotic therapy (OR = 3.11; 95% CI 1.10–8.76; p = 0.03) during the first 6 months of life and the use of probiotics at the age of 9–12 months (OR = 4.32; 95% CI 1.07–17.45; p = 0.04). Moreover, the risk of developing AD was shown to increase over 20-fold in genetically predisposed patients, with a positive family history (OR = 20.46; 95% CI 2.73–153.15, p < 0.01).[12]

The risk of developing an allergy when one of the parents has an allergy ranges from 35 to 40%; however, when both parents have been diagnosed with atopy, the risk can increase up to 60%. Moreover, a predilection for developing allergies is usually inherited by the son when the father has an allergy and, correspondingly, by the daughter when the mother has an allergy. Scientific literature contains considerably more evidence documenting a higher risk of allergy in the offspring, irrespective of sex, from the affected mother than from the affected father (40% in the case of maternal allergies, approx. 30% in the case of paternal allergies). Conversely, our analysis clearly indicates higher rates of allergies inherited from the father's side. Recent attempts to estimate the risk of developing allergy via hereditary means have been using filaggrin (an amino acid precursor with molecular weight of 35–37 kDA that is a component of a natural moisturizing factor), particularly its mutations in the *2282del4* and *R501X* genes.[13] A study by Ponińska et al. (one of the studies on the role of filaggrin *2282del4* in inheriting allergic diseases), conducted on a population of 3,802, estimated the risk of developing AD (OR = 2.01; 95% CI 1.20–3.36; p = 0.007), AR (OR = 1.69; 95% CI 1.12–2.54; p = 0.011) and atopic asthma (OR = 2.22; 95% Cl 1.24–3.96; p = 0.006) in patients with mutations in this gene.[13] We observed no correlation between the presence of gene mutations and serum sIgE levels. Conversely, Filipowska-Grońska et al. demonstrated filaggrin gene mutations in 11.4% out of all 205 study subjects with AD with concomitantly increased total and specific serum IgE levels.[15]

Subsequent attempts at estimating the effectiveness of probiotic supplementation in patients with AR and BA also yielded inconsistent evidence. A meta-analysis by Zajac et al., including 23 randomized studies conducted on 1,919 subjects to assess the rationale for probiotic use in 3 aspects – Rhinitis Quality of Life (RQLQ), Rhinitis Total Symptom Scores (RTSS) and sIgE levels, demonstrated the preventive effect of probiotics in 17 studies and a protective effect in 6 studies. The probiotics used in the studies significantly improved quality of life (SMD –2.23; p = 0.02), with no effect on rhinitis symptoms or total serum IgE levels (SMD 0.01; p = 0.94). Moreover, we observed a decreasing trend in sIgE levels (SMD 0.20; p = 0.06) vs placebo.[16] Giovannini et al. showed a significant beneficial effect of *Lactobacillus casei* supplementation on the symptoms of AR (n = 131), with a lack of effectiveness on the rates of dyspneic episodes in atopic asthma (n = 119).[17] These finding were consistent with those of Wheeler et al., who evaluated the effect of *Lactobacillus bulgaricus* in a group of 15 patients diagnosed with moderate asthma and demonstrated no significant changes in the assessed lung function parameters and total serum IgE levels.[18]

This first cross-sectional study on probiotic supplementation in the Polish population suggested a need for further prospective, cohort studies. The observed preventive effects of live bacterial cultures in patients aged over 14 years, diagnosed with allergic diseases may be explained by a change in dietary preferences – making own decisions, e.g., to augment one's diet to include cultured dairy products (yogurts, kefirs) with a composition recommended by the Food and Agriculture Organization of the United Nations and World Health Organization (FAO/WHO).

There is no consistent evidence for the effectiveness of consuming live bacterial cultures in the prevention or treatment of allergic diseases, especially in subjects under 14 years of age. Probiotic supplementation while changing dietary habits and full maturation of microbiota may have a protective effect against the development of allergic diseases. Further prospective studies are needed to evaluate the effectiveness of the potential probiotic-induced prevention of allergic diseases.

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Genetic polymorphisms and their involvement in the regulation of the inflammatory response in asthma and COPD

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Abstract

Asthma and chronic obstructive pulmonary disease (COPD) are widely documented diseases with an inflammatory component. Asthma is a heterogeneous disorder of the airways that involves chronic inflammation, decline of the airway function and tissue remodeling. Chronic obstructive pulmonary disease is a preventable and treatable disease, which is characterized by persistent limited airflow, and is usually progressive with an increased inflammatory response in the airways. The inflammatory response is evoked by the stimulus of noxious particles and gases. Inflammation is a natural process in response to injury, but in asthma and COPD patients it occurs as an abnormal immune response to pathogenic stimuli which induce chronic inflammation, a key process in the pathogenesis of both diseases. However, the inflammatory process is different in both diseases, and is involved in several release patterns of inflammation mediators. It is not entirely clear whether these proteins are simply markers of the inflammatory process that accompanies a chronic disease or if they play a major role in the pathogenesis of the disease. The main proteins which have been described in these illnesses are: IL-4, IL-6, IL-8, and TNF-α. In addition, polymorphisms have been described in genes encoding these proteins that alter the transcription and susceptibility associated with these diseases. In this review, we will focus on asthma and COPD, and the involvement of these proteins and their genetic polymorphisms.

Key words: asthma, chronic obstructive pulmonary disease, gene regulation, single nucleotide polymorphism, tumor necrosis factor alpha

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Background

Inflammation is a common process in chronic respiratory diseases. The presence of increased levels of systemic inflammatory markers is a recurrent finding in laboratory tests. In particular, asthma and chronic obstructive pulmonary disease (COPD) are diseases with a widely documented inflammatory component and have been discussed in various studies; however, given the transversal nature of most of the studies conducted so far and the possible confusion regarding a number of external factors related to lifestyle associated with levels of inflammatory biomarkers, which in turn, are generally nonspecific, it is not entirely clear whether these proteins are simply markers of the inflammatory process that accompanies a chronic disease or if they play a major role in the pathogenesis of the disease. In this review, we will focus on asthma and COPD as study models.

Asthma

Asthma is a heterogeneous disorder of the airways that involves chronic inflammation, decline of the airway function and tissue remodeling.[1] The overall prevalence varies between 1% and 18% of the population in different latitudes.[2] In developed countries, asthma is found in about 10% of adults, while in emerging countries, the prevalence is lower, but rapidly increasing, most likely due to underdiagnosis. The World Health Organization (WHO) estimates that 300 million people worldwide are affected by the disorder and this number is estimated to increase to 400 million by 2025.[3] Although there are no specific genetic or environmental factors conclusive, genetic predisposition to increased immunoglobulin E (IgE) in local mucosa (atopy) is a strong risk factor for developing the disease.[4,5] It has been proposed that asthma develops from a complex interplay between genetic and environmental factors, such as the dose of allergens and respiratory tract infections.[4] This culminates with an abnormal inflammatory response directed by Th2 cells to normally innocuous allergen content in the air.[6]

Chronic asthma immunopathogenesis

Inflammation is a natural process in response to injury, but it occurs in asthmatic patients as an abnormal immune response to pathogenic stimuli which induces chronic inflammation, a key process in the pathogenesis of the disease.[7] Another crucial event in the development of asthma is the recruitment of leukocytes mediated by chemokines, which produce an inappropriate immune activation, believed to be in part responsible for the chronic allergic asthma.[8] In chronic asthma there is an accumulation of CD4 + T cells in airway.[9] These cells typically exhibit an immune response called Th2 cytokine profile characterized by the production of interleukins (IL): IL-4, IL-5 and IL-13, which contribute to the recruitment of eosinophils and the development of airway hyperresponsiveness.[10–12] Moreover, in recent years research has suggested the involvement of Th17 cells, which secrete IL-17A, believed to contribute to the pathogenesis of the disease, however, its mechanism remains obscure.[13] The initiation of the allergic response in the airways begins with epithelial cells, which release thymic stromal lymphopoietin (TSLP), IL-33 and IL-25 in response to allergens. TSLP regulate the migration of dendritic cells (DCs), present antigen and promote Th cell differentiation. Th cells mediate the IgE isotype switching in B cells. The antigen-specific IgE response leads to the recruitment of mast cells and basophils into the airways, increasing the local allergic response. Additionally, IL-33 and IL-25 induce the release of IL-13 and IL-5 from CD25 + Th cells into the airway, which promotes Th2 cells differentiation and local production of cytokines, such as IL-4, IL-5 and IL-13.[14] In this process, B-cells and memory T cells are generated, which facilitates a faster response to repeated stimulation, thereby causing the chronicity of the disease.[15] Knowledge of asthma molecular phenotypes and the molecular pathways involved in asthma, in particular cytokines which are involved in them, has allowed us to create subgroups of asthma patients based on the activity (or inactivity) of specific cytokine pathways. There have been several studies of gene expression based on the fact that epithelial cells respond to stimuli of various cytokines and they found various overexpressed genes. The calcium-activated chloride channel accessory 1 (*CLCA1*), periostin (*POSTN*), serpine peptidase inhibitor (*SERPINB3*), are all involved in the regulation of IL-13 and IL-4, and therefore Th2 inflammation.[16,17] Other genes that have been found over-expressed in asthmatic patients are *ACACA*, *TPSAB1* and proteins which are secreted by mast cells. These cells have been implicated in airway hyperresponsiveness, although it has been reported that this is a variable in asthmatic patients.[18,19] The stimulation of bronchial epithelial cells with IL-13 induces the expression of stem cell factor, which is a growth factor and a mast cell attractant. This induction of stem cell factor provides a mechanism for increasing the number of intraepithelial mast cells, which are of particular importance in severe asthma.[20] Genetic polymorphisms in asthma/asthma-like diseases may increase the risk for developing these diseases that are determined by environmental factors. In fact, twin studies have estimated heritability ranging from 35–90%.[21] Different genome-wide association studies (GWAS) have found different genes related to the development and phenotypic features of the disease, as *ORMDL3* (ORM1-like 3), *PDE4D* (phosphodiesterase 4D, cAMP-specific), *IL-1RL1* (interleukin-1 receptor-like 1), *IL-18R1* (interleukin-18 receptor 1), *HLA-DQ*, *IL33*, *SMAD3* (SMAD family member 3), and *IL-2RB* (interleukin-2 receptor beta).[22–24] Moreover, through genetic association studies and family-based

information, in certain specific protein coding genes it was identified that single nucleotide polymorphisms (SNP) are related to the development of asthma or phenotypes responding to treatment differently. Among thes, human leukocyte antigen (HLA), T-cell receptor (TCR), and cytotoxic T-lymphocyte-associated antigen (CTLA-4) may be mentioned. Additionally, genetic variations have been found in genes that encode proteins involved in the inflammatory process, as IL-4, -9, -13, and their respective receptors and intracellular signaling molecules, such as signal transducer and activator of transcription (STAT-6), suppressor of cytokine signaling (SOCS-1).[26] Although many of these findings have been replicated in other populations, some of the studies failed to replicate the same results. This is of vital importance because the studies replicate different polymorphisms (within the same chromosome) forming haplotypes and how they contribute to or elucidate a set of genes that may be involved in the development and the progression of the disease.

Chronic obstructive pulmonary disease

Chronic obstructive pulmonary disease (COPD) is defined by the Global Initiative for Obstructive Lung Disease (GOLD) as a preventable and treatable disease, which is characterized by persistent limited airflow, which is usually progressive with an increased inflammatory response in the airways, the response is to the stimulus of particles and gases. Exacerbations and co-morbidities contribute to the individual illness severity (GOLD 2011). Cigarette smoking is the major environmental risk factor for developing COPD in emerging countries like Nepal, Colombia and Mexico and is also associated with exposure to wood smoke.[28,29] The worldwide prevalence of COPD ranges between 5% and 10% (it has increased in recent decades) and is more common in men than in women in the case of exposure to cigarette smoke. The Latin American Project for the Investigation of Obstructive Lung Disease (PLATINO) obstructive pulmonary research, conducted by the Latin American Thoracic Association (ALAT), determined the prevalence of COPD in 5 Latin American countries and found that the percentages vary between countries from 7.8% in Mexico City to 17% in Montevideo.[30]

Inflammation in COPD

COPD is a condition characterized by inflammation and airway remodeling, as well as inflammation and the destruction of lung parenchyma, resulting in the obstruction of expiratory airflow, lung hyperinflation retractability, loss of elasticity, and finally altered gas exchange. Lung remodeling and tissue damage coupled with wall thickening, inflammation and fibrosis of the small airways seem to play an important role in the pathogenesis of COPD.[31] Pulmonary parenchymal inflammation, oxidative stress, apoptosis, and proteolysis eventually result in emphysematous destruction of the alveolar wall.[32]

Inflammatory mediators involved in COPD

The family of proteins known as chemokines and chemokine receptors is considered to be key mediators in inflammatory cell recruitment. Chemokine receptors play an important role in the trafficking of immune cells to sites of injury and inflammation after an antigen encounter. Approximately, 50 chemokines and their 20 receptors have been associated with COPD. Among its functions is the ability to drive the migration of leukocytes involved in proliferation, differentiation, survival, and cellular retention.[33] In addition, chemokine receptors 5 (CCR5) and 3 (CCR3) have been implicated in COPD, since the expression of these receptors on T cells infiltrated in COPD patients has been demonstrated. Il-8, growth related oncogene (GRO-alpha), and extractable nuclear antigen (ENA)-78 may be involved in the increased numbers of PMN in smokers' airspaces, whereas greater concentrations of macrophages, neutrophils, IL-1β and IL-8 are elevated in the pulmonary microenvironment of smokers in a cigarette dose-dependent manner.[35,36] IL-8 is a potent neutrophil chemoattractant found in elevated levels of this cytokine in induced sputum of COPD patients and was correlated with a high number of neutrophils.[37,38] Concentrations of IL-8 are even higher in emphysema patients, where there is a-1 antitrypsin deficiency.[39]

Inhibitory cytokines, including IL-10, transforming growth factor β1 (TGF-β1), IL-11, and the receptor antagonist of IL-1 are also delivered to limit the duration and extent of the inflammatory response in the lung of patients with COPD, but there is limited information. IL-10 is particularly useful as an inhibitor of various inflammatory processes, low levels of IL-10 positive cells in sputum of COPD patients have been reported.[36]

Genetic polymorphisms associated with COPD

As mentioned previously, COPD is characterized by chronic inflammation based on an abnormal inflammatory response. Studies found different polymorphisms in genes encoding inflammatory mediators that have been implicated in the pathogenesis of the disease, such as tumor necrosis factor (*TNF*), *IL-8* and *TGF b*, and others. Regarding the *TNF* gene, as previously described, one SNP in the promoter region of the gene, which has been identified directly, affects the transcriptional regulation of the same.[40] Studies show some relevance to the SNP-308G/A *TNF* in the Asian populations but not in Caucasian populations; for example, Japanese and Taiwanese populations show an increased prevalence of COPD in relation to their respective control groups, but these results have

not been confirmed in other populations.[41] Recent studies have shown a relationship between metabolic alterations and changes in the levels of TNF-α in the systemic circulation of patients with COPD, where TNF-α is associated with accelerated metabolism and protein loss of skeletal muscle and adipose tissue.[42,43] Moreover, IL-8 is a chemokine which mediates the activation and migration of neutrophils from peripheral blood to tissue. This plays an important role in initiating the amplification of the inflammatory response. A transversion (A→G) at position -351 of the promoter of IL-8 has been positively associated with bronchiolitis reported, but inversely associated with bronchial asthma.[44–46] There are many genetic association studies of polymorphisms in genes whose protein products are involved in the inflammatory process, although their participation in the disease is not clear. Table 1 summarizes the main findings in COPD and in asthma.

Major inflammatory mediators and regulatory processes

A number of polymorphisms in genes encoding key proteins in the inflammatory process are found in promoter regions, including *IL4*, *IL6*, *IL8* and *TNF*, to name a few, so that it is important to meet regulatory aspects of these genes and their relationship to the pathologies in question. *IL4* is a glycoprotein of molecular weight approximately 15 kDa. Its expression is restricted to activated T cells, mast cells, basophils and eosinophils. The *IL-4* was discovered as a cofactor in the proliferation of resting B cells stimulated with anti-IgM.[68,69] IL-4 acts as a differentiation factor for B lymphocytes by regulating the IgG4 isotype switching to IgE and induces an increased expression of major histocompatibility complex class II (MHC II). It also promotes Th2 differentiation from Th0 cells, stimulates their growth and proliferation and inhibits the development of Th1 cells.[70] *IL-4* is shown to be increased in bronchoalveolar lavage (BAL) fluid and serum of allergic patients. People with atopy have altered regulation of the production of *IL-4* in response to bacterial antigens and dust mites. Furthermore, atopic patients have a higher number of T cells secreting *IL-4* compared with normal subjects. It also increases the release of chemokines such as CCL11 and expression of adhesion molecules such as VCAM-1 on lung fibroblasts, thereby promoting swelling of the airway, and inhibits the apoptosis of eosinophils and Th2 lymphocytes by expression of the Bcl-2 protein.[71] The findings and potential roles in COPD are still unknown, although there are some reports of increased serum levels in relation to the degree of smoking, but these findings were found in African population and not replicated in Mexican mestizo population.[72]

Gene structure and regulation of expression of interleukin 4

IL4 gene consists of 4 exons and 3 introns, located on the long arm of chromosome 5 in cytogenetic bands q23–31; it has potential binding sites for several transcription factors with positive or negative regulatory sequences, depending on the action generated after transcription factor binding. Among the main factors that interact with positive regulatory sequences are POS-1 and POS-2, which need to be assembled and which need to interact with different transcription factors such as C/EBP- g, NF-IL6, NF- IL6/3, Jun or NF-AT, depending on the cellular expression.[73,74]

Interleukin 6

This is a pleiotropic cytokine that plays an important role in regulating the immune and inflammatory response. It is produced by T cells, monocytes, fibroblasts, endothelial cells and keratinocytes. It also stimulates B cell differentiation and antibody production in synergy with IL-3 in the development of megakaryocytes and platelet production. It induces the expression of hepatic acute phase protein and has been associated with impaired functional capacity, reduced the daily physical activity and general deterioration of the health status.[29,75,76] Before the nomenclature of IL-6, it was known in a variety of names, such

Table 1. Main findings related to IL-4, IL-6, IL-8 and TNF in COPD and asthma

Protein	COPD	Asthma	References
IL-4	no studies	SNP associated with allergic rhinitis, serum levels and induced sputum ↑↑	47, 48
IL-6	IL-6 ↑↑ in induced sputum, BAL, and exhaled air concentration during exacerbations, SNPs associated with COPD	serum levels and BAL ↑↑ in patients with no allergic asthma	49–52, 54–56
IL-8	serum levels and induced sputum ↑↑, several SNPs associated with COPD	levels ↑↑ in BAL, in asthmatic patients with *C. pneumoniae* infection	37, 38, 57–59
TNF-α	serum levels and induced sputum ↑↑, SNPs associated with COPD and clinic phenotypes	↑↑ miRNAs involved in *TNF* regulation, serum levels ↑↑ in asthmatic patients without eosinophils	31, 41, 60–67

↑↑ – raised; BAL – bronchoalveolar lavage; SNP – single nucleotide polymorphism; miRNA – microRNA.

as IFN-b2, T cell replacement factor (TFR), B-cell differentiation factor (BCDF), B-cell stimulating factor, and hybridoma plasmacytoma growth (HPGF or IL-HP1).[77–81] In relation to their association with disease, asthma patients showed elevated serum levels of IL-6 and of bronchoalveolar lavage fluid, compared with nonsmokers with asthma whose results were as stable as non-asthmatics.[49,50] Additionally, a study conducted by Neveu et al. in 2010 showed an increase in levels of IL-6 and IL-13 in sputum from patients with allergic asthma; interestingly IL-1 b and TNF-α were not increased, which suggests an increase in IL-6 independent of the degree of inflammation.[51] In COPD, increased IL-6 concentrations are found in induced sputum, bronchoalveolar lavage and concentrated exhaled air from COPD patients, particularly during exacerbations.[52–54] IL-6 is also increased in plasma during exacerbations.[55,56] There is genetic evidence regarding the involvement of certain *IL-6* gene polymorphisms SNPs type involved in the increased production of the protein and its association with phenotypic traits, in both asthma and COPD.[82–85]

Gene structure and regulation of expression of interleukin 6

IL-6 gene is about 5 kb and consists of 4 exons and 6 introns, and is located on the short arm of chromosome 7 in the region p15–21.[86,87] The expression control depends on several different stimuli and cellular mechanisms that can act individually or in concert to activate transcription. The first to be shown to induce the production of IL-6 are the phorbol ester, IL-1 and TNF-α.[88] Within the promoter region of IL6, there are regions of the target signal transduction. These targets include DNA binding regions which are specific for nuclear factors such as NFIL-6 (protein binding CCAAT elements), nuclear factor kappa light chain in B cells (NFkB), activator protein-1 (AP-1), protein binding cAMP response element (CREB), and glucocorticoid receptor (GR), these sequences can be found at 200bp upstream of the transcription start site.[89,90]

Interleukin 8

IL-8 is a chemokine, a member of the CXC family, which is produced by macrophages, epithelial cells and fibroblasts in response to bacterial or viral stimulation or cellular stress response. It has been involved in the development of various biological processes such as repair, angiogenesis and inflammation.[91,92] Its main function is the chemotaxis of neutrophils and lymphocytes; it exerts its biological activity through 2 high affinity receptors designated as CXCR1 and CXCR2. Regarding the association of this chemokine with COPD, its function is still unknown, although studies of both genetic association and/or serum levels with this disease have been established. For example, Yamamoto et al. also reported elevated concentrations in sputum in COPD patients compared to different control groups.[38] Consistent with this finding, Keatings et al. described elevated IL-8 in induced sputum of COPD patients compared to different control groups.[37] Jeremy Hull et al. found polymorphism associated with bronchiolitis IL-8–251A in a family study conducted in the UK.[57] Yet, there are no studies that prove the role of these polymorphisms in this disease. Recently, Alfredo de Diego et al. conducted a study in Valencia, Spain, in which the values determined in sputum of different cytokines, including IL-8, resulting in such high values. Additionally, cultured bronchial epithelial cells, which were stimulated with cigarette smoke extract, for the purpose of measuring mRNA levels, resulted in an increased amount of mRNA and TNF-α, IL-8, which suggests that these molecules may increase in COPD, in response to the stimulus of cigarette smoke.[58] Regarding the most important findings of IL-8 and asthma, the findings are limited; however, in 2010, Patel et al. found an increase in bronchoalveolar lavage *IL8* mRNA in asthmatic patients with *C. pneumoniae*.[59] No results are available on polymorphisms in *IL-8* gene with asthma.

Gene structure and regulation of expression of interleukin 8

IL-8 gene consists of 4 exons and 3 introns, with a total length of 5.25 kbp, and is located on the long arm of chromosome 4 in the region (q12–21).[93] The 5' flanking region contains several *IL-8* gene regulatory elements, e.g., binding sites for transcription factors such as NF-kB, NF-IL6, AP-1, AP-2, AP-3, interferon regulatory factor-1 (IRF-1) and glucocorticoid response elements (GRE).[93] Transcriptional activation can occur after the stimulation with IL-lα IL-1β, TNF-α, bacterial endotoxin, reactive oxygen species and nitrogen intermediates.[94] *IL-8* can also be regulated at a post-transcriptional level, because in the 3' flanking region contains a repeat motif ATTTA, which is responsible for mRNA destabilization of several different cytokines.[95] TNF-α is the primary mediator of the immune response to gram-negative bacteria and other infectious organisms. The release of TNFα produced the local activation of vascular endothelium, with a release of nitric oxide, vasodilation and increased vascular permeability, leading to the recruitment of inflammatory cells, immunoglobulins and complement, causing the activation of T and B lymphocytes. It also increases adhesion and platelet activation.[96] Tumor necrosis factor alpha is a critical molecule in the regulation of inflammation, inducing a cascade of other inflammatory cytokines, chemokines and growth factors.[97] The results of several studies in vivo and in vitro indicate that the increased production of TNF-α leads to an increase in inflammation

and pro-oxidative response. TNF-α mediates inflammation and is thought to play a key role in respiratory and systemic features of COPD.[60] Jardim et al. found increased expression of the miRNAs involved in the regulation of *TNF*, *IL-8* and COX2 in epithelial cells of asthmatic patients.[61] In another study, Waserman et al. found an increase in serum levels of Th1-type cytokines, including TNF-α, in asthmatic patients without eosinophilia.[62] A result which correlates with these findings on this molecule has been described previously. With respect to TNF-α and its association with COPD in 2010, Tanni et al. conducted a study that showed high serum levels of TNF-α in patients with COPD and healthy chronic smokers compared with nonsmokers.[63] In another study, Gan et al. found elevated levels of TNF-α in bronchoalveolar lavage and induced sputum of COPD patients compared to the control group. Genetic association studies of *TNF* in COPD show some significance for rs1800629 (position -308 G/A) in Asian populations, but not in Caucasian populations.[41] However, these results are contradictory, because they could not be confirmed in other populations. In 2012, a study was conducted in the Taiwanese population, which identified TNF-863 (rs1800630) with an improvement in FEV1/FVC and with increasing BMI.[64] Another gene that affects the expression of TNF-α is lymphotoxin alpha gene (*LTA*). The rs909253 (G⟶A) in LTA has been implicated in gene regulation and reported associations with asthma and COPD.[31,65,66] Elevated serum TNF-α levels have been associated with SNPs in LTA.[67]

Structure and regulation of gene expression of tumor factor necrosis

TNF gene was cloned in 1984 and mapped along with the major histocompatibility complex on chromosome 6p21.3, along with genes encoding LT-a and LT-b.[98–100] The *TNF* gene consists of 4 exons and 3 introns, of which the last exon encodes over 80% of the secreted protein.[101] The major regulatory gene elements of *TNF* are the elements of response to NFkB important factor involved in LPS conferred inducibility. However, many other factors may be involved in the selective activation and expression of *TNF*. The mRNAs of TNF and LT, like many other cytokines, have AU-rich sequences in the 3'UTR region of the mRNA, which decreases its stability.[102] These sequences represent recognition sites for specific mRNA processing proteins. In 1988, Beutler et al. identified a ribonuclease that was isolated from mouse macrophages, which specifically destabilizes mRNA containing the sequence UUAUUUAU in the 3'UTR.[103,104] Interestingly, LT mRNA lacks these AU regions. Additionally, TNF-α induces several proteins involved in inflammation, tissue repair, hematopoiesis, immune response and anti-tumor effects. Some of these genes encode proteins called "TNF resistance proteins" which may inhibit TNF cytotoxicity.[105] Examples of these proteins include superoxide dismutase, protein A-20 zinc finger and the heat shock protein-70 (HSP70).[106–108] In Table 2, one can observe some polymorphisms in genes encoding the aforementioned proteins and their involvement in the expression.

Conclusions

Asthma and COPD are lung diseases, which represent a major public health problem and our country is no exception. Both disease entities share a common mechanism to inflammation, which acts differently in both pathologies. In this regard, there are several studies that have explored the levels of proteins involved in inflammation, both systemically and locally. According to these results, we can see that there are mediators that are shared in both diseases, such as IL1β, IL-6 and TNF-α, plus some others which differ, such as IL-13, IL-4, IL-5, and IL-8. For this reason, to understand the regulatory mechanisms that lead to the expression of these gene products as well as the research studies which analyze the genetic variations and their relationship with the phenotype expressed, it is vital to differentiate the genetic and molecular mechanism of both illnesses and to provide more effective treatment alternatives that contribute to the improvement of the patient.

Table 2. Polymorphisms in genes associated to inflammation and its biological implication

Gene	Polymorphism	Position	Change	Biological implication	Reference
TNF	rs1800629	-308	G⟶A	increases transcription and protein levels	109
	rs361525	-238	G⟶A	increases transcription	110
	rs1800630	-863	C⟶A	decreased binding capacity of NFkB	111
IL-4	rs2243250	-589	C⟶T	increases transcriptional activity	112, 113
	rs2070874	-33	C⟶T	increases the amount of protein	114
IL-6	rs1800795	-174	G⟶C	increased plasma levels	115
	G/G/A	-174/-572/-597	GGG/A	haplotype associated with increased mRNA	116
IL-8	rs4073	-251	A⟶T	increases the protein expression up to 5 folds	44, 117

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The significance of anthocyanins in the prevention and treatment of type 2 diabetes

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Abstract

Anthocyanins are food compounds which belong to polyphenols and can mainly be found in dark fruits (e.g., blueberries, black currants, cranberries) and vegetables (e.g., red cabbage, radish, eggplant). The results of large research have shown that these compounds play an important role in the prevention of type 2 diabetes (T2D). In rodent studies and in studies with isolated omental adipocytes, it was observed that anthocyanins regulated the carbohydrate metabolism in the body due to the upregulation of GLUT4 (insulin-regulated glucose transporter) translocation, increased activation of PPARγ (peroxisome proliferator-activated receptor-γ) in adipose tissue and skeletal muscles as well as increased secretion of adiponectin and leptin. Moreover, these compounds reduced the inflammation status in the body. Studies conducted on humans and experimental animals showed that anthocyanins decrease insulin resistance. This effect may be achieved by the upregulation of GLUT4 gene expression, activation of AMP-activated protein kinase and downregulation of retinol binding protein 4 (RBP4) expression. Anthocyanins also increased the uptake and utilization of glucose by tissues in streptozotocin-induced diabetic rats and mice, and they also protected pancreatic cells against necrosis induced by streptozotocin. Another mechanism that might explain the lower glucose level in the blood after a meal with anthocyanins compared to a meal without them is the inhibition of intestinal α-glucosidase and pancreatic α-amylase by these compounds. Moreover, anthocyanins improve insulin secretion, which can have a special meaning for people with T2D. The evidence from the presented studies suggests that foods rich in anthocyanins may be one of the diet elements supporting the prevention and treatment of T2D.

Key words: insulin resistance, type 2 diabetes, anthocyanins, postprandial glycemia, cyanidin-3-O-glucoside

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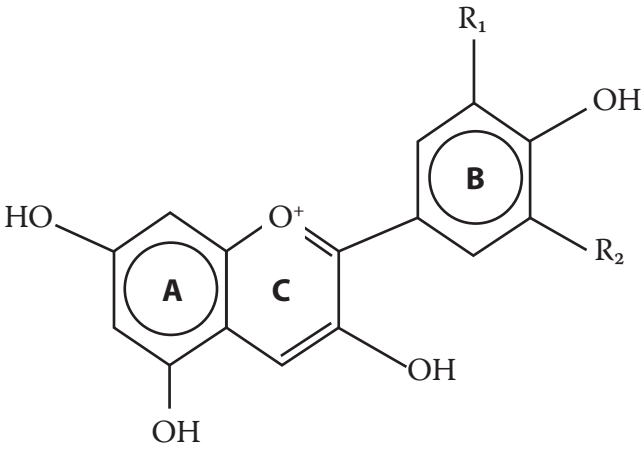
Background

Flavonoids are a numerous group of plant compounds that cannot be synthesized in the human body. Their main chemical structure consists of phenolic and pyrane rings. The variety of flavonoids is determined by the type of substituent, the number of hydroxyl and methoxyl groups in the molecule, and the location of its addition. Flavonoids exhibit antioxidant, antiradical and chelating abilities. These compounds are usually responsible for the color and flavor of plant foods. Moreover, they prevent fat oxidation and protect vitamins and enzymes. In plants, these compounds occur mainly as glycosides.[1–3]

One of the subclasses of flavonoids are anthocyanins. Among these compounds there are: cyanidin, delphinidin, malvidin, pelargonidin, peonidin, and petunidin. The chemical structure of anthocyanins is presented in Fig. 1. These compounds have 4, 5 or 6 hydroxyl groups in the molecule, while some of them have a methoxyl group in chain B.[4]

The main source of anthocyanins are dark fruits such as blackberries, blueberries, cranberries, black and red currants, red grapes, raspberries, and vegetables such as red cabbage and radish, but also some types of nuts and red wine.[4] The content of these compounds in selected food products is presented in Table 1.

An association between a higher intake of total flavonoids, or their subclasses, and a lower risk of hypertension, myocardial infarction or stroke was found in numerous epidemiological studies.[5–7] Particularly noteworthy, however, is the role of anthocyanins as dietary components in the protection against the development of type 2 diabetes (T2D). The results of Nurses' Health Study I and II and Health Professionals Follow-Up Study showed that a lower risk of T2D was associated with higher anthocyanin content in the diet.[8]



Cyanidin (R_1=H, R_2=OH)
Delphinidin (R_1=R_2=OH)
Malvidin (R_1=R_2=OMe)
Pelargonidin (R_1=R_2=H)
Peonidin (R_1=H, R_2=OMe)
Petunidin (R_1=OH, R_2=OMe)

Fig. 1. Basic structure of anthocyanins[4]

The aim of this study was to review the literature on the importance of anthocyanins in the regulation of the carbohydrate metabolism and reduction of insulin resistance in the body, as major factors decreasing the risk of type 2 diabetes.

Insulin resistance

Weakening the sensitivity of cells to insulin is one of the factors contributing to the development of T2D. In the development of insulin resistance, many mechanisms associated with improper functioning of some enzymes and hormones may be involved. An increased risk of insulin resistance is related to an excessive level of visceral fat in the body, which leads to the dysregulation of the carbohydrate metabolism, a decrease in insulin sensitivity of tissues, the development of hyperglycemia and inflammatory status and, as a consequence, an increased risk of developing T2D. Adipocytes of visceral fat are metabolically active. They secrete, among others, hormones such as adiponectin, leptin, resistin, and proinflammatory cytokines such as TNF-α (tumor necrosis factor) or IL-6 (interleukin-6).[9]

Scazzocchio et al. analyzed the influence of cyanidin-3-O-β-glucoside (C3G) and metabolite protocatechuic acid (PCA) on the activation of glucose transport in human omental adipocytes and mice cells (3T3-L1).[10] Initially, the cells were incubated with oxidized LDL (oxLDL), which caused a decrease in glucose uptake by 40% and a decrease in GLUT4 (insulin-regulated glucose transporter) concentration by 60%. After that, the cells were treated with 50 μmol/L C3G and 100 μmol/L PCA. It was then observed how these compounds affected the uptake of [^{3}H]-2-deoxyglucose, GLUT4 translocation, secretion of adiponectin, and activation of peroxisome proliferator-activated receptor-γ (PPAR-γ), which participate in adipocyte differentiation and maturation, and increased insulin sensitivity. Both of the studied phenolic compounds counteracted the decline in glucose uptake and reversed defective GLUT4 translocation in cells treated and not treated with insulin. C3G and PCA overcame the negative influence of oxLDL on mRNA PPAR-γ expression and PPAR-γ activity. The beneficial effect of anthocyanins on PPAR activity in adipose tissue and skeletal muscles was also observed by other authors in a study conducted on rats.[11]

Tsuda et al. also found anthocyanins to be compounds that might be of importance in T2D prevention.[12] A study on isolated rat adipocytes demonstrated enhanced adiponectin and leptin secretion, in cells treated with cyanidin. Moreover, increased concentration of adiponectin mRNA in white adipose tissue was observed in rats after 12 weeks of being fed a diet enriched with C3G,

as compared to the control group. Low levels of adiponectin in the serum of people with T2D correlates with insulin resistance and decreased insulin sensitivity.[13–15] Adiponectin regulates glucose homeostasis in an organism through many mechanisms in adipose tissue, e.g., in the pancreas and the liver. It also prevents apoptosis in INS-1 β cells, which promotes proper insulin secretion by the pancreas.[15] Leptin is a hormone which takes part in the regulation of food intake by reducing appetite, thereby reducing body weight gain, but its activity in obese people is usually limited. However, in studies conducted on diabetic people without obesity, leptin significantly enhanced insulin sensitivity, improved glycemic control and reduced triglyceride concentration in the blood and adipose tissue. Moreover, treatment with leptin caused a mild reduction of blood glucose level without causing hypoglycemia.[16] Liu et al. proved that C3G increased serum adiponectin concentrations in diabetic mice, which also improved the endothelial function, thereby reducing the risk of developing a cardiovascular disease.[17]

In mice fed with a high-fat diet (60% energy from fats) there was observed an increase in the expression of inflammatory markers such as TNF-α, IL-6, MCP-1 (monocyte chemoattractant protein 1), iNOS (inducible nitric oxide synthase), and increased oxidative stress.[18] In mice fed with a high-fat diet supplemented with blueberry powder (31.44 g of anthocyanins/1 kg dry weight), such irregularities were not noticed. Also in a randomized, single-blind, placebo-controlled crossover trial, conducted on a group of overweight and obese people, it was found that anthocyanins from strawberries decreased the inflammatory status (defined as C-reactive protein and IL-6 levels) after a meal rich in carbohydrates, with moderate fat content.[19]

A randomized, double-blind, placebo-controlled study showed that anthocyanin supplementation reduced fasting plasma glucose levels and decreased insulin resistance in diabetic patients. The beneficial effect of these compounds was associated, among others, with enhanced adiponectin synthesis. In addition, patients in the anthocyanin group showed higher β-hydroxybutyrate concentrations compared to the placebo group, though still in the normal range, implying an increase in total body energy expenditure. This, however, did not increase the risk of ketoacidosis in diabetic patients.[20] Other authors explain that β-hydroxybutyrate decreases the risk of insulin resistance by, among others, reducing the glycation of insulin and reducing the generation of insulin advanced glycation end products.[21]

The authors of another randomized, double-blind, placebo-controlled clinical trial analyzed the influence of supplementation with bioactives from blueberries on whole-body insulin sensitivity in obese (insulin resistant and nondiabetic) adults. After 6 weeks of study, the supplemented group showed increased insulin sensitivity compared

Table 1. The content of anthocyanins in selected food products [mg/100g][4]

Product	Cyanidin	Delphinidin	Malvidin	Pelargonidin	Peonidin	Petunidin	Total
Blackberries	99.95	0.0	0.0	0.45	0.21	0.0	100.61
Blueberries	8.46	35.43	67.59	0.0	20.29	31.53	163.30
Cherries	32.57	nd	nd	nd	0.87	nd	33.44
Cranberries	46.43	7.67	0.44	0.32	49.16	0.0	104.02
Black currants	62.46	89.62	nd	1.17	0.66	3.87	157.78
Red currants	65.54	9.32	nd	nd	0.16	nd	75.02
Red grapes	1.16	2.27	39.00	0.02	3.62	1.97	48.04
Raspberries	45.77	1.32	0.13	0.98	0.12	0.31	48.63
Strawberries	1.68	0.31	0.01	24.85	0.05	0.11	27.01
Gooseberries	8.73	0.01	nd	nd	0.77	nd	9.51
Apples	1.57	0.0	0.0	0.0	0.02	0.0	1.59
Bananas	0.0	7.39	0.0	0.0	0.0	0.0	7.39
Red cabbage	209.83	0.10	nd	0.02	nd	nd	209.95
Radish	0.0	0.0	0.0	63.13	0.0	0.0	63.13
Eggplant	nd	85.69	nd	nd	nd	nd	85.69
Red onion	3.19	4.28	nd	0.02	2.07	nd	9.56
Beans (black, mature seeds, raw)	nd	18.50	10.61	nd	nd	15.41	44.52
Almonds	2.46	0.0	0.0	0.0	0.0	0.0	2.46
Pistachios	7.33	0.0	0.0	0.0	0.0	0.0	7.33
Red wine (Cabernet Sauvignon)	nd	4.18	26.24	nd	1.85	3.32	35.59
Red wine (sweet)	nd	3.90	94.83	nd	3.93	6.63	109.29

nd – no data.

to the placebo group.[22] Jennings et al. observed an improvement in insulin resistance related to a higher intake of foods rich in anthocyanins and flavones.[23] The effect of anthocyanins on improving insulin resistance may be associated, among others, with the upregulation of GLUT4 gene expression, as was found for C3G.[24]

The authors of the rodent studies explained the amelioration of insulin sensitivity through anthocyanins activating AMP-activated protein kinase (AMPK), which stimulated glucose uptake and insulin secretion by pancreatic β cells. Among others, the activation of AMPK was accompanied by upregulation of GLUT4 in white adipose tissue and skeletal muscles, and downregulation of gluconeogenesis in the liver.[25,26] The activation of AMPK is associated with the phosphorylation of Thr172 in the activation loop of AMPK.[27] After 3–5 weeks, the diabetic mice fed with a diet consisting of anthocyanin-rich bilberry extract had significantly lower blood glucose levels than mice from the control group. The effect of decreasing glucose level in the blood after 30 min, 90 min and 120 min of insulin injection was significantly greater in the study group compared to the control. The mice fed with a high anthocyanin diet, as compared to the control group, had increased total AMPKα and phosphorylation of AMPKα at Thr172 in white adipose tissue. Increased phosphorylation of AMPKα was also observed in skeletal muscles and the liver.[25] Tsuda et al. also observed increased phosphorylation of AMPKα at Thr172 in rat adipose cells treated with cyanidin and C3G, as compared to the control group.[12] The gene expression level of GLUT4, both in white adipose tissue and in skeletal muscles, was significantly higher in mice fed with a diet consisting of anthocyanin-rich bilberry extract compared to the control group. On the other hand, there were no differences between the groups in the gene expression level of adiponectin and adiponectin receptors (AdipoR$_1$ and R$_2$) in the liver and skeletal muscles, the concentration of adiponectin in serum, the levels of tyrosine phosphorylation of insulin receptor substrate-1 (IRS-1) and serine phosphorylation of Akt (the phosphoprotein:total protein ratio) in the liver, skeletal muscles and white adipose tissue.[25] Kurimoto et al. also found increased insulin sensitivity and reduced hyperglycemia in diabetic mice due to the activation of AMPK, after a diet supplemented with black soybean seed coat extract, rich in anthocyanins.[26]

Sasaki et al. suggest another mechanism that can explain why anthocyanins may reduce hyperglycemia and improve insulin sensitivity.[28] They analyzed the influence of C3G on retinol binding protein 4 (RBP-4) expression in a study conducted on diabetic mice. RBP-4 is an adipokine; its higher concentration is correlated with insulin resistance. Diabetic mice were fed a control diet or a control + 0.2% C3G diet. At the beginning of the study, mice from the control group and C3G group had similar glucose levels in serum. After 3 weeks, the animals from the treatment group had significantly lower fasting glucose in serum compared to the control group (300.1 md/dL vs 393.9 mg/dL), and the difference persisted after further 2 weeks of study (356.5 mg/dL vs 454.2 mg/dL). The amelioration of insulin sensitivity was also observed in mice from the treatment group. These changes were not associated with the expression of adiponectin and its receptors. However, it was found that C3G significantly upregulated GLUT4 and downregulated RBP-4 in white adipose tissue.[28]

Seymour et al. observed the beneficial impact of blueberry extract on reducing insulin resistance and fasting insulin levels in obese rats.[11] These effects were related to enhanced adipose and skeletal muscle PPAR activity.

De Furia et al. conducted a study on mice, which were divided into 3 groups and fed for 8 weeks with 3 types of diet: A – low-fat (10% of energy from fats); B – high-fat (60% of energy from fats); and C – high-fat with 4% extract from blueberries.[18] The mice from group C had significantly lower insulin resistance than the mice from group B, and similar to that observed in group A. It was noted that the increase in insulin resistance was accompanied by the death of adipocytes, which was offset by the berry extract. Moreover, in the mice from group B, compared to C, increased M1-polarized adipose tissue macrophages (CD11c+) were observed, which is considered a marker of human insulin resistance.[18,29]

Other authors also proved the protective role of anthocyanins with regard to insulin resistance.[30] The 1st group of rats was fed with a high-fructose diet (630 g/kg), while the 2nd was fed with a high-fructose diet with an anthocyanin-rich extract from black rice (5 g/kg high-fructose diet). After 4 weeks, rats from the 2nd group had lower insulin resistance compared to those from the 1st group. At a further stage of the study, rats with established insulin resistance were treated with the anthocyanin-rich extract in the amount of 5 g/1 kg high-fructose diet or with pioglitazone (a drug that increases insulin sensitivity, reduces insulin resistance in adipose tissue, skeletal muscle and the liver, decreases the concentration of free fatty acids and glucose in the blood) at an amount of 270 mg/1 kg high-fructose diet. Both of these therapies reduced glucose intolerance, but only pioglitazone reversed the fructose-induced hyperinsulinemia.

The results of the presented studies are summarized in Table 2.

Postprandial glycemia

Anthocyanins were analyzed in view of their importance in the regulation of postprandial glycemia. Törrönen et al. conducted a study among healthy adult volunteers to assess the influence of berries on the postprandial plasma glucose response to sucrose.[31] The study group consumed a purée made of bilberries, blackcurrants, cranberries, strawberries and 35 g of sucrose, while the control group only sucrose. Plasma glucose concentration in the study group at 15 min and 30 min after a meal was significantly lower than in the control group, while at 150 min it was

significantly higher. At 3 h after a meal there was no difference in plasma glucose between the groups. The maximum plasma glucose concentration was reachedat 45 min after the berry meal and at 30 min after the sucrose alone. The results of the study indicate reduced absorption of glucose from a meal containing berries, and a consequent delay in glycemic response after a meal. Similar results were also observed in a later study, where the effects of 35 g of sucrose consumption with blackcurrants, lingonberries and berry nectars on postprandial glucose and insulin were investigated.[32] Volunteers who consumed sugar with fruits, compared to those who consumed sugar alone, had lower glucose and insulin concentrations during the first 30 min, and a slower decline during the following 90 min. Thereby, improved glycemic response prevented a hypoglycemic state in volunteers from groups which were given fruits or nectar.

Törrönen et al. also investigated the effects of different berries consumed with wheat bread or rye bread on postprandial glucose in healthy women.[33] After the volunteers consumed wheat bread with a fruit mixture (strawberries, bilberries, cranberries, blackcurrants), the 0–30 min area under their blood glucose curve (AUC) was decreased by 32% in comparison to AUC after wheat bread

Table 2. Results of the selected studies on the role of anthocyanins in the prevention of type 2 diabetes

No.	Anthocyanin/product tested	Mechanism of action	Ref.
1	C3G and PCA	improved GLUT4 translocation, secretion of adiponectin and activation of PPAR-γ; counteracted decline in glucose uptake	10
2	Blueberry extract	reduced insulin resistance and fasting insulin levels in obese rats; enhanced adipose and skeletal muscle PPAR activity	11
3	Cyanidin	enhanced adiponectin and leptin secretion; increased phosphorylation of AMPKα at Thr172 in rat adipose cells	12
4	Blueberry powder	reduced insulin resistance, inflammatory marker expression and oxidative stress	18
5	Anthocyanins from strawberries	decreased inflammatory status	19
6	Anthocyanins from blueberries	increased insulin sensitivity in obese adult subjects (insulin resistant and nondiabetic)	22
7	Food rich in anthocyanins	improved insulin resistance	23
8	C3G	upregulated GLUT4 gene expression	24
9	Anthocyanin-rich bilberry extract	increased total AMPKα and phosphorylation of AMPKα at Thr172 in white adipose tissue; increased phosphorylation of AMPKα in skeletal muscles and the liver; enhanced gene expression level of GLUT4; ameliorated insulin sensitivity	25
10	Black soybean seed coat extract	increased insulin sensitivity and reduced hyperglycemia in diabetic mice due to the activation of AMPK	26
11	C3G	ameliorated insulin sensitivity; upregulated GLUT4 and downregulated RBP-4 in white adipose tissue	28
12	Anthocyanin-rich extract from black rice	reduced insulin resistance	30
13	Purée made of bilberries, blackcurrants, cranberries, strawberries	reduced absorption of glucose and delay in glycemic response after a meal	31
14	Fruit mixture (strawberries, bilberries, cranberries, blackcurrants)	decreased area under the blood glucose curve (AUC) after the consumption of bread with fruits compared to bread alone; improved glycemic profile	33
15	Black soybean seed coat extract (C3G, delphinidin-3-glucoside, petunidin-3-glucoside)	reduced blood glucose level in diabetic rats; increased expression and translocation of GLUT4; activated insulin receptor phosphorylation; increased uptake and utilization of glucose by cells; prevention of streptozotocin-induced apoptosis in pancreatic cells	35
16	C3G	prevention of pancreatic cell death; decreased mitochondrial production of ROS; increased IGF-II, gene transcript levels and insulin protein in INS-1 cells	36
17	Cyanidin-3-rutinoside	α-glucosidase inhibition	37
18	Cyanidin and its glycosides	intestinal sucrose inhibition: cyanidin-3-galactoside > C3G > cyanidin > cyanidin-3.5-diglucosides; C3G – the most effective inhibitor for pancreatic α-amylase	38
19	Cyanidin-diglucoside and pelargonidin-3-rutinoside	inhibitors for α-glucosidases, but not for pancreatic α-amylase and lipase	39
20	9 anthocyanin compounds (glycosides and aglycones)	stimulation of insulin secretion with 4 mM glucose concentration: delphinidin-3-glucoside > cyanidin > pelargonidin > delphinidin > C3G; stimulation of insulin secretion with 10 mM glucose concentration: C3G > delphinidin-3-glucoside > cyanidin-3-galactoside > pelargonidin-3-galactoside > cyanidin	40

C3G – cyanidin-3-O-β-glucoside; PCA – protocatechuic acid; PPAR-γ – peroxisome proliferator-activated receptor-γ; AMPKα – AMP-activated protein kinase; RBP-4 – retinol binding protein 4; ROS – reactive oxygen species; IGF-II – insulin-like growth factor II.

consumption, and by 27% in comparison to AUC after rye bread consumption. A significant improvement in glycemic profile (the time [min] during which the plasma glucose was above the fasting concentration divided by the incremental peak glucose value [mmol/L]) was observed after wheat bread consumption with strawberries and fruit mixture (by 36% and 38%, respectively) and after rye bread consumption with fruit mixture (by 19%) in comparison to glycemic profile after the consumption of bread without fruits.

Jayaprakasam et al. showed that anthocyanins extracted from Cornelian cherries amend glucose tolerance in mice.[34] The 1st group of mice was fed a high-fat diet (60% energy from fats), the 2nd was fed a high-fat diet supplemented with an extract from Cornelian cherries (1 g of anthocyanins/1 kg high-fat diet), and the 3rd was fed a high-fat diet supplemented with ursolic acid, which demonstrates potential anti-diabetic properties (500 mg/1 kg high-fat diet). The control group was fed a standard rodent diet (10% energy from fats). A glucose tolerance test was performed after 6 weeks. The results showed that both anthocyanins and ursolic acid significantly improved glucose tolerance compared to a high-fat diet alone.

The results of another study proved that anthocyanins may also play a role in the regulation of the plasma glucose level in streptozotocin-induced diabetic rats.[35] The diet used in this study contained black soybean seed coat extract (50 mg/kg), which consisted of cyanidin-3-glucoside (72%), delphinidin-3-glucoside (20%) and petunidin-3-glucoside (6%). It was observed that anthocyanins significantly reduced the blood glucose level in diabetic rats. Anthocyanins also increased the expression and translocation of GLUT4 as well as enhanced the activation of the insulin receptor phosphorylation, thereby increasing the uptake and utilization of glucose by cells. It was also found that anthocyanins may prevent streptozotocin-induced apoptosis in pancreatic cells. Sun et al. conducted a study on streptozotocin-induced diabetic mice.[36] The authors observed that C3G had a protective effect on pancreatic cells by preventing their death, increasing cellular viability and decreasing the mitochondrial production of reactive oxygen species. Chinese bayberry extract, rich in C3G, improved glucose tolerance in diabetic mice. This extract also caused an increase in insulin-like growth factor II (IGF-II), gene transcript levels and insulin protein in INS-1 cells.[36]

Other mechanisms that may explain lower blood glucose level after a meal with anthocyanins compared to a meal without these compounds is the inhibition of intestinal α-glucosidase and pancreatic α-amylase.[37–39] The effectiveness of cyanidin-3-rutinoside (C3R) in α-glucosidase inhibition was observed in a study on normal rats with an oral maltose and sucrose tolerance test. Moreover, C3R exhibited a synergistic effect with acarbose, used in the treatment of T2D.[37] Akkarachiyasit et al. showed that cyanidin and its glycosides are more specific inhibitors of intestinal sucrase than maltase.[38] The highest inhibition activity against intestinal sucrose was shown with cyanidin-3-galactoside, followed by C3G, cyanidin and cyanidin-3.5-diglucosides. Cyanidin glucosides also exhibited a synergistic effect with acarbose in the inhibition of sucrase and maltase, but such an effect was not observed for cyanidin aglycon. C3G was the most effective inhibitor for pancreatic α-amylase, while cyanidin-3-galactoside and cyanidin-3.5-diglucosides were not so powerful. The results of the study also showed a synergistic inhibition for a combination of cyanidin or C3G with acarbose against α-amylase. Zhang et al., however, found, that anthocyanins are inhibitors for α-glucosidases, but not for pancreatic α-amylase and lipase.[39] The most effective were cyanidin-diglucoside and pelargonidin-3-rutinoside as well as 2 other polyphenol compounds – catechin and ellagic acid.

The results of the selected studies discussed above are presented in Table 2.

Postprandial insulin secretion

Törrönen et al. conducted a study on a group of healthy women to investigate the effects of different berries consumed with wheat bread or rye bread on postprandial insulin.[33] The 0–60 min area under the insulin curve (AUC) after wheat bread consumption with strawberries and chokeberries, in comparison to AUC after wheat bread consumption alone, was decreased by 24%, while with bilberries and lingonberries, AUC decreased by 19% and 20%, respectively. However, raspberries and cloudberries did not exhibit such effects. After the consumption of wheat or rye bread with a fruit mixture (strawberries, bilberries, cranberries, blackcurrants), the 0–60 min AUC was decreased by 25% in comparison to the consumption of bread alone. Moreover, a lower insulin level in the blood was observed at 15 min and 30 min after a meal, but higher at 120 min after wheat or rye bread consumption with a fruit mixture in comparison to the consumption of bread alone. Edirisinghe et al. showed decreased insulin levels in the blood at 60 min and 180 min after the consumption of a beverage rich in anthocyanins (containing a strawberry extract) compared to the placebo.[19]

Other authors in an in vitro study investigated the influence of 9 compounds (glycosides and aglycones) on insulin secretion by rodent pancreatic β cells (INS-1 832/13) treated with 4 mM and 10 mM glucose concentrations.[40] The most effective in the stimulation of insulin secretion with 4 mM glucose concentration was delphinidin-3-glucoside (1.8-fold increase in insulin secretion) followed by: cyanidin, pelargonidin, delphinidin, and C3G (a 1.5-, 1.4-, 1.3-, and 1.3-fold increase in insulin secretion, respectively). C3G was also tested in different concentrations of the compound investigated and, interestingly, the stimulation of insulin secretion was not related to the C3G dose applied (5, 10, 50, 100, and 250 μg/mL).

With 10 mM glucose concentration, the highest increase in insulin secretion (1.43-fold) was observed for C3G, followed by: delphinidin-3-glucoside (1.4-fold), cyanidin-3-galactoside and pelargonidin-3-galactoside (1.2-fold) and cyanidin (1.1-fold). The influence of cyanidin-3-galactoside and pelargonidin-3-galactoside on insulin secretion with 4 mM glucose concentration was not observed, nor was the influence of malvidin and petunidin with 4 mM and 10 mM glucose concentrations.[40] The improvement in insulin secretion caused by anthocyanins may have special significance for people with T2D, whose pancreatic activity is damaged and insufficient.

The results of the selected studies presented in this manuscript are summarized in Table 2.

Summary

Anthocyanins are food compounds which belong to polyphenols and they might have special significance in the prevention of type 2 diabetes. Numerous studies, both with humans and experimental animal subjects, were conducted to explain the mechanisms of anthocyanin function, by which they regulate the carbohydrate metabolism in the body and reduce insulin resistance.

There are many ways in which these compounds interact in the body. Anthocyanins regulate GLUT4 gene expression and translocation, increase the activation of PPARγ in adipose tissue and in skeletal muscles, increase the activation of AMP-activated protein kinase, enhance the secretion of adiponectin and leptin, reduce retinol binding protein 4 expression, and, moreover, are inhibitors for intestinal α-glucosidase and pancreatic α-amylase. Anthocyanins also improve insulin secretion by rodent pancreatic β cells. It was also found that these compounds protect pancreatic cells against necrosis induced by streptozotocin in diabetic rodents. However, it was observed that the individual anthocyanins and their glycosides have different activity. It is, therefore, necessary to include a variety of plant products in the daily diet, because they contain various anthocyanins. For example, blackberries and red cabbage contain mainly cyanidin, eggplant – delphinidin, blueberries and red grapes – malvidin, while radish – pelargonidin.

Currently, there are no recommendations regarding the optimal content of flavonoids and their subclasses in a diet, neither for healthy nor for sick people. However, the results of the presented studies proved a potential beneficial role of anthocyanins in the prevention and treatment of T2D. The sources of these compounds are mainly fruits and vegetables. Therefore, these products should be included in the everyday diet in the amount of at least 600 g, of which approx. 3/4 should be vegetables. An important source of anthocyanins are the following vegetables: red cabbage, eggplant, radish and red onion. These products contain less than 10% of carbohydrates and, therefore, can be the main vegetables in a diet. The following fruits are an important source of anthocyanins: blueberries, strawberries, raspberries, blackberries, cranberries, gooseberries or cherries, that all of which also contain less than 10% of carbohydrates and, therefore, can be consumed in the recommended amounts of up to approx. 150 g/day. Other fruits, such as red grapes, black currants, plums or bananas, are also sources of anthocyanins, but they contain more than 10% of carbohydrates and, therefore, should be consumed occasionally and in limited quantities.

Although the development of type 2 diabetes may be due to a number of factors, the evidence from the studies presented by many authors, considering the impact of anthocyanins on the regulation of glycemia and reduction of insulin resistance, is worth emphasizing. Therefore, it appears that the consumption of foods rich in these compounds may be included in recommendations as one of the elements supporting the prevention and treatment of type 2 diabetes.

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Post-ERCP pancreatitis: Pathophysiology, early identification and risk stratification

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Abstract

Acute pancreatitis is the most common and feared complication of endoscopic retrograde cholangiopancreatography (ERCP). The aim of the study was to review the current knowledge on the nomenclature, etiology, pathophysiology, clinical presentation, diagnostic workup, and risk stratification of post-ERCP pancreatitis (PEP). A structured search in PubMed and Scopus databases was performed using search terms related to the subject of diagnosis, pathophysiology, risk stratification of post-ERCP pancreatitis, including full text articles and abstracts in the English language. Several causes, operating both at a local and systemic level, might play an important role in the pathogenesis of PEP. Different patient-related risk factors can help predict post-ERCP pancreatitis; diagnosis depends on clinical presentation, imaging and laboratory investigations. As an outpatient procedure, post-ERCP pancreatitis may be safe in a selected group of low-risk patients. Further investigation of the etio-pathogenesis of post-ERCP pancreatitis is required in order to improve diagnosis and treatment. Early identification and severity stratification of post-ERCP pancreatitis greatly affects the patient's outcome. There is still controversy concerning the risk factors related to PEP. More studies are needed to clarify early and definite diagnosis, risk and severity stratification, as well as treatment of post-ERCP pancreatitis.

Key words: pancreatitis, endoscopic retrograde cholangiopancreatography, risk score, post-ERCP pancreatitis

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Introduction

Acute pancreatitis is the most common post-procedural complication following endoscopic retrograde cholangiopancreatography (ERCP). Its incidence is reported between 2.1% and 24.4%, with such variability being attributable to heterogeneous patient populations, differing levels of endoscopic expertise, procedural differences, disparate definitions of post-ERCP pancreatitis (PEP) and its severity.[1–13] Although, whilst the final pathogenic mechanisms of pancreatic damage are similar regardless of the causative factor, it has been suggested that non-ERCP-induced acute pancreatitis and PEP are different clinical entities with different outcomes in both mild and severe forms.[14] PEP prevail over acute pancreatitis developed under the influence of factors other than ERCP in rates of developing infected necrosis; the rate of postoperative pancreatic and enteric fistula is also higher in PEP compared to acute pancreatitis due to other causes than ERCP; patients suffering PEP constitute a younger cohort and have increased residual long-term morbidity compared to non-ERCP acute pancreatitis. On the other hand, the mortality rate is higher in cases of acute pancreatitis induced by non-ERCP related causes compared to PEP.[15] This review summarizes and critically appraises recent major published studies devoted to the issue of pathophysiology, early identification and risk stratification of PEP. This review was prepared as part of the 3rd year of the MSc in Surgical Sciences or Edinburgh Surgical Sciences Qualification.

The pathophysiology of PEP

The pathophysiology of PEP is not entirely clear with a multi-factorial concept being held. This involves a combination of chemical, thermal, mechanic, hydrostatic, enzymatic, allergic, and microbiological insults that result from papillary instrumentation and/or hydrostatic injury from the overfilling of the pancreatic duct with contrast material. The influence of these factors leads to a cascade of events resulting in premature intracellular activation of pancreatic proteolytic enzymes, autodigestion, and the release of inflammatory cytokines that produce both local and systemic effects.[2,12,17–19]

Among pathogenic factors of PEP, cannulation trauma to the papilla is the most common cause of sphincter of Oddi spasm and/or edema of the papilla. It creates an obstacle to the flow of pancreatic juice, and subsequently determines an acute pancreatic inflammation.[20]

Another important factor is the contrast media used with its osmolarity and ionic nature believed to be the major factors responsible.[21,22]

Injection pressure during contrast media injection into the pancreatic duct contributes to ductal epithelial or acinar injury. This injury is believed to happen from the disruption of cellular membranes or tight junctions between the cells and the backflow of the intraductal contents, especially into the interstitial space.[22] The role of intestinal enzymes refluxed into the pancreatic ductal system by ERCP maneuvers has been suggested as another possible trigger.[23]

It has also been suggested that bacteria may play a role in the induction of PEP, where bacterial-specific enzymes, toxins or activators of bacterial origin may initiate cytokine release from immune cells which will subsequently initiate pancreatic cell damage.[24,25]

Finally, genetic abnormalities should be noted as a risk factors as well. Homozygous alpha-1-anti trypsin deficiency causing increased rates of hemorrhagic PEP compared to the general cohort is a known example.[26]

Definition of post-ERCP pancreatitis

The consensus definition of PEP consists of the following criteria: serum amylase at least 3 times above the upper limit of normal 24 h post-procedure level accompanied by new abdominal pain consistent with pancreatitis and symptoms severe enough to require a hospital stay or to extend the length of stay of already hospitalized patients, and/or abdominal computer tomography scan (CT) consistent with the diagnosis of acute pancreatitis.[9] The classification has been widely accepted as it allows standardized reporting of the incidence and severity of PEP.

The severity of attack was graded by the proposed classification of mild, moderate and severe based on needed duration of hospital stay, presence of local or systemic complications, which may be also estimated using the revised Atlanta classification consensus.[27]

Various alternative diagnostic criteria for PEP were proposed since the first encounter with ERCP complications has happened. Testoni et al. concluded that the level of serum amylase measured 4 h after endoscopic sphincterotomy was a sufficiently reliable indicator of PEP, as more than two-thirds of the cases involving pancreatitis occurred among the patients whose 4-h amylase level was higher than 5 times the normal upper limit.[28]

The subsequent study conducted by Testoni et al. indicated that serum amylase levels at 24 h after the procedure appear to be more sensitive than those at 4 h.[10] Authors declared that pain at 24 h associated with amylase levels greater than 5 times the normal upper limit is the most reliable indicator of PEP.

Ito et al. has stressed the importance of a dynamic rise of serum amylase between 3 and 6 h post procedure in the diagnosis of PEP.[29] He suggested that when hyperamylasemia (higher than 2 times the normal upper limit) is observed at 3 h after ERCP, serum amylase concentration should be measured at 6 h after the procedure. A decrease in serum amylase level at 6 h after ERCP indicates the absence of PEP. Gottlieb et al. proposed ruling out the diagnosis of PEP

in accordance with 2-h serum amylase and lipase values.[30] Monitoring the intensity of patients' pain in the first 6 h after the ERCP procedure using visual analog scales (VAS) was also proposed as one of the early independent PEP's diagnosis criteria by a single center case control study.[31]

Both Cotton's and the revised Atlanta classification consensuses are agreed by the Revised European Society of Gastrointestinal Endoscopy Guidelines 2014 (ESGE) as PEP's definition statements and severity assessors, although notice is given that 2 definition statements poorly correlate with each other.[9,27,32]

With regards to post-procedural prediction of PEP, ESGE suggest testing serum amylase or lipase 2–6 h after ERCP in patients presenting with pain and those who are to be discharged on the day of ERCP. It is reported that patients with amylase or lipase values less than 1.5 and 4 times the upper normal limit, respectively, can be discharged without concern about the risk of PEP.[32]

Efforts have been made by other authors trying to identify alternative biochemical markers for PEP diagnosis. Among markers which were proven to be associated with PEP by a small observational series were: trypsinogen, trypsinogen activation peptide, C-reactive protein (CRP), serum elastase-1, erythrocyte sedimentation rate (ESR), chemerin, and various interleukins such as IL-6 and IL-10.[11,33–38]

Yet, the distinction between hyperamylasemia with transient abdominal discomfort (TAD) due to post-procedural intestinal distension and PEP remains difficult to establish during the first 24 h after the procedure.

Is ERCP an outpatient procedure?

There is a lack of randomized comparative trials to compare ERCP as an outpatient or inpatient procedure in terms of safety, efficacy, cost-effectiveness. ERCP as an outpatient procedure is widely utilized and relatively safe, but results in a significant number of readmissions due to complications. The main factor in favor of same-day discharge ERCP is that it is cost-effective as it avoids unnecessary hospital admissions. The main advantage of in-patient ERCP care is that it eliminates the risks related to ERCP complications, which may develop under unsupervised non-clinical setting and late readmission. A selective policy for early discharge and identification of those who possess a high risk of PEP, based on 2–6 h post-ERCP monitoring and assessment of risk factors, has been proposed to address the existing disadvantages of ERCP as an outpatient procedure.[39–41]

Risk stratification

Early and accurate post-procedural PEP diagnosis is aided by a pre-procedural risk stratification that would allow us to clearly establish low-risk, while identifying patients with a higher risk.

There is a lack of uniformity between different observational studies in defying risk factors for PEP. Where some risk factors have been widely accepted by the majority of observational studies, some factors continue to show conflicting evidence between different studies as to whether they are related to increased incidence of PEP. Among the recent studies, a retrospective cohort study by Cheng et al., which included a total of 1,115 patients, revealed a suspected dysfunction of the sphincter of Oddi (SOD), a history of post-ERCP pancreatitis and the age of 60 years and above to be risk factors of PEP.[8] A retrospective cohort study by Katsinelos et al., which included a total of 2,715 patients, revealed by both univariate and multivariate analysis that the history of acute pancreatitis is the only significant risk factor, thus denying the role of age and gender in the development of PEP.[42]

ESGE indicates SOD, female gender, younger age, and previous history of pancreatitis as risk factors for PEP, based on data from the meta-analysis, plus those from 7 prospective, multicenter studies that analyzed potential risk factors for PEP using multivariate analysis.[2,4–6,32,43–45]

A different age cutoff was used to investigate the correlation between age and the occurrence of PEP. The most common cutoff adopted is 60 years, with 70 years holding the 2nd place in the literature references.[5–8,45–47]

Risk factors for PEP were shown to be independent by a multivariate analysis and are reported to increase PEP's rate synergistically, hence they might have a cumulative effect. Freeman et al. calculated the adjusted odds ratio (OR) for various combinations of risk factors by using data prospectively collected from about 2,000 ERCPs: the highest risk of PEP (42%) was found in female patients with a normal serum bilirubin level, SOD, and difficult biliary cannulation.[2]

The list of recognized risk factors is not exhaustive, because not all potential risk factors have been analyzed. For example, the underlying presence of cirrhosis, primary sclerosing cholangitis (PSC), chronic (autoimmune) hepatitis, Crohn's disease, and obesity were found to be independent predictors of post-ERCP complications, including PEP on the basis of small prospective studies.[48–50]

Individual singularities of the anatomy of the pancreatic duct and second part of duodenum have been shown to affect the risks of PEP. Where SOD has been widely agreed as a risk factor, and the presence of a peripapillary diverticulum was reported to be a risk factor by a few observational studies, pancreas divisum has been found, in contrast, to be a protective factor.[51–54]

One study has shown the predictive quality of pre-ERCP blood urine nitrogen (BUN) and hematocrit (HCT) level as potential predictors of PEP.[55] Higher pre-procedure BUN and HCT level were found to be associated with a higher incidence of PEP.

Another case-control study enrolling 6,505 patients found that smoking, former drinking and diabetes are independent risk factors.[56]

Study by Freeman et al. showed that the presence of at least 1 of the independent risk factors (suspected sphincter of Oddi dysfunction, cirrhosis, difficult bile duct cannulation, precut sphincterotomy, or combined percutaneous endoscopic procedure) significantly increases the risk of overall complications, including PEP. This has lead to justifying overnight stays for post ERCP patients who exhibited one of the listed risk factors.[57]

Based on the retrospective case control study involving 1,372 ERCPs, where predictors of PEP were evaluated in a multivariable analysis, and supported by existed evidence of risk factors from the literature review, a prognostic model offering eligibility criteria for early discharge was proposed by Jeurnink et al.[47] The prognostic model based on patient- and procedure-related factors that are associated with PEP is reported to be able to identify patients who can be safely discharged within 6 h after ERCP.

Risk factors included are (precut) sphincterotomy, suspected SOD, younger age (<60 years), PSC, female gender, history of pancreatitis, pancreas divisum, and difficult cannulation (>10 min attempting to cannulate). Each of the included factors is worth 1 point and PSC is worth 2 points. The sum score for each of the risk factors allows us to allocate patients to the high-risk group (overall sum score >3) or a low to intermediate risk group (overall sum score ≤3). Based on that, a 6 h post procedure discharge plan can be executed.

Procedural risk factors and prophylaxis of PEP

Procedure-related risk factors are similarly important as patient-related factors in determining the incidence and severity of post-ERCP pancreatitis. However, technical factors, as well as those dependent on the surgeon, are controversial. The obvious fact is that a minimized number of cannulation and injections and a minimal amount of contrast medium cause less papillary trauma and are therefore important in preventing PEP. ESGE have defined definitive procedural risk factors: cannulation attempts whose duration exceeds 10 min, pancreatic guidewire passages more than 1 time, pancreatic injection. Those considered to be likely risk factors are: precut sphincterotomy, pancreatic sphincterotomy, biliary balloon sphincter dilation, failure to clear bile duct stones, intraductal ultrasound.[32] At the same time, it is agreed that temporary stenting with 5-Fr stent of the pancreatic duct is a protective measure which can reduce the risk of pancreatitis after ERCP in high-risk patients.[32,58,59]

Several agents have been tested experimentally and in clinical trials for potential efficacy in the prevention of PEP, including antibiotics, heparin, corticosteroids, nifedipine, octreotide and somatostatin derivatives, trinitrin, lidocaine spray, gabexate, secretin, topical epinephrine, and cytokine inhibitors. Among all these, sufficient evidence of the efficiency was reached only for nonsteroidal anti-inflammatory drugs (NSAIDs). ESGE recommend routine rectal administration of 100 mg of diclofenac or indomethacin immediately before or after ERCP in all patients without contraindication. Sublingually administered glyceryl trinitrate or 250 µg somatostatin given in bolus injection are considered optional in high-risk cases if NSAIDs are contraindicated.[32]

Recent observational studies have revealed a protective role of aggressive hydration in the development of PEP.[60–62] Large-scale randomized controlled trials to establish an evidence-based approach to intensive hydration are needed before the strategy is applied in clinical practice. Once the new strategy has emerged, it may backshift the trend towards the prioritization of inpatient management of ERCP patients.

Conclusions

The etiology of PEP is multi-factorial. The pathophysiology has not yet been studied entirely. Patient physiological characteristics and co-morbidities, procedural features, post-procedural factors are influential in the pathogenesis of PEP and may be used to determine the risk of its appearance. The prediction and early identification of PEP is challenging. Despite various diagnostic techniques and different attempts at establishing scoring models of early PEP recognition, they are all flawed and the task of improving risk stratification and early diagnosis is still relevant. Various diagnostic approaches and scoring systems have been devised that aim to stratify those at high risk of developing PEP. Recently, PEP's risk stratification and early identification strategies have been proposed as being based on grouping clinical and procedural factors and generating single integral diagnostic model. It is anticipated that next guidelines on the prognosis, diagnosis, prophylaxis, and management of PEP will include a complex prognostic model for early discharge post-ERCP, which will be able to distinguish event-free cases early and with the highest level of sensitivity and specificity.

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