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## Reviews

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# Application of an anionic polymer in the formulation of floating tablets containing an alkaline model drug

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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.** Gastric residence time is the key factor affecting the bioavailability of active pharmaceutical ingredients absorbed mainly through the gastric mucous membrane and influencing the local activity of some drugs.

**Objectives.** The aim of this study was the development of a new composition of non-effervescent floating tablets and the evaluation of the effect of an anionic polymer and compressive force on the floating properties and release characteristics of tablets containing a model alkaline drug, chlorhexidine (CHX).

**Material and methods.** Direct compression was applied to a polyacrylic acid derivative and sorbitol to fabricate the tablets. Drug release was analyzed using several kinetic models. The formulations floated on the surface of the fluid for 24 h. The values of the rate constants, statistical parameters, and half-release time ($t_{0.5}$) were calculated.

**Results.** The diffusion coefficient n falls between 0.54 ±0.02 and 0.81 ±0.03 for most formulations. The floating time (FT) and floating lag time (FLT) were found to depend on the amount of polymer incorporated in the formulations. A high compressive force sustained the release of the drug but reduced the FT and FLT. Based on the FT and $t_{0.5}$, it was determined that the C1 composition is the optimal formulation with FT >24 h and $t_{0.5}$ between 113 ±2 and 144 ±13 min, depending on the drug release model.

**Conclusions.** The application of an anionic polymer results in a prolonged release of the drug from the tablets and allows them to float on fluid surfaces.

**Key words:** chlorhexidine, carbopol, floatation, kinetics of drug release

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# Introduction

Oral delivery is a simple, convenient and patient-friendly route for drug delivery. There are many factors affecting the bioavailability of orally administered drugs. Numerous active pharmaceutical ingredients (APIs) are absorbed in only specific regions of the gastrointestinal tract (GIT). Thus, gastric residence time (GRT) is a factor affecting the bioavailability of APIs absorbed through the gastric mucous membrane, as well as through further parts of the GIT.[1] Several systems have been developed to prolong the retention time of drug dosage in the stomach, including bioadhesive, swellable, or floating systems. Floating methods seem to be promising in controlling gastroretention, ensuring that the level of drug concentration in the plasma remains therapeutically effective over a longer period.[2] Diet and complex gastric motility play an important role in gastric retention behavior.[3–6] Several studies have been conducted on the formulations of effervescent tablets and non-effervescent tablets with prolonged GRTs.[7–15] Floating effervescent tablets contain sodium bicarbonate and citric acid that enable floatage of the tablet on the surface of the gastric fluid by releasing carbon dioxide upon contact with water in the GIT. Consequently, the density of the tablet decreases, resulting in floatation. However, the device may rapidly exit the stomach prior to becoming buoyant as the system does not float immediately after administration.[16] Non-effervescent systems do not produce carbon dioxide and remain buoyant, even after 24 h.[11,14,15] Their floating capabilities are due to a specific combination of polymers, which include gel forming hydrocolloid polymers, viz polycarbonates and polystyrenes, as well as bioadhesive polymers, such as chitosan. There are several types of floating systems: single layer and bilayer tablets, alginate beads or hollow microspheres, e.g., micro-balloons. Single layer tablets are formulated by mixing the drug with the gel-forming hydrocolloid. After oral administration of such a dosage form, the tablet swells upon contact with the gastric fluid and attains a decreased volume density. Floatation results from air trapped in the expanding gel structure. The gel serves as a reservoir and controls the drug release from the hydrogel matrix.[12,13]

Sauzet et al. investigated single layer polymer tablets composed of theophylline as a model drug, silicon dioxide as a hydrophobic dusty powder, polyvinyl pyrollidone K30 as a binder, and stearic acid as a controlled release agent.[11] Such a constitution and porous structure in the tablets ensured drug release in the stomach and upper part of the gastrointestinal tract. Studies on bilayer floating polymeric tablets of hydroxyl propyl methyl cellulose (HPMC) containing metoprolol tartrate as a model drug with a prolonged gastric retention time were conducted by Narendra et al.[10] The results suggest that total polymer content to drug ratio and polymer to polymer ratio have a significant impact on the floating time (FT) and release properties, but that the HPMC viscosity grade had no effect. Jeganathan et al. studied the non-effervescent floating tablets of tramadol hydrochloride composed of polyethylene oxide (PEO) and a combination of cationic and anionic polymethacrylate polymers Eudragit® EPO (EE) and Eudragit® L100–55 (EL).[15] It was revealed that the optimized formulation is a combination of PEO and a mixture of EE and EL. Although there are numerous papers and patents concerning non-effervescent floating tablets, few of them address polyacrylates.[10–15] In the present study, the authors propose an application of polyacrylic acid in combination with granulated sorbitol for preparation of tablets presumed for prolonged release of a model alkali drug. The parallel usage of 2 effects, floatation and ionic bonding of alkali molecules, is supported in the tablets by the addition of a soluble filler – sorbitol. The sorbitol gradually dissolves in the polymeric matrices, and may favor the prolonged release of the drug from the tablets in the stomach.

The aim of the study was to develop a new composition of non-effervescent floating tablets, and the evaluation of the effect of an anionic polymer – crosslinked polyacrylic acid (PA) and compressive force on the floatation properties and release characteristics of tablets containing the model alkaline drug, chlorhexidine (CHX).

# Material and methods

## Material

A hydrochloric acid solution with a concentration of 1 mol/L (Stanlab, Gliwice, Poland), chlorhexidine 98% (Sigma-Aldrich, Poznań, Poland), sorbitol P 30/60 (Roquette, Lestrem Cedex, France), Carbopol 934 NF (Lubrizol, Wickliffe, USA), and magnesium stearate (Sigma Aldrich, Poznań, Poland) were procured for use in this work.

## Composition and production of the tablets

The tablets were prepared using the compounds listed in Table 1. The powders were mixed in a Turbula double cone blender (WAB, Muttenz, Switzerland), and then the tablets were made with a Fette tablet press Exacta 11 to control the compressive force in a suitable manufacturing environment with a humidity of 50% and a temperature of 22°C. Formulations A1 and A2 were prepared without anionic polymers, and served as the control formulations.

## Main physical parameters: Uniformity of mass, friability, resistance to crushing, and size

The selected physical parameters were assessed on the basis of the European Pharmacopoeia (Ph. Eur.) to evaluate the mechanical properties of the prepared tablets from batches A1, A2, B1, B2, C1, and C2.[17]

Table 1. Composition of the evaluated floating tablets

| Formulation code | Components | | | | TW (g) | CF (kN) |
|---|---|---|---|---|---|---|
| | SL (g) | PA (g) | MS (g) | CHX (g) | | |
| A1* | 0.440 | 0.000 | 0.005 | 0.005 | 0.450 | 2.5 |
| A2* | 0.440 | 0.000 | 0.005 | 0.005 | 0.450 | 25.0 |
| B1 | 0.417 | 0.023 | 0.005 | 0.005 | 0.450 | 2.5 |
| B2 | 0.417 | 0.023 | 0.005 | 0.005 | 0.450 | 25.0 |
| C1 | 0.395 | 0.045 | 0.005 | 0.005 | 0.450 | 2.5 |
| C2 | 0.395 | 0.045 | 0.005 | 0.005 | 0.450 | 25.0 |

SL – sorbitol; PA – crosslinked polyacrylic acid; MS – magnesium stearate; CHX – chlorhexidine; TW – total weight; CF – compression force;
* control formulations.

The uniformity of mass of the tablets was assessed on 20 tablets taken at random and weighed individually. The average mass, was determined, and the respective percentage deviation was calculated. The friability, mass, and size were evaluated according to the Ph. Eur.[18] The friability was assessed on 12 tablets from every batch in a tablet friability apparatus compatible with the Ph. Eur. The rotational speed was set to 100 rpm. Tablet resistance to crushing was also performed in accordance with the respective Ph. Eur. monograph using a system with a precision of 1 N. The measurements were performed on 10 tablets, placed with the tablet plane parallel to the jaws of the device.[19] The size of the tablets produced was assessed using a Mitutoyo (Kawasaki, Japan) digital micrometer with a precision of 0.001 mm.

## Scanning electron microscopy

The cross-sections of formulations A1–C2 were imaged using scanning electron microscopy. A LEO 1525 (Zeiss GmbH, Jena, Germany) was used for the microphotographs with an extra high tension (EHT) of 10.00 kV.

## Matrix integrity and water content of the evaluated tablets

The matrix integrity of the tablets was monitored visually and recorded on macrophotographs. After 24 h, the height and diameter of the B1, B2, C1, and C2 tablets were assessed. Then, the tablets were removed from the solution, excess water was removed with a paper towel, and the tablets were weighed before being dried and weighed again.

## Evaluation of floatation ability

The floatation ability was evaluated via measurements of the floating lag time (FLT) and floatation duration (floating time, FT). FLT was assessed as the time between the introduction of the tablet into the hydrochloric acid solution and the buoyancy in the solution. FT was measured as the time that the dosage form constantly remained on the surface of the medium.

## Release studies

Release studies were performed in vitro, using the Ph. Eur.-compliant Symphony 7100 type-2 paddle apparatus.[20] The kinetics of CHX release from the individual tablets were determined at 37°C in a volume of 1000 mL of the hydrochloric acid solution with a pH = 1. The rotational speed was set to 50 rpm. The experiment was conducted for 2 h. Samples of the solution with the released API for tablets A1 and A2 were taken every 2 min during the first 10 min, and every 10 min thereafter. In the case of tablets B1, B2, C1, and C2, 3 mL samples of the fluid were extracted every 10 min (1$^{st}$ hour), and every 20 min (2$^{nd}$ hour). The further stages of release were estimated from kinetic calculations. The amount of the released substance was determined using a UV-VIS T60 spectrophotometer (PG Instruments, Cincinnati, USA) interfaced with a PC to record the data, according to previously performed spectrophotometrical studies of CHX.[21]

## Kinetic calculations and statistical methods

The in vitro release data was fitted to various kinetic models including: zero-order, first-order, Korsmeyer-Peppas, Hixon-Crowell, and Higuchi models.[22–24] Based on the obtained release equations, $t_{0.5}$ of the process was calculated for all formulations. The kinetic parameters, determination coefficient R2 and the probability p of making a type I error were calculated. Curvilinear regression and ANOVA tests were performed using STATISTICA v. 10 software.[25] The descriptive data is presented as arithmetic averages with respective standard deviations.

# Results

## Main physical parameters: Mass distribution, hardness, friability, and size

The average mass of the tablets was 449.5–451.0 mg. No more than 2 of the individual tablet masses deviated from the average mass by more than 5%, and none deviated by more than 10%. The friability did not exceed 1% for all the evaluated samples. The hardness of the tablets obtained was 85–322 N for the evaluated batches A1–C1, whereas for the batch C2, the hardness was greater than 350 N. The diameter of the tablets produced was 9.969–10.019 mm, whereas the thickness of the evaluated tablets was 4.152 ±0.007 mm, 3.007 ±0.031 mm,

5.02 ±0.012 mm, 4.089 ±0.012 mm, 4.706 ±0.048 mm, and 4.169 ±0.008 mm, for A1, A2, B1, B2, C1, and C2, respectively.

## Matrix integrity and water content

Images of the cross-sections of the tablets obtained via scanning electron microscopy are presented in Fig. 1.

The mean initial mass ($M_0$) and volume ($V_{T0}$) of the assessed tablets, the volume of the wet tablets after 24 h of incubation in an acceptor fluid ($V_{T24}$), the mean volume of water absorbed by the tablets over 24 h ($V_{W24}$), and the mean mass of the dry residue of the tablets after 21 days ($M_{21}$) were gathered and are presented in Table 2. The volume of the tablets containing the polymer increased from 2.2 times for formulation B1 to 2.9 times for formulation C2.

Furthermore, the tablets formed with a compressive force of 25 kN (B2 and C2) increased in volume 2.4 and 2.9 times, respectively, whereas the volume of formulations B1 and C1 formed with 10 times less compressive force increased 2.2 and 2.6 times, respectively.

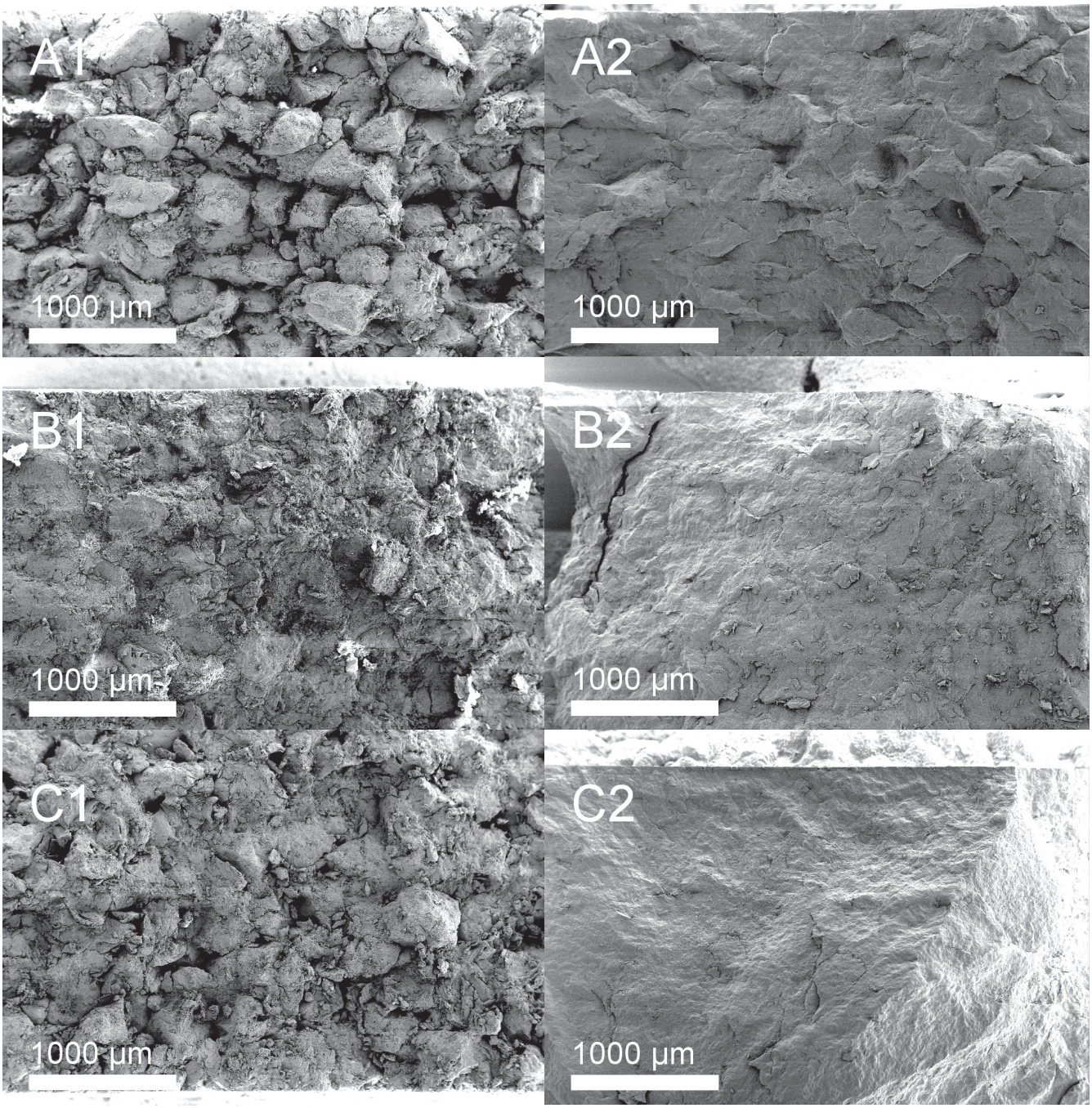


**Fig. 1.** Microphotographs obtained from SEM studies

The bar represents 1000 µm; the left column represents tablets formed with a 2.5 kN compressive force (A1, B1, and C1), whereas the right column shows tablets formed with a compressive force of 25 kN (A2, B2, and C2).

**Table 2.** Water intake of the assessed tablets after 24 h and dry residue of the tablets after 21 days of desiccation

| Formulation code | | A1 | A2 | B1 | B2 | C1 | C2 |
|---|---|---|---|---|---|---|---|
| parameter | unit | | | | | | |
| $M_0$ | (mg) | 450 | 450 | 450 | 450 | 450 | 450 |
| $M_{21}$ | | – | – | 30.0 | 24.2 | 50.4 | 49.6 |
| $V_{T0}$ | ($mm^3$) | 392.5 | 314.0 | 392.5 | 314.0 | 392.5 | 314.0 |
| $V_{T24}$ | | – | – | 870 | 754 | 1010 | 913 |
| $V_{W24}$ | | – | – | 564 | 496 | 951 | 855 |

$M_0$ – initial mass of the tablet; $M_{21}$ – mass of the tablet after 21 days of desiccation; $V_{T0}$ - initial mean volume of the evaluated tablets; $V_{T24}$ – mean volume of the wet tablets after 24 h of incubation in an acceptor fluid; $V_{W24}$ – mean volume of water absorbed by the tablets after 24 h of incubation in an acceptor fluid.

## Floating characteristics

The tablets swelled rapidly in the radial and axial dimensions during the in vitro study. Consequently, the density of the tablets decreased, and the tablets floated. The floatation properties of all tablet types are collected and presented in Table 3.

**Table 3.** Floating properties of the evaluated tablets

| Formulation code | A1 | A2 | B1 | B2 | C1 | C2 |
|---|---|---|---|---|---|---|
| FLT (min) | 15 | 31 | 61 | 89 | 80 | 105 |
| FT (h) | * | ** | >24 h | <24 h | >24 h | <24 h |
| Observation after 24 h | *** | | FS | B | FS | B |

FT – floating time; FLT – floating lag time; * dissolved completely after 22 min; ** dissolved completely after 43 min; *** white film on the surface of the fluid; FS – floating on the fluid surface; B – on the bottom of the vessel.

Formulations A1 and A2 dissolved in the acceptor medium. Tablet A1 lifted and floated on the surface of the solution after approximately 15 min. However, after the next 7 min, it dissolved completely. Tablet A2 floated on the surface of the fluid after 31 min and gradually dissolved. Both A1 and A2 left a white film of magnesium stearate as shown in Fig. 2A.

The tablets containing the polymer (B1, B2, C1, and C2 – Fig. 2) did not dissolve and floated on the surface of the solution. Tablets B1 and C1, formed with a compressive force of approximately 2.5 kN, floated on the surface after 61 min and 80 min, respectively. Tablets B2 and C2, which were formed with a compressive force of 25 kN, appeared on the surface of the solution after 89 min and 105 min, respectively. Tablets B1 and C1 floated on the surface of the fluid for over 24 h, whereas tablets B2 and C2 sank to the bottom of the vessel. The FLT and FT were determined for the tablets, as shown in Table 3.

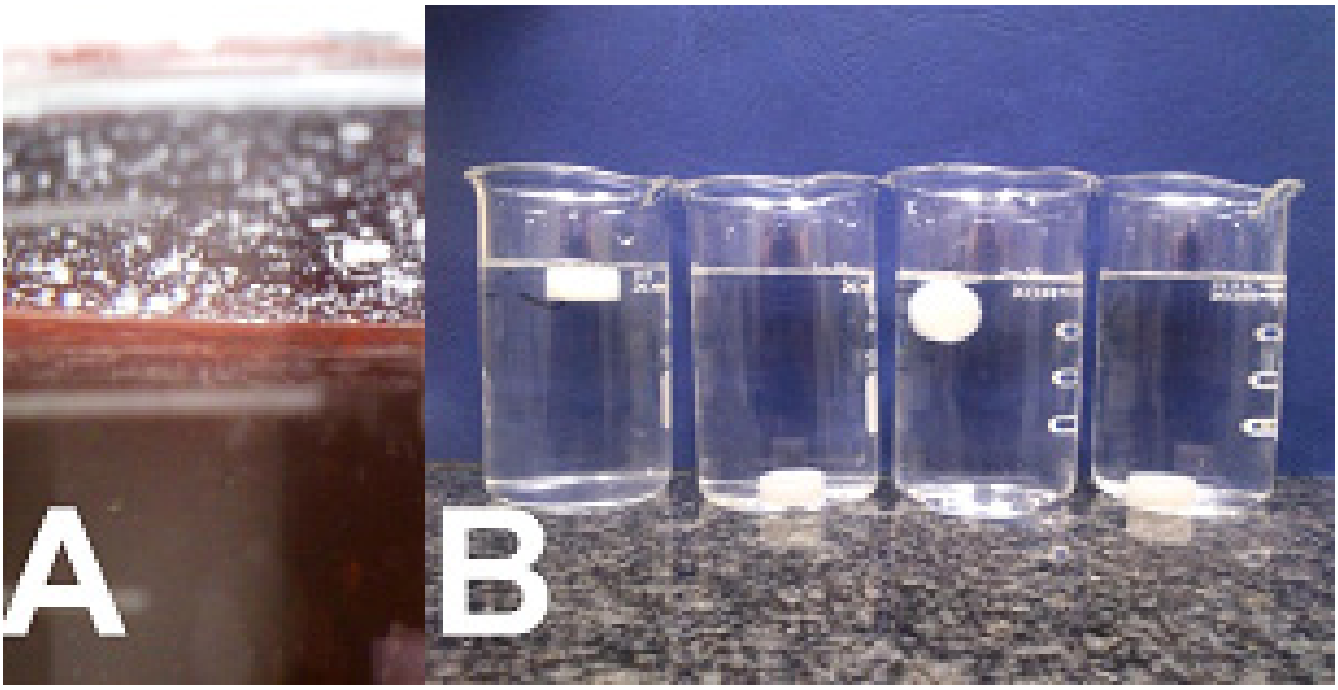


**Fig. 2.** The appearance of the tablets after 24 h of incubation in 0.1 mol/L hydrochloric acid

A – the remains of the dissolved A1; B – B1, B2, C1, and C2 formulations, from left to right, respectively.

## In vitro drug release kinetics study

The dissolution profiles of CHX obtained from the formulations studied are presented in Fig. 3.

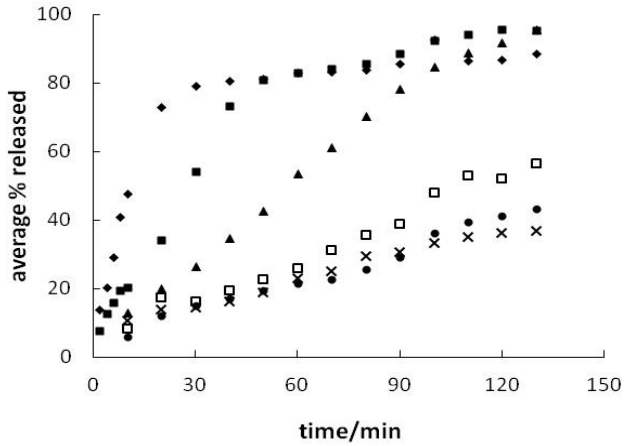


**Fig. 3.** The in vitro release curves of CHX from formulations A1 (♦), A2 (■), B1 (▲), B2 (×), C1 (□), and C2 (●)

**Table 4.** Kinetics of the in vitro drug release for the evaluated formulations and fitted to selected models

| M | P | Formulation code | | | | | |
|---|---|---|---|---|---|---|---|
| | | A1 | A2 | B1 | B2 | C1 | C2 |
| ZO | $K_0 \times 10^3$ ($min^{-1}$) | 11.1 ±1.3 | 10.9 ±0.9 | 6.6 ±0.2 | 3.3 ±0.1 | 4.4 ±0.1 | 3.4 ±0.1 |
| | $R^2$ | 0.8233 | 0.9161 | 0.9933 | 0.9828 | 0.9952 | 0.9889 |
| | p | 3.3 $\times 10^{-7}$ | 4.1 $\times 10^{-9}$ | 1.7 $\times 10^{-13}$ | 2.9 $\times 10^{-11}$ | 2.6 $\times 10^{-14}$ | 2.6 $\times 10^{-12}$ |
| FO | $K_1 \times 10^3$ ($min^{-1}$) | 56.2 ±3.5 | 42.7 ±4.4 | 11.1 ±0.3 | 3.9 ±0.1 | 5.9 ±0.1 | 4.1 ±0.1 |
| | $R^2$ | 0.9599 | 0.8715 | 0.9939 | 0.9912 | 0.9938 | 0.9909 |
| | p | 1.6 $\times 10^{-8}$ | 2.4 $\times 10^{-7}$ | 9.4 $\times 10^{-14}$ | 7.2 $\times 10^{-13}$ | 1.0 $\times 10^{-13}$ | 8.9 $\times 10^{-13}$ |
| KP | $K_{KP} \times 10^2$ ($min^{-n}$) | 20.2 ±2.6 | 8.7 ±0.7 | 1.6 ±0.2 | 1.7 ±0.1 | 1.7 ±0.3 | 1.3 ±0.2 |
| | n | 0.37 ±0.03 | 0.54 ±0.02 | 0.81 ±0.03 | 0.64 ±0.02 | 0.69 ±0.04 | 0.70 ±0.03 |
| | $R^2$ | 0.8842 | 0.9738 | 0.9880 | 0.9896 | 0.9599 | 0.9781 |
| | p | 2.03 $\times 10^{-8}$ | 1.81 $\times 10^{-12}$ | 6.35 $\times 10^{-12}$ | 2.87 $\times 10^{-12}$ | 4.93 $\times 10^{-9}$ | 1.77 $\times 10^{-10}$ |
| HC | $K_\beta \times 10^3$ ($min^{-1}$) | 11.14 ±0.5 | 8.08 ±0.45 | 3.08 ±0.04 | 1.24 ±0.04 | 1.78 ±0.03 | 1.27 ±0.03 |
| | $R^2$ | 0.9662 | 0.9560 | 0.9977 | 0.9888 | 0.9961 | 0.9911 |
| | p | 1.02 $\times 10^{-11}$ | 1.42 $\times 10^{-15}$ | 8.19 $\times 10^{-11}$ | 2.03 $\times 10^{-13}$ | 1.63 $\times 10^{-10}$ | 3.67 $\times 10^{-11}$ |
| H | $K_H \times 10^2$ ($min^{-0.5}$) | 11.2 ±0.6 | 10.2 ±0.3 | 6.3 ±0.3 | 3.1 ±0.1 | 4.2 ±0.2 | 3.2 ±0.1 |
| | $R^2$ | 0.9551 | 0.9899 | 0.9792 | 0.9930 | 0.9765 | 0.9821 |
| | p | 1.02 $\times 10^{-11}$ | 1.42 $\times 10^{-15}$ | 8.19 $\times 10^{-11}$ | 2.03 $\times 10^{-13}$ | 1.63 $\times 10^{-10}$ | 3.67 $\times 10^{-11}$ |
| BF | | HC | H | HC | H | HC | HC |

M – model; P – parameter; ZO – zero-order; FO – first-order; KP – Korsmeyer-Peppas; HC – Hixon-Crowel; H – Higuchi; $K_0$, $K_1$, $K_{KP}$, $K_\beta$, and $K_H$ – the respective release rate constants; BF – best fit; $R^2$ – determination coefficient; p – the probability p of making a type I error; the value following the ± symbol represents the standard deviation.

The release of CHX varied in each of the formulations assessed. The release kinetics of the CHX for formulations A1 and A2, which do not contain the polymer, were significantly different over the initial stage from those of formulations B1, B2, C1, and C2. The release of the drug from formulation A1 was rapid over the initial phase before reaching a plateau after approx. 20 min, whereas for formulation A2, a plateau was observed after approx. 40 min. The API release rate from formulations B1, B2, C1, and C2, containing the polymer, was much slower, and no plateau phase was observed. The results of the kinetic tests are reported in Table 4.

For example, graphs are shown of the release kinetics, evaluated using the various methods, for the B1 formulation. Fig. 4 shows plots for the first-order, Korsmeyer-Peppas, Hixon-Crowell, and Higuchi models.

The half-release time values for the models presented are gathered together and presented in Table 5.

# Discussion

## Matrix integrity and intersection visualization

The amount of the polymer incorporated in the formulations and the compressive force of the press affect the floating properties of the tablets being examined here. From Fig. 1, the visualization enables the tracking of the evaluated tablets in an aqueous environment. The huge granules represent the sorbitol, which was completely dissolved after the tests due to the loss of mass (Table 2: $M_0$–$M_{21}$). The residual mass of the tablet is a result of the anionic polymer, and may represent insoluble magnesium stearate and a residual part of the active substance. The visualization indicates that higher compression results in a highly compact structure which should be more resistant to water inflow. The increase in the polymer content leads to a visual homogenization of the structure at higher pressures.

When floating on the surface of the solution, tablet formulations B1, B2, C1, and C2 exhibited good matrix integrity. The increase in the tablet volume in water depends on the quantity of the polymer within the formulation, whereas the increased pressure during the tableting process decreases the volume of the tablet after incubation

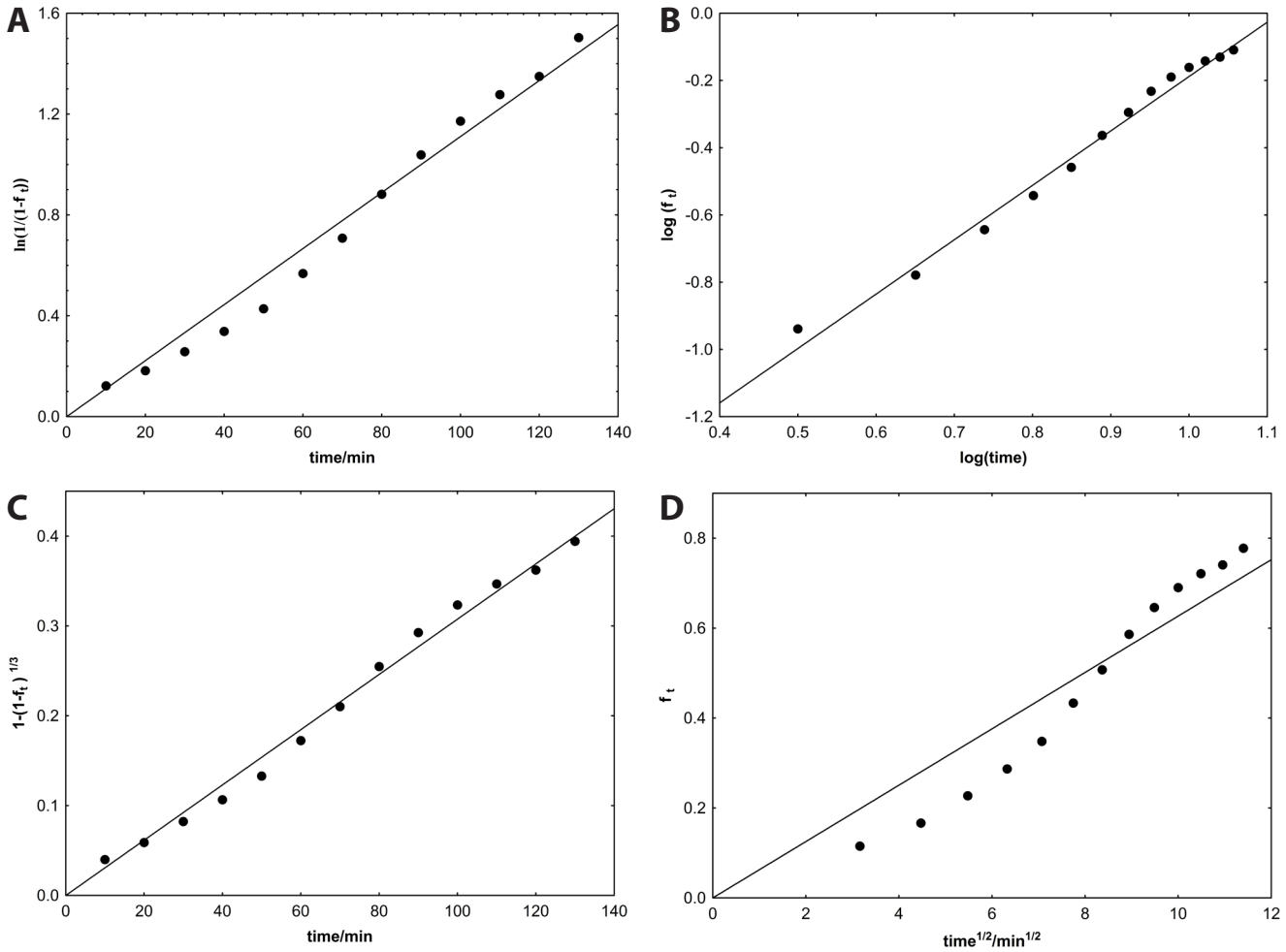


Fig. 4. Graphs of the release kinetics for the B1 formulation

A – first-order model; B – Korsmeyer-Peppas model; C – Hixon-Crowell model; D – Higuchi model.

Table 5. Half-release times for the evaluated formulations (details shown in the text)

| M | Formulation code | | | | | |
|---|---|---|---|---|---|---|
| | A1 | A2 | B1 | B2 | C1 | C2 |
| | $t_{0.5}$ (min) | | | | | |
| ZO | 45 ±5 | 46 ±4 | 75 ±2 | 152 ±6 | 113 ±2 | 149 ±5 |
| FO | 12 ±1 | 16 ±2 | 62 ±1 | 175 ±5 | 117 ±3 | 169 ±5 |
| KP | 11 ±4 | 25 ±4 | 72 ±10 | 202 ±26 | 128 ±33 | 185 ±34 |
| HC | 19 ±1 | 26 ±1 | 67 ±1 | 167 ±5 | 116 ±2 | 162 ±4 |
| H | 20 ±2 | 24 ±1 | 64 ±5 | 255 ±12 | 144 ±13 | 249 ±19 |

M – model; $t_{0.5}$ – in vitro half-release time; ZO – zero-order; FO – first-order; KP – Korsmeyer-Peppas; HC – Hixon-Crowell; H – Higuchi; the value after the ± symbol represents the standard deviation; n = 5.

in the acceptor fluid. The $M_{21}$ parameter reflects the residual amount of polymer forming in the tablets, whereas the difference between $M_0$ and $M_{21}$ illustrates the amount of dissolved sorbitol (Table 2). This data correlates with the initial amount of polymer and sorbitol in the formulation (Table 1).

## Floatation

The tablets produced using a force of 2.5 kN exhibited shorter FLTs than tablets produced using a force of 25 kN. Bijumol et al. revealed that an increase in the hardness of the tablets resulted in a significant increase in the FLT by 15–30 min, which is in good agreement with the results obtained in this study.[14] Furthermore, formulations made with a compression force of 2.5 kN (B1 and C1) floated on the surface of the solution for a much longer period than tablets B2 and C2, which were formed with a compressive force of 25 kN, and exhibited an FT that was less than 24 h. These observations are well correlated with previous results reported by Jeganathan et al.[15] The tablets fabricated with the highest compressive force were found to sink in aqueous conditions, suggesting that the total tablet density may be greater than 1.0 mg/mm$^3$. Otherwise, formulations B1–C2, which contain PA, exhibited a more controlled release profile than formulations without the polymer, such as A1 and A2, which were almost completely dissolved after 22 and 43 min,

respectively. It is worth noting that the FT for formulations B1 and C1 were the longest obtained in this work, and simultaneously higher than that reported in other works concerning non-effervescent floating tablets.[11,14,15]

The floating properties of the tablets may be ascribed to the properties of the PA, i.e., to the hydrogen bonding groups, strong anionic charges and high molecular weight.[26] PA resins are hydrophilic substances that are insoluble in water and swell when dispersed in water to form colloidal suspensions. When they come into contact with water, PA polymers increase their original volume and diameter. Because the pKa of these polymers is approximately 6.0, carboxyl groups in the main chain of the polymer may undergo ionization in an aqueous environment. The polymer swells as a result of the electrostatic repulsion between anionic groups.[27] However, the acidic pH should hinder this process. In the present study, the polymer matrix of the tablet, with implemented alkali CHX in the acidic acceptor fluid, is penetrated by the solvent. After being placed into a solution, the dimensions of the polymer particles increase as a result of limited polymer relaxation, initially resulting in a gel formation around the tablet, which was shown as an increase in the tablet volume, as shown in Table 2 – VT24 for the B1–C2 formulations. A high compressive force during tablet formation has a negative impact on the floating properties by decreasing the free space between the polymer chains. Consequently, limited penetration of the solvent into the particles results in prolonged matrix swelling.

## Release study

The diffusion coefficient n determined via the Korsmeyer-Peppas model was less than 0.45 (0.37 ±0.03) for only the control formulation A1.[22] For the other 5 formulations, the value of the diffusion coefficient n was between 0.54 ±0.02 and 0.81 ±0.03. These values imply that the mechanism behind the CHX release in tablets B1, B2, C1, and C2 is not based solely on diffusion. The value of the release exponent n obtained indicates a coupling of the diffusion and erosion mechanisms, which corresponds to the so-called anomalous transport or non-Fickian model. The API release is rapid over the initial stage because the drug dissolves on the surface of the tablet, and a gel layer is formed with time as a result of the hydrophilic polymer making contact with water. Other factors that affect the flotation properties of the tablets and drug release rate may include electrostatic interactions within the tablet and the environmental pH.

Other authors examined the impact of the rate of water intake by a polymer matrix composed of Carbopol 934 P and verapamil hydrochloride, an alkaline drug, on the swelling of the polymer matrix.[27] The results showed that the drug-to-polymer ratio had an important impact on both the interaction between the drug and the polymer, and on the rate of water absorption by the polymer. However, pH has a significant impact on the drug release rate. PA is an anionic polymer, and changes its solubility with the environmental pH. An increase in the viscosity coefficient can be observed with the increase of pH, according to the ionization and, consequently, the swelling of the polymer net.[28] The structure of CHX contains 10 nitrogen atoms that enable bonding with the carboxyl groups in the PA. In a previous work, CHX with PA at a pH of approximately 5 formed a strong insoluble salt complex, resulting in a very weak CHX release, which is within the error range.[29] In the current work, the pH value was similar to that found in the stomach, namely approx. 1. This may slow the dissociation of PA and lead to the retarded release of CHX from the tablets due to the formation of a relatively low ionized polymeric matrix. Thus, a limited affinity of the cationic groups of the CHX to the negatively charged carboxyl groups may be expected. Binding of the functional groups of the polymer, and the ionic sites of CHX can result in the delayed release of the drug from the polymeric matrix. The formation of a complex may be responsible for longer CHX retention. Ionic interaction between the CHX and polycarboxylate containing the carboxylic groups was proposed, based on the Raman spectra obtained by Jones et al.[30]

The in vitro drug release data was subjected to a goodness of fit test using a linear regression analysis according to zero-order, first-order, Korsmeyer-Peppas, Hixon-Crowell, and Higuchi models to determine the mechanism behind the drug release.[22–24] The results of this analysis are listed in Table 4. Based on the determination coefficients (R2), the best fitted models were determined. For the control formulation A1 and formulations B1, C1, and C2, the highest determination coefficient R2 was obtained for the Hixon-Crowell model, whereas formulations A2 and B2 are best described by the Higuchi model. Furthermore, the probability of making a type I error p is small and ranges between 1.42 × 10–15 and 3.3 × 10–7. Thus, this probability is significantly lower than the level of significance $\alpha = 0.05$, which suggests a correlation between the released drug fraction and time in virtually all models. Table 5 lists the values of $t_{0.5}$ determined for all the formulations and all models of drug substance release kinetics.

The results show that, similarly to the FLT, $t_{0.5}$ is the shortest for the control formulation A1 and falls between 11 ±4 min and 45 ±5 min, depending on the drug substance release kinetics model. However, A1 tablets were almost completely dissolved after 22 min. Therefore, the $t_{0.5}$ = 45 ±5 min obtained for the zero-order kinetics model shows that the model is not well fitted for this formulation. A similar phenomenon occurs for the control formulation A2. The determined $t_{0.5}$ value falls between 16 ±2 min and 46 ±4 min, whereas at 43 min, the tablets were almost completely dissolved. The $t_{0.5}$ = 46 ±4 min calculated using the zero-order kinetics model seems very unlikely.

The longest half-release time was noted for formulations B2 and C2. Considering the error limits of $t_{0.5}$ for these formulations in the case of all the kinetics models, the $t_{0.5}$ values obtained are similar for tablets B2 and C2. Of the tablets containing the polymer, the smallest $t_{0.5}$ value falling within the range from 62 ±1 min to 75 ±2 min was noted for formulation B1. The analysis of the half-release time of the tablets floating for over 24 h (B1 and C1) showed that $t_{0.5}$ for formulation C1 is longer and falls between 113 ±2 min and 144 ±13 min, which suggests that C1 is the optimal formulation in terms of the FT and $t_{0.5}$.

According to the in vitro data obtained, the proposed compositions may enable prolonged release of cationic drugs, e.g., some antibiotics, antihistaminic drugs, or neutralizing agents, in the cases when long and local activity of the drug in the stomach is demanded. The therapeutic target, after expanded studies, including ex vivo and in vivo research, may cover i.a. patients with chronic gastric diseases.

# Conclusion

We demonstrated that tablet buoyancy is promoted by a low compressive force as the solvent particles may freely penetrate the free spaces within the tablet. This phenomenon is indirectly confirmed via SEM photographs. The FT and FLT were found to be dependent on the amount of polymer incorporated in the formulations. Introduction of the polymer into formulations B1, B2, C1, and C2 provides the desired floating ability and prolonged drug release. C1 is determined to be the optimal formulation. The results of the current study indicate that PA offers a good control over the release of CHX from the tablets and can be suggested for therapies that require prolonged treatment covering a daily time period.

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# The neuroprotective effect of N-acetylcysteine in spinal cord-injured rats

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## Abstract

**Background.** Spinal cord injury (SCI) is an important cause of impairment of sensory and motor nerve function. It has been shown that free-radical species play an important role in the pathogenesis of acute tissue trauma after SCI. There are no proven pharmacological therapies that provide neuroprotection and stimulate axonal growth after trauma.

**Objectives.** The aim of this study was to investigate the neuroprotective effect of N-acetylcysteine (NAC) on the regeneration of spinal cord injuries in rats.

**Material and methods.** A total of 20 male Wistar C rats were subjected to SCI and divided into control and experimental groups. In the control group (n = 10) trepanation and SCI by means of a pressure impactor was performed without any therapy. In the study group (n = 10), 1 dose of NAC was applied intraperitoneally (150 mg/kg b.w.) immediately after SCI, and another one after 24 h. The functional outcome on the Basso-Beattie-Bresnahan (BBB) scale and sciatic functional index (SFI) and morphological features of regeneration were analyzed during a 12-week follow-up. The spinal cords and brains were collected 12 weeks after SCI for histopathological and immunohistochemical analyses.

**Results.** The rats treated with NAC presented some improvement in locomotor activity and spinal cord morphology when compared to the control group. Namely, the hind paw angle of rotation was significantly lower in the NAC group than in the control group. No differences were observed between the control and study groups in terms of interlimb coordination. The area of the main lesion was only slightly decreased in the NAC group as compared to the control group. The length of lesions in the injured spinal cord in the NAC group was diminished in comparison to the control group. The number of FG-positive cells was higher in the NAC group than in the control group.

**Conclusions.** The study showed that the neuroprotective activity of NAC had limited positive influence on the regeneration of the isolated SCI in rats.

**Key words:** neuroprotection, N-acetylcysteine, spinal cord injury, neuroregeneration

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Spinal cord injury (SCI) is an important cause of impairment globally, with an incidence between 236 and 1,009 cases per million people.[1] The usual causes of spinal cord injury are motor crashes, sport-related accidents, and incidents associated with community violence and the workplace. Injury of the spinal cord causes sensory and motor dysfunction distally to the place of trauma.[2]

There are 2 main mechanisms that lead to spinal cord injury. The first is related to mechanical damage to the spinal cord structure. The other is secondary injury, which plays a dominant role in a cascade of biochemical events at the cellular level. It has been shown that free-radical species play an important role in the pathogenesis of acute tissue trauma after spinal cord impact injury.[3]

Many authors have suggested that this oxidative stress is associated with such processes as edema, hypoperfusion, conduction disturbances, impairment of metabolism, and Wallerian degeneration of neurons.[4,5] It has been shown that neutralization of reactive oxygen species (ROS) and nitrogen in the first 3–4 h after the onset of trauma or ischemia reduces oxidative stress in neurons and shows a neuroprotective effect.[6,7]

Currently, there are no proven pharmacological therapies for spinal cord injury that provide neuroprotection and stimulate axonal growth after trauma.[8,9]

N-acetylcysteine (NAC) is a thiol compound possessing antioxidant properties and the precursor of glutathione.[10,11] It acts by scavenging reactive oxygen species and inhibits the activity of cyclooxygenase-2 and membrane lipid peroxidation induced by inflammation.[12] Some studies have indicated that NAC might have neuroprotective effect following brain ischemia or traumatic brain injury in rats.[13,14]

The aim of this study was to investigate the neuroprotective effect of NAC therapy on the regeneration of isolated spinal cord injuries in rats.

# Material and methods

All the procedures were performed in accordance with EU animal protection laws and were approved by the Local Animal Research Ethics Committee. Twenty adult male Wistar C rats (approximate body weight: 300 g) were randomly divided into 2 groups: 1) the control group (n = 10) – animals subjected to trepanation of the vertebra and a single injury blast of the spinal cord (explained in detail below) without any repair therapy; and 2) the NAC group (n = 10) – animals subjected to trepanation and injury in the same way as the animals in the control group and then administered intraperitoneal doses of 2.55% N-acetylcysteine solution, both immediately and 24 h after the injury, at a dose of 150 mg/kg b.w.

## Spinal cord injury technique

Focal spinal cord injury was performed using the authors' original apparatus – a pressure impactor producing a precisely controlled air blast.[15] After intraperitoneal anesthesia with ketamine (100 mg/kg) and xylazine (10 mg/kg), the animals were placed on a heated plate and immobilized by means of head holding bars and spine clamps at the level of Th-9 and Th-11, making Th-10 stable and easily accessible for further steps. The skin was then incised over the spinous processes and the vertebral surface was exposed dorso-laterally from Th-9 to Th-11. Under control of a stereomicroscope (Nikon, Tokyo, Japan), a 2 mm diameter opening was drilled in the Th-10 vertebral arch on the right side. To avoid overheating and thermal lesion, the trepan was cooled down with chilled phosphate-buffered saline (PBS). Then, the impactor tip was placed close to the drilled opening and adjusted by means of a micromanipulator. The penetration depth of the tip was set up to establish contact with the dura mater but without exerting any pressure on it. After setting the impact parameters (150 kPa pressure; 0.1 s duration), the air blast system was activated. The "shot" was observed under the stereomicroscope and recorded by the attached camera (Nikon, Tokyo, Japan). After the procedure was complete, the hole was secured with wax, the muscles were sutured in layers, the skin was closed, and the wound was covered with a sterile bandage. To avoid dehydration, all the animals were subcutaneously injected with 2 mL of sterile saline. Because the autonomic function of the urinary bladder was impaired due to the spinal shock, it was emptied manually twice a day until the recurrence of bladder function. To prevent pain, 400 mg of paracetamol (in a 125 mg/5 mL suspension) was dissolved in 100 mL of drinking water, providing an average drug dosage of 200 mg/kg b.w. per day.

## Assessment of locomotor function

All the animals were observed and analyzed regarding development or regression of neurological deficits for 12 weeks following the SCI procedure. Behavioral observations included gait analysis (the footprint test) and open field tests using the Basso-Beattie-Bresnahan (BBB) locomotor rating scale.[16]

## Footprint tests

Footprint tests were carried out in the 1$^{st}$, 4$^{th}$, 7$^{th}$ and 12$^{th}$ week postoperatively. The animals were tested on a 100 cm long and 7 cm wide runway with side walls and a transparent floor. The walk of a rat along the runway was recorded with a digital camera and automatically analyzed frame by frame (Catwalk XT 8.1, Noldus Information Technology BV, Wageningen, Netherlands). Each animal was tested 3 times consecutively. Framed foot images were analyzed with respect to the foot rotation angle (the angle made by 2 lines connecting the 3$^{rd}$ toe and the stride line at the center of the paw) of the right hind paw (ipsilateral to the injury site), and interlimb coordination (the smallest distance between the middle point of the hind paw and the forepaw on the same side).

## BBB open field test

Open field tests were carried out in the classical manner on a plexiglass surface. During the test, the motor function of the joints was analyzed to assess the stepping ability of the animal. Additionally, the general coordination and stability of the body were evaluated. The value range was 0–22, where 0 was a complete lack of motor capability and 22 indicated normal locomotion. The results obtained were presented as average values from both hind extremities.

## Retrograde neuroanatomical tracing

Retrograde neuroanatomical tracing was used to determine the extent to which supraspinal axons regenerate to reach spinal cord segments caudal to the injury. The application of Fluoro-Gold (FG) (Fluorochrome Inc., Englewood, USA) was performed using methods originally described by Coumans et al.[17]

Only 4 animals in each group were randomly selected for this procedure. The remaining animals from each group were used for functional testing, due to restrictions of the Local Animal Research Ethics Committee limiting the number of animals used in all the experiments. For retrograde tracing, 1 week before the end of the experiment, anesthesia was administered as described above, then the spinal cord was exposed by laminectomy below the injury site and 2 microcrystals of Fluoro-Gold were placed bilaterally inside the spinal cord 10 mm caudally from the injury site. To ensure that there was no unintended diffusion of FG into the spinal cord rostral to the injury, the injection sites and lesions were examined in all the animals. None of the animals had any spread of the tracer.

To quantify the number of FG-positive neurons present in the brain stem (red nucleus) and primary motor cortex, on the last day of experiment, the animals were rapidly perfused with a bolus of cold PBS, and whole brains were carefully dissected, dehydrated in 15% sucrose, embedded in Tissue-Tek matrix (Sakura Finetechnical Co., Tokyo, Japan), frozen, and then cut coronally into 10 µm sections. Every 6$^{th}$ slide was analyzed, giving 18–22 sections per animal. The sections were viewed under UV excitation (at a 365 nm wavelength) in a fluorescent microscope using the appropriate filter (Labophot 2, Nikon, Tokyo, Japan) and photographed. Microphotographs were taken near the 4 corners and the center of the areas where the most cells were present. The number of cells from each section was then summed for each rat for both the brain stem and the motor cortex; the number of cells per mm$^2$ was calculated and finally averaged for the whole group.

## Histopathologic examination

After 12 weeks, the animals were re-anesthetized and perfused transcardially with 100 mL PBS (pH = 7.4) followed by 100 mL of 4% paraformaldehyde solution in the same buffer. Fragments of spinal cord (~2 cm) incorporating the injured area were dissected and dehydrated in 20% sucrose in PBS for 24 h at 4°C. The spinal cords were then embedded in Tissue-Tek matrix (Sakura Finetechnical Co., Tokyo, Japan), frozen, and cut sagitally or transversally into 10 µm sections mounted on SuperFrost Plus slides (Thermo Fisher Scientific, Waltham, USA). The slides were subjected to routine hematoxylin-eosin staining, 1% toluidine staining or immunohistochemical labeling. The anti-rat antibodies used were rabbit GFAP (for astrocytes) and mouse GAP-43 (both from Chemicon Europe Ltd., Southampton, UK). The sections were incubated with the primary antibody overnight at 4°C, and then, after triple rinsing in PBS, they were treated with secondary goat anti-rabbit or anti-mouse IgG antibodies conjugated with Alexa 488 and/or Alexa 568 (Molecular Probes, Eugene, USA). Sections coverslipped in VectaShield with DAPI (Vector Laboratories, Burlingame, USA) were examined under a confocal laser scanning microscope (FluoView, Olympus Corporation, Tokyo, Japan). The images were digitally stored and then analyzed. Twelve weeks after surgery the total average lengths and sizes of lesions (cavity areas) were measured.

## Statistical analysis

The ANOVA and nonparametric Kruskal-Wallis ANOVA were used to analyze normally distributed data and non-continuous data, respectively. Differences between the group means in footprint test results, histological and BBB scores at each time point after the injury were identified using the Student's t-test. The level of statistical significance was set at $p \leq 0.05$. All data was expressed as mean ±SD.

# Results

## Functional tests

All the functional tests were performed by the same person, properly trained and experienced in conducting this type of research. Immediately after SCI most of the animals were monoplegic. The hind paw angle of rotation increased in all the animals in the 1$^{st}$ week after the injury, reaching 19.6 ±1.2° in the control group at the end of the 12-week observation, indicating significant deterioration of locomotor function following the injury. In the NAC group, this parameter was significantly lower by the 12$^{th}$ week (15.1 ±1.2°; $p < 0.05$). The differences appeared in the 7$^{th}$ week of observation (Fig. 1).

Interlimb coordination in the animals treated with NAC did not improve, reaching a value of 2.17 ±0.26 cm, which was comparable with the results in the control group (2.13 ±0.27 cm) (Fig. 2).

The analysis of the BBB tests revealed comparable dynamics of recovery of function in the NAC group and in the control group. BBB scores 1 week after SCI were lower than 10 in all the animals, and after 12 weeks they had not significantly improved in either the control group (10.3 ±0.6) or the NAC group (11.9 ±0.7; $p > 0.05$) (Fig. 3).

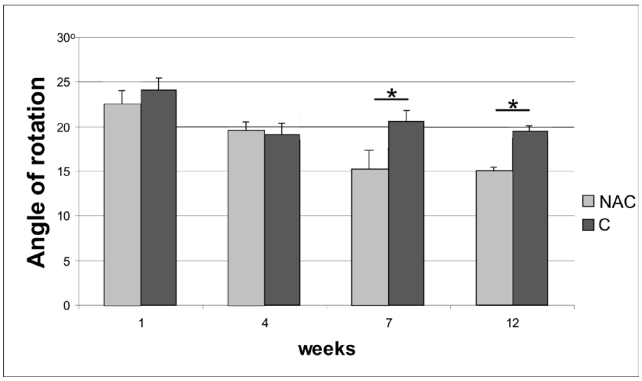


**Fig. 1.** Results of the measurements of the angle of hind paw rotation

NAC – study group; C – control group; * indicates statistical significance ($p \leq 0.05$).

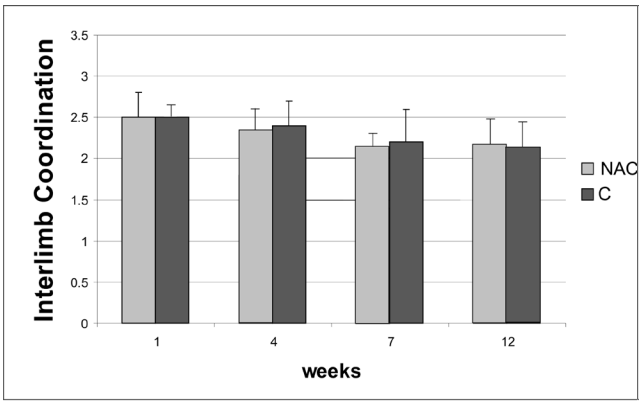


**Fig. 2.** Results of the study of interlimb coordination

NAC – study group; C – control group.

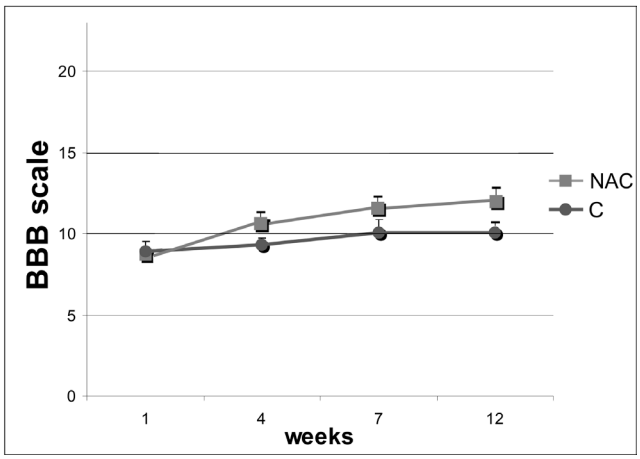


**Fig. 3.** Results of the BBB tests

NAC – study group; C – control group.

The area of the main lesion (cavity area) was slightly decreased in the NAC group (0.81 ±0.29 $mm^2$) as compared to the control group (1.03 ±0.21 $mm^2$) ($p > 0.05$) (Fig. 4). These values, however, were not significantly different.

## Morphology

The length of the spinal cord lesions differed between the 2 groups. In the control group it was 6.11 ±2.1 mm, which was significantly higher than in the NAC group (4.07 ±0.56 mm; $p < 0.05$) (Fig. 5).

## Neuronal tracing

FG-positive cells in the brain stem and primary motor cortex, proving the survival of long-tract neurons, were more numerous in the NAC group (96.7 ±43.1) than in the control group (17.3 ±2.6), and this difference was significant ($p < 0.05$) (Fig. 6).

# Discussion

Spinal cord injury leads to a loss of motor and sensory function distally to the site of the trauma. The most important goal in limiting spinal cord injury should be reducing edema and free radical generation in damaged neurons and controlling inflammation in order to decrease secondary injury to the spinal cord parenchyma after the initial insult, promoting neural growth and demyelination repair to reduce conduction deficits.[2] In the present study, NAC was used because of its ability to reduce oxidative stress by interfering with free radicals or up-regulating antioxidant systems.[18,19]

Some beneficial effects of NAC application in SCI in an animal model were observed in the present study. The angle of hind paw rotation was significantly lower in the NAC group than in the control group. However, interlimb coordination did not improve in the animals treated with NAC in comparison to the control group. Karimi-Abdolrezaee et al. revealed that after trauma, the normal rotation angle (about 8°) increased to 30°, while interlimb coordination was about 1.5 cm in healthy rats and increased to 3 cm or more after injury.[20] Similarly, the area of the main lesion and the length of lesion in the injured spinal cords were only slightly decreased in the NAC group as compared to the control group.

There are only a few articles about the protective role of NAC on injured spinal cord neurons in vivo. Hanci et al. investigated the effectiveness of methylprednisolone, NAC and a combination of both compounds in spinal cord injury in rats. In that study, injuries were performed extradurally with aneurysm clips at the T4–T5 level. After injury, methylprednisolone was applied intraperitoneally in the 1st group (30 mg/kg and maintenance doses of 5.4 mg/kg); in the 2nd group NAC (150 mg/kg)

was administered; and in the 3rd group both compounds were administered in the doses given above. The authors showed that administration of methylprednisolone, NAC and a combination of both compounds prevented secondary trauma in experimental spinal cord injury in the rats.[21]

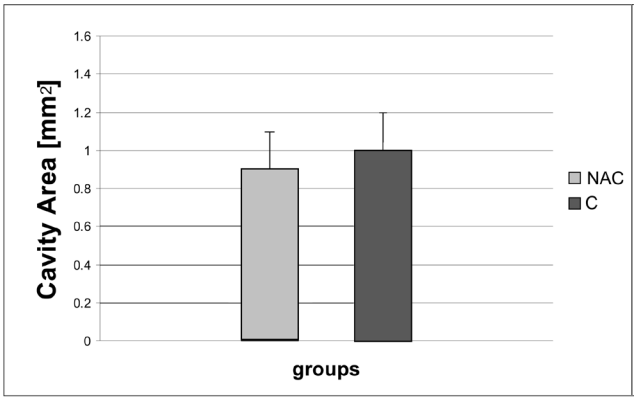


Fig. 4. Results of the measurements of the cavity area

NAC – study group; C – control group.

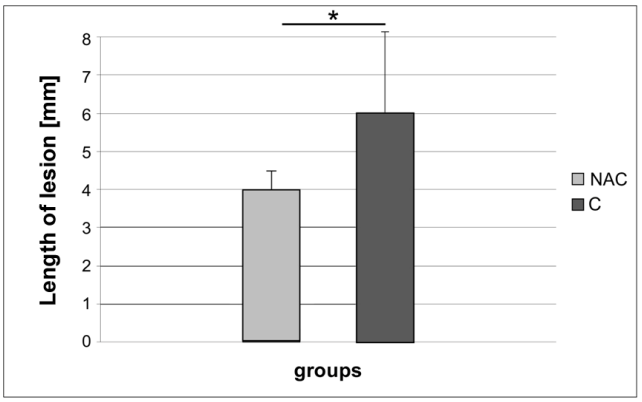


Fig. 5. Measurements of the length of the spinal cord lesion

NAC – study group; C – control group; * indicates statistical significance ($p \leq 0.05$).

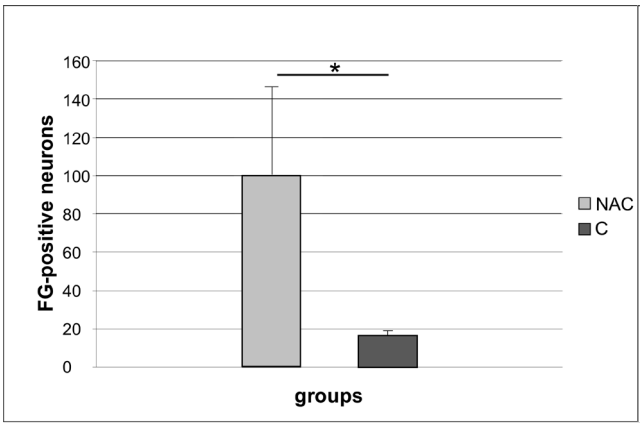


Fig. 6. Results of retrograde neuronal tracing with Fluoro-Gold

NAC – study group; C – control group; * indicates statistical significance ($p \leq 0.05$).

Karalija et al. demonstrated the neuroprotective efficacy of NAC and acetyl-L-carnitine in cases of spinal motoneuron degeneration. They showed that both substances restored the density of dendritic branches and axons in the ventral horn of hemisected rat spinal cords.[22] However, Kaynar et al. reported that a single dose of NAC administered intraperitoneally was ineffective after experimental spinal cord injury with an aneurysm clip in rats.[23]

Naziroglu et al. examined the effects of NAC and selenium on apoptosis and oxidative stress in the hippocampus of rats following brain injury. NAC and selenium were administered intraperitoneally in 32 rats. The authors observed that both substances had a protective effect against oxidative stress and apoptosis in the hippocampus, and that the effect of NAC was greater than that of selenium.[14]

Some authors have shown that N-acetylcysteine has favorable effects on brain ischemia and ischemia/reperfusion injury. Khan et al. investigated the neuroprotective potential of NAC administered intraperitoneally (150 mg/kg) in rats with temporary focal cerebral ischemia immediately and 6 h after the reperfusion. They observed a significant reduction in the infarct area and volume, and also an improvement in neurologic scores. Their results showed that NAC protected against free oxygen radical injury, apoptosis, and inflammation.[24] However, Thomale et al. found that after brain contusion, NAC was not effective at reducing the area of injury and did not influence post-traumatic brain edema formation.[25]

Cakir et al. investigated the effect of NAC on spinal cord ischemia-reperfusion injury in rabbits. Ischemia was induced by clamping the aorta both below the left renal artery and above the aortic bifurcation. They observed that administering 50 mg/kg of NAC resulted in significant reduction of motor dysfunction, and that a combination of hypothermia and NAC led to complete recovery of motor function in the animals. The authors stated that NAC and hypothermia following ischemia and reperfusion of the spinal cord protected against spinal cord injury.[26]

Hicdonmez et al. assessed the effects of a single dose of NAC on tissue malondialdehyde (MDA), superoxide dismutase (SOD), glutathione peroxidase and catalase activity in rats subjected to experimental closed head trauma. They reported favorable effects of NAC treatment on the oxidative brain tissue injury induced by the trauma.[27] However, in a study on rats with moderate left focal cortical contusion trauma, Thomale et al. found that 163 mg/kg of NAC applied intraperitoneally 2–4 h after the brain injury was ineffective against post-traumatic perfusion, brain edema or contusion volume.[28]

Cuzzocrea et al. investigated the effect of NAC on brain ischemic injury in gerbils. Ischemia-reperfusion injury was induced in Mongolian gerbils by a single bilateral occlusion of the common carotid arteries, and NAC (20 mg/kg) was given intraperitoneally 30 min before and 1 h, 2 h, and 6 h after reperfusion. The authors observed a reduction

in brain edema after the injury, and an increase in MDA and myeloperoxidase (MPO) levels in the hippocampus. They showed that NAC administration increased survival and reduced the hyperactivity of neurons associated with post-traumatic neurodegeneration, and also caused a reduction in neuronal loss in the treated animals.[29]

In the present study, the neuroprotective activity of NAC had limited positive influence on the regeneration of isolated spinal cord injuries in rats. This may be due to the relatively low permeability of NAC through the blood-brain barrier. The ability of NAC to cross the blood-brain barrier is questionable and could be dependent on the dose and schedule of administration.[30]

Further confirmation of the neuroprotective efficacy of NAC in spinal cord injury is needed. It is important to establish the optimal dose of this compound, especially in comparison to other substances with antioxidant properties previously studied in animal models.

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# Transcriptomic analysis of the PI3K/Akt signaling pathway reveals the dual role of the c-Jun oncogene in cytotoxicity and the development of resistance in HL-60 leukemia cells in response to arsenic trioxide

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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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**Conflict of interest**
None declared

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## Abstract

**Background.** Arsenic trioxide (ATO) is a well-recognized antileukemic drug used for the treatment of newly diagnosed and relapsed acute promyelocytic leukemia (APL). A major drawback of therapy with ATO is the development of APL cell resistance, the mechanisms of which are still not clear.

**Objectives.** The aim of this study was to investigate the role of the PI3K/Akt signaling pathway in ATO-treated human acute myeloid leukemia (HL-60) cells and in ATO-resistant clones.

**Material and methods.** The cytotoxicity of ATO was assessed using Trypan blue staining or a WST-1 reduction assay. The Akt phosphorylation level was measured by immunofluorescent staining and flow cytometry. Gene expression analysis was performed using real-time polymerase chain reaction (PCR).

**Results.** The clones derived by culturing for 8–12 weeks in the presence of 1.75, 2.5, and 5 μM ATO were characterized by high viability but a slower growth rate compared to the parental HL-60 cells. The flow cytometry analysis showed that in the parental cells the levels of p-Akt were undetectable or very low, and that ATO had no effect on the level of p-Akt in either the ATO-treated parental cells or the clones. The gene expression analysis revealed that some of the genes involved in the Akt pathway may play a key role in the induction of resistance to ATO, e.g., genes encoding cyclin D1 (CCND1), fork head box O1 (FOXO1), Jun oncogene (JUN), protein kinase C isoform B1 (PRKCB1), because their expression profiles were predominantly changed in the clones and/or the ATO-treated parental HL-60 cells.

**Conclusions.** The overall results indicate that CCND1, FOXO1, and JUN may contribute to the induction of resistance to ATO, and that the C-Jun N-terminal kinase (JNK) signaling pathway may have greater significance than the phosphoinositide 3-kinase (PI3K)/Akt pathway in mediating the cytotoxic effects of ATO and the development of resistance to ATO in the HL-60 cell line.

**Key words:** Akt kinase, C-Jun, arsenic trioxide, HL-60 cells, arsenic resistant clones

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Arsenic trioxide (ATO) is a widely recognized antileukemic drug used for the treatment of newly diagnosed and relapsed acute promyelocytic leukemia (APL).[1] ATO effectively induces the differentiation and apoptosis of APL cells.[2,3] A major drawback of therapy with ATO is the development of APL cell resistance, the mechanisms of which are still not clear.Some data suggests that mutations in the B2 domain of the *PML/RARA* fusion gene and a mutation in the *PML* gene that was not rearranged play an important role in the development of ATO resistance.[4,5] Signal transduction studies indicate that the phosphoinositide 3-kinase (PI3K)/Akt pathway contributes to the development of resistance. Tabellini et al. reported high levels of the phosphorylated form of Akt in the leukemic NB4 clones resistant to ATO.[6] This observation was concordant with many studies confirming the important role of the Akt kinase in promoting cell survival and malignant transformation through diverse mechanisms.[7] Additionally, the PI3K/Akt pathway has been shown to be modulated by ATO in various cancer cells originating from both solid tumors and leukemia, e.g., in acute lymphoblastic leukemia cells, B-cell chronic leukemia cells, and acute promyelocytic leukemia cells.[8–11]

In a previous study, the current authors identified several target genes involved in the PI3K/Akt pathway that might mediate ATO cytotoxic effects and/or the resistance to the drug in Jurkat cells derived from human acute lymphoblastic leukemia.[12] The present study examines the effect of ATO on the growth of HL-60 cells originating from APL, but lacking the typical t(15;17) chromosomal translocation, and characterizes the contribution of the PI3K/Akt signaling pathway in the development of ATO resistance in the cells.

# Material and methods

## Chemicals and reagents

Arsenic trioxide was purchased from Sigma-Aldrich Co. (St. Louis, USA; PubChem CID: 24852110). Stock solutions of ATO prepared in 1M NaOH were diluted in Dulbecco's Phosphate Buffered Saline (PBS, Sigma-Aldrich Co., St. Louis, USA) to a concentration of 10 mM, aliquoted and stored at 4–8° C. Cell Proliferation Reagent WST-1 and 0.4% solution of Trypan blue were purchased from Roche Diagnostics GmbH (Mannheim, Germany) and Sigma-Aldrich Co. (St. Louis, USA), respectively.

## Cell culture

The HL-60 (human acute myeloid leukemia; #ACC 3) cell line was obtained from the German Collection of Microorganisms and Cell Cultures (DSMZ) and maintained in cRPMI (RPMI 1640 medium with Gibco® GlutaMAX™, Thermo Fisher Scientific, Inc., Waltham, USA), supplemented with Gibco® 10% heat-inactivated fetal bovine serum (Thermo Fisher Scientific, Inc., Waltham, USA), 100 U/mL penicillin and 100 µg/mL streptomycin (Sigma-Aldrich Co., St. Louis, USA). The cell line was screened for Mycoplasma sp. infection using MycoProbe Mycoplasma Detection Kit (R&D Systems, Minneapolis, USA).

## Establishing ATO-resistant clones from HL-60 cells

HL-60 cells were cultured in the presence of ATO at selected concentrations of 1.75, 2.5 or 5 µM until resistance developed. Every 3–4 days of the culture, the cells were collected, centrifuged, and re-suspended in fresh culture medium with ATO added.

Because the developing clones grew slowly, particularly at the higher ATO concentrations, a higher initial density of HL-60 cells was used for the development of ATO resistance than is normally used for parental cells (i.e., $2 \times 10^5$ cells/mL as opposed to $0.5–1 \times 10^5$ cells/mL). The cells were changed every 3–4 days to maintain a density of $2–8 \times 10^5$ cells/mL. During the initial steps, the exposure to ATO was discontinued temporally when the Trypan blue exclusion test showed that the viability of the cells decreased below 10% (often observed at the highest concentration of ATO), and restarted when the viability attained over 80%. Usually, the cells became resistant to ATO within 8–12 weeks, which was reflected by their high viability (>85%).

## WST-1 reduction assay

In brief, HL-60 cells were grown in 96-well microplates ($1.5 \times 10^3$ cells/well) and exposed continuously to the test compounds for 72 h. Afterwards, 10 µL WST-1 reagent was added to all the wells for 1.5 h. The optical density (OD) of the formazan product was measured with a Multiscan RC spectrophotometer (Labsystems Diagnostics Oy, Helsinki, Finland) at 450 nm with a reference filter of 620 nm. The results were expressed as percent of cell survival, i.e., the ratio of the OD of exposed vs the OD of non-exposed control cells.

## Measurements of Akt phosphorylation level by flow cytometry

The preparation of the cells and the analysis were performed following the protocol described in a previous publication.[12] In brief, for the immunofluorescent staining, rabbit monoclonal antibodies were used (anti-phospho-Akt (Ser473) and anti-Akt from Cell Signaling Technology, Inc., Danvers, USA). Alexa Fluor 488-labeled anti-rabbit IgG (Cell Signaling Technology, Inc., Danvers, USA) was used as a secondary antibody. A flow cytometry analysis was performed using FACS Canto II (BD Biosciences, San Jose, USA). Data analysis (~10,000 cells

analyzed) was performed using WinMDI v. 2.8 software. The level of activated Akt kinase (phospho-Akt or p-Akt) normalized to the level of total Akt kinase was calculated using the geometric mean intensity of fluorescence (MIF) according to the formula:

$$\text{p-Akt/Akt} = \frac{\text{MIF of cells stained with p-Akt - MIF of isotype control cells}}{\text{MIF of cells stained with Akt - MIF of isotype control cells}}$$

## Real-time PCR analysis of gene expression

The preparation of the cells and the analysis were performed following the protocol described in a previous publication.[12] In brief, HL-60 cells and ATO-resistant clones ($1.2 \times 10^6$ cells/6 mL) were treated with ATO for 16 h. The real-time PCR (RT-PCR) analysis was performed in 2 steps: 1) screening of genes involved in the PI3K/Akt pathway, using the RT Profiler PCR Array Human PI3K-AKT Signaling Pathway (SABiosciences, Germantown, USA); and 2) the analysis of selected genes using iQ™ SYBR Green SuperMix (Bio-Rad, Hercules, USA) and the primers presented in Table 1. The Ct values for each test gene were normalized using 3 housekeeping genes (*ACTB*, *B2M*, *GAPDH*; Table 1). For the 2nd stage of the study, genes were selected based on ≥1.7-fold up-regulation or ≤0.6-fold down-regulation compared to untreated cells. The results for gene expression are shown as geometric mean ±geometric standard error.

## Statistical analysis

Bartlett's test of homogeneity of variances was used to determine if the results had equivalent variances at the $p < 0.05$ level. The results were compared using a standard one-way analysis of variance (ANOVA) with Tukey's test for post-hoc comparisons. All the calculations, including doubling time, were performed using GraphPad Prism 6.01 software (GraphPad Software Inc., San Diego, USA).

# Results

## Characterization of the HL-60 derived ATO-resistant clones

ATO-resistant clones were generated from HL-60 cells after 8–12 weeks of continuous exposure to ATO. All the clones, in contrast to the parental cells, showed a high viability (>80%) when cultured in the presence of ATO (Fig. 1). However, the growth rate of the clones was considerably lower than that of the parental cells. For example, the doubling time of HAs5 (the clone resistant to the highest ATO concentration of 5 μM) was more than twice as long as that calculated for the parental HL-60 cells (66 h vs 25 h, respectively).

## Levels of Akt kinase in HL-60 cells exposed to ATO and in ATO-resistant clones

In the flow cytometry analysis, the parental HL-60 cells showed constitutively no or very little p-Akt immunostaining. After 4 h of exposure, ATO had no effect on the p-Akt levels, either in the parental cells or in the ATO-resistant clones (Fig. 2).

**Table 1.** The official symbols and names of the test genes and the primer sequences used for real-time PCR in the 2nd stage of the study (the confirmation analysis of selected genes)

| Accession No. | Gene symbol | Gene name | Sequence 5′ → 3′ of forward primer<br>Sequence 5′ → 3′ of reverse primer |
|---|---|---|---|
| NM_000061 | *BTK* | bruton agammaglobulinemia tyrosine kinase | 5′-TGCAAGGATGTCTGTGAAGC-3′<br>5′-GGACAGGCCGAAATCAGATA-3′ |
| NM_053056 | *CCND1* | cyclin D1 | 5′- ACAAGCTCAAGTGGAACCTG-3′<br>5′-ATCTGTTTGTTCTCCTCCGC-3′ |
| NM_002015 | *FOXO1* | forkhead box O1 | 5′-ATGGACAACAACAGTAAATT-3′<br>5′-CCAGTTATCAAAGTCATCAT-3′ |
| NM_005311 | *GRB10* | growth factor receptor-bound protein 10 | 5′-GTCAAATGGCAGTCAAACCC-3′<br>5′-TCCATGATTTCTTTCCCAGC-3′ |
| NM_002228 | *JUN* | jun oncogene | 5′-CCCCAAGATCCTGAAACAGA-3′<br>5′-CCGTTGCTGGACTGGATTAT-3′ |
| NM_002738 | *PRKCB* | protein kinase C, beta 1 | 5′-TGAAGGGGAGGATGAAGATG-3′<br>5′-GTGTTTGGTCATCAGCCCTT-3′ |
| Housekeeping genes | | | |
| NM_001101 | *ACTB* | actin beta | 5′-TGACTGACTACCTCATGAAGATCC-3′<br>5′-CCATCTCTTGCTCGAAGTCC-3′ |
| NM_004048 | *B2M* | beta-2-microglobulin | 5′-TGCTGTCTCCATGTTTGATGTATCT-3′<br>5′-TCTCTGCTCCCCACCTCTAAGT-3′ |
| NM_002046 | *GAPDH* | glyceraldehyde-3-phosphate dehydrogenase | 5′-GACCTGCCGTCTAGAAAAACC-3′<br>5′-GTTGAAGTCAGAGGAGACCACC-3′ |

**Table 2.** Gene expression changes in HL-60 cells exposed to 2.5 μM ATO for 16 h and in the clone resistant to the same 2.5 μM ATO concentration (H.As2.5) – the 1st screening of genes

| Gene symbol | HL-60 cells exposed to 2.5 μM ATO | H.As2.5 clone resistant to 2.5 μM ATO |
|---|---|---|
| *ADAR* | 1.4 | 1.4 |
| *AKT1* | 1.5 | 1.4 |
| *AKT2* | 1.4 | 1.6 |
| *APC* | 1.6 | 1.3 |
| *BAD* | 1.3 | 1.4 |
| *BTK* | 0.9 | 2.1 |
| *CASP9* | 1.0 | 1.3 |
| *CCND1* | 2.4 | 0.2 |
| *CD14* | 0.9 | 0.7 |
| *CDC42* | 1.1 | 1.2 |
| *CDKN1B* | 1.0 | 0.8 |
| *CHUK* | 1.1 | 1.1 |
| *CSNK2A1* | 0.8 | 1.1 |
| *CTNNB1* | 1.1 | 1.0 |
| *EIF2AK2* | 0.9 | 1.1 |
| *EIF4E* | 0.9 | 1.0 |
| *EIF4EBP1* | 0.7 | 0.9 |
| *ELK1* | 1.0 | 1.0 |
| *FASLG* | 0.9 | 1.8 |
| *FKBP1A* | 0.6 | 0.9 |
| *FOS* | 0.7 | 1.4 |
| *FOXO1* | 2.0 | 0.6 |
| *FOXO3* | 1.0 | 1.3 |
| *FRAP1* | 0.7 | 1.2 |
| *GRB10* | 0.5 | 0.9 |
| *GRB2* | 0.9 | 1.5 |
| *HSPB1* | 1.0 | 1.2 |
| *IGF1* | 1.2 | 0.9 |
| *IGF1R* | 0.6 | 0.7 |
| *IRAK1* | 0.8 | 1.1 |
| *IRS1* | 1.2 | 0.6 |
| *ITGB1* | 1.0 | 1.0 |
| *JUN* | 0.8 | 0.4 |
| *MAP2K1* | 1.2 | 1.2 |
| *MAPK1* | 0.9 | 1.2 |
| *MAPK14* | 1.0 | 1.2 |
| *MAPK3* | 1.0 | 1.5 |
| *MAPK8* | 1.0 | 1.0 |
| *MYD88* | 1.1 | 1.2 |
| *NFKBIA* | 0.9 | 0.9 |
| *PABPC1* | 0.7 | 1.3 |
| *PAK1* | 1.0 | 0.8 |
| *PDGFRA* | 0.8 | 0.9 |
| *PDK1* | 0.8 | 1.0 |
| *PDPK1* | 0.9 | 1.2 |
| *PRKCA* | 1.0 | 1.1 |
| *PRKCB1* | 2.9 | 2.7 |
| *PRKCZ* | 1.1 | 1.0 |
| *PTEN* | 1.0 | 1.0 |
| *PTPN11* | 0.8 | 0.7 |
| *RAC1* | 0.6 | 0.8 |
| *RASA1* | 0.7 | 1.0 |
| *RBL2* | 1.3 | 1.2 |
| *RHOA* | 0.7 | 0.9 |
| *RPS6KA1* | 1.0 | 1.6 |
| *RPS6KB1* | 0.9 | 1.3 |
| *SHC1* | 1.2 | 0.9 |
| *SOS1* | 0.8 | 0.7 |
| *SRF* | 0.7 | 0.9 |
| *TIRAP* | 1.4 | 1.4 |
| *TLR4* | 1.1 | 0.7 |
| *TOLLIP* | 0.9 | 0.8 |
| *TSC1* | 1.2 | 1.4 |
| *TSC2* | 0.8 | 1.2 |
| *WASL* | 0.7 | 1.1 |
| *YWHAH* | 0.8 | 0.8 |

The results are presented as the $2^{-ddCt}$ values from 1 experiment; light gray shadow – mean values ≤0.5; dark gray shadow – mean values ≥2.

## Expression of genes involved in the PI3K/Akt signaling pathway in ATO-treated HL-60 cells and in the corresponding ATO-resistant clones

Based on the expression analysis of 66 genes involved in the PI3K/Akt signaling pathway in the parental cells exposed for 16 h to 2.5 μM ATO and in the corresponding resistant clone (HAs2.5), 6 genes whose expression was changed compared to non-exposed HL-60 cells were selected: *BTK, CCDN1, FOXO1, GRB10, JUN* and *PRKCB1* (Table 2).

In the subsequent analysis, these 6 genes were verified in the parental HL-60 cells exposed for 16 h to 1.75 μM or 5 μM ATO, and in the clone resistant to 1.75 μM ATO (HAs1.75). The results of the analysis (Table 3) indicated that the expression of *FOXO1* was up-regulated in a concentration-dependent manner in the ATO-treated parental cells, and down-regulated at least 2-fold ($2 \geq 2^{-\Delta\Delta Ct} \leq 0.5$, where Ct is the threshold cycle; according to the delta-delta Ct method) in the ATO-resistant cells. Strong down-regulation of *CCND1* and *JUN* was shown only in the ATO-resistant cells. In the ATO-exposed

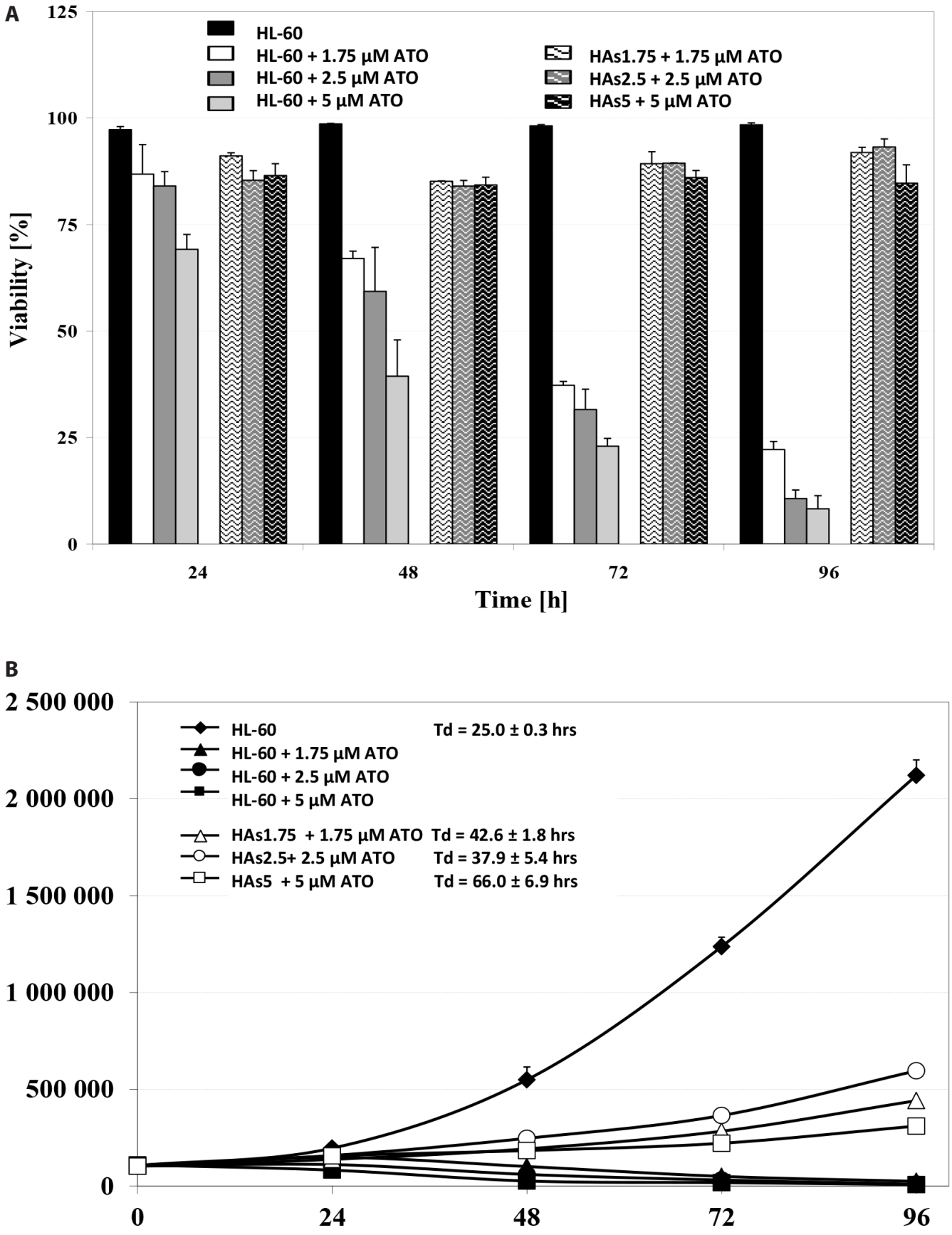


**Fig. 1.** The viability and growth of HL-60 cells exposed to the indicated concentrations of ATO

The viability (A) and number (B) of the parental HL-60 cells and THE ATO-resistant clones derived from this cell line (HAs1.75, HAs2.5 and HAs5) were determined using the Trypan blue exclusion test after 96 h of incubation with or without ATO. Based on the results doubling time (Td) was calculated for untreated HL-60 cells as well as their ATO-resistant clones.

HL-60 parental cells, the expression of *CCND1* and *JUN* was increased only after treatment with the highest concentration of the drug (5 μM), which was in contrast to concentration-dependent decreased expression observed in the resistant clones. The expression of *PRKCB1* was strongly up-regulated both in the ATO-treated parental cells and the ATO-resistant clones. *BTK* and *GRB10* showed a tendency toward increased expression in the ATO-resistant clones, but the increase was less than 2-fold.

## Discussion

A previous study by the current authors identified several genes involved in the PI3K/Akt pathway associated with ATO cytotoxic effects and/or the resistance to the drug in Jurkat cells.[12] This concurred with other data indicating that dysregulation of PI3K/Akt signaling pathway may be the reason for apoptosis resistance in leukemic cells.[6,8,13–15] In the present study, the focus was on the effect of ATO on the PI3K/Akt signaling pathway in HL-60 acute promyelocytic leukemia cells, which – similarly to NB-4 cells but in contrast to other leukemic cell lines (e.g., Jurkat, CEM, and MOLT-4) – do not display phosphorylated (activated) Akt (p-Akt).[6,12]

The present study has shown that prolonged incubation of HL-60 cells in the presence of clinically relevant ATO concentrations of 1–5 μM induced resistance to the drug.[16,17] In contrast to the NB-4-derived ATO-resistant clones, the HL-60-derived clones in the present study did not display elevated levels of p-Akt.[6] Similarly, a short (4-hour) exposure of the parental HL-60 cells to ATO did not induce any detectable changes in the level of activated Akt. This contrasts with the findings of Choi et al., who observed a decrease in p-Akt levels associated with apoptosis induction in ATO-treated U937 cells.[18]

The present study identified 4 gene targets for ATO that might mediate its cytotoxic effect and the development of ATO resistance in HL-60 cells, i.e., *CCND1, FOXO1, JUN*, and *PRKCB1*. The expression of these genes changed at least 2-fold after 16 h of exposure to clinically relevant ATO concentrations, but only *PRKCB1* (the beta isoform of protein kinase C, or PKC beta) was up-reg-

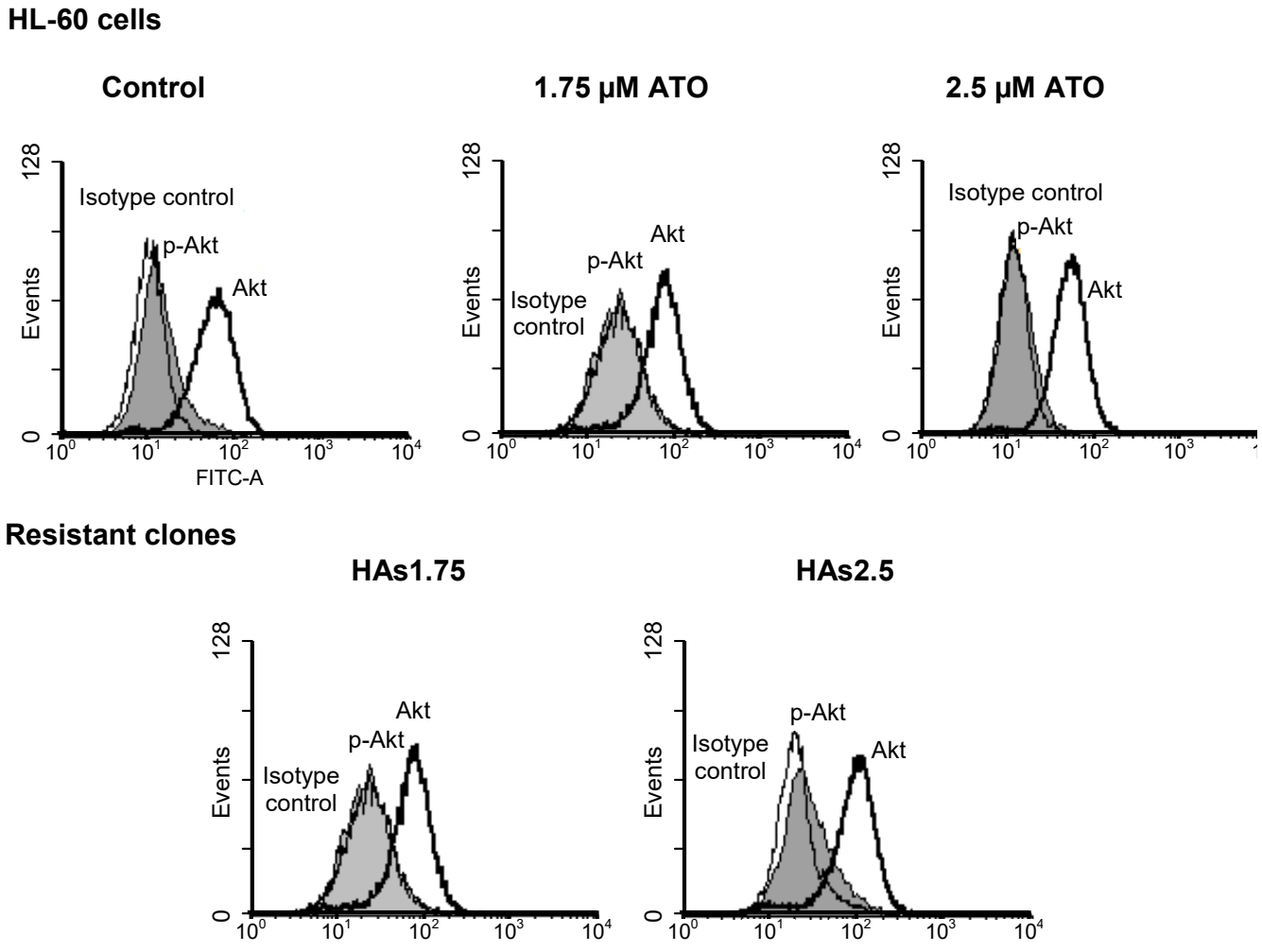


**Fig. 2.** The effect of ATO on Akt kinase in parental cells and HAs1.75 and HAs2.5 clones

The graph presents the mean level of p-Akt kinase normalized to the value of the total Akt kinase level (p-Akt/Akt) ±SD (representative histograms for 2–3 independent experiments).

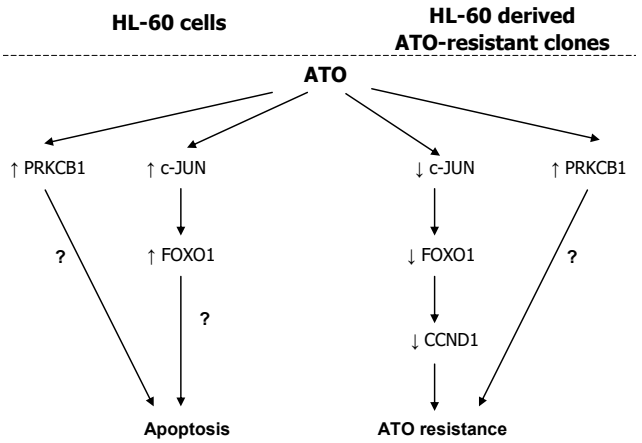


Fig. 3. The possible signaling pathways activated in HL-60 cells in response to arsenic trioxide (ATO)

ulated in both ATO-treated and ATO-resistant cells. Although there exists data that demonstrates the role of PKC beta in the induction of apoptosis, the results of the present study show that the role of this kinase is far-reaching and cell-dependent.[19] The results in HL-60 cells implicate elevated levels of the kinase in the development of ATO resistance, while the authors' previous work showed that in Jurkat cells down-regulation of *PRKCB1* expression was responsible for the development of ATO resistance.[12] Other data links PKC beta with increased invasion and proliferation of cancer cells.[20]

*JUN*, the gene encoding the c-Jun oncogene, seems to play a dual role in cell response to ATO. In the present study, it was up-regulated in ATO-treated HL-60 cells and down-regulated in ATO-resistant clones. Elevated levels of mRNA for c-Jun leading to cell death were also observed in Jurkat cells in the authors' previous study.[12] In addition, other authors, e.g., Redondo-Munoz et al. and Wu et al., have demonstrated that ATO induced the activation of c-Jun N-terminal kinase (JNK, an upstream activator of c-Jun), leading to Akt inactivation and induction of apoptosis in various cell systems.[9,21] The c-Jun protein, along with other members of the Jun family (JunB and JunD) and Fos family members (c-Fos, FosB, Fra1, and Fra2) form dimerized transcription factors: activator proteins 1 (AP-1). The AP-1 proteins have been implicated in the regulation of many important biological responses, including opposing cellular responses such as cell cycle progression, transformation, differentiation, and apoptosis. The activity of the AP-1 dimers is defined by their composition.[22] In addition, the activity of c-Jun may be regulated at both the transcriptional level (changes in *JUN* expression) and post-transcriptional level (phosphorylation of c-Jun protein mediated by JNK). The molecular mechanism by which c-Jun triggers apoptosis remains unclear. It is speculated that c-Jun may activate cell death by acting as a transcriptional regulator leading to changes in the ratio of death-inhibiting vs death-enhancing Bcl-2 proteins.[23] On the other hand, c-Jun may also activate members of the IL-1 beta converting enzyme (ICE)-related proteases, known to be overexpressed in apoptotic cells and required in Fas- or TNFR1-mediated apoptosis.[24]

The role of c-Jun in promoting cell growth has been well documented in several studies showing that it participates in at least 2 signaling pathways.[25] The c-Jun proteins may activate cyclin D1 transcription, leading to a progression from G1 to S phase, or may be involved in the negative regulation of p53 transcription and its downstream target, the cyclin-dependent kinase inhibitor 1A (p21). In the present study, the proliferation of ATO-resistant clones was accompanied by strong down-regulation of *JUN*; in addition, changes in *CCND1* (the gene encoding cyclin D1) expression seemed to be c-Jun-dependent. ATO up-regulated the level of *CCND1* mRNA (although to a much lower degree than *JUN*) in HL-60 cells exposed to the highest ATO concentration, and strongly down-regulated it in ATO-resistant clones. Since cyclin D1 is an important regulator of G1/S phase transition, and has been reported to be a direct target of c-Jun transcriptional activation, this observation suggests an important role of cyclin D1 in the development of resistance to ATO. The role, however, is not clear at the moment. The results of the current authors' previous study, in which Jurkat cells showed a strongly elevated level of mRNA for cyclin D1 in the clones resistant to higher ATO concentrations (2.5–5 μM), suggest that different cell type-dependent mechanisms may be induced in the development of resistance to ATO.[12]

In the present study, the transcription of *FOXO1* (the gene encoding forkhead box O1) showed strong up-regulation in ATO-treated HL-60 cells and strong down-regulation in HL-60-derived ATO-resistant clones. FOXO proteins are activators of transcription. They interact with the DNA binding to the insulin response sequence (IRS) that was identified in promoters of genes encoding important proteins involved in the regulation of apoptosis (e.g., Fas ligand, Bim, Bcl-XL) and cell cycle progression (e.g., cyclin-dependent kinase inhibitor 1B, also referred to as p27Kip1, cyclin D1).[26] The involvement of proteins belonging to the FOXO family in apoptosis induction has been clearly demonstrated by others.[27] In addition, FOXO proteins can be regulated at the post-transcriptional level through phosphorylation in 2 signaling pathways, leading to opposite effects. Akt-mediated phosphorylation has been shown to inactivate FOXO proteins, in contrast to c-Jun N-terminal kinase (JNK)-mediated phosphorylation, which activates FOXO.[28] In the present study, the strong up-regulation of *FOXO1* and *JUN* expression detected in ATO-treated HL-60 cells suggests that the JNK pathway might play a role in the induction of apoptosis in these cells. The observation that prolonged exposure to ATO led to down-regulation of *FOXO* in HL-60-derived clones is also noteworthy, although no Akt kinase activation was detected in ATO resistant cells. These results

are consistent with the data published by Grabiec et al., who observed the Akt-independent, but JNK-dependent, reduction of FOXO1 mRNA that was required for the survival of fibroblast-like synoviocytes in rheumatoid arthritis.[29]

This study demonstrated that prolonged incubation of HL-60 cells in the presence of ATO at clinically relevant concentrations induced resistance to the drug. Apparently, this effect was not dependent on Akt activation. Although ATO modified the expression of some genes involved in the PI3K/Akt signaling pathway in both parental HL-60 cells and resistant clones (Fig. 3), the results suggest that the JNK signaling pathway has a stronger impact on the induction of cytotoxicity and resistance to ATO in HL-60 cells than the PI3K/Akt signaling pathway.

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# Higher concentrations of osteoprotegerin in type 1 diabetic patients are related to retinopathy: Results from the Poznań Prospective Study

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## Abstract

**Background.** Osteoprotegerin (OPG) is an arterial calcification marker which has been associated with vascular damage. Elevated OPG concentrations associated with low-grade inflammatory processes are found in diabetic subjects.

**Objectives.** The aim of the study was to assess concentrations of OPG in relation to the presence of diabetic complications in patients with diabetes type 1 (DM 1) participating in the Poznań Prospective Study (PoProStu).

**Material and methods.** The study included 74 patients with DM1 (48 men) with a median age of 39 years (interquartile range [IQR]: 34–43) and a median 15-year history (IQR: 14–16) of diabetes, who were participants in the PoProStu. Serum OPG concentration was measured using the ELISA method, and serum concentration of C-reactive protein was measured with a high sensitivity test (hsCRP). The visceral adipose index (VAI) was used to determine indirect markers of insulin resistance (IR). The prevalence of microangiopathic diabetes complications was assessed.

**Results.** Retinopathy was diagnosed in 28 patients (38%), diabetic kidney disease (DKD) in 28 (38%) patients, and neuropathy in 17 (23%) patients. The median OPG level was 43.8 (28.0–74.0) pg/mL. Patients with retinopathy had higher levels of OPG than those without retinopathy: 47.5 (35.0–88.0) vs 35.4 (24.7–69.4) pg/mL ($p = 0.04$). Positive correlations were observed between OPG concentration and hsCRP ($Rs = 0.53$; $p < 0.001$), HbA1c level ($Rs = 0.36$; $p = 0.002$), VAI ($Rs = 0.23$; $p = 0.04$) and waist circumference ($Rs = 0.24$; $p = 0.04$).

**Conclusions.** Higher concentrations of osteoprotegerin in DM1 patients are related to the presence of retinopathy. The study results indicate that OPG might play a role in the pathogenesis of vascular complications in association with hyperglycemia and low-grade inflammatory processes.

**Key words:** inflammation, hyperglycemia, osteoprotegerin, diabetic angiopathies, diabetes mellitus type 1

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Osteoprotegerin (OPG) is a glycoprotein originally described as an inhibitor of bone resorption.[1] It is a member of the tumor necrosis factor receptor superfamily, and it binds to receptor activator of nuclear factor κB ligand (RANKL) and TNF-related apoptosis-inducing ligand (TRAIL). The effects of OPG prevent RANK-mediated kappa B activation, thus influencing bone metabolism and immune responses, and inhibiting TRAIL-related apoptosis.[2] Studies have linked higher levels of OPG with cardiovascular disease (CVD) and arterial calcification.[3] OPG has been well described as a biomarker for endothelial dysfunction and a predictor of CVD in the general population, in obese subjects and in type 2 diabetic (DM 2) patients.[4–6] Chronic diabetes complications are still a significant cause of morbidity and mortality in DM 1 patients.[7] There is good evidence that hyperglycemia and low-grade inflammatory processes play an important role in the pathogenesis of the vascular complications of diabetes.[8] Recent large studies have also reported OPG as an independent predictor of cardiovascular complications in a large cohort of patients with DM 1.[9]

Knowledge of the potential role of OPG in diabetic microangiopathy is limited. Knudsen et al. found increased levels of OPG in the plasma of DM 2 patients with albuminuria.[10] Higher plasma OPG concentrations were also observed in subjects with peripheral neuropathy in a study by Nybo et al.[11] However, this association was statistically significant only in patients with DM 2. Therefore, it would be valuable to determine whether serum OPG level is related to microangiopthy in DM 1 subjects. Potentially, OPG could be a biomarker to identify patients with diabetes and microvascular complications.

The aim of this study was to assess the concentrations of OPG in DM 1 patients participating in the Poznań Prospective Study in relation to the presence of microangiopathy.

# Material and methods

## Study design

The Poznań Prospective Study (PoProStu) is a prospective observational study of patients with DM1 uniformly treated with intensive functional insulin therapy (IFIT) from the onset of the disease. The PoProStu enrolled 100 consecutive patients with newly diagnosed DM 1, aged below 35 years, hospitalized due to diabetic ketoacidosis at the Department of Internal Medicine and Diabetology at the Poznan University of Medical Sciences (Poland) from 1994 to 1999, as described by Grzelka et al.[12] From the onset of the disease, all the patients have been treated with IFIT, defined as a treatment method requiring multiple insulin injections during the day, counting carbohydrate equivalents, and considering upcoming physical activity. Annual follow-ups were conducted. Of the original cohort, 26 patients were lost to follow-up; consequently, 74 cases were examined. The data presented in this paper – anthropometric measurements, laboratory results, and microangiopathic complication status – was derived from follow-ups conducted from October to December 2012. The 74 DM 1 patients included in this cross-sectional analysis comprised 26 women and 48 men, with a median age of 39 years (interquartile range [IQR]: 34–43 years) and a median 15-year (IQR: 14–16) history of diabetes. All the subjects were informed of the aim of the study and gave their consent. The study was approved by the local Ethics Committee. The study is registered in the ClinicalTrials.gov database (NCT01411033). Table 1 shows the relevant clinical and demographic characteristics of the group.

Table 1. Clinical characteristics of the study group

| Characteristic | Study group |
|---|---|
| No. of patients | 74 |
| Sex (M/F) | 48/26 |
| Age (years) | 39 (34–43) |
| Disease duration (years) | 15 (14–16) |
| Smoking n (%) | 22 (30) |
| Hypertension n (%) | 29 (39) |
| SBP (mm Hg) | 131 (120–142) |
| DBP (mm Hg) | 83 (77–90) |
| BMI (kg/m$^2$) | 24.7 (22.5–27.7) |
| Waist circumference (cm) | 87.3 (82.5–96.0) |
| WHR | 0.86 (0.82–0.93) |
| Daily insulin dose (U/kg/d) | 45.5 (36.0–52.0) |
| VAI | 1.86 (1.50–2.90) |
| HbA1c (%) | 7.7 (7.1–8.7) |
| Total cholesterol (mmol/L) | 5.2 (4.5–5.8) |
| LDL cholesterol (mmol/L) | 3.1 (2.3–3.8) |
| HDL cholesterol (mmol/L) | 1.7 (1.4–2.0) |
| Triglicerydes (mmol/L) | 0.97 (0.7–1.3) |
| eGFR (mL/min/1,73 m$^2$) | 90.9 (85.1–109.6) |
| hsCRP (mg/L) | 1.12 (0.62–2.70) |
| Retinopathy n (%) | 28 (38) |
| Diabetic kidney disease n (%) | 28 (38) |
| Neuropathy n (%) | 17 (23) |
| Any microangiopathy n (%) | 38 (51) |
| Macroangiopathy n (%) | 3 (4) |

Data shown as median (IQR); n (%) – number (%) of patients; SBP – systolic blood pressure; DBP – diastolic blood pressure; BMI – body mass index; WHR – waist-to-hip ratio; eGDR – estimated glucose disposal rate; VAI – visceral adipose index; LDL – low-density lipoprotein; HDL – high-density lipoprotein; eGFR – estimated glomerular filtration rate; hsCRP – high sensivity C-reactive protein.

## Data collection procedures

All the patients underwent a complete physical examination with anthropometric measurements and a blood pressure check. Blood pressure was measured twice

by the Korotkov method in a sitting position, after 10 min at rest, using a mercury manometer. Arterial hypertension was diagnosed if the mean blood pressure was more than 140/90 mm Hg or if the patient had had arterial hypertension diagnosed previously and had received appropriate treatment. Body mass index (BMI) was calculated from the following equation: BMI = weight (kg)/squared height ($m^2$). Visceral adiposity index (VAI) was assessed using the following formula: for women VAI = [waist circumference/36.58 + (1.89 × BMI)] × (TG/0.81) × (1.52/HDL); for men VAI = [waist circumference/39.68 + (1.88 × BMI)] × (TG/1.03) × (1.31/HDL).

Blood samples were collected in a fasting state. Glycated hemoglobin (HbA1c) was measured using high performance liquid chromatography with the VARIANT Haemoglobin A1c Program (Bio-Rad Laboratories, Hercules, USA). Serum C-reactive protein (hsCRP) concentration was assessed by highly sensitive microparticle enzyme turbidimetric immunoassay. Plasma glucose was measured using the hexokinase-mediated reaction. Total cholesterol, high density lipoprotein (HDL) cholesterol, low density lipoprotein (LDL) cholesterol, and triglyceride levels were determined using commercially available assay kits (F. Hoffman-LaRoche AG, Basel, Switzerland). Creatinine levels were measured using the kinetic method. The estimated glomerular filtration rate (eGFR) was calculated using the Modification of Diet in Renal Disease Study formula.

OPG levels were measured using enzyme linked immunosorbent assay kits (eBioscience Human Osteoprotegerin Instant ELISA, Thermo Fisher Scientific, Waltham, USA). OPG presenting in the serum was bound by antibody precoated on the wells. Captured protein was detected by adding a biotinylated antibody followed by streptavidin-horseradish peroxidase (SA-HRP). A substrate of SA-HRP–3,3,5,5'tetramethylbenzidine (TMB) was then added to the wells. The intensity of the color was measured spectrophotometrically at 450 nm, using a Zenyth 200 Microplate Reader (Thistle Scientific, Glasgow, UK), and the concentration of OPG was read off from a standard curve. The detection limit was 2.5 pg/mL, the intra-assay coefficient of variation (CV) was 7%, and the inter-assay CV was 8%.

## Microangiopathic complications at follow-up

Diabetic retinopathy was diagnosed using direct ophthalmoscopy through dilated pupils, followed by fundus photography in all the patients. The fundus examinations were performed using an indirect Volk lens (Volk Optical, Inc., Mentor, USA). Two fundus photographs of each eye were taken with a 45° digital camera (VISUCAM, Zeiss AG, Oberkochen, Germany), 1 centered on the fovea and 1 centered on the optic disc. The evaluations of both photographs were performed by the same ophthalmologist experienced in diabetic retinopathy. Diabetic retinopathy was graded according to the classification of the American Academy of Ophthalmology: no retinopathy or mild nonproliferative retinopathy; moderate nonproliferative retinopathy; severe nonproliferative retinopathy; and proliferative retinopathy.[13] The severity of retinopathy was recognized based on the presence of microaneurysms, retinal hemorrhages and hard exudates. Nonproliferative retinopathy is differentiated based on the presence of retinal bleeding, venous beading, cotton-wool spots or intraretinal microvascular anomalies.

Diabetic kidney disease (DKD) was detected at the stage of albuminuria. Urinary albumin excretion was assessed in a 12 h collection and recalculated for 24 h. Albuminuria was defined as an urinary albumin excretion rate between 30 and 300 mg/24 h in 2 out of 3 samples collected over a 3-month period, after the exclusion of secondary causes of proteinuria (urinary tract infection, heart failure, acute febrile illness, hematuria or excessive physical activity). DKD was defined as the presence of albuminuria in connection with a 10-or-more-year history of diabetes or with diagnosed diabetic retinopathy (KDOQI 2007).[14]

Neuropathy assessment was performed using pressure sensation (10 g monofilament perception), vibration perception (a 128 Hz tuning fork) and ankle reflex tests. Diabetic neuropathy was diagnosed in patients with 2 or more of the 4 following: the presence of typical symptoms of neuropathy, the absence of ankle tendon reflexes, and/or abnormal scores for pressure and/or vibration perception.

Microangiopathy was defined as the presence of at least 1 of the following microvascular complications: retinopathy, DKD or neuropathy. Macroangiopathy was defined as the presence of either coronary heart disease, a known episode of myocardial infarction, stroke or the presence of peripheral artery disease.

## Statistical analysis

The statistical analysis was run using STATISTICA v. 8.0 software (StatSoft, Tulsa, USA). To test for normality, the Kolmogorov-Smirnov test with the Lilliefors correction was used. The results for continuous variables are shown as median values and IQR, and for categorical data the results are shown as numbers and percentages of patients. To compare subgroups, the Mann-Whitney U test was performed. To compare qualitative data, the $\chi^2$ test was used. The Spearman rank correlation method was used to test relationships between OPG and other variables. To determine factors connected with diabetic microangiopathy, univariate logistic regression was used. P-values <0.05 were considered statistically significant.

# Results

Retinopathy was diagnosed in 28 patients (38%), DKD in 28 patients (38%), and neuropathy in 17 patients (23%) (Fig. 1). Macroangiopathy was diagnosed in 3 cases (4%).

The median HbA1c level was 7.7% (7.1–8.7). The median OPG level was 43.8 pg/mL (28.0–74.0).

With regard to the metabolic control assessment in relation to the presence of microvascular complications, the only positive finding was higher triglyceride levels in patients with retinopathy (p = 0.002), DKD (p = 0.02), and when any microangiopathy was considered (p = 0.04) than in patients without these complications. Significantly higher HbA1c levels were found in patients with retinopathy (p < 0.001), DKD (p < 0.001), and neuropathy (p = 0.01) than in patients without these complications (Table 2).

Higher OPG levels were observed in patients with diabetic retinopathy, DKD, peripheral neuropathy, and when any microangiopathy was considered, as compared to subjects without vascular complications (Table 2). However, the results were statistically significant only in relation to retinopathy (47.5 pg/mL [35.0–87.8] vs 35.4 pg/mL [24.7–69.4]; p = 0.04) (Fig. 2).

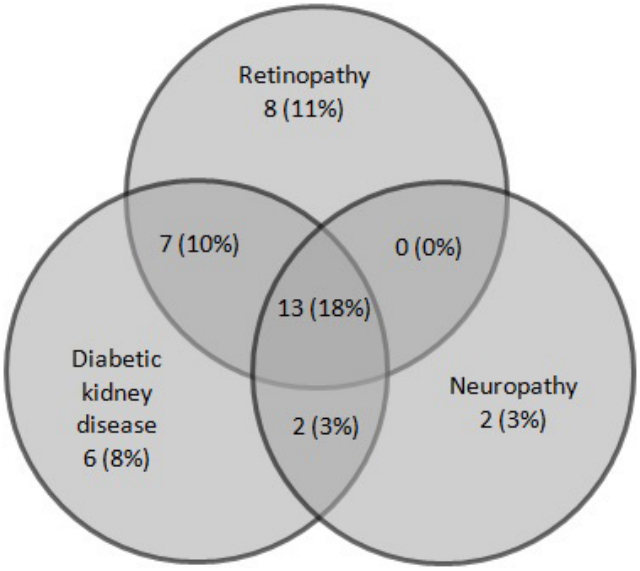


Fig. 1. Microangiopathic complications in the study group

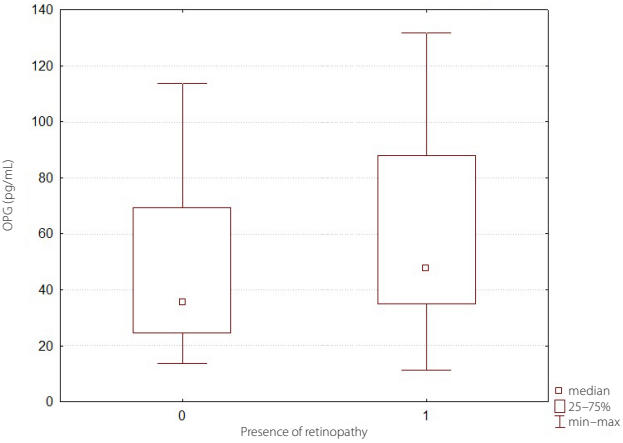


Fig. 2. OPG concentration in relation to the presence of retinopathy

In the univariate logistic regression, OPG levels were significantly associated with retinopathy (OR 1.02, 95% CI 1.01–1.04; p = 0.03). Positive correlations were found between OPG concentration and hsCRP (Rs = 0.53; p < 0.001), HbA1c level (Rs = 0.36; p = 0.002), VAI (Rs = 0.23; p = 0.04) and waist circumference (Rs = 0.24; p = 0.04).

## Discussion

OPG is a bone-related peptide that can be found in different tissues, including bone, heart, and vascular endothelial cells.[11] Hofbauer and Schoppet wrote that endothelial cells activated by pro-inflammatory cytokines might be a potential source of circulating OPG.[15] This mechanism could explain the link between low-grade inflammation and elevated OPG levels in patients with DM 2 and DM 1.[16,17] Recent studies have shown elevated serum OPG concentrations in patients with microangiopathic diabetes complications.[9,11,18,19] Endothelial dysfunction and vascular damage is regarded as possibly being the linking pathway between vascular complications and higher OPG levels.[10] There are few studies exploring the role of OPG in the pathogenesis of microangiopathy in DM 1 patients. In the Finnish Diabetic Nephropathy Study, the authors speculate on the possible role of inflammation in the increased expression of OPG.[9] However, there is no literature concerning this issue among patients treated with IFIT from the onset of the disease.

The present study showed that, among the microangiopathic complications under consideration, it was only in diabetic retinopathy that the OPG concentration was significantly higher than in patients without retinopathy. To the authors' knowledge, this is the first study showing this correlation in patients with DM 1. Yu et al. showed elevated serum and vitreous concentrations of OPG in patients with DM 2 with retinopathy.[18] In another study of patients with DM 2, the authors revealed a positive association between an OPG gene polymorphism and diabetic retinopathy.[20] Therefore, the role of OPG in diabetic complications might be partially explained by genetic factors.

Furthermore, strong associations between OPG, DKD and neuropathy have been described.[9,11] The lack of statistical significance in the results of the present study might be explained by the whole study group having quite good metabolic control of their diabetes. However, in the subgroups with complications, significantly higher levels of HbA1c were observed. Hyperglycemia and the associated activation of inflammatory process appear to be the main mechanisms leading to the production of OPG. The HbA1c values presented were measured during the patients' final follow-up, and were remarkably lower than described in papers by Gordin et al. and Knudsen et al., who did not describe the treatment method.[9,10] The patients in the PoProStu group have been treated

**Table 2.** Clinical characteristics of the patients in relation to the presence of microvascular complications

| Characteristic | No retinopathy | Retinopathy | p-value | No DKD | DKD | p-value | No neuropathy | Neuropathy | p-value | No microangiopathy | Any microangiopathy | p-value |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| N | 46 | 28 | – | 46 | 28 | – | 57 | 17 | – | 36 | 38 | – |
| Sex (M/F) | 26/20 | 22/6 | ns | 26/20 | 22/6 | ns | 33/24 | 15/2 | 0.02 | 20/16 | 28/10 | ns |
| Age (years) | 39.0 (35.0–43.0) | 38.0 (33.5–41.5) | ns | 38.0 (35.0–41.0) | 39.0 (34.0–44.0) | ns | 39.0 (34.0–41.0) | 40.0 (37.0–46.0) | ns | 39.0 (35.0–43.0) | 38.0 (34.0–42.0) | ns |
| Disease duration (years) | 15.0 (14.0–16.0) | 15.0 (14.0–17.0) | ns | 15.0 (14.0–16.0) | 15.0 (14.0–16.5) | ns | 15.0 (14.0–16.0) | 15.0 (14.0–17.0) | ns | 15.0 (14.0–16.0) | 15.0 (14.0–16.0) | ns |
| Smoking n (%) | 9 (20) | 13 (46) | 0.01 | 7 (15) | 15 (54) | <0.001 | 12 (21) | 10 (59) | 0.003 | 6 (17) | 16 (42) | 0.02 |
| Hypertension n (%) | 12 (26) | 17 (61) | 0.003 | 10 (22) | 19 (68) | <0.001 | 16 (28) | 13 (77) | <0.001 | 9 (25) | 20 (53) | 0.02 |
| SBP (mmHg) | 129 (119–138) | 135 (125–152) | ns | 125 (118–137) | 138 (129–154) | 0.002 | 130 (121–139) | 136 (126–153) | ns | 129 (120–139) | 134 (122–147) | ns |
| DBP (mmHg) | 81 (74–89) | 85 (81–93) | 0.04 | 81 (74–89) | 86 (81–94) | ns | 82 (77–89) | 85 (79–93) | ns | 81.5 (76.5–89.7) | 84.7 (79.0–91.3) | ns |
| BMI (kg/m$^2$) | 25.8 (23.0–28.0) | 23.8 (21.9–25.7) | ns | 24.2 (22.7–27.7) | 25.3 (22.2–28.0) | ns | 24.9 (22.7–28.0) | 24.2 (22.4–26.1) | ns | 25.2 (22.8–28.4) | 24.1 (22.2–27.0) | ns |
| Waist circumference (cm) | 88 (81–96) | 87 (83–96) | ns | 88 (79–94) | 92 (86–99) | 0.03 | 87 (81–96) | 88 (85–93) | ns | 87.6 (77.0–96.0) | 87.0 (84.2–96.0) | ns |
| WHR | 0.87 (0.81–0.92) | 0.87 (0.84–0.94) | ns | 0.86 (0.81–0.90) | 0.90 (0.83–0.96) | ns | 0.86 (0.81–0.92) | 0.90 (0.84–0.93) | ns | 0.85 (0.79–0.92) | 0.89 (0.84–0.93) | ns |
| VAI | 1.73 (1.36–2.6) | 1.93 (1.79–3.41) | 0.04 | 1.80 (1.36–2.61) | 2.04 (1.62–3.92) | ns | 1.84 (1.49–2.89) | 1.89 (1.66–2.73) | ns | 1.78 (1.39–2.63) | 1.88 (1.57–3.06) | ns |
| HbA1c (%) | 7.3 (6.8–7.9) | 8.2 (7.7–9.5) | <0.001 | 7.4 (6.9–8.1) | 8.2 (7.6–9.4) | <0.001 | 7.4 (6.9–8.5) | 8.2 (7.7–9.4) | 0.01 | 7.3 (6.9–8.2) | 7.9 (7.3–9.4) | 0.01 |
| Total cholesterol (mmol/L) | 5.19 (4.30–5.83) | 5.23 (4.67–5.80) | ns | 5.15 (4.35–5.83) | 5.28 (4.68–5.93) | ns | 5.28 (4.53–5.91) | 5.08 (4.35–5.39) | ns | 5.32 (4.39–5.94) | 5.15 (4.46–5. 70) | ns |
| LDL cholesterol (mmol/L) | 3.06 (2.20–3.76) | 3.06 (2.72–3.59) | ns | 3.06 (2.31–3.86) | 3.06 (2.70–3.42) | ns | 3.06 (2.36–3.86) | 2.95 (2.20–3.26) | ns | 3.19 (2.31–3.91) | 2.98 (2.60–3.37) | ns |
| HDL cholesterol (mmol/L) | 1.67 (1.40–2.02) | 1.61 (1.41–1.96) | ns | 1.67 (1.43–1.97) | 1.61 (1.35–1.98) | ns | 1.68 (1.43–2.02) | 1.61 (1.40–1.71) | ns | 1.59 (1.37–1.98) | 1.68 (1.43–1.97) | ns |
| Triglicerydes (mmol/L) | 0.91 (0.67–1.05) | 1.12 (0.88–1.43) | 0.002 | 0.94 (0.70–1.09) | 1.15 (0.83–1.76) | 0.02 | 0.97 (0.73–1.21) | 0.98 (0.86–1.40) | ns | 0.91 (0.68–1.08) | 1.04 (0.82–1.40) | 0.04 |
| hsCRP (mg/L) | 1.1 (0.59–1.6) | 1.74 (0.76–3.83) | ns | 1.1 (0.51–2.17) | 1.32 (0.87–5.06) | ns | 1.16 (0.60–2.48) | 1.05 (0.87–3.24) | ns | 1.10 (0.42–2.30) | 1.32 (0.87–3.01) | ns |
| OPG (pg/mL) | 35.4 (24.7–69.4) | 47.5 (35.0–87.8) | 0.04 | 38.0 (26.6–73.6) | 47.5 (33.6–83.4) | 0.15 | 49. 9 (29.8–74.1) | 36.4 (23.8–69.4) | 0.57 | 35.2 (26.3–73.6) | 45.2 (33.6–80.1) | 0.18 |

Data shown as median (IQR); n (%) – number (%) of patients; SBP – systolic blood pressure; DBP – diastolic blood pressure; BMI – body mass index; WHR – waist-to-hip ratio; VAI – visceral adipose index; LDL – low-density lipoprotein; HDL – high-density lipoprotein; hsCRP – high sensivity C-reactive protein; OPG – osteoprotegerin (Mann–Whitney and $\chi^2$ tests).

from the onset of the disease with IFIT, ensuring better metabolic control and thus a reduction in the prevalence of complications.[21]

The current study group's relatively short history of diabetes might be another reason for the relatively low OPG levels found. Higher concentrations of OPG might occur in the more advanced stages of microvascular complications. In studies by Gordin et al. and Nybo et al., a significantly longer history of diabetes results in greater prevalence of microangiopathy.[9,11] Finally, significantly elevated OPG levels were described in the presence of macroangiopathy by Doherty et al.[3] The limited cases of macroangiopathic complications in the present study, even when microangiopathy was diagnosed, might have resulted in the relatively low OPG concentration as compared to the data in the literature.

Low-grade inflammation has been associated with macro- and microvascular complications in DM 1 subjects.[22] Serum hsCRP concentration has been directly proven to be a strong predictor of microangiopathic complications in diabetes.[23–25] It is also a well-known marker for the inflammation response. Yaturu et al. found correlations between OPG and insulin resistance, CRP, TNF-alpha concentrations, and showed elevated OPG in diabetic patients compared to control subjects.[26] The present study found a positive correlation between concentrations of OPG and hsCRP. Pro-inflammatory cytokines might promote OPG production by endothelial cells and thus cause intensification of the calcification process and vascular damage. Recently, it has been shown that OPG expression can be induced by glucose and insulin, and OPG itself increases the production of IL-6 and IL-8.[27] This finding confirms the fact that OPG is involved in the pathogenesis of both endothelial dysfunction and inflammation. Hyperglycemia contributes to the activation of inflammatory processes and to the pathogenesis of complications. The present study found a positive correlation between OPG and HbA1c levels. Similar results were observed by Knudsen et al.[10] As in previously published data, the present study showed a positive correlation between OPG and insulin resistance, assessed using the visceral adiposity index and waist circumference.[28] Plasma concentrations of hsCRP are elevated in insulin resistant patients in the work by Festa et al. and Ndumele et al.[24,29] Low-grade inflammation might be regarded as an underlying pathogenetic mechanism for the relationship between OPG and IR. Additionally, considering the confirmed relationship between IR and cardiovascular disease, this finding supports the growing concept that OPG might be used as a serum biomarker identifying patients at risk for adverse outcomes.[30] The correlations described above could also support the idea that upregulation of OPG might be explained by pathways altered by IR and low-grade inflammation.

Some limitations of the present study should be mentioned. Firstly, the study group is relatively small. Further investigations of a larger cohort of type 1 diabetes patients in the Poznań Clinical Database of Diabetes are planned. Secondly, there was considerable variability in the participants' OPG measurements, which might have influenced the results. Finally, retinopathy was evaluated by a single ophthalmologist, which leaves the potential for a diagnostic bias. However, every patient had fundus photographs taken to support the ophthalmologist's assessment.

In conclusion, higher concentrations of OPG in DM 1 patients might be related to the presence of retinopathy. The study results indicate that OPG may play a role in the pathogenesis of diabetic retinopathy in association with hyperglycemia and low-grade inflammatory processes.

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# Oral mucositis and saliva IgA, IgG and IgM concentration during anti-tumor treatment in children suffering from acute lymphoblastic leukemia

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## Abstract

**Background.** Oral mucositis is a problem occurring within the oral cavity, which is the most difficult to deal with during anti-tumor treatment. The first symptom reported by the patient is discomfort. Salivary immunoglobulins play an important role in pathological processes occurring in the oral cavity.

**Objectives.** The study objective was to assess the occurrence of oral mucositis and to assess changes in the saliva IgA, IgG, and IgM concentration in children with acute lymphoblastic leukemia during anti-tumor treatment.

**Material and methods.** The study included 78 children with acute lymphoblastic leukemia (ALL) and a control group of healthy children. All the participants underwent 3 examinations.

**Results.** Mucosal opacity followed by redness usually occurred within 2–4 days after the methotrexate infusion. The most severe lesions of the oral mucosa were observed after the 1st month of chemotherapy. Correlations were found between hard-to-heal wounds and ulcers and blood morphology parameters. The mean saliva IgA concentration in children with ALL during chemotherapy was significantly lower than in children in the control group. A comparison of the mean saliva IgG in a given patient in subsequent examinations revealed a significant saliva IgG decrease occurring between the 1st and 3rd examinations.

**Conclusions.** Wounds and ulcers that were difficult to heal were related to blood morphology parameters. A low salivary IgA concentration in children with ALL may result in the development and potentiation of oral lesions typical of mucositis during anti-tumor treatment. A significant decrease in salivary IgG and IgM concentrations in children with ALL during chemotherapy may cause potentiation of pathological lesions in the oral mucosa.

**Key words:** children with oncological diseases, oral mucositis, saliva immunoglobulins, acute lymphoblastic leukemia

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Oral mucositis is a problem occurring within the oral cavity that is extremely difficult to deal with during anti-tumor treatment. Generally, the first symptom reported by the patient is discomfort. This is followed by burning of mucosa, dry mouth, mucosal erosion, and ulceration, which causes strong pain. Pain caused by oral mucositis may hinder proper nutrition, which in turn leads to weight loss, cachexia, and dehydration of the body, which is already weakened by disease.[1–3] Pain caused by mucositis also affects drug administration, speech, and breathing.[4–6] Oral mucosa may become deteriorated to such an extent that the patients require changes in the anti-tumor treatment and/or administration of parenteral analgesia. Observations reveal that oral mucositis occurs more often in children than in adults with a similar tumor disease. Oral inflammation is also more often observed in patients after bone marrow transplantation.[7,8] Early diagnosis and prompt treatment of oral mucositis that leads to patient deterioration are of crucial importance in the multidisciplinary treatment of patients. Although the symptoms of mucositis have been known for a long time and a 5-grade pathomechanism of these changes has been developed, there is no effective method to treat and eliminate the pain related to oral mucosa lesions.[8–10] Both the disease and treatment dramatically change the oral environment. Insufficient defense mechanisms are an important factor in the pathogenesis of numerous diseases. Pathological lesions of the oral mucosa observed in children with acute lymphoblastic leukemia are often caused by impaired functioning of the immune system.[11,12] Salivary immunoglobulins play an important role in pathological processes occurring in the oral cavity.[13] It is also believed that a tendency to develop mucositis symptoms is related to poor oral hygiene prior to treatment.[14,15]

The aim of the study was to assess the occurrence of oral mucositis and to evaluate changes in the level of immunoglobulins A (IgA), G (IgG) and M (IgM) in the saliva of children with acute lymphoblastic leukemia during anti-tumor treatment.

## Material and methods

The study included 78 children (34 girls and 44 boys) aged from 2 to 18 years with acute lymphoblastic leukemia (ALL) and a control group of 78 age- and gender-matched healthy children. The mean age of the subjects was 8.14 ±4.61 years. The study was carried out among patients who were diagnosed and undergoing anti-neoplastic treatment according to the protocols/blocks for risk groups in the ALL-IC BFM 2002 trial. The study was conducted at the Department of Paediatric Haematology and Oncology and Transplantation at the Medical University of Lublin (Poland), which belongs to the multicenter Polish Pediatric Group for Leukemia and Lymphoma and the Polish Pediatric Group for the Treatment of Solid Tumors (PPGLBC). This center coordinates a nationwide program of treatment for acute lymphoblastic leukemia. Under this program, children are stratified into 3 risk groups: standard risk (SR), intermediate risk (IR), and high risk (HR). Children from the SR group accounted for 25.4% of the study group; children from the IR group accounted for 50.79%; and children from the HR group accounted for 23.81%. In the study group, 5 children had cerebrospinal ALL recurrence; 2 had bone marrow recurrence; 7 had their CNS affected; and 3 had Down's syndrome. The children with ALL were examined in 3 stages: examination 1 was conducted prior to chemotherapy, examination 2 was conducted between 2 days and 5 months after the onset of chemotherapy, and examination 3 was conducted between 6 and 18 months of anti-tumor treatment. The 2$^{nd}$ and 3$^{rd}$ stages of saliva sample collection were always carried out with the consent of the pediatric hematologists who were treating the patients. Saliva collections were carried on the most secure day, ensuring no impact on the sick children. In the vast majority of the children, the 2$^{nd}$ saliva sample was taken in between the 6$^{th}$ and 8$^{th}$ week of chemotherapy, while in the case of 4 children, because of the general condition of the patients, the 2$^{nd}$ saliva sample was collected after 4.5 months of therapy. The stomatological clinical study was conducted by a dentist with the use of basic diagnostic tools in artificial lighting. The condition of the oral mucosa of the children in the study group was assessed with the use of a 5-grade WHO classification of oral mucositis.[2] Changes in oral mucosa were monitored every day.

The control group consisted of 78 generally healthy children, matched for age and gender. These children were patients at the Department of Paedodontics at the Medical University of Lublin. The children were undergoing check-ups or were being treated for dental caries. The control group consisted of 34 girls and 44 boys, and their ages ranged from 3 to 18 years, with a mean age of 8.51 ±2.38 years.

Unstimulated saliva was collected from the children in the study in the morning, 2 h after eating, in order to determine immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) concentration. The collected saliva was centrifuged for 15 min at 5000 rpm. The centrifuged saliva was frozen to a temperature of -80°C until the laboratory test was conducted. The concentrations of the selected parameters were determined with commercially available ELISA tests for determining immunological parameters in biological fluids (DRG Diagnostics GmbH, Marburg, Germany). IgA concentrations were determined by means of the Salivary IgA ELISA; IgM concentrations by the CIC IgM ELISA; and IgG concentrations by the IgG CIC ELISA.

The findings were analyzed statistically using STATISTICA v. 10.0 software (StatSoft Inc., Tulsa, USA). The characteristics of the measurable parameters were presented as mean, median, minimum and maximum

values, and standard deviation. The Mann-Whitney U test was used to compare 2 independent groups. The Wilcoxon signed-rank test was used to compare dependent groups. A logistic regression analysis was also performed. The level of statistical significance was set at $p < 0.05$, and statistically significant differences are marked hereafter with asterisks (*).

## Results

Lesions of the mucositis type were observed in the ALL children in the period from 48 h to 6 months after the initiation of chemotherapy. The lesions were of various intensities; and there were periods without pathological lesions, which were related to the intensity of the chemotherapy. Mucosal opacity followed by redness usually occurred within 2–4 days after a methotrexate infusion. The most severe lesions of the oral mucosa were observed after the 1st month of chemotherapy. Changes of varying severity were observed in the oral mucosa: localized erythema of the mucosa (grade I) were noted in 35% of the children; pseudomembranous mucosa (grade II) in 18%; ulcers with extensive erythema (grade III) in 40%; massive mucosal ulcers and tissue necrosis (grade IV) in 4%.

Hard-to-heal wounds and ulcers were related to the blood parameters. It was observed that healing was faster, especially with regard to oral mucosa ulceration, when blood morphological parameters were improved. Lesions of the mucositis type were also dependent on the level of neutropenia. Neutropenia was recognized when the number of neutrophils was <1500/μL. Data on blood cell counts was obtained from case records. Each child with neutropenia also had fungal complications in the oral mucosa.

In the periods between protocols of chemotherapy, there were usually no lesions.

After 6 months of chemotherapy, lesions in the oral mucosa were less intense and were observed in 3.17% of the study children. The lesions were usually observed as redness and erosion. No ulcers in the oral cavity were observed.

For prophylactic purposes, the children were directed to regularly rinse the oral cavity with preparations of chlorhexidine (Corsodyl, GlaxoSmithKline PLC, London, UK) or benzydamine hydrochloride (Tantum verde, Angelini S.P.A., Ancona, Italy), as well as with a herbal mixture (Dentosept, Phytopharm Klęka SA, Nowe Miasto nad Wartą, Poland). When lesions appeared in the oral mucosa, children were administered a mixture for oral swabbing containing bicarbonate, gentamicin, colimycin, and nystatin. When massive ulceration in the oral cavity occurred, the children received Solcoseryl ampules intravenously and Solcoseryl adhesive paste (Meda Pharma GmbH & Co. KG, Bad Homburg, Deutschland) on the oral mucosa. In cases of massive milky white opacities, the treatment included antifungal preparations of the azole group, e.g., fluconazole (10 mg/kg/day).

The findings concerning saliva IgA, IgG, and IgM levels in the study participants are presented in Table 1. The mean saliva IgA concentration in children with ALL during chemotherapy was significantly lower than in the control group (Table 1). Saliva IgA measured in subsequent examinations of a given ALL patient did not reveal significant differences between the study stages being compared (Wilcoxon signed-rank analysis) (Fig. 1).

In examination 1, the mean saliva IgG concentration in the children with ALL was significantly higher than in the healthy children, which was also confirmed by a logistic regression analysis (logistic regression: $\chi^2(1) = 13.5820$; $p = 0.0002$*). During the anti-tumor therapy, however, the mean saliva IgG in the study children gradually decreased; and in examination 3 it was lower than in the control group (Table 1). A comparison of mean saliva IgG in subsequent examinations of any given patient revealed a significant saliva IgG decrease between examinations 1 and 3 (Wilcoxon signed-rank test: $Z = 2.303654$;

**Table 1.** Salivary immunoglobulin A (IgA), immunoglobulin G (IgG), and immunoglobulin M (IgM) concentrations (μg/mL) in the children with ALL and in the healthy controls

| Saliva Ig | Studied group of children | | Mean value | Me | Min | Max | SD | Mann-Whitney U Test | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | | | | | Z | p-value |
| IgA μg/mL | all | exam 1 | 218.1 | 245.8 | 8.89 | 458.2 | 153.9 | 3.631 | 0.0003* |
| | | exam 2 | 225.8 | 233.7 | 5.68 | 450.8 | 118.4 | 4.099 | 0.00004* |
| | | exam 3 | 188.5 | 171.0 | 8.14 | 480.0 | 147.2 | 4.937 | 0.000001* |
| | healthy | | 353.1 | 337.2 | 15.27 | 804.7 | 171.2 | the above values refer to healthy children | |
| IgG μg/mL | all | exam 1 | 4.8 | 5.2 | 0.00 | 12 | 4.6 | -2.83 | 0.0046* |
| | | exam 2 | 2.3 | 1.3 | 0.05 | 13 | 3.3 | -0.8019 | 0.423 |
| | | exam 3 | 1.5 | 0.8 | 0.0 | 6 | 1.9 | 1.8192 | 0.0689 |
| | healthy | | 2.1 | 0.8 | 0.16 | 12 | 2.7 | the above values refer to healthy children | |
| IgM μg/mL | all | exam 1 | 31.5 | 28.1 | 15.15 | 62 | 12.6 | -8.2676 | 0.00001* |
| | | exam 2 | 22.3 | 22.7 | 7.57 | 66 | 13.4 | -4.8644 | 0.000001* |
| | | exam 3 | 20.0 | 13.0 | 6.49 | 66 | 13. 6 | -2.3299 | 0.019811* |
| | healthy | | 11.7 | 11.9 | 0.0 | 31 | 6.1 | the above values refer to healthy children | |

exam – examination; Me – median; Min – minimum; Max – maximum; SD – standard deviation; p – significance level; * statistically significant value.

p = 0.0212*). These findings were also confirmed by a logistic regression analysis (logistic regression: $\chi^2(3) = 20.592$; p = 0.00013*) (Fig. 2).

Saliva IgM concentration in the ALL patients was at all stages significantly lower than in the control group (Table 1). A comparison of the mean saliva IgM in subsequent examinations of any given patient revealed significant differences between examinations 1 and 2 (Wilcoxon signed-rank test: Z = 3.4098; p = 0.0006*) and between examinations 1 and 3 (Wilcoxon signed-rank test: Z = 3.0544; p = 0.0022*). These findings were confirmed by a logistic regression analysis (logistic regression: $\chi^2(3) = 74.952$; p < 0,0001*) (Fig. 3).

## Discussion

In the present study, mucositis symptoms were correlated with blood parameters. Similar correlations have also been observed by other authors.[16–20] Mucositis is most frequently induced by a reduced number of white blood cells, the use of cytotoxic antibiotics and by alkylating factors.[16,17] A significant decrease in salivary S-IgA following chemotherapy in children with leukemia was reported by Karolewska et al., although the authors did not report differences in the S-IgA concentration in comparison with the control group prior to anti-tumor treatment. According to those authors, a statistically significant low salivary S-IgA level was maintained after chemotherapy.[18] A statistically significant lower level of saliva IgA in patients with leukemia (2.9–1.9 μg/mL) as compared to controls (5.4 μg/mL) was reported by Thomaz et al.[19] Månsson-Rahemtulla et al. also reported that the value of saliva IgA in patients with ALL undergoing chemotherapy was significantly reduced. The authors revealed that at the time of diagnosis, salivary IgA in patients with ALL was 0.183 ±0.193 mg/mL, and that it was higher than in the control group. After 3 and 5 weeks of chemotherapy, salivary IgA levels were statistically significantly reduced in those patients, both as compared to patients with acute myeloid leukemia and to healthy patients.[20] A low level of saliva IgA and IgG has been reported to result from the effects of chemotherapy on salivary gland secretion, which in turn leads to impairment of the local immune system. Local disturbances of the immune system may affect systemic immunity of patients undergoing chemotherapy.[18]

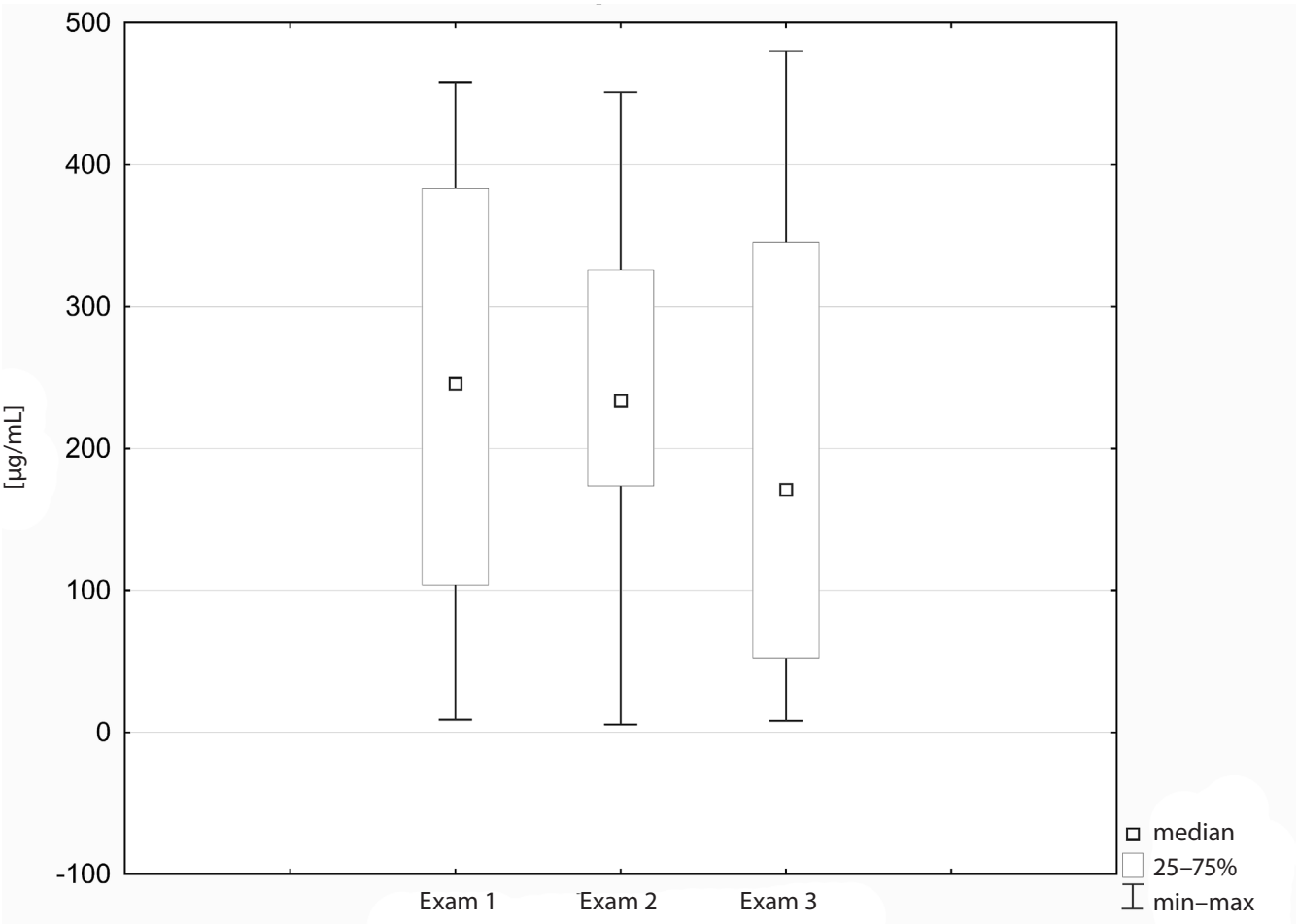


Fig. 1. Saliva IgA (μg/mL) concentration in children with ALL in subsequent tests

According to a study by Pajari et al., neither children with the remission of ALL, nor children in the acute phase of the disease showed a significant difference in saliva IgA, IgG and IgM as compared to the control group, but patients with other hematological neoplasms had significantly higher levels of salivary IgG and IgM than the healthy controls.[21] These authors stated that the significant reduction in salivary IgG and IgM observed in the children cured of neoplastic diseases indicates a probability that the treatment and/or disease may impair the immune response in these patients. Other authors studying children with ALL observed normal serum IgG, IgA and IgM levels at the time of diagnosis, whereas during chemotherapy, they observed a decrease in the concentration of 1 or more immunoglobulins; the greatest decreases were observed for IgG and IgM. In those studies, the permanent IgM deficit was related to a higher risk of disease recurrence and death in the ALL children. In patients with good prospects for remission and recovery, the immunoglobulins normalized within a year following the completion of treatment.[22–24]

Human IgA occurring in serum induces a significant increase in interleukin-1 receptor antagonist (IL-1RA) excretion by mononuclear cells of the peripheral blood, as well as increased adherence of monocytes. By inducing IL-1RA and decreasing excretion of pro-inflammatory cytokines such as IL-1β, TNF-α and IL-6, IgA contributes to regulating the inflammatory response. According to a pathomechanism outlined by Sonis et al., a low S-IgA concentration in patients undergoing anti-tumor treatment could be partially responsible for a higher risk of mucositis development.[8,18] In my own previous studies, decreases in salivary TNF-α and IL-2 was observed in patients with ALL as compared to healthy children.[25,26]

In an effort to minimize the side effects of radiation therapy and chemotherapy used in cancer treatment, there have been studies aimed at developing therapeutic strategies to regenerate oral mucosal tissues affected by mucositis.[3,5] Certain agents have been examined for prevention or treatment of oral mucositis caused by chemotherapy or radiotherapy, but none of them has been confirmed as fully effective.[27] A study by Pereira Pinto et al. revealed that in a group of children with ALL who used 0.12% chlorhexidine gluconate to rinse the mouth, 26% developed mucositis in comparison with 80% who did not use the oral rinse.[28] Initially, the lesions were

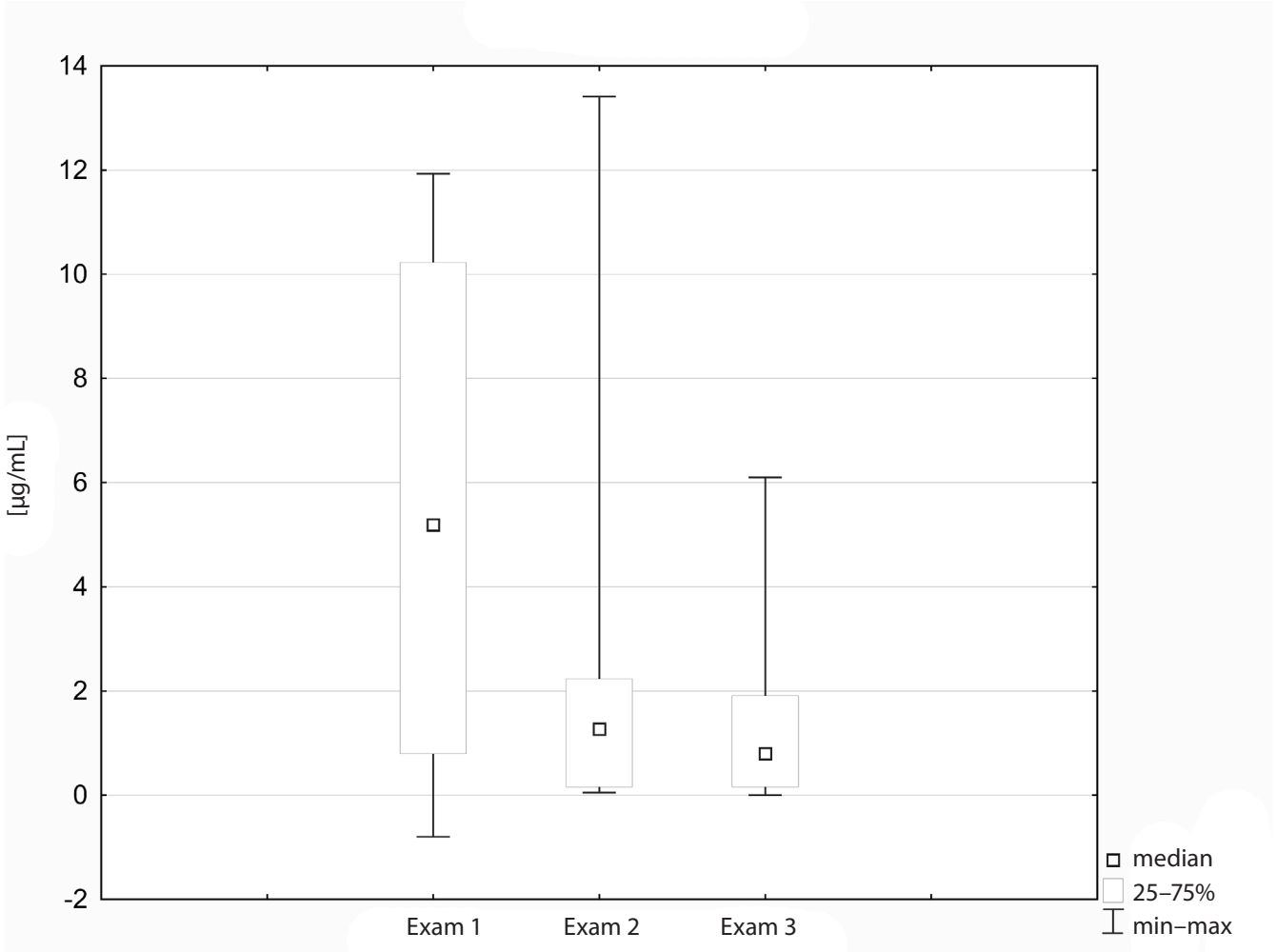


Fig. 2. Saliva IgG (µg/mL) concentration in children with ALL in subsequent tests

manifested as a characteristic erythema on the buccal and labial mucosa, followed by edema and ulceration. Lesions of the mucositis type developed within 2–4 days after administration of methotrexate, and made normal nutrition impossible. In a study by de Oliveira Lula et al., patients with acute lymphoblastic leukemia treated according to the GBTLI-93 and the BFM protocols developed oral problems irrespective of the chemotherapy protocol. Pathological lesions were mainly observed shortly after the initiation of anti-tumor therapy. Mucosal pallor occurred in 20% of the subjects treated according to the GBTLI-93 protocol and in 23.3% of patients treated according to the BFM protocol. Ulceration was observed at the same level in both groups of patients. Bleeding gums, candidiasis, and xerostomia occurred mainly in patients treated according to the GBTLI-93 protocol.[29] Some scientists suggest applying chlorhexidine when used in a 0.1% or 0.12% solution to prevent and mitigate pain during chemotherapy as a way to avoid grade II and III oral mucositis.[28,30] According to some researchers, both chlorhexidine and benzydamine contribute to the reduction of oral mucositis during chemotherapy, but only in children over 6 years of age.[2,18,31–35] Currently, opioids seem to be the most effective in the treatment of mucositis-induced pain. However, patients develop tolerance to opioids relatively quickly, and responses to their analgesic effect vary.[5] Despite the use of opioids, pain related to oral lesions of the mucositis type in patients subject to hematopoietic stem cell transplantation was significantly correlated with increased expression of the TNF-α gene in buccal cells on the 9$^{th}$ day of therapy in comparison with the baseline.[33]

Randomized trials do not allow recommendations, but may suggest possible preparations for use in the treatment of mucositis. Only palifermin is recommended by both the US Food and Drug Administration and the European Medicines Agency for the treatment of oral mucositis.[9] Prevention of mucositis in patients with hematological neoplasms who have undergone stem cell transplantation is based on palifermin, which contains the recombinant human keratinocyte growth factor (KGF). Following the use of palifermin in these patients, grade III and grade IV oral mucositis was significantly reduced, and the average duration of its occurrence was shorter than in the control group.[36] Palifermin appears to reduce the incidence of oral mucositis in patients treated for head

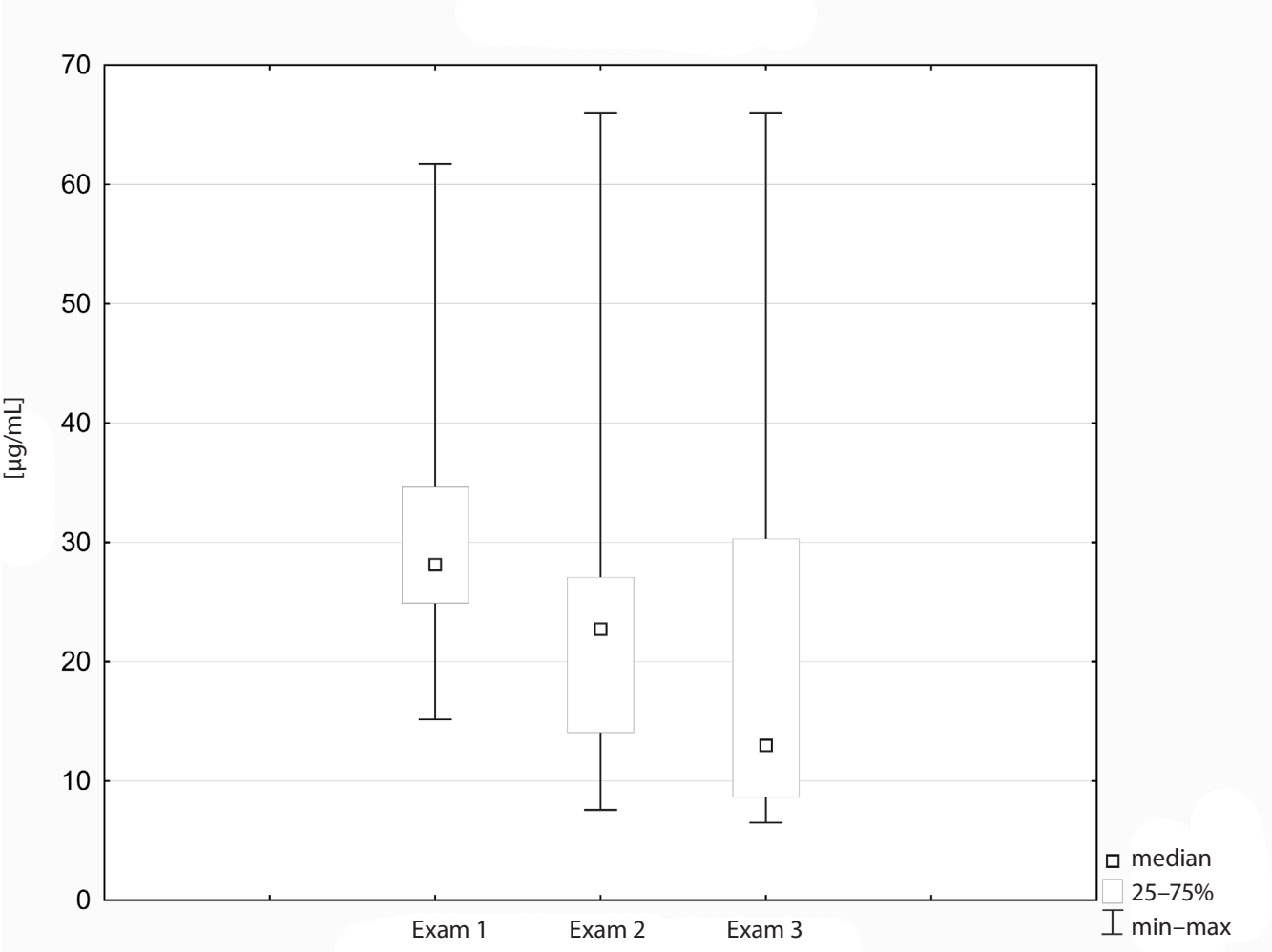


Fig. 3. Saliva IgM (µg/mL) concentration in children with ALL in subsequent tests

and neck cancer, but its position in therapy has not yet been established.[37] The efficacy of the preparation has been confirmed for intravenous administration, whereas local administration seems to be less effective.[5,10,15,34,38,39] Palifermin is an expensive preparation, whose use is difficult to justify when the risk of severe oral mucositis is low. However, if physicians could predict which patients would suffer from oral mucositis after standard doses of chemotherapy and/or radiotherapy, then, from a pharmaco-economic point of view, the use of new and more expensive preparations could be well justified.[35] In certain cases, oral mucositis makes it necessary to change or stop antitumor therapy. Moreover, the additional costs of treating oral mucositis has economic consequences that can be difficult for national health funds to bear.[5,9,10]

One factor reducing the risk of oral complications that is especially important, and simple at the same time, is regular oral hygiene. Motivating the patient to properly clean all tooth surfaces and oral soft tissues, to brush the teeth at least twice a day, and to rinse the oral cavity may contribute to reducing the incidence of oral mucositis or mitigate its symptoms.[9,10,35] The participation of dentists in multidisciplinary teams treating children with ALL can greatly contribute to better health protection for these patients.[16] At the same time, it must be emphasized that the immunoregulatory mechanisms of cytokines and immunoglobulins involved in oral inflammations play a special role in protecting the host and maintaining oral homeostasis. Saliva may become a good source for detecting pro-inflammatory markers. In the future, salivary cytokines and immunoglobulins might play an important role as replacement biomarkers in assessing the efficacy of chemotherapy.[40]

In conclusion, the findings of this study demonstrate that the condition of the oral mucosa in children with acute lymphoblastic leukemia was not satisfactory during chemotherapy. Low salivary IgA concentrations in children with ALL may result in the development and potentiation of oral lesions of the mucositis type during anti-tumor treatment, and significant decreases in salivary immunoglobulin G and immunoglobulin M concentrations in children with ALL during chemotherapy may cause a potentiation of pathological lesions in the oral mucosa.

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# An inverse relationship between plasma glutathione concentration and fasting glycemia in patients with coronary artery disease and concomitant type 2 diabetes: A pilot study

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## Abstract

**Background.** There have been occasional reports indicating that plasma concentrations of reduced glutathione (GSH) may be associated in some way with blood glucose. This relationship, however, has not hitherto been explored in the blood plasma of patients with coronary artery disease (CAD).

**Objectives.** The aim of this study was to evaluate potential associations of fasting glycemia and peripheral blood plasma GSH concentrations in CAD-free and CAD-affected subjects.

**Material and methods.** In blood samples obtained from patients with CAD, defined by coronary angiography and/or echocardiography, and from an age-matched control group of patients with a confirmation of no coronary artery occlusion and with no history of cardiovascular events, plasma concentrations of glucose and reduced glutathione were analyzed by routine laboratory diagnostic methods and high performance liquid chromatography (HPLC), respectively.

**Results.** The results showed that in the CAD patients, but not in the non-CAD controls, fasting glycemia is negatively associated with plasma levels of GSH ($r = -0.328$; $p = 0.011$). Moreover, in the CAD-affected subjects (but not in the controls) the presence of type 2 diabetes mellitus significantly discriminated plasma levels of GSH ($r_P = -0.125$; $p = 0.350$, between GSH and glucose adjusted for the occurrence of diabetes).

**Conclusions.** The study suggests that GSH may be an important factor contributing to glucose metabolism in CAD patients. Hence, it may be considered a significant therapeutic target in strategies aimed at improving glycemic control in CAD-affected subjects.

**Key words:** atherosclerosis, glucose, glutathione, cardiovascular risk factors, diabetes mellitus

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# Introduction

Impaired fasting glycemia is associated with the severity of coronary artery disease (CAD).[1,2] Maintaining proper proportions between glucose utilization in essential cell metabolism and glucose supply in the daily diet is one of the key factors protecting against the development of CAD, as well as minimizing the risk of CAD aggravation in the cases of existing CAD. The prime example of the association between glucose metabolism and CAD is diabetes mellitus (DM), which is considered one of the main CAD risk factors.[2–4]

One of the leading hypotheses elucidating the relationship between disturbances in glucose metabolism and CAD posits that oxidative stress is a link between hyperglycemia and CAD. This association, which is observed in subjects with disturbed glucose metabolism, is also seen in the general population.[5,6] Despite huge progress in understanding hyperglycemia-induced cardiovascular pathogenesis, the exact biochemical pathway(s) linking glucose with oxidative stress in CAD are still not completely understood.[7] Particular attention in this regard can be paid to glutathione (GSH), one of the low-molecular-weight thiols, formed by 3 amino acids (tripeptide). GSH constitutes the main "ingredient" of both extracellular redox buffers (including the blood plasma) and intracellular ones. Its importance in the pathogenesis, progress and treatment of CAD has been shown in a few recent works.[8–10] Some reports clearly suggest a relationship between glycemia and GSH.[7,11] Nevertheless, the possible association of plasma GSH levels and glucose concentration has not yet been evaluated in CAD patients with and without concomitant type 2 diabetes mellitus. The aim of the present study was to evaluate this relationship.

# Material and methods

## Chemicals

Sodium hydroxide (NaOH), hydrochloric acid (HCl), sodium hydrogen phosphate heptahydrate ($Na_2HPO_4{\cdot}7H_2O$), sodium dihydrogen phosphate dihydrate ($NaH_2PO_4{\cdot}2H_2O$) and HPLC-grade acetonitrile were obtained from J.T. Baker Chemicals (Deventer, the Netherlands). Trichloroacetic acid (TCA), perchloric acid (PCA) and tris-(2-carboxyethyl)phosphine (TCEP) were obtained from the Merck Group (Darmstadt, Germany). Sodium dodecyl sulfate (SDS), Ellman's reagent – 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB), glutathione (reduced), 2,4-dinitrophenylhydrazine (Brady's reagent, DNPH), ethanol, ethyl acetate and guanidine hydrochloride were from Sigma-Aldrich Poland (Poznań, Poland). The Pierce™ BCA Protein Assay Kit was from Thermo Fisher Scientific Inc. (Waltham, USA). All the chemicals used throughout the study were of analytical-reagent grade except for the derivatization reagent 2-chloro-1-methylquinolinium tetrafluoroborate (CMQT), which was synthesized in our laboratory as described in a previous publication.[12]

## Subjects

All the experiments were done in agreement with the Declaration of Helsinki (2008) of the World Medical Association. The study was approved by the Local Ethics Committee of the Medical University of Lodz (Poland). All the participants were informed of the terms of their involvement in the study and gave their informed consent before the study.

The group of 59 CAD-affected patients were recruited after the verification of the occurrence of coronary artery disease on the basis of echocardiography and/or angiography. Those with at least 50% occlusion in at least 1 coronary vessel were considered to be suffering from coronary artery disease. Fifty-four non-CAD control subjects were recruited from individuals whose echocardiography and/or angiography results showed that none of their coronary arteries were 50% occluded.

The exclusion criteria were similar to those used in earlier studies, with some modifications.[13,14] Individuals who suffered from unstable angina pectoris, chronic renal failure, type 1 diabetes mellitus, allergic diseases, autoimmune diseases, infectious diseases, acute infection in the previous 2 weeks, acute coronary syndrome within the previous 6 months, those with any kind of inflammatory disease or non-chronic inflammation, malignancies, alcohol abusers, those who used alcohol the day before the experiment, drug abusers, those suffering from psychiatric disorders or any chronic diseases other than CAD, and those on specific diets or taking diet supplements were excluded. Patients whose clinical status was difficult to diagnose and pregnant women were also excluded.

The following data were recorded for each participant: age, gender, height, weight, hip and waist dimensions, smoking (past or current), exposure to stress, kind of professional activity (physical, mental, mixed, none), frequency of physical activity (high, moderate, none), the occurrence of myocardial infarction in the past (with the number of infarctions), ischemic stroke or transient ischemic attack (with the number of cerebrovascular events), the occurrence of cardiovascular procedures such as percutaneous coronary intervention (PCI) and/or coronary bypass grafting (CABG), other concomitant diseases and current pharmacotherapies (antihypertensive, hypolipidemic and antidiabetic drugs).

## Measurements of blood morphology and biochemistry

Samples of peripheral blood were taken (always between 8:00 and 10:00 am) from a forearm vein, following

at least 12 h of overnight fasting. For anticoagulation 0.105 M buffered citrate was used in the case of plasma samples used for the analysis of thiol concentrations, while EDTA was used in the case of samples for the morphology analysis. Glucose was assessed in serum samples. To obtain plasma, the blood samples were centrifuged (2000 × g/15 min/4°C) immediately after blood withdrawal. The plasma was portioned and the aliquots were stored at -70°C until further use, without any thawing before testing. To obtain blood serum, the blood samples were collected in tubes with a clotting activator, and were left undisturbed at room temperature for 30 min, then centrifuged (2000 × g/15 min/4°C) and stored at -70°C until further use, without any thawing before measurements.

Blood morphology and biochemical serum/plasma parameters were measured using the 5-Diff Sysmex XS-100i (Sysmex, Kobe, Japan) and the DIRUI CS 400 analyzer (Dirui, Changchun, China), respectively.

## HPLC measurements of low-molecular-weight thiol concentrations

A 2-fold dilution of plasma aliquots with phosphate buffer (pH = 7.6; 0.2 mol/L) was followed by sample supplementation with TCEP phosphate buffer solution (0.25 mol/L). The mixture obtained was vortexed and put aside for 10 min and then supplemented with CMQT (0.1 mol/L). After 2 more min, 3 mol/L PCA was added to precipitate plasma proteins, which were further spun down (10 min, 12000 × g). The supernatant obtained was transferred into a vial, and a 20 µL aliquot was injected into the ZORBAX SB C18 column (150 × 4.6 mm) packed with 5 µm particles.

The composition of the mobile phase was as follows: 0.1 mol/L TCA (solution A), adjusted to pH = 1.65 with 1 mol/L NaOH solution, and acetonitrile (solution B); flow rate 1 mL/min; temperature 25°C.

The elution profile was as follows: 0–8 min, 11–40% B; 8–12 min, 40–11% B; (A/B, v/v). Detection and quantification were conducted by UV absorbance at 355 nm. Identification of the peaks was based on comparisons of retention times and diode-array spectra, taken at the real time of analysis, with a corresponding set of data obtained by analyzing the authentic compounds.

## Measurements of concentrations of free sulfhydryl groups and carbonyl groups in blood plasma proteins

The concentrations of free sulfhydryl groups in plasma proteins were assessed according to the method reported in a previous publication, originally developed by Ando and Steiner.[13,15] The concentration of carbonyl groups in plasma proteins was measured with the use of a spectrophotometric assay, according to the protocol reported in papers by Levine et al. and Rice-Evans et al.[16,17] The concentration of plasma protein was assessed with the Pierce™ BCA Protein Assay Kit, in accordance with the manufacturer's instructions.

## Statistical analysis

Outliers, normal data distribution and homogeneity of variance were verified with Grubbs' test, the Shapiro-Wilk test and Levene's tests, respectively. Depending on the departures from normal distributions, data was presented as mean ±SD, or median and interquartile range (IQR: lower quartile, LQ [25%], to upper quartile, UQ [75%]). Some variables were transformed using the Box-Cox method. The unpaired Student's t-test on transformed data or the Mann-Whitney U test were employed to evaluate the significance of differences between the groups of non-CAD and CAD individuals. Multiple regression or Pearson's correlation (on raw data or the Box-Cox-transformed data) and an analysis of covariance (ANCOVA) were used to estimate the associations between the variables. All the statistical calculations were performed using STATISTICA.PL, v. 12.5 (StatSoft Polska, Kraków, Poland) and StatsDirect, v. 2.7.7 (StatsDirect Ltd, Cheshire, UK).

# Results

## Basic characteristics of the study groups: Demography, anthropometry, socioeconomics, peripheral blood morphology and plasma biochemistry

The patients affected by CAD had higher counts of total white blood cells (WBC) than the controls (Table 1). The counts of subpopulations of lymphocytes, neutrophils and monocytes were significantly increased in the CAD patients compared to the controls (data not shown).

Plasma creatinine and C-reactive protein (CRP) concentrations were increased in the patients suffering from CAD; these differences, however, did not reach statistical significance. Significantly higher fasting glucose was recorded in the CAD patients, and the difference remained significant when patients with diabetes were excluded from the CAD and non-CAD groups. Increased levels of triglycerides were noted in the CAD group. Total cholesterol and its lipoprotein subfractions were significantly lower in the CAD group than in the non-CAD controls. A comparison of low-molecular-weight thiols revealed significantly higher plasma homocysteine concentrations in the CAD-affected patients ($p < 0.005$ in the Mann-Whitney U test). The concentrations of cysteine, cysteinylglycine and glutathione were not significantly different between the non-CAD control group and the CAD subjects (Table 1).

**Table 1.** Demographic, clinical, social and biochemical characteristics of investigated populations of patients

| Variable | non-CAD | CAD |
|---|---|---|
| **Demographic** | | |
| Age (years) | 60.4 ±9.4 | 64.8 ±10.8[a#] |
| Sex (male/female) | 33/21 | 44/15 |
| **Clinical** | | |
| BMI [kg/m$^2$] | 27.2 ±3.5 | 29.4 ±4.5 |
| Diabetes mellitus (type 2) [n and %] | 18 (33.8) | 34 (57.6) |
| WHR | 0.9 ±0.2 | 0.9 ±0.1 |
| Previous MI [%] | 0 | 56 |
| PCI [%] | 0 | 69.5 |
| CABG [%] | 0 | 16.9 |
| **Social and lifestyle** | | |
| Current smoking [%] | 5.6 | 6.7 |
| Past smoking [%] | 86.8 | 19 |
| **Medication** | | |
| **Antiplatelet drugs** | | |
| acetylsalicylic acid [%] | 18.5 | 59.3 |
| purinergic receptors antagonists [%] | 0 | 32.3 |
| **Hypolipidemic drugs** | | |
| statins [%] | 25.9 | 83.0 |
| fibrates [%] | 0 | 3.4 |
| **Antihypertensive drugs** | | |
| ACE-inhibitors [%] | 44.4 | 64.4 |
| β-blockers [%] | 10.2 | 79.6 |
| Ca-channel blockers [%] | 2.2 | 1.7 |
| **Hypoglycemic drugs** | | |
| insulin [%] | 5.5 | 20.3 |
| sulfonylureas [%] | 9.2 | 3.4 |
| metformin [%] | 3.7 | 11.9 |
| **Blood morphology** | | |
| RBC [x 10$^6$/μL] | 4.8 ±0.46 | 4.7 ±0.4 |
| HGB [g/dL] | 14.5 ±1.3 | 14.2 ±1.1 |
| HCT [%] | 42.9 ±3.8 | 41.9 ±3.2 |
| PLT [x10$^3$/μL] | 217 (178; 267) | 226 (184.7; 265.7) |
| WBC [x10$^3$/μL] | 5.7 (5.05; 7.1) | 6.95 (5.6; 7.9)[b*] |
| **Selected blood plasma metabolites** | | |
| Creatinine [μmol/L] | 93 (82; 105) | 95 (86; 104.7) |
| Total cholesterol [mmol/L] | 5.4 (4.5; 6.1) | 4.7 (3.9; 5.3)[c*] |
| HDL cholesterol [mmol/L] | 1.4 (1.2; 1.6) | 1.2 (1.04; 1.5)[a*] |
| LDL cholesterol [mmol/L] | 3.5 (2.9; 4.5) | 3.06 (2.4; 3.7)[b*] |
| Triglycerides [mmol/L] | 1.3 (1.0; 1.7) | 1.5 (1.2; 2.06) |
| hs-CRP [mg/mL] | 1.4 (0.7; 2.9) | 1.7 (0.7; 3.3) |
| Glucose [mg/dL] | 116 (101.5; 129.9) | 126 (114; 159.8)[*] |
| Homocysteine [μmol/L] | 7.3 (6.2; 8.2) | 8.5 (6.6; 10.2)[*b] |
| Glutathione [μmol/L] | 4.9 ±1.3 | 5.0 ±1.2 |
| Cysteine [μmol/L] | 159.5 ±23.9 | 25.7 ±5.2 |
| Cysteinylglycine [μmol/L] | 26.4 ±5.0 | 167.5 ±29.3 |
| Glycated hemoglobin (all pts)/excl. DM pts [%] | 6.0 (5.7; 6.7) 5.8 (5.5, 6.2) / 5.8 (5.4; 6.1) | 5.8 (5.3; 6.7) 5.9 (5.4; 6.6) / 5.8 (5.4; 6.3) |

Variables presented as means ±SD, median and interquartile range (from lower quartile, LQ to upper quartile, UQ) or percentage fractions of whole groups of investigated patients (n = 54 for non-CAD and n = 59 for CAD patients). Comparisons between non-CAD and CAD groups performed with the use of unpaired Student's t-test (#) or Mann-Whitney U test (*). [a] $p < 0.05$; [b] $p < 0.005$; [c] $p < 0.001$; BMI – body mass index; CABG – coronary artery bypass grafting surgery; CAD – coronary artery disease; DM – diabetes mellitus; excl. – excluding; HCT – hematocrit; HDL – high-density lipoprotein; HGB – hemoglobin; hs-CRP – high-sensitivity C-reactive protein; MI – myocardial infarction; LDL – low-density lipoprotein; PCI – percutaneous coronary intervention; PLT – platelet count; pts – patients; RBC – red blood cell count; WBC – white blood cell count; WHR – waist-hip ratio.

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## Associations between plasma GSH levels, fasting glycemia and the occurrence of type 2 diabetes mellitus in the non-CAD and CAD patients

When the patients in the CAD group were divided into 2 subgroups according to their plasma GSH levels – patients with plasma GSH lower than $Me_{GSH}$ = 5.12 µmol/L (GSH group 1) and patients with plasma GSH higher or equal to the median value (GSH group 2), the patients with lower GSH demonstrated average fasting glycemia higher (by 13%) than the patients with higher plasma GSH levels (p = 0.037 in the unpaired Student's t-test on the Box-Cox-transformed data) (Fig. 1A).

A similar analysis in the non-CAD control subjects, aimed at comparing fasting glucose levels in relation to GSH plasma concentrations, revealed no significant differences between the fasting glucose values of the patients with low GSH (plasma concentrations lower than $Me_{GSH}$ = 4.84 umol/L) and those with plasma GSH levels higher or equal to the median value (glucose concentrations of 118 [100–134] mg/dL vs 110 [103–126] mg/dL; p = 0.434 in the unpaired Student's t-test on the Box-Cox-transformed data) (Fig. 1A).

An analogical comparison between the control participants and the CAD-affected patients, with each group subdivided into 2 subpopulations according to the median values of blood glycemia (114 and 126 mg/dL in non-CAD and CAD subjects, respectively), revealed that the mean plasma GSH concentrations were approximately equal (ca. 5 µmol/L) in both subpopulations of the non-CAD controls stratified according to median glycemia, but they were lower in the subpopulation of CAD patients with higher glycemia (≥126 mg/dL): 4.8 µmol/L vs 5.3 µmol/L GSH; p = 0.04 in the non-paired, one-sided Student's t-test).

Plasma GSH and fasting glycemia were adjusted for age and the occurrence of diabetes mellitus, 2 variables that differed significantly between the non-CAD and CAD individuals (Fig. 1B). The adjusted values of plasma GSH

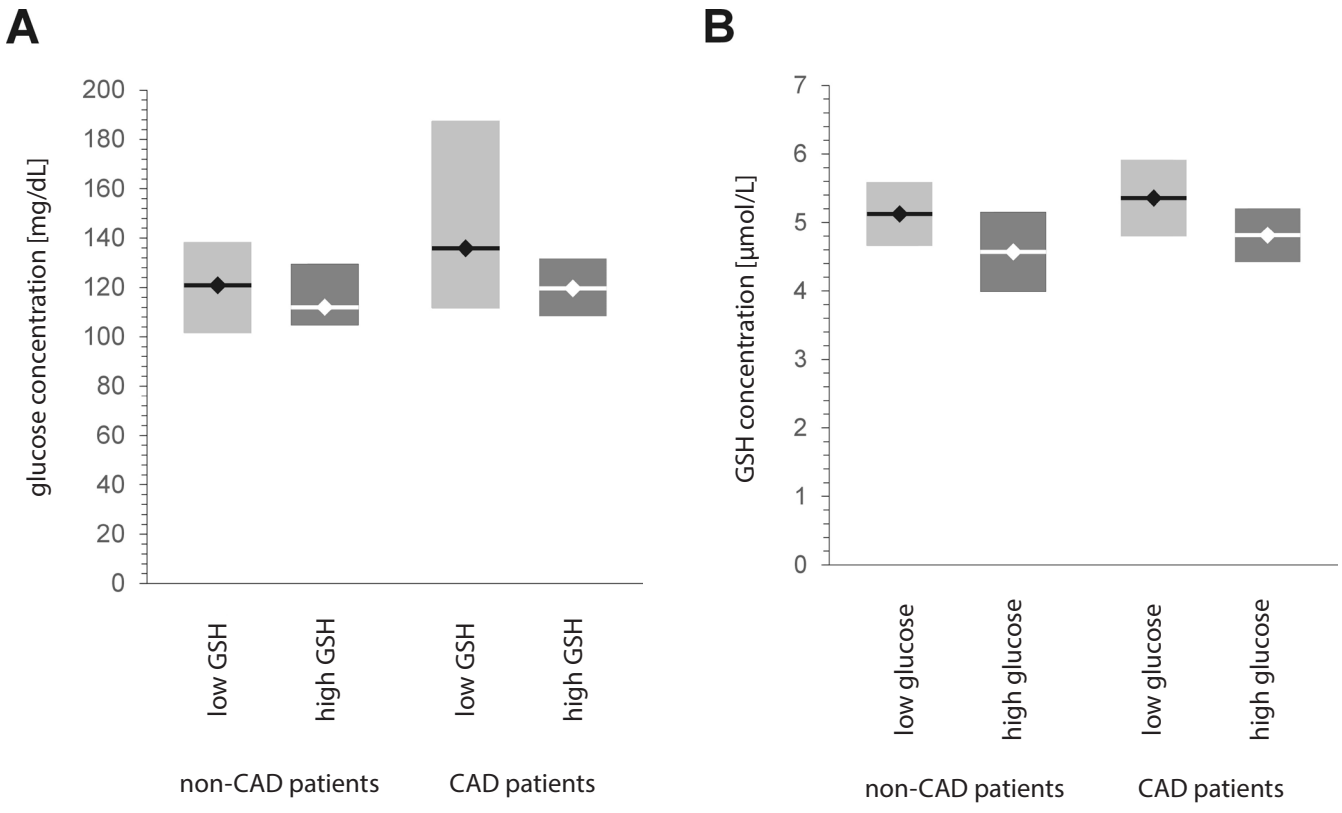


**Fig.1.** Glucose and reduced glutathione (GSH) concentrations in stratified groups of non-CAD controls and CAD patients

Data is presented as median and interquartile range for glucose (A) or mean ±95% CI for GSH (B) in the subgroups of non-CAD controls and CAD subjects, stratified according to plasma GSH and fasting glycemia, respectively. For glucose, non-CAD controls and CAD patients were stratified into "low GSH" subgroups (n = 27 and n = 29, respectively) with GSH levels lower than the group median ($Me_{GSH}$ = 4.807 µmol/L for the controls and $Me_{GSH}$ = 5.154 µmol/L for the CAD patients), and "high GSH" subgroups (n = 27 and n = 30, respectively) with GSH levels higher or equal to the established GSH median value. For GSH, non-CAD controls and CAD patients were stratified into "low glucose" subgroups (n = 27 and n = 29, respectively) with fasting glycemia lower than the group median ($Me_{Glucose}$ = 117.1 mg/dL for the controls and 122.8 mg/dL for the CAD patients), and "high glucose" subgroups (n = 27 and n = 30, respectively) with fasting glycemia higher or equal to the established median value. Both GSH and glucose concentrations were adjusted for age and the occurrence of diabetes with the use of ANCOVA. The significance of changes was estimated with 2-way ANOVA and the post-hoc Fisher's LSD test. For glucose: the effect of CAD, $p < 0.01$; the effect of GSH stratification, $p < 0.05$; CAD, low GSH > CAD, high GSH, $p < 0.02$. For GSH: the effect of CAD, p = 0.325; the effect of glucose stratification, $p < 0.03$.

and fasting glycemia demonstrated a significant association in the cohort of all the study participants (both non-CAD and CAD) (r = -0.232; p = 0.014). No significant association between plasma GSH and fasting glycemia was found in the non-CAD controls (r = -0.190; p = 0.169). In the group of CAD-affected subjects, plasma GSH concentrations and glycemia were significantly negatively correlated (r = -0.328; p = 0.011) (Fig. 2A). In the CAD-affected patients, but not in the control subjects, the occurrence of type 2 diabetes mellitus significantly discriminated plasma GSH levels (r = -0.125; p = 0.350, between GSH and glucose adjusted for the occurrence of diabetes) (Fig. 2).

However, further subdivision of CAD- and DM-affected patients into subgroups according to the degree of impairment of glucose metabolism – normal glycemia (<88 mg/dL), higher normal glycemia (89–99 mg/dL), impaired fasting glycemia (100–125 mg/dL), diabetes mellitus (>126 mg/dL) – revealed a significant association between glycemia and plasma GSH levels only in the DM group ($r_P$ = -0.297; p < 0.05), pointing to the significant role of DM in the occurrence of a GSH-glucose relationship. Further support for this hypothesis may be derived from the correlation calculus with and without standardization for CAD and DM. The overall association between GSH and glucose, without standardization for CAD or DM, was $r_P$ = -0.236 (p < 0.02). When another variable was added to the model (CAD or DM), it appeared that only the first resulted in the maintenance of this significant association: $r_{Psemipartial}$ = -0.265 (p < 0.005) for the GSH-glucose correlation standardized for CAD and $r_{Psemipartial}$ = -0.119 (p = 0.199) for the GSH-glucose correlation standardized for DM. This clearly indicates that in both the CAD and non-CAD participants, the association between GSH and glucose is maintained, while DM reorients the extent of this association: it is maintained for DM patients, while it disappears for non-DM patients.

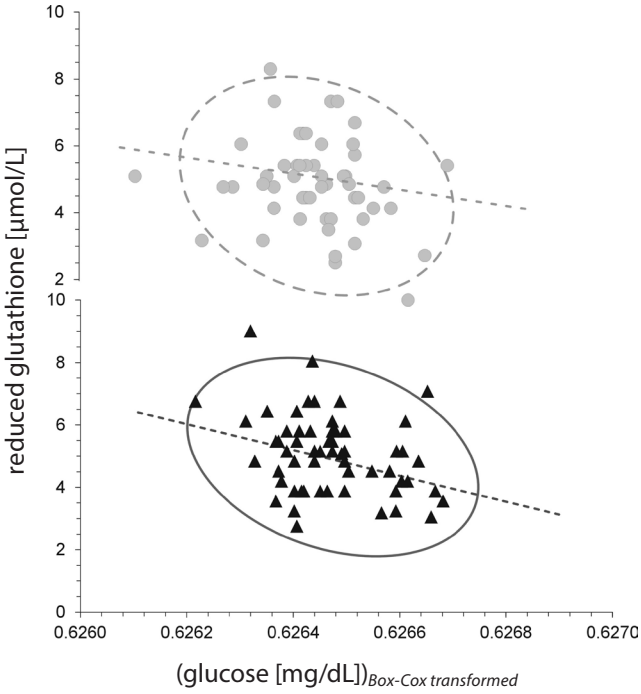


Fig. 2. Associations between plasma reduced glutathione (GSH) and fasting glycemia in non-CAD controls and CAD patients

Correlations between the concentration of GSH and fasting glycemia in non-CAD controls (grey circles) and CAD patients (black triangles), assessed by Pearson's linear correlation on Box-Cox transformed data. Both GSH and glucose concentrations were adjusted for age and the occurrence of diabetes with the use of ANCOVA. Scatter ellipses were estimated with the use of 95% CI. Regression equations were: 1630.9–2595.9x for non-CAD (r = -0.190; p = 0.169) and 2554.3–4069.3x for CAD patients (r = -0.328; p = 0.011); for both pooled groups: 1842.6–2933.4x for CAD patients (r = -0.232; p = 0.014).

## Concentrations of sulfhydryl and carbonyl groups in blood plasma proteins and their association with levels of low-molecular-weight thiols in non-CAD and CAD patients

To evaluate the pro-oxidant status of the peripheral blood plasma of the recruited subjects, the levels of carbonyl groups and free protein sulfhydryl groups in blood plasma proteins were measured. The concentrations of plasma protein carbonyls were not significantly different between the non-CAD and CAD patients: 0.10 (0.08–0.14) µmol/mg of plasma protein in non-CAD subjects compared to 0.11 (0.08 – 0.19) µmol/mg in CAD-affected subjects (p > 0.05 in the Mann-Whitney U test). Likewise, the levels of free sulfhydryl groups in plasma proteins were not different between non-CAD and CAD individuals (28.07 [19.51–41.17] µmol/mg of plasma protein vs 27.72 [22.50–45] µmol/mg in non-CAD and CAD-affected patients, respectively; p > 0.05 in the Mann-Whitney U test).

Neither marker of oxidative damage to blood plasma proteins was significantly associated with plasma levels of any of the low-molecular-weight thiols tested (GSH, Hcy, Cys or CysGly – data not shown) nor with glycemia, regardless of the inclusion or exclusion of DM-affected subjects.

# Discussion

GSH seems to play some role in controlling plasma glucose levels and it reduces the progression of some hyperglycemia-associated cardiovascular complications, such as hypertension.[18] It is possible that the development of cardiovascular complications might be facilitated in patients with higher glycemia and lower GSH concentrations, and these complications might therefore be more probable than in individuals with higher GSH.

The inverse relationship between plasma GSH and glucose concentrations in CAD reported in the present study is in agreement with results showing that hyperglycemia induces oxidative stress due to reductions of the pool of antioxidants like vitamin E, uric acid and vitamin C.[19,20] On the basis of the present results, as well as those shown

by others, it can be suggested that – very much alike α-tocopherol, ascorbic acid and uric acid – GSH is very likely to be decreased by higher concentrations of glucose, as demonstrated in the CAD subjects in the current study, especially those with concomitant T2DM.[21] Tessier et al. noted that the GSH/GSSG (glutathione disulfide) ratio is significantly lower in diabetic patients compared to healthy controls, either at baseline or after a glucose challenge.[20] This clearly supports the current authors' assumption that the inverse association between glycemia and plasma GSH levels derives from the negative influence of high glucose on GSH levels. Hence, it can be confirmed that increased glucose concentrations are associated with decreased plasma GSH levels, and that this association appears to be independent of the concomitance of CAD. This conclusion, however, should be treated with caution, since the subpopulations of diabetic patients constituted only part of the non-CAD and CAD individuals in the present study (33.8% of the controls and 57.6% of the CAD subjects).

The study shows that the stratification of the CAD patients according to plasma GSH levels significantly differentiates this group into those with lower and higher glucose. On the other hand, however, the inverse operation, i.e., the stratification according to fasting glucose levels, also discriminates the CAD patients into those with significantly lower and those with significantly higher plasma GSH concentrations. Thus, plasma GSH levels and fasting glycemia may simply be considered a 2-way interplay: high glucose decreases the concentrations of GSH, while GSH reduces fasting glycemia. The authors therefore suggest, probably for the first time, that, contrary to previous reports, it is also probable that GSH may be the key factor determining glucose metabolism, and not only that glucose is a factor contributing to a diminished GSH pool. This conclusion may be supported by results demonstrating that an increased GSH/GSSG ratio, like that induced by vitamin E supplementation, positively associates with whole-body glucose disposal (WBGD) in hypertensive patients.[22]

This finding also implies that the link between GSH and glycemia may be not 1-directional but 2-way: high plasma glucose depletes the plasma GSH concentration, but an increased GSH concentration also improves glucose metabolism. Since GSH is a potent antioxidant, it seems reasonable to expect that lowering the GSH concentration should be connected with the presence of abundant markers of oxidative damage to biomacromolecules. Nevertheless, no significant increase in the levels of plasma protein carbonyls or decreased plasma protein free sulfhydryls was found in subjects with reduced concentrations of GSH and higher glycemia. This suggests that oxidative stress should not be considered the main mediator of the impact of glycemia on GSH and vice versa.

The present results should be considered with caution, since the papers cited present results from samples originating only from diabetic patients and mainly concern GSH concentrations inside erythrocytes rather than in blood plasma, whereas the results of the current study are all from samples of peripheral blood plasma in subgroups including some patients with diabetes mellitus. The authors can suggest that the distinct associations between blood glucose and GSH are less evident in subjects with apparently undisturbed (nondiabetic) glucose metabolism.

Thus, observed correlations between fasting glycemia and plasma GSH levels found in CAD-affected patients are strongly associated with coexistence of T2DM.

The inverse relationships between blood GSH and glucose offer some potentially interesting therapeutic clues. Strict control of GSH, or more broadly a proper balance of all low-weight thiols in human tissue fluids, may potentially significantly decrease the progression of CAD through the reduction of glucose levels. One promising compound in this regard seems to be N-acetylcysteine, a known precursor of GSH that has been used successfully in the treatment of a few diseases, including vascular disturbances.[23–25]

The results presented in this paper should be treated with caution for at least 2 reasons. Firstly the study involved quite few control and CAD-affected subjects, so the results certainly need to be reevaluated with a higher number of patients. Secondly as it can be seen in Table 1, the patients suffering from CAD were taking different drugs, often simultaneously. Thus, it cannot be excluded that the observed relationship between glucose and GSH should not be ascribed only to CAD and DM, but may also be at least partly a result of exposure to certain drugs and their metabolites. This issue should be also clarified in further studies.

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# Dietary patterns and breast or lung cancer risk: A pooled analysis of 2 case-control studies in north-eastern Poland

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## Abstract

**Background.** Breast cancer in women and lung cancer in men are the most prevalent cancers in Poland and worldwide. Evidence of the impact of food groups and nutrients on the risk of breast and lung cancer is limited and lacking conclusions. Studies on food consumption and breast or lung cancer are limited.

**Objectives.** Assessment of the association between dietary patterns and the prevalence of breast and lung cancers in adult Poles.

**Material and methods.** The study involved a pooled analysis of 2 case-control studies on 320 subjects aged 50–70 years from north-eastern Poland (160 women, 160 men). Breast cancer cases in 80 women and lung cancer cases in 80 men were diagnosed. The food consumption frequency for 21 selected foods was collected using the Questionnaire of Eating Behaviors (QEB). Principal component analysis and multiple logistic regression analysis were used. The odds ratio (OR) and 95% confidence interval (95% CI) were calculated.

**Results.** Three dietary patterns (DPs) were identified: 'Prudent', 'Processed & fast food', and 'Traditional Polish'. In the pooled analysis for both cancers, the ORs were from 0.35 (95% CI: 0.20–0.61; $p < 0.05$ with adjustment for age) to 0.48 (95% CI: 0.26–0.88; $p < 0.05$ with adjustment for age, socioeconomic status index, physical activity, smoking, and abuse of alcohol) in the upper tertile of the 'Prudent' DP in comparison to the absence of cancers (OR = 1.00). The ORs of both cancers were 1.83 (95% CI: 1.06–3.16; $p < 0.05$ with adjustment for age) in the upper tertile of the 'Processed & fast food' DP. The ORs of both cancers for the 'Traditional Polish' DP were insignificant.

**Conclusions.** In the pooled analysis, a strong inverse relation was found between the 'Prudent' dietary pattern, characterized by higher frequency of dairy, fruit, vegetables, wholemeal bread, fish and juices consumption, and breast or lung cancer prevalence, irrespective of age, socioeconomic status, physical activity, smoking, alcohol abuse, and type of cancer in Polish adults from north-eastern Poland.

**Key words:** lung cancer, adults, breast cancer, PCA, dietary patterns

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# Introduction

In industrialized countries, cancers are the second leading cause of death in humans, just after cardiovascular diseases.[1] Breast cancer in women and lung cancer in men are the most prevalent cancers in Poland and worldwide.[1] In Poland, in 2010, breast cancer accounted for 22% of all diagnosed cancers in women and lung cancer accounted for 21% of all cancers in men.[2] Out of the 16 regions in Poland, Warmia and Mazury had the highest incidence of lung cancer in men and was 6$^{th}$ in terms of the incidence of breast cancer in women in 2010.[2] The highest lung cancer mortality was recorded in men aged over 50 years old, while 50% of cases occurred after 65 years of age.[2] The highest mortality of breast cancer in women was recorded in peri- and postmenopause, at the age of 50–69 years.[2] Recently, an increase has been observed in the incidence of breast cancer in women aged 20–49 years. In Poland, the number of cases of breast cancer per 100,000 women increased from 20 in 1980 to 34 in 2010.[2]

The development of cancer in the human body depends on the interactions between the immune system, individual genetic predisposition and outside environmental factors.[3] From among the modifiable environmental factors, lifestyle is very important, including nutrition and quality of food consumed, as well as the degree of environmental pollution, region of residence and related social and cultural conditions.[1,4] It is estimated that the role played by diet in cancer development, depending on the location, may be at the level of 10–70%.[3] Convincing evidence has only been obtained for alcoholic drinks as a factor increasing the risk of breast cancer and for beta-carotene supplements for smokers as a factor increasing the risk of lung cancer.[1,5] Fruit and food containing carotenoids probably decrease the risk of lung cancer.[1] There is limited evidence suggesting that non-starchy vegetables, foods containing selenium and quercetin decrease, while red meat, processed meat, total fat, butter and retinol supplements (for smokers only) increase the risk of lung cancer.[1] Evidence of the impact of other food groups and nutrients on the risk of breast and lung cancer is limited, and no conclusions have yet been drawn.[1,5]

Because of the complex character of the daily diet, apart from estimating the impact of the consumption of individual food groups or nutrients on cancer incidence, it is important to assess food consumption comprehensively. One of the generally accepted ways of assessing the type of most commonly consumed foods is by identifying dietary patterns.[6] Currently, there are no conclusive results of research on the effects of nutrition and dietary patterns on the prevalence of breast and lung cancers, especially among Polish research in a regional perspective. Knowledge of nutritional factors associated with the risk of cancer growth is very important, both in primary and secondary prevention of cancer diseases. The similar epigenetic mechanisms of breast and lung cancers indicate common dietary causes.[7]

To provide a more precise evaluation of the association between dietary patterns and the prevalence of breast and lung cancers in adult Poles, a pooled analysis of 2 case-control studies in north-eastern Poland was performed.

# Material and methods

## Ethical considerations

These studies were approved by the Bioethics Committee of the Faculty of Medical Sciences, University of Warmia and Mazury in Olsztyn (Poland), on October 2, 2013 (resolution No. 29/2013). All participants gave their voluntary and written consent to take part in the studies and were informed that the information obtained was confidential and used only for scientific purposes.

## Study design and sample characteristics

These studies were conducted in years 2013–2015 among adults from the Warmia and Mazury region in north-eastern Poland. The main inclusion and exclusion criteria of the sample collection and study design are shown in Fig. 1. All subjects had current results (obtained not earlier than 6 months before inclusion in the study) of ultrasonography (USG) and/or mammography of the breast (in women), and of a digital X-ray examination (RTG) and/or a computer tomography and/or bronchoscopy of the chest (in men). Subjects with breast or lung cancer, confirmed by a biopsy and/or histopathology, were included in the cancer sample (160 patients, including 80 women and 80 men, aged 50–70 years), and those without cancer were included in the control sample (160 patients, including 80 women and 80 men, aged 50–70 years). The control sample was matched in size, age and gender to the cancer sample. The 15 cases of non-malignant breast cancer in women were excluded (Fig. 1). In the end, the cancer-control sample involved 320 subjects, aged 50–70 years (61.2 ±4.7). The characteristics of the cancer and control samples are shown in Table 1.

The cancer and control samples were chosen in a non-random and convenient selection. Patients with breast cancer were recruited at the surgical oncology ward of the Ministry of Internal Affairs Hospital with the Warmia and Mazury Oncology Center in Olsztyn. Patients with lung cancer were recruited at the pulmonary and oncology hospital wards in the Independent Public Complex of Tuberculosis and Lung Diseases in Olsztyn. The control sample consisted of women who came for breast screening at the Center for Prevention and Breast Diagnostics in Olsztyn, and men who came for lung screening at selected health clinics in the Warmia and Mazury region. All participants were informed of the study aim and signed the consent form to participate in the study.

**Table 1.** Cancer and control sample characteristics (%)

| Category | Cancer-control sample | Cancer sample | Control sample | p-value |
|---|---|---|---|---|
| Size (n) | 320 | 160 | 160 | – |
| Gender | | | | |
| female | 50.0 | 50.0 | 50.0 | ns |
| male | 50.0 | 50.0 | 50.0 | |
| Age (years*) | 61.2 ±4.7 | 61.8 ±4.8 | 60.6 ±4.6 | 0.0133 |
| BMI (kg/m$^2$*) | 27.7 ±4.8 | 27.6 ±5.1 | 27.9 ±4.6 | ns |
| Place of residence | | | | |
| village | 29.7 | 35.0 | 24.4 | ns |
| town (<20,000 inhabitants) | 24.4 | 22.5 | 26.3 | |
| town (20,000–100,000 inhabitants) | 23.8 | 21.3 | 26.3 | |
| city (>100,000 inhabitants) | 22.2 | 21.3 | 23.1 | |
| Education level | | | | |
| primary | 19.7 | 27.5 | 11.9 | 0.0002 |
| secondary | 63.1 | 61.3 | 65.0 | |
| higher | 17.2 | 11.3 | 23.1 | |
| Economic situation | | | | |
| below the average | 18.1 | 23.1 | 13.1 | 0.0367 |
| average | 69.1 | 66.9 | 71.3 | |
| above average | 12.8 | 10.0 | 15.6 | |
| SES index[1] | | | | |
| low | 14.4 | 20.6 | 8.1 | 0.0034 |
| average | 38.8 | 38.8 | 38.8 | |
| high | 46.9 | 40.6 | 53.1 | |
| Physical activity at work[2] | | | | |
| low | 50.6 | 62.5 | 38.8 | 0.0001 |
| average | 34.7 | 27.5 | 41.9 | |
| above average | 14.7 | 10.0 | 19.4 | |
| Physical activity in leisure time[3] | | | | |
| low | 26.9 | 30.6 | 23.1 | 0.0495 |
| average | 60.9 | 61.3 | 60.6 | |
| above average | 12.2 | 8.1 | 16.3 | |
| Overall physical activity[4] | | | | |
| low | 51.3 | 63.8 | 38.8 | <0.0001 |
| average | 44.1 | 32.5 | 55.6 | |
| above average | 4.7 | 3.8 | 5.6 | |
| Smoking currently | | | | |
| no | 65.0 | 60.6 | 69.4 | ns |
| yes | 35.0 | 39.4 | 30.6 | |
| Smoking in the past | | | | |
| no | 27.8 | 20.6 | 35.0 | 0.0214 |
| yes (<5years) | 5.3 | 6.3 | 4.4 | |
| yes (5–10years) | 5.3 | 4.4 | 6.3 | |
| yes (>10years) | 61.6 | 68.8 | 54.4 | |
| Abuse of alcohol[5] | | | | |
| no | 94.4 | 90.0 | 98.8 | 0.0163 |
| yes | 5.6 | 10.0 | 1.2 | |

n – sample number; % – sample percentage; * expressed as median and quartile deviation; the level of significance was assessed by Kruskal-Wallis test, $p \leq 0.05$; SES – socioeconomic status; [1] calculated on the basis of place of residence, education level and declared economic situation (description in the Material and methods section); [2] physical activity at work: low – more than 70% of working time spent sedentary; average – approx. 50% of working time spent sedentary and 50% of working time spent in an active manner; above average – approx. 70% of working time spent in an active manner or physical work related to great exertion; [3] physical activity in leisure time: low – sedentary for most of the time, watching TV, reading books, walking 1–2 h per week; average – walking, cycling, gymnastics, gardening, light physical activity performed 2–3 h per week; above average – cycling, jogging, gardening, sport activities involving physical exertion performed more than 3 h weekly; [4] after combining data based on declared physical activity at work and physical activity in leisure time; [5] at least 1 bottle (0.5 L) of beer, or 2 glasses of wine (300 mL), or 2 drinks (300 mL), or 2 glasses of vodka (60 mL) per day.

## Food frequency

Information on the consumption of selected 21 food groups (Table 2) in the last 12 months before involvement in this study was obtained by the food frequency method, using an interviewer-administrated QEB questionnaire (Questionnaire of Eating Behaviors) of great internal reliability with Fleiss' kappa from 0.64 to 0.84.[8,9] The frequency of consumption was expressed in 6 categories: never, 1–3 times per month, once per week, several times per week, daily, several times per day. The frequency of consumption was then expressed as times/day and assigned the following values: never = 0; 1–3 times per month = 0.06; once per week = 0.14; several times per week = 0.5; daily = 1; several times per day = 2.

## Confounders

Respondents were asked about 3 single factors of their socioeconomic status (SES). Numerical values were assigned to each response category as follows (in brackets):

- place of residence: village (1), town with <20,000 inhabitants (2), town with 20,000–100,000 inhabitants (3), city with >100,000 inhabitants (4);
- educational level: primary (1), secondary (2), higher (3);
- economic status (self-declared): below average (1), average (2), above average (3).

The SES index was calculated as the sum of the values assigned to the individual response categories to each SES factor. The SES index values were logarithmized, and then

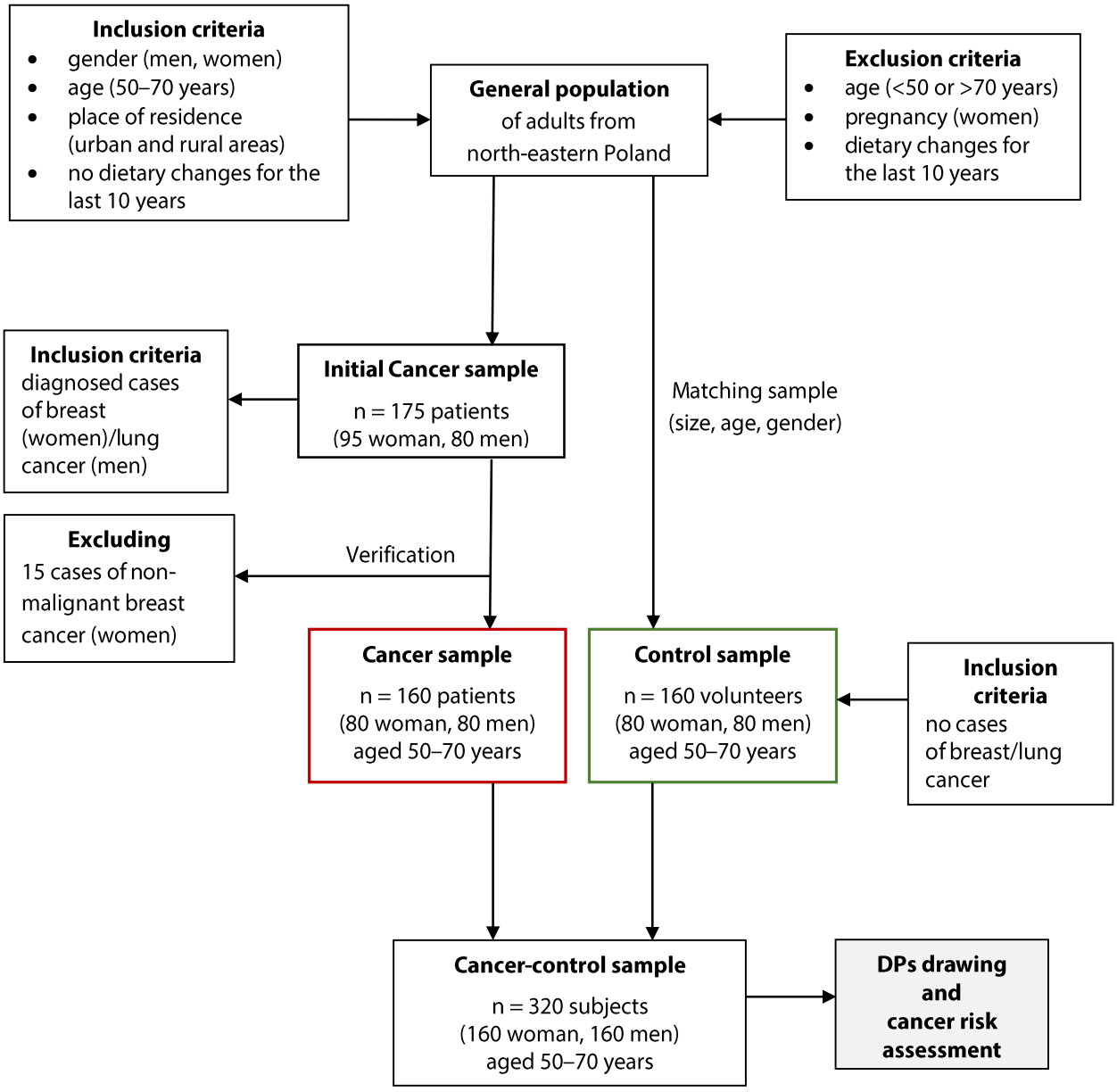


Fig. 1. Flow chart of sample collection and study design

the tertiles of the SES were created to identify respondents with low, average, and high SES.

Respondents were asked about their physical activity at work. Numerical values were assigned to each response category as follows (in brackets): low – more than 70% of working time spent sedentary (1); average – approx. 50% of working time spent sedentary and 50% of working time spent in an active manner (2); above average – approx. 70% of working time spent in an active manner or physical work related to great exertion (3).[10] Respondents were also asked about their physical activity in leisure time. Numerical values were assigned to each response category as follows (in brackets): low – sedentary for most of the time, watching TV, reading books, walking 1–2 h per week (1); average – walking, cycling, gymnastics, gardening, light physical activity performed 2–3 h per week (2); above average – cycling, jogging, gardening, sport activities involving physical exertion performed more than 3 h weekly (3).[10] The data based on the physical activity declared at work and in leisure time was combined, then 3 categories of overall physical activity were created, with numerical values assigned as follows (in brackets): low (1); average (2); above average (3).[11]

Respondents were asked about smoking currently: no (1), yes (2); smoking in the past: no (1), yes <5years (2), yes 5–10years (3), yes >10years (4); and abuse of alcohol: no (1), yes (2), defined as intake of at least 1 bottle (0.5 L) of beer, or 2 glasses of wine (300 mL), or 2 drinks (300 mL), or 2 glasses of vodka (60 mL) per day.

## Statistical analysis

For the cancer-control sample, the consumption frequency (times/day) of 21 selected food groups was expressed as a mean value, and then was standardized and included in the Principal Component Analysis (PCA) with varimax rotation.[12] Three dietary patterns (DPs) were identified a posteriori based on the factor loadings for standardized mean values of food consumption frequency and Scree plot for eigenvalues of factors, and the sum of explaining the variance (Fig. 1). The value |0.3| was accepted as the cut-off point of factor loadings, and the tertile intervals were calculated for each of the 3 dietary patterns. We compared the percentage distribution of the prevalence of breast or lung cancer in tertiles of DPs by Pearson $\chi^2$ test with Yates' correction as necessary. The prevalence of breast or lung cancer as a categorical independent dichotomous variable in the upper and middle tertile in comparison to the bottom tertile of DPs was assessed. A logistic regression analysis was performed. The odds ratio (OR) and 95% confidence interval (95% CI) were calculated. The reference groups were subjects without cancer (OR = 1.00) and subjects in the bottom tertile of each DPs (OR = 1.00). Five models were created: model 1 – without adjustment for confounding variables; model 2 – with adjustment for age as a continuous variable; model 3 – with adjustment for age and SES index; model 4 – with adjustment for age, SES index, overall physical activity, smoking in the past and abuse of alcohol; and model 5 – with adjustment for age, SES index, overall physical activity, smoking in the past, abuse of alcohol and type of cancer. The significance of the odds ratio was assessed by Wald's statistics.[12] The statistical analysis was performed using STATISTICA v. 10.0 PL (StatSoft Inc., USA, Tulsa; StatSoft Polska, Kraków). A p-value <0.05 was considered statistically significant.

Table 2. The values of factor loadings for selected food groups in dietary patterns – PCA

| Food groups# | Dietary Patterns | | |
|---|---|---|---|
| | 'Prudent' | 'Processed & fast food' | 'Traditional Polish' |
| Curd cheese (including homogenized cheese) | **0.67** | | |
| Fermented milk drinks | **0.60** | | |
| Fruit | **0.55** | | |
| Wholemeal bread | **0.52** | -0.26 | -0.24 |
| Vegetables | **0.47** | -0.23 | 0.37 |
| Fish and fish dishes | **0.47** | | |
| Cheese (including cream cheese) | **0.47** | 0.26 | 0.27 |
| Milk | **0.37** | | |
| Fruit, vegetable or vegetable-fruit juices | **0.36** | | 0.31 |
| Soups (instant, ready to eat) | | **0.71** | |
| Canned meat, canned fish or canned vegetable-meat | | **0.58** | |
| Alcoholic drinks | | **0.53** | |
| Fast food | | **0.48** | |
| Potatoes | | | **0.65** |
| Sweets, confectionery | | | **0.58** |
| Sweetened carbonated beverages | | | **0.39** |
| Meat and meat dishes | 0.21 | | **0.30** |
| Fried foods | | 0.24 | 0.22 |
| Canned vegetables or fruit, pickles | 0.23 | | 0.20 |
| Legume-based dishes | 0.22 | | |
| Energy drinks | | | -0.16 |
| **Share in explaining the variance (%)** | **13** | **8** | **7** |

# PCA was performed on standardized variables (frequency of consumption expressed as times/day) for cancer-control sample (n = 320).

# Results

## Food consumption frequency and dietary patterns

These studies found 3 main dietary patterns. The 'Prudent' DP was positively correlated with the frequency of consumption of: curd cheese (r = 0.67), fermented milk drinks (r = 0.60), fruit (r = 0.55), wholemeal bread (r = 0.52), vegetables, fish and fish dishes, cheese (r = 0.47), milk (r = 0.37), fruit, vegetable or vegetable-fruit juices (r = 0.36) (Table 2). The 'Processed & fast food' DP was positively correlated with the consumption frequency of: instant soups, concentrated ready-made soups (r = 0.71), canned meat, fish or canned vegetable-meat (r = 0.58), alcoholic drinks (r = 0.53), and fast food (r = 0.48) (Table 2). The 'Traditional Polish' DP was positively correlated with the frequency of consumption of: potatoes (r = 0.65), sweets (r = 0.58), sweetened carbonated beverages (r = 0.39), meat and meat dishes (r = 0.30) (Table 2). The shares in explaining the variance for 'Prudent', 'Processed & fast food' and 'Traditional Polish' DPs were 13%, 8% and 7%, respectively (Table 2).

## Dietary patterns and breast or lung cancer prevalence

There was a statistically significant decrease of the percentage of breast or lung cancer cases in tertiles of the 'Prudent' DP (p = 0.0010) (Table 3). In the upper tertile

**Table 3.** Dietary patterns and the prevalence of breast or lung cancer

| Dietary patterns | Cancer (% of the sample) | | | p-value | p-trend |
|---|---|---|---|---|---|
| | tertiles of dietary patterns | | | | |
| | bottom | middle | upper | | |
| 1 – 'Prudent' (curd cheese, fermented milk drinks, fruit, wholemeal bread, vegetables, fish and fish dishes, cheese, milk, fruit/vegetable/vegetable-fruit juices) | (n = 106) 41.3[a] | (n = 108) 34.4 | (n = 106) 24.4[a] | 0.0010 | ns |
| 2 – 'Processed&fast food' (instant soups/concentrated, ready-made soups, canned meat/fish/vegetable-meat, alcoholic drinks, fast food) | (n = 107) 28.8 | (n = 106) 32.5 | (n = 107) 38.8 | ns | ns |
| 3 – 'Traditional Polish' (potatoes, sweets, sweetened carbonated beverages, meat and meat dishes) | (n = 106) 33.8 | (n = 108) 35.6 | (n = 106) 30.6 | ns | ns |

n – sample size; ns – the differences were not statistically significant (the level of significance was assessed by Pearson's $\chi^2$ test, $p \leq 0.05$); [a] statistically significant differences between the pairs of dietary pattern tertiles, $p \leq 0.05$.

**Table 4.** Odds ratio (OR) and 95% confidence interval (95% CI) of breast or lung cancer prevalence in relation to dietary patterns

| Dietary patterns | OR (95% CI) | | | | | |
|---|---|---|---|---|---|---|
| | without cancer (n = 160) | cancer (n = 160) | | | | |
| | | model 1 | model 2 | model 3 | model 4 | model 5 |
| 1 – 'Prudent' | | | | | | |
| bottom tertile | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| middle tertile | 1.00 | 0.63 (0.36–1.09) | 0.59 (0.34–1.04) | 0.60 (0.34–1.05) | 0.70 (0.39–1.27) | 0.64 (0.35–1.16) |
| upper tertile | 1.00 | 0.35*** (0.20–0.62) | 0.35*** (0.20–0.61) | 0.38*** (0.21–0.66) | 0.48* (0.26–0.88) | 0.41** (0.22–0.78) |
| 2 – 'Processed & fast-food' | | | | | | |
| bottom tertile | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| middle tertile | 1.00 | 1.28 (0.74–2.20) | 1.29 (0.74–2.25) | 1.22 (0.69–2.16) | 1.16 (0.64–2.10) | 1.10 (0.61–1.97) |
| upper tertile | 1.00 | 1.83* (1.06–3.15) | 1.83* (1.06–3.16) | 1.66 (0.95–2.90) | 1.53 (0.82–2.86) | 1.60 (0.87–2.97) |
| 3 – 'Traditional Polish' | | | | | | |
| bottom tertile | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 | 1.00 |
| middle tertile | 1.00 | 1.08 (0.63–1.84) | 1.08 (0.63–1.87) | 1.12 (0.64–1.96) | 1.10 (0.62–1.97) | 1.08 (0.61–1.91) |
| upper tertile | 1.00 | 0.83 (0.48–1.42) | 0.92 (0.53–1.60) | 0.79 (0.44–1.42) | 0.75 (0.40–1.38) | 0.68 (0.38–1.21) |

model 1 – without adjustment for confounding variables; model 2 – with adjustment for age; model 3 – with adjustment for age and SES index; model 4 – with adjustment for age, SES index, overall physical activity, smoking in the past and abuse of alcohol; model 5 – with adjustment for age, SES index, overall physical activity, smoking in the past, abuse of alcohol and type of cancer; the level of significance was assessed by Wald's test, * $p \leq 0.05$, ** $p < 0.01$, *** $p < 0.001$.

of the 'Prudent' DP in comparison to the bottom tertile, there was a significantly lower percentage of breast or lung cancer cases reported (24.4% vs 41.3%) (Table 3). There were no significant differences reported in the percentage of breast or lung cancer cases within the tertiles of the 'Processed & fast food' DP and the 'Traditional Polish' DP (Table 3).

Two out of the 3 identified dietary patterns, 'Prudent' and 'Processed & fast food', showed a significant association with the prevalence of breast or lung cancers in a logistic regression analysis (Table 4). Subjects in the upper tertile of the 'Prudent' DP in comparison to the bottom tertile had a lower risk of breast or lung cancers, from 52% (model 4: OR = 0.48; 95% CI: 0.26–0.88; $p < 0.05$) to 65% (model 2: OR = 0.35; 95% CI: 0.20–0.61; $p < 0.001$) (Table 4). Subjects in the upper tertile of the 'Processed & fast food' DP in comparison to the bottom tertile had almost 2-fold higher risk of breast or lung cancers (model 2: OR = 1.83; 95% CI: 1.06–3.16; $p < 0.05$) (Table 4) . There was no significant association reported between the 'Traditional Polish' DP and the risk of breast or lung cancers (Table 4).

## Discussion

This work presents the results of the first study on dietary patterns and breast or lung cancer prevalence in Poland. We found a strong inverse association between the 'Prudent' DP and the prevalence of breast or lung cancer cases, irrespective of age, socioeconomic status, physical activity, smoking, abuse of alcohol, and type of cancer as confounding variables. Inversely, the 'Processed & fast food' DP was weakly associated with an increased risk of breast or lung cancers. There was no evidence of an association between the 'Traditional Polish' DP and the prevalence of breast or lung cancer in female and male adults from north-eastern Poland.

In the study, the 'Prudent' DP was characterized by a high frequency of consumption of dairy products, fruit, vegetables, wholemeal bread, fish and juices, and significantly reduced risk of breast or lung cancer (from 52% to 65%, depending on confounders incorporated into the model). A similar trend has been observed in studies conducted in many countries around the world. However, not all studies adjusted the results for the many confounders such as alcohol consumption, smoking or physical activity, as in our study. The DPs characterized by high consumption of fruit and vegetables, such as 'Plant-based', 'Fruit and salad' and 'Antioxidants', were associated with a 15–56% lower risk of breast cancer in women and with a 39% lower risk of lung cancer in men.[13–15] In a systematic review, in 10 out of 26 studies, a significant association was found between the 'Mediterranean' DP, comprised of vegetables, fruit, legumes, fish and olive oil, and a reduced risk of breast cancer in women on various continents (from 27% to 86%).[16] In a meta-analysis, in a combination of 8 case-control and 10 cohort studies, it was shown that the 'Prudent/Healthy' DP, rich in fruit, vegetables, poultry, fish, low-fat dairy and whole grains, reduced the risk of breast cancer by 11%.[17] In the Netherlands Cohort Study, the 'Salad vegetables' DP, comprised of vegetables, fruit, pasta, rice, poultry, fish and oil, reduced the risk of lung cancer by 25%.[18] This protective effect probably resulted from a high-quality diet, rich in bioactive compounds including specific peptides, fatty acids, phenolics, vitamins, minerals and fiber. Conversely, in a North American study, the 'Prudent' DP, comprised of low-fat dairy products, whole grains, vegetables, fruit, legumes and vegetable or fruit juices, increased the risk of breast cancer 1.42 times.[19] This result is contrary to conventional wisdom and to the results of other studies. In the USA, the 'Prudent' DP diet is relatively higher in carbohydrates and fat than the diet of 'Prudent/Healthy' DPs in European countries. In some studies, there was no association found between breast cancer risk and the 'Cereals/Milk/Dairy' DP, 'Vegetable' DP and 'Prudent' DP rich in low-fat dairy products, juices, whole grains, vegetables and fruit.[13,20,21] The differences in these associations could result from differences in the study designs, study populations, secular trends in food supply or different definitions of 'Prudent/Healthy' diet and characteristics for their foods.[19]

In these studies, the 'Processed & fast food' DP was characterized by a higher frequency of consumption of alcoholic drinks and processed food such as fast food, instant soups and canned goods, which increased the risk of breast or lung cancers almost 2 times, but this relation was weaker and disappeared after the adjustment for many confounders. Many studies performed in different countries such as Germany, Italy and Korea, did not report a statistically significant association between the 'Western' DP and breast cancer.[22–24] However, the 'Drinkers' DPs, including alcoholic beverages such as wines, beers and spirits, were associated with increased risk of breast cancer, from 12% in the California Teachers' Study, through 21% in a meta-analysis of 4 studies and 40% in Uruguayan women, to 2.5 times in French women.[13,17,25,26] Alcohol is a proven risk factor for breast cancer.[1] The 'Western/Unhealthy' DPs, which were characterized by a high consumption of processed meat, fast food, canned goods, mayonnaise, butter, high-fat dairy, refined grains, sweets and alcoholic beverages, increased the risk of breast cancer from 20% in the French Cohort Study to 31% in a meta-analysis.[17,27] In a Spanish study, the 'High-meat' DP, rich in processed meat, fried red meat and alcoholic beverages, increased the risk of breast cancer approx. 3.5 times.[21] In the Netherlands Cohort Study, the 'pork, processed meat and potatoes' DP increased the risk of lung cancer 2.67 times.[18] However, this negative effect probably resulted from a diet rich in foods with high energy density, with high glycemic index (GI) or glycemic

load (GL) such as processed food, because of their high fat and sugar content.[28] The differences in associations could result, as mentioned above, from differences in the study designs, study populations, trends in food supply or different definitions of 'Western/Unhealthy' diet and characteristics of their food.[19]

In our studies, the 'Traditional Polish' DP was characterized by a higher frequency of potatoes, sweets, beverages, and meat consumption, mainly as fried foods, which are typical foods in the Polish diet. There was no significant effect found for the 'Traditional Polish' DP on the prevalence of breast or lung cancers in Polish adults from north-eastern Poland. The 'Traditional Polish' DP included both food with potentially beneficial effects on health such as potatoes, and food with potentially negatively effects on health such as fried meat, sweets and sweetened carbonated beverages, which may determine its neutral character in relation to the prevalence of cancer cases. As in our studies, in many studies there was no evidence of an association between 'Traditional' DP and risk of breast or lung cancers. Dietary patterns such as the Australian 'Meat' (meat, fried dishes, cooked potatoes and pickled vegetables), the Greek 'Meat/Potatoes', the Californian 'High-protein' (meat, fried foods and fat), and the 'Ethnic' (legumes, soy-based foods, rice and leafy vegetables) were not significantly associated with breast cancer risk.[13,14,20] To the contrary, in Asian-American women, the 'Ethnic meat/starch' DP, comprised of vegetable soups, pork, dried and salted fish and fried rice, increased the risk of breast cancer almost 1.5 times.[29] Among Uruguayan men, the 'High-meat' DP, characterised by high consumption of meat, dairy products, eggs and desserts, increased the risk of lung cancer about 3 times.[15] Some of the 'Traditional' DPs decreased the risk of breast cancer, from 22% for the 'Traditional southern US' (cooked greens, beans, legumes, mixed vegetables, fried fish and chicken) to 47% for the 'Uruguayan' (cooked red meat, cereals, cooked legumes and tubers).[30,31] Differences in associations between the 'Traditional' DPs and the prevalence of breast or lung cancers could result from the different characteristics of the typical foods in the diet of a given country/region.

The present study provides new and interesting insights regarding dietary patterns and breast or lung cancer risk. It was found that the protective effect of a diet composed of dairy, fruit, vegetables, wholemeal bread, fish and juices was stronger than the negative effects of a diet containing alcoholic drinks and processed food on cancer prevalence. This effect did not depend upon the presence of many confounders, which included age, socioeconomic status, physical activity, abuse of alcohol, smoking, and type of cancer. It may be supposed that a regular diet composed of dairy, fruit, vegetables, wholemeal bread, fish and juices may reduce the negative effects of the socioeconomic and lifestyle risk factors of cancer. Furthermore, we found this diet composition using a data-driven approach by drawing dietary patterns from a data-set.[12,32] So, there is strong evidence that such dietary patterns exist in real life and may be found within Polish adults from north-eastern Poland. It is also possible that similar dietary patterns may be found among other people living in central and eastern Europe.[33] Thus, our results may go beyond national significance. Finally, these findings may be helpful in making public dietary recommendations when improving strategies to promote a healthy diet and decrease the risk of breast and lung cancer.

## Study strengths and weaknesses

The major weakness of these studies is a lack of quantitative data regarding food and nutrient intake. However, current evidence shows the limitations of a single-nutrient component focus.[32] We collected data concerning the frequency of food consumption, which reflected the usual intake, and then identified the dietary patterns. Dietary patterns represent the overall combination of foods usually consumed, which together produce synergistic health effects.[32] The strength of the study was that we used the validated, interviewer-administrated FFQ of greater internal repeatability than the self-administrated FFQ.[9] Moreover, the prevalence of cancer incidence was confirmed by histopathology results. These studies were interdisciplinary studies including 2 scientific areas: human nutrition and medicine – oncology, which is rare in Polish studies. An interesting area of these studies was to show the dietary patterns and prevalence of breast and lung cancers in a pooled analysis across a wide area of north-eastern Poland.

# Conclusions

There was a strong inverse relation between the 'Prudent' dietary pattern and breast or lung cancer prevalence, irrespective of age, socioeconomic status, physical activity, smoking, alcohol abuse or type of cancer in Polish adults from north-eastern Poland. For cancer prevention, one should start a diet composed of dairy products, fruit, vegetables, wholemeal bread, fish and juices. Our approach is focused on the foods and overall dietary patterns that exist in the real life of people living in north-eastern Poland, not on single isolated nutrients. So, this food-based approach is better fitted to making public dietary recommendations and individual behavioural counseling.

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# The influence of vitamin D deficiency on eradication rates of *Helicobacter pylori*

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## Abstract

**Background.** *Helicobacter pylori* eradication therapy improves the healing of various gastro-duodenal diseases such as chronic gastritis and peptic ulcer, and also reduces gastric cancer incidence. Several studies have reported on risk factors other than antibiotic resistance related to *Helicobacter pylori* eradication failure.

**Objectives.** In this study, we aimed to investigate whether or not the serum levels of 25-hydroxy-vitamin D (25(OH)D) influence eradication rates of *H.pylori*.

**Material and methods.** 220 patients diagnosed with *H.pylori* gastritis using endoscopic biopsy had their 25-OH vitamin D levels measured via the electrochemiluminescence method before beginning eradication therapy of *H.pylori*. Gastric biopsies obtained at endoscopy were examined for *H.pylori* strains and histopathologic findings. All patients were treated with bismuth-containing quadruple therapy for 14 days. *H.pylori* eradication was determined via the 14C-urea breath test performed 4 weeks after the end of therapy. Based on the 25-OH vitamin D levels, the patients were divided into 2 groups: group 1 (deficient) had a vitamin D level of $<10$ ng/mL, while group 2 (sufficient) had a vitamin D level of $\geq 10$ ng/ mL.

**Results.** Eradication was successful in 170 (77.2%) patients and failed in 50 (22.7%) patients. The prevalence of 25(OH)D deficiency was 30.5%. Mean 25(OH)D levels were significantly lower in the eradication failure group compared to the successful treatment group ($9.13 \pm 4.7$ vs $19.03 \pm 8.13$; $p = 0.001$). There were significantly more patients with deficient 25(OH)D levels in the failed treatment group compared to the successful treatment group ($p = 0.001$).

**Conclusions.** Our findings suggest that 25-OH vitamin D deficiency may be considered a risk factor related to eradication failure of *H.pylori*, which may lead to a need for supplementation of vitamin D before eradication of *H.pylori*.

**Key words:** vitamin D, *Helicobacter pylori*, *Helicobacter pylori* eradication

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# Introduction

*Helicobacter pylori* (*H.pylori*) is a gram-negative bacterium that colonizes the human stomach; it is also increasingly prevalent, ranging from 25% in developed countries to 90% in developing countries.[1] *H.pylori* is a main causative factor in various gastrointestinal diseases such as chronic gastritis, peptic ulcers, mucosa-associated lymphoid tissue lymphoma (MALT) and gastric cancer.[2] *H.pylori* eradication significantly affects the treatment of both peptic ulcers and gastric lymphoma.[3] Therefore, successful eradication of *H.pylori* infection may prevent the development of gastric cancer. Clearly, it is important to eradicate this bacterium and its related risk factors.[4] Bacterial and host factors in *H.pylori* eradication therapy include antibiotic resistance, virulence factors and host-related genetic disorders (CYP2C19, IL-1B, multidrug-resistant transporter-1).[5] Host immunity also plays an important role against an infectious disease such as *H.pylori* infection. Meanwhile, vitamin D is responsible for regulating calcium and phosphorus metabolism, both of which are needed for bone formation. Beyond its well-known role in bone formation, vitamin D also has an immunomodulator role in targeting various immune cells, including monocytes, macrophages and dendritic cells, as well as T-lymphocytes and B-lymphocytes.[6] Hence, vitamin D deficiency may increase the incidence of immune system disorders and may be a risk factor for the progression of an infectious disease.[7]

Vitamin D deficiency might increase the risk of *H.pylori* infection, yet this association has yet to be evaluated. Therefore, in this study, we aimed to evaluate the association between vitamin D deficiency and the treatment of *H.pylori* infection.

# Material and methods

## Patients

Patients complaining of dyspeptic symptoms for at least 1 month underwent diagnostic esophago-gastro-duodenoscopy. All patients were non-ulcer dyspeptic patients. The study included 220 sequential patients diagnosed with *H.pylori* gastritis by endoscopic biopsy for prospective observation in a gastroenterology clinic between September 2014 and December 2015. The research excluded patients who had previously received *H.pylori* eradication treatment, vitamin D supplements, corticosteroids/immuno-suppressive treatment, antibiotics, or anti-inflammatory or acid suppressive treatment in the prior 2 months. It also excluded those with a history of systemic inflammatory or autoimmune disorders, gastric surgery, renal failure, liver cirrhosis, and malignancies. The study was planned according to the ethics guidelines of the Helsinki Declaration, informed consent was obtained from all the participants, and the research was approved by our hospital's Institutional Research Ethics Board.

## Endoscopic evaluation

Endoscopy was conducted with Olympus Evis Exera 160 videoendoscopes (Olympus America Inc., Center Valley, USA). Two biopsy specimens were obtained from the antrum and 2 from the corpus for histological examination.

## Histopathologic examination

The biopsy samples were fixed in 10% formalin before being sliced into 4–6 mm pieces, dehydrated in ethanol, embedded in paraffin wax, sectioned (5 µm thick), and stained with hematoxylin and eosin (H&E) for histological examination and Giemsa stain for *H.pylori* identification. A blinded histopathologist examined all specimens and diagnosed cases as active or chronic gastritis. The updated Sydney system was used to grade the activity of gastritis, inflammation, atrophy and *H.pylori* density.[8] Thus, mucosal atrophy was defined by the loss of glandular tissue; inflammation of gastric mucosa was defined by the presence of an inflammatory infiltrate composed of lymphocytes and plasma cells; and activity of gastric mucosa was defined by the presence of neutrophil cells at superficial or deep layers. The degree of activity, inflammation, atrophy and *H.pylori* density were classified into 4 categories, scored on a scale of 0–3 (0 = none; 1 = mild; 2 = moderate; 3 = severe).

## Treatment protocol

All infected patients were treated for 14 days with bismuth-containing quadruple eradication therapy consisting of colloidal bismuth sub-citrate 300 mg q.i.d., pantaprazole 40 mg b.i.d., tetracycline 500 mg q.i.d., and metronidazole 500 mg t.i.d.

## Confirmation of *Helicobacter pylori* eradication (14C-urea breath test)

A 14C-urea breath test was performed at least 4 weeks after treatment completion. Following overnight fasting, patients used 25 mL of water to swallow 37 kBq (1 mCi) of an encapsulated form of 14C-urea/citric acid composition (Helicap, Noster System AB, Stockholm, Sweden). Breath samples of the patients were collected with a special dry cartridge system (Heliprobe BreathCard, Noster System AB) at 10 min. Patients exhaled gently into the cartridge mouthpiece until the indicator membrane changed color from orange to yellow. The breath-card was inserted into a special small desktop Geiger-Muller counter (Heliprobe analyzer, Noster System AB), and activity was counted for 250 s. The results were expressed both as counts per minute (HCPM) and as a grade

(0 – not infected, CPM < 25; 1 – equivocal, CPM 25–50; 2 – infected, CPM > 50). This procedure adhered to manufacturer guidelines regarding the counts obtained from the cartridges, and a negative test result was defined as *H.pylori* eradication.

## Laboratory measurements

Patients who had the histopathological diagnosis of *H.pylori* infection underwent the assessment of serum 25-hydroxyvitamin D3 levels and CagA seropositivity before *H.pylori* treatment. Serum 25(OH)D3 levels were measured using an electrochemiluminescence method (Roche Diagnostics GmBH, Mannheim, Germany), with inter-assay and intra-assay coefficients of variation (CVs) of 2.4% and 5.7%, respectively. Sera obtained by centrifugation were stored at -20°C and analyzed simultaneously by technicians who were blind to group allocation. Serologic assays for specific IgG antibodies against CagA protein were analyzed by enzyme immunoassays (DIA.PRO Diagnostic Bioprobes S.r.l, Milan, Italy). CagA antibody titers (≥8 U/mL) were classified as positive, per manufacturer instructions. Vitamin D deficiency was defined as a condition in which the 25(OH)D serum level was lower than 10 ng/mL.[9] Before beginning *H.pylori* treatment patients were divided into 2 groups as follows: group 1 (vitamin D deficient) had a vitamin D level of <10 ng/mL, and group 2 (vitamin D sufficient) had a vitamin D level of ≥10 ng/ mL.

## Statistical analysis

SPSS for Windows v. 17.0 was used for the statistical analyses of our study data. Mean standard deviations (SD) were used to identify the data related to the continuous variables, and categorical variables were provided as percentages. The Kolmogorov-Smirnov normalizing test was used to determine whether the continuous variable data fit a normal distribution. The comparison of the variables with normal distribution was tested with an unpaired t-test, and the comparison of the variables without normal distribution was tested with a Mann-Whitney U test. The categorical variables were compared with Pearson's $\chi^2$ test, and a p-value <0.05 was considered statistically significant.

# Results

The study involved 220 patients. In 170 (77.2%) patients, *H.pylori* was eradicated successfully, while in 50 (22.7%) patients, eradication failed. At the end of therapy, all patients' compliance with the drug protocol was excellent. There were no significant differences between the eradication successful and eradication failure groups regarding age or sex (p = 0.54 and p = 0.44, respectively) (Table 1). We evaluated the relationship of histopathologic findings and eradication rates between the eradication successful and eradication failure groups. In the successful treatment group, the degree of activity and inflammation were significantly higher than that in the failed treatment group (both values of p = 0.001). However, no significant differences were seen between the 2 eradication groups in terms of the degree of atrophy and *H.pylori* density (both had values of p > 0.05) (Table 2). The *H.pylori* virulence marker CagA was positive in 108 (63.5%) patients in the successful treatment group, and it was positive in 8 (16%) patients in the failure treatment group. We found CagA-positive strains to have significantly higher eradication rates compared to negative ones (p = 0.001) (Table 2). We divided patients into 2 groups according to vitamin 25(OH)D status; mean vitamin 25(OH)D levels were significantly lower in the eradication failure group compared to the successful treatment group (9.13 ±4.7 vs 19.03 ±8.13; p = 0.00) (Table 1). In addition, all patients had an overall 30.5% vitamin 25(OH)D deficiency. We found 42 (84%) patients in the failed treatment group and 25 (14.7%) patients in the successful treatment group to be vitamin 25(OH)D deficient. As shown in Fig. 1, vitamin 25(OH)D deficiency was significantly higher in the failed treatment group compared to the successful treatment group (p = 0.001).

# Discussion

In this study, we found *H.pylori* eradication rates to be significantly lower in patients with low vitamin D levels. Bacterial and host-related risk factors such as antibiotic resistance, virulence factors and host-related genetic disorders are associated with *H.pylori* eradication failure.[5]

**Table 1.** Demographic and vitamin 25(OH)D level differences between the successful and failed eradication groups of *H.pylori*

| Variable | Failure (n = 50) | Successful (n = 170) | p-value |
|---|---|---|---|
| Age (mean ±SD) | 47.3 ±14.3 | 47.5 ±13.9 | 0.546 |
| Male (n, %) | 27 (54%) | 102 (60%) | 0.449 |
| Vitamin 25(OH)D (mean ±SD) | 19.0 ±8.1 | 9.1 ±4.7 | 0.001 |

**Table 2.** Histological scores and CagA seropositivity between the successful treatment group and the failed *H.pylori* eradication group (mean ±SD)

| Variable | Failure (n = 50) | Successful (n = 170) | p-value |
|---|---|---|---|
| Activity | 1.5 ±0.6 | 1.0 ±0.2 | 0.001 |
| Inflammation | 1.7 ±0.7 | 1.1 ±0.2 | 0.001 |
| Atrophy | 1.3 ±0.5 | 1.4 ±0.5 | 0.360 |
| *H.pylori* density | 1.3 ±0.5 | 1.5 ±0.7 | 0.058 |
| CagA seropositivity (n, %) | 8 (16%) | 108 (63.5%) | 0.001 |

CagA – cytotoxin-associated antigen; *H.pylori* – *Helicobacter pylori*; SD – standard deviation.

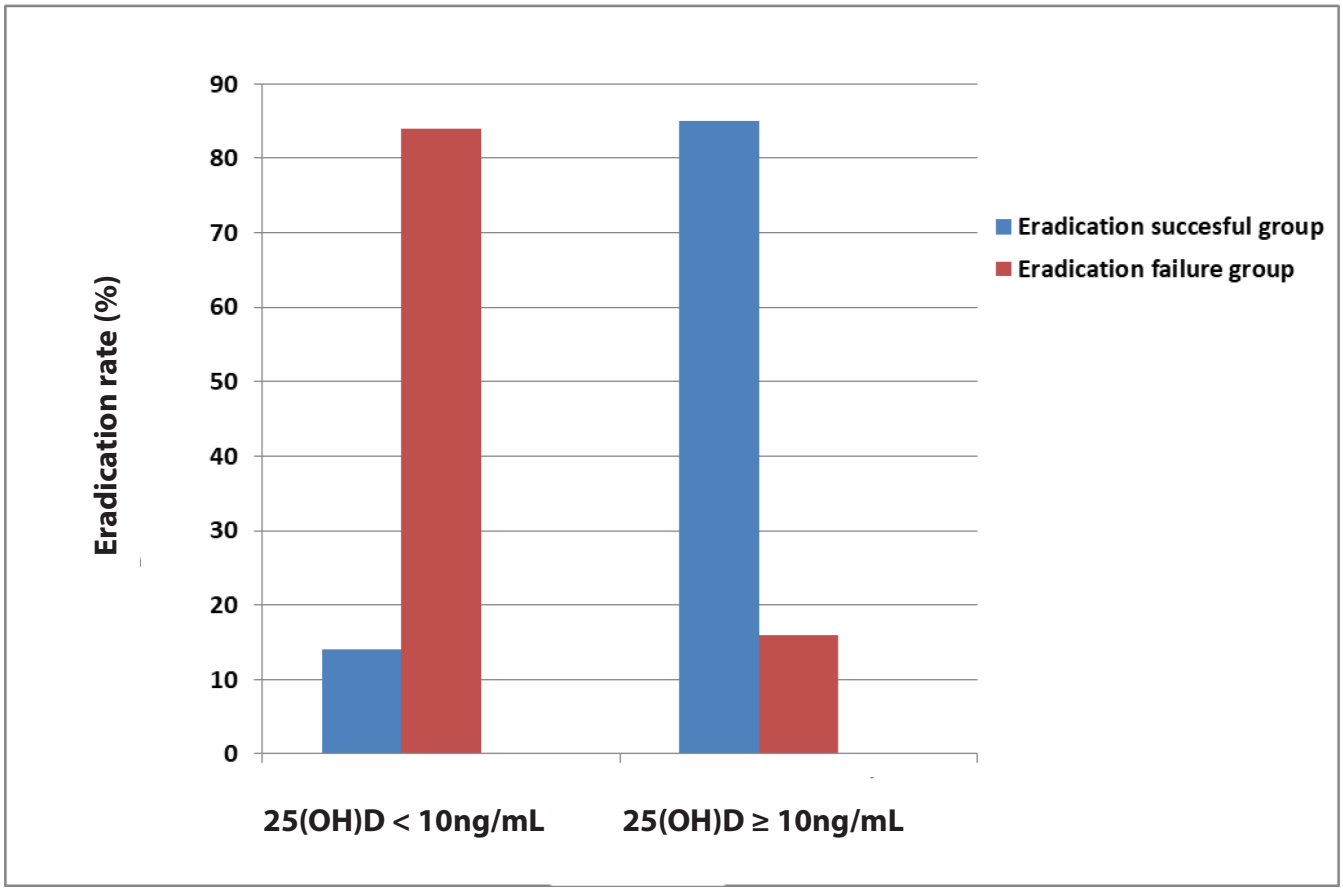


Fig. 1. Comparison of successful and failure rates of *H.pylori* eradication according to the vitamin 25(OH)D levels

Determining the antibioticresistance of *H.pylori* strains is the most important factor for achieving effective eradication therapy. Worldwide, antibiotic resistance has been reported at an increasing rate (16.2–60.7%), particularly to clarithromycin and metronidazole.[10] Increasing the susceptibility of some antibiotics, another important factor is maintaining a high intragastric pH for 24 h. Although several studies have shown that standard triple therapies are effective in most patients as a first-line treatment, we treated the patients with a bismuth-containing quadruple eradication therapy, which is recommended as a first-line treatment in regions where clarithromycin resistance is >15%.[11] CagA expression in CagA-positive *H.pylori* strains is another bacterial factor in *H.pylori* eradication therapy. Research has shown it to be associated with the host inflammatory response and an increased risk for clinical outcomes.[12] Van Doorn et al. have shown an association between lower eradication efficacy and *H.pylori* strains missing the CagA gene, while Scholte et al. did not find this relation.[13,14] The present study indicated that *H.pylori* eradication rates were significantly higher in patients infected with CagA-positive strains compared to negative ones (CagA-positive, 63.5%; CagA-negative, 36.5%; p = 0.001). It is possible that CagA-positive strains cause more intense gastric mucosal inflammation, which may play a role in eradication by increasing the blood flow, thus improving the flow of antibiotics.[15] Recent studies have shown higher *H.pylori* density by histopathology to be related with complications while indicating a negative correlation with *H.pylori* eradication rates.[16,17] Our study, however, found no significant difference. It is important to note that both of these studies used the triple therapy. Bacterial density does not seem to negatively affect differing treatment of bismuth + quadruple therapy adapted in our study. Previous studies have reported histopathological findings predicting *H.pylori* treatment failure.[18,19] Our study demonstrated that high histological scores of gastritis and activity are effective in determining eradication success. However, there was no significant difference in eradication rates of *H.pylo*ri according to the severity of atrophy in the antrum. The host immune system has been hypothesized to affect pharmacological treatment in *H.pylori* eradication. Some authors have found that impaired mucosal immune response may contribute to eradication failure in *H.pylori* infection.[20,21] Vitamin D has a long tradition of playing a role in regulating calcium and phosphorus metabolism, but it has also proven effective as a potent immune modulator of the adaptive immune system, stimulating the innate immune response upon infection.[22] Recent studies have demonstrated the relationship between vitamin D deficiency and infectious diseases.[23,24] Vitamin D regulates the innate immune system in macrophages against *Myco-*

*bacterium tuberculosis* through the mechanisms of activated toll-like receptors (TLRs), leading to the induction of antimicrobial peptide cathelicidin that kills the organism.[25] A recent meta-analysis has found that low serum 25(OH)D levels are associated with a higher risk of active tuberculosis.[26] We have evaluated the possible association between vitamin D levels and *H.pylori* infection.

Our study primarily demonstrates the relationship between *H.pylori* eradication rates and low vitamin D levels. We found that *H.pylori* eradication rates were significantly lower in patients with vitamin D deficiency. A potential pathogenic mechanism explaining the observed association between vitamin D status and eradication rates is impairment of the vitamin D signal immune function, which may lead to inadequate immune response. There is limited data demonstrating the relationship between vitamin D and *H.pylori* infection. One in vitro study showed the selective antibacterial effect of vitamin D3 decomposition product (VDP1) against *H.pylori*.[27] Vitamin D is also known to regulate the expression of antimicrobial peptides – cathelicidin and β-defensin, which kill the bacteria. Although the effect of cathelicidin has been demonstrated only in macrophages infected with *M. tuberculosis*, antibacterial action against gram-negative and gram-positive bacteria has also been reported.[28,29] Another antimicrobial peptides β-defensin, which is secreted in the gastric mucosa after infection by *H.pylori*, constitutes immune defense against this bacterial pathogen at the mucosal surface.[30] In a vitamin D-deficient state, the infected macrophage is unable to produce sufficient 1,25-(OH)D2 to upregulate the production of cathelicidin and β-defensin, thus rendering them unable to kill the *H.pylori* strains.

In this paper, we have demonstrated low eradication success in infected patients with vitamin D deficiency. Vitamin D deficiency may be a risk factor associated with *H.pylori* infection treatment failure and may lead to a need for supplementation of vitamin D before *H.pylori* eradication therapy. More prospectively designed clinical trials considering pre-treatment vitamin D levels are needed to further evaluate the relationship between vitamin D status and *H.pylori* infection.

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# The usefulness of routinely used malnutrition screening tools in predicting anemia in lung cancer patients

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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.** Anemia and malnutrition are frequently observed during lung cancer development, and the associations between them have been researched. However, no study concerning the utility of routinely used nutritional screening tools in predicting anemia in lung cancer has been performed.

**Objectives.** The aim of this study was to assess the usefulness of routinely used malnutrition screening tools in predicting anemia in lung cancer patients.

**Material and methods.** Eighty-five male patients were recruited to this study. Blood counts, serum iron concentration, total iron binding capacity (TIBC) and serum transferrin saturation (STS), measurements of selected anthropometric parameters, Mini Nutritional Assessment (MNA) and Glasgow Prognostic Score (GPS) were performed for the subjects. To evaluate the differences in the distribution of hematological and iron status parameters according to nutritional status, a t-test (Mann-Whitney U test for non-parametric data) and an analysis of variance (ANOVA) were performed. Tukey's post hoc test was performed for inter-group comparison of parametric data. The sensitivity, specificity, positive and negative predictive values of MNA and GPS were compared to blood counts and biochemical parameters of iron status.

**Results.** Using the MNA test, we observed that ca. 60% of subjects had deteriorated nutritional status. About half of the patients had inflammation cumulated with malnutrition. A similar part of the subjects had anemia. The MNA test showed a significant difference in the distribution of Hb and Htc, while GPS showed the distribution of Fe and TIBC among lung cancer patients. We did not observe any influence of fat-free mass index (FFMI) on hematological and iron status parameters. The MNA test had very high specificity and positive predictive values (PPV) for all the hematological parameters evaluated as well as GPS for serum Fe concentration and TIBC.

**Conclusions.** Our data demonstrates that an evaluation of nutritional status with the MNA test can provide additional predictive information regarding anemia, while GPS may do the same with type of anemia in lung cancer patients.

**Key words:** lung cancer, malnutrition, anemia, MNA, FFMI

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## Introduction

In the course of lung cancer, accompanying symptoms are often as important as the underlying disease and may influence the treatment schedule. Among a whole range of paraneoplastic symptoms, malnutrition and hematological disturbances, e.g., cancer-related anemia (CRA), often appear, especially among patients in the later stages of lung cancer, those with metastatic disease or among the elderly.[1–3] The pathomechanism of CRA is different from iron-deficiency anemia (IDA) and similar to anemia of chronic disease (ACD). ACD is usually normochromic and normocytic anemia, and tends to be more severe in cancer patients, where IDA may additionally coexist. The reported prevalence of CRA is about 30%, however data indicates that 60% or even 90% of patients with tumors may suffer from anemia.[4–7] Severe anemia is diagnosed in about 10–20% of these cases.[8] This pathological condition generally occurs more often among patients with gastrointestinal tumors or lung cancer patients than others.[6,9,10] The results of the European Cancer Anemia Survey showed that during diagnosis about 38% of lung cancer patients were anemic and the prevalence of anemia is associated with the clinical stage of the disease and further the type of treatment.[10] The pathogenesis of CRA is multifactorial and can result from cancer progression, a coexisting inflammatory process, oncological treatment or the kidney and bone marrow injuries.[7,11] Decreased erythropoiesis, a predominant mechanism of CRA, is a result of several factors, e.g., reduced erythropoietin synthesis (the kidney injuries or an inflammatory process), and iron, folate and vitamin B12 deficiencies (lack of appetite or deteriorated intestinal absorption and metabolism). Pure red cell aplasia is, in general, observed in patients with hematological malignances and rather does not occur in patients with solid tumors, except thymoma. Destruction or loss of red blood cells (RBC) due to e.g., intestinal bleeding, are other factors that mostly influence the risk of CRA.[7] Despite the many factors leading to the development of CRA, it is believed that the influence of the inflammatory process is one of the key components of its pathomechanism.[12,13] The main impact of proinflammatory cytokines in CRA is the disruption in Fe metabolism. Proinflammatory mediators increase hepcidin expression, which blocks Fe flows into plasma, with the resulting effect of the unavailability of Fe for erythropoiesis.[14] These mediators of inflammation are also, in turn, independent risk factors of cancer malnutrition and cachexia.[15,16]

Malnutrition is common in progressive advanced lung cancer but it may also occur in the early stages of the disease. The prognosis and therapeutic outcome of undernourished lung cancer patients are generally poor. In addition, they are at risk of impaired response to chemo- or radiotherapy, increased susceptibility to chemotherapy-induced toxicity, higher incidence of post-operative complications and generally deteriorated quality of life and shorter lifespan.[17] Malnutrition as well as hematological disorders may negatively affect the clinical decision about oncological treatment.[18] A vicious circle may arise between anemia and nutritional disturbances in lung cancer. The altered nutritional status may potentiate the risk of cancer-related anemia due to insufficient nutrient intake and cachexia, which in turn disturbs the metabolism of macro- and micronutrients. On the other hand, anemia may influence the nutritional status due to e.g., loss of appetite.[19]

Awareness of the symptoms associated with lung cancer can be useful for clinicians in the planning and choosing treatment regimens. Since both hematological and nutritional disturbances occur in the course of lung cancer growth and then may potentiate during treatment, it is interesting to evaluate the relationship between them as early as at the stage of disease diagnosis, with simple, routinely used tools evaluating nutritional status. Knowledge of the relationship between anemia and malnutrition, and finding simple tools useful for nutrition evaluation that might also predict anemia incidence, may help to better and more quickly evaluate the condition of patients with lung cancer and, eventually, influence oncological treatment.

The aim of this study was, therefore, to assess the relationship between nutritional status and anemia in lung cancer patients at the stage of diagnosis, using simple, commonly used tools, and to assess the predictive values of the nutritional tools compared to parameters related to anemia.

## Material and methods

Eighty-five male subjects (aged 65.5 ±8.6 years, range: 50–81 years) with newly-diagnosed lung cancer were recruited to this study from the Lower Silesian Center of Lung Diseases. Anthropometric parameters and the MNA test were performed on the day of admission to the hospital. Non-confirmed lung cancer patients were excluded from the study. The majority of patients (74.1%) suffered from non-small-cell lung cancer (NSCL). The vast majority of recruited subjects (70.7%) were at clinical stage III and IV lung cancer; about 20.0% at stage II and a few subjects (9.3%) at stage I of the disease. Concomitant diseases were reported as follows: cardiovascular diseases (44.7%), dyslipidemia (35.3%), impaired fasting glucose (18.8%), diabetes mellitus type 2 (5.7%), gastritis and/or stomach ulcers (11.8%), chronic obstructive pulmonary disease (COPD) (11.8%), and thyroid diseases (7.1%). Subjects chronically used the following drugs: cardiovascular agents (48.2%), statins (16.5%), H2 blockers and/or proton pump inhibitors (12.9%), inhaled agents (glucocorticoids and/or beta2-agonists) (12.9%), oral antidiabetic agents (7.1%), insulin (4.8%), thyroid hormone replacement therapy (3.5%), and thyreo-

statics (2.4%). The study was approved by the First Local Ethics Commission (approval No. 540/2013) and it conforms to the provisions of the Declaration of Helsinki. Informed consent forms were signed by the subjects who volunteered to participate in the study.

## Hematological and iron status parameters

The parameters related to red blood cells, hemoglobin (Hb) concentration, hematocrit, RBC, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH) and mean corpuscular hemoglobin concentration (MCHC) were performed with an automated Sysmex XT-1800i analyzer (Roche Diagnostics, Indianapolis, USA) for recruited patients on the day after admission to the hospital. Serum iron concentration and total iron binding capacity (TIBC) were measured spectrophotometrically using commercial test kits, iron ferrozine (Applied Biosystems cat. No. 11509, Barcelona, Spain) and total iron binding capacity (TIBC) (Applied Biosystems cat. No. 11554, Barcelona, Spain). Blood was taken from the elbow vein between 6:00 and 7:00 am and the parameters related to red blood cells were analyzed the same day. For determination of serum iron concentration and total iron binding capacity, albumin and CRP serum was separated and frozen at -80˚C until analysis.

## Anthropometric measurement variables

Body mass and height were self-reported. BMI was calculated as the ratio of the body mass to body height squared and expressed as kg/m$^2$. Waist and arm circumference were measured twice for every patient on the day of admission to the hospital. Waist circumference (WC) [cm] was measured at the minimum circumference between the iliac crest and the rib cage, and the waist-height ratio (WHtR) was calculated as the ratio of waist circumference divided by the height. Upper arm circumference was measured with the left arm hanging relaxed. The measurement was taken midway between the tip of the acromion and olecranon process. To evaluate fat-free mass index (FFMI) as a sign of muscle wasting, body fat percentage (BFP) [%] was determined by a bioelectric impedance analysis using a body fat analyzer (Omron BF 306, Kyoto, Japan) 2 times for every patient. Then FFMI was calculated using the following formula:

$$FFMI = (100\% - BFP)/100 \times \text{body mass}/(\text{height})^2$$

## Mini Nutritional Assessment

The Mini Nutritional Assessment (MNA) questionnaire is composed of 18 items and involves anthropometric, general, dietary and subjective assessments. Although the questionnaire was originally validated for use in elderly non-malignant patients, some authors have also adapted it for the assessment of cancer patients' nutritional status.[3,20,21] The questionnaire consists of 2 main parts: screening and assessment. Screening includes questions related to changes in weight loss, oral intake, mobility, stress, etc. Assessment additionally includes medical history, some questions related to eating habits and measurements of arm and calf circumferences. A total score >23.5 indicates adequate nutritional status, 17.0–23.5 denotes a risk of malnutrition, while <17.0 indicates malnutrition.[20]

## Glasgow Prognostic Score

Glasgow Prognostic Score (GPS) is a cumulative prognostic score based on the systemic inflammatory response and albumin concentration.[22] Patients with both an elevated C-reactive protein (>10 mg/L) and hypoalbuminemia (<35 g/L) were assigned a score of 2 (group 2). Patients in whom only 1 of these biochemical disturbances was found were allocated a score of 1 (group 1). Patients in whom neither of these abnormalities were present were assigned a score of 0 (group 0).

## Statistical analysis

To evaluate the differences in the distribution of hematological and iron status parameters according to nutritional status, a t-test (Mann-Whitney U test for non-parametric data) and an analysis of variance (ANOVA) were performed. Tukey's post hoc test was performed for intergroup comparison of data. All statistical analyses were performed using STATISTICA v. 12.0 (StatSoft Inc., Tulsa, USA). P-value less than 0.05 was considered to indicate a statistically significant difference.

Sensitivity, specificity, positive (PPV) and negative (NPV) predictive values of the MNA test and GPS were compared to biochemical parameters and calculated from the following formulas:

$$\text{Sensitivity} = a/a + b \quad \text{Eq. (1)}$$
$$\text{Specificity} = c/c + d \quad \text{Eq. (2)}$$
$$PPV = a/a + d \quad \text{Eq. (3)}$$
$$NPV = c/c + b \quad \text{Eq. (4)}$$

a – the number of patients who had values of the MNA test or GPS indicating malnutrition or systemic inflammation, respectively, and at the same time a particular biochemical parameter below the reference value (true positive);

b – the number of patients who had values of the MNA test or GPS indicating proper nutritional status or lack of systemic inflammation, respectively, and at the same time a particular biochemical parameter below the reference value (false negative);

c– the number of patients who had values of the MNA test or GPS indicating proper nutritional status or lack of systemic inflammation, respectively, and at the same time correct particular biochemical parameters (true negative);

d – the number of patients who had values of the MNA test or GPS indicating malnutrition or systemic inflammation, respectively, and at the same time correct particular biochemical parameters (false positive).

In calculating sensitivity, specificity, PPV and NPV, the MNA test < 17.0 and GPS = 2 were considered as parameters explicitly indicating malnutrition and systemic inflammatory cumulated with malnutrition, respectively. The altered hematological and iron status parameters were defined as those below the reference values (Table 1).

# Results

The baseline nutritional, hematological and iron status of lung cancer patients is presented in Table 1. The MNA test was found to be the most sensitive in screening for malnutrition; ca. 60% of the group had impaired nutritional status. However, using BMI and AC, only a very small percentage of the subjects studied were found to be malnourished – ca. 1% for both parameters. About half of the group had both elevated C-reactive protein and hypoalbuminemia expressed as GPS = 2. Impaired hematological parameters related to anemia were observed in a significant proportion of the group. More than 40% of the subjects had Hb below reference values.[23] Low Htc was observed in ca. 70% of patients and low RBC in about half of the group.[24] Moreover, low TIBC, which is mostly seen in anemia of chronic disease, was found in almost 60% of the group.[25]

## Prevalence of anemia according to nutritional status

The high sensitivity of the MNA test gave us the opportunity to evaluate the distribution of hematological and iron status parameters according to the results of this test (Table 2). Indeed, we found that Hb concentration and Htc were significantly lower in malnourished patients compared to well-nourished ones. Similar trends were observed in the other evaluated parameters, except MCH and MCHC, however these observations were not statistically significant. The distributions of all the hematological and iron status parameters according to FFMI did not differ significantly between the groups (Table 3). Even so, almost all the parameters tended to be higher in the well-nourished group vs the group with risk of malnutrition. The distribution of the parameters according to the prevalence of systemic inflammation disclosed a significantly lower serum iron concentration and TIBC in the group with systemic inflammation (Table 4). Interestingly, we did not find any meaningful differences in the remaining parameters, e.g., Hb concentration, Htc, RBC and the parameters of red blood cells.

## Sensitivity, specificity, PPV and NPV of the MNA test and GPS compared to hematological and iron status

The MNA test had very low sensitivity, oscillating around 20%, when compared to blood parameters (Table 5). However, this test was shown to have high specificity, close to 90% or even 100%, when compared to TIBC. Additionally, we observed high PPV for the MNA test when compared to TIBC (100%) and Htc (ca. 85%). The sensitivity, specificity, PPV and NPV of GPS were low in general when compared to the parameters related to red blood cells and iron status. However, relatively high PPV was found when compared to Fe (ca. 73%) and TIBC (75%).

# Discussion

Lung cancer represents a significant clinical concern, accounting for the highest mortality and morbidity of all cancers, especially among men.[26] Anemia and malnutrition were recognized in several studies as good indicators of lung

**Table 1.** Baseline nutritional, hematological and iron status of male lung cancer patients

| Parameter | N | Reference values | Range | Mean (95% CI) | % of group under (*over) the reference values |
|---|---|---|---|---|---|
| BMI [kg/m$^2$] | 85 | 18.5–25.0 | 17.9–45.8 | 25.9 (24.9–26.9) | 1.2 |
| FFMI [kg/m$^2$] | 80 | >17.0 | 14.9–25.4 | 18.9 (18.5–19.5) | 22.5 |
| AC [cm] | 85 | >21 | 19–36.5 | 27.2 (26.5–27.9) | 1.2 |
| WHtR | 84 | ≤0.5 | 0.42–0.79 | 0.59 | 89.4* |
| MNA [points] | 82 | >23.5 | 9–28.5 | 21.4 (20.4–22.4) | 58.9 |
| GPS 0/1/2 [%] | 53 | – | 35.8/14.2/50.9 | – | – |
| Hb [g/dL] | 85 | >13 | 8.3–16.9 | 13.1 (12.7–13.4) | 44.7 |
| Htc [%] | 85 | >41 | | 38.9 (38.0–39.8) | 69.4 |
| RBC | 85 | >4.4 | 2.9–5.4 | 4.4 (4.3–4.5) | 51.8 |
| MCV [fL] | 26 | >82 | 74.6–96.8 | 87.2 (85.3–89.0) | 8.2 |
| MCH [pg] | 26 | >27 | 23.9–33.7 | 29.2 (28.2–30.2) | 18.9 |
| MCHC [g/dL] | 26 | >31.5 | 30.8–37.3 | 33.5 (32.8–34.2) | 8.2 |
| Fe [µg/dL] | 65 | 65–175 | 11.6–226.3 | 78.9 (65.5–92.3) | 40.0 |
| TIBC [µg/dL] | 64 | 250–450 | 23.6–379.1 | 190.6 (170.5–210.8) | 58.8 |
| STS [%] | 64 | 20–50 | 5.5–90.7 | 35.6 (29.9–41.4) | 16.5 |

CI – confidence interval; BMI – body mass index; FFMI – fat-free mass index; AC – arm circumference; WHtR – waist-height ratio; MNA – Mini Nutritional Assessment; GPS – Glasgow Prognostic Score; Hb – hemoglobin; Htc – hematocrit; RBC – red blood cell; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; TIBC – total iron binding capacity; STS – serum transferrin saturation.

**Table 2.** Distribution of hematological parameters according to MNA test results

| Hematological parameter | Well-nourished | Risk of malnutrition | Malnutrition |
|---|---|---|---|
| Hb [g/dL] | 13.6 (13.0–14.2) ±1.6[a] | 12.8 (12.3–13.4) ±1.5[ab] | 13.6 (13.0–14.2) ±1.7[b] |
| Htc [%] | 40.4 (39.0–41.8) ±4.1[a] | 38.2 (36.9–39.5) ±3.7 [ab] | 37.3 (35.0–39.7) ±4.0 [b] |
| RBC | 4.5 (4.1–4.6) ±0.5 | 4.3 (4.1–4.4) ±0.5 | 4.3 (4.1–4.6) ±0.5 |
| MCV [fL] | 88.4 (86.0–90.8) ±3.4 | 87.1 (84.2–89.9) ±4.5 | 84.3 (73.2–95.5) ±7.0 |
| MCH [pg] | 27.3 (22.6–32.1) ±3.0 | 29.3 (27.8–30.9) ±2.5 | 29.9 (28.4–31.4) ±2.1 |
| MCHC [g/dL] | 33.8 (32.4–34.6) ±1.6 | 33.6 (32.6–34.6) ±1.6 | 33.8 (32.4–35.1) ±1.8 |
| Fe [µg/dL] | 83.4 (61.8–105.0) ±58.8 | 78.9 (59.1–98.8) ±48.1 | 63.2 (20.2–106.1) ±55.9 |
| TIBC [µg/dL] | 211.8 (185.9–237.6) ±70.4 | 177.1 (139.2–214.9) ±89.6 | 153.9 (95.3–212.5) ±76.2 |
| STS [%] | 36.2 (27.2–45.1) ±23.1 | 35.9 (25.40–046.6) ±21.7 | 33.2 (20.9–45.6) ±14.8 |

MNA – Mini Nutritional Assessment; Hb – hemoglobin; Htc – hematocrit; RBC – red blood cell; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; TIBC – total iron binding capacity; STS – serum transferrin saturation; a, b – statistically significant differences in hematological parameters between patients with different nutritional status according to the MNA test (ANOVA test).

**Table 3.** Distribution of hematological parameters according to FFMI results

| Hematological status | Well-nourished | Risk of malnutrition |
|---|---|---|
| Hb [g/dL] | 13.2 (12.8–13.6) ±1.7 | 12.5 (11.7–13.3) ±1.6 |
| Htc [%] | 39.2 (38.2–40.2) ±4.0 | 37.7 (35.5–39.8) ±4.3 |
| RBC | 4.4 (4.3–4.5) ±0.4 | 4.2 (3.9–4.5) ±0.6 |
| MCV [fL] | 87.9 (86–89.8) ±4.1 | 84.2 (77.3–91.1) ±5.6 |
| MCH [pg] | 29.6 (28.5–30.6) ±2.3 | 27.9 (24.1–31.7) ±3.1 |
| MCHC [g/dL] | 33.6 (32.8–34.3) ±1.6 | 33.1 (30.7–35.6) ±2.0 |
| Fe [µg/dL] | 75.5 (61.6–89.3) ±48.7 | 90.2 (51.5–128.9) ±69.9 |
| TIBC [µg/dL] | 196.9 (173.8–220.0) ±80.4 | 170.2 (125.3–215.0) ±81.0 |
| STS [%] | 32.9 (27.1–38.7) ±18.7 | 44.5 (28.1–60.8) ±27.1 |

FFMI – fat-free mass index; Hb – hemoglobin; Htc – hematocrit; RBC – red blood cell; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; TIBC – total iron binding capacity; STS – serum transferrin saturation.

**Table 4.** Distribution of hematological parameters according to GPS results

| Hematological status | Score 0–1 | Score 2 |
|---|---|---|
| Hb [g/dL] | 13.4 (12.7–14.1) ±1.7 | 13.1 (12.4–13.7) ±1.6 |
| Htc [%] | 39.7 (38.2–41.2) ±3.6 | 39.3 (37.6–40.9) ±4.2 |
| RBC | 4.5 (4.3–4.6) ±0.3 | 4.4 (4.2–4.6) ±0.5 |
| MCV [fL] | 87.5 (85.2–89.8) ±3.0 | 87.1 (84.1–80.1) ±5.4 |
| MCH [pg] | 30.0 (28.2–31.9) ±2.5 | 28.8 (27.4–30.2) ±2.5 |
| MCHC [g/dL] | 34.3 (32.8–35,8) ±1.9 | 33.0 (32.2–33.8) ±1.5 |
| Fe [µg/dL] | 89 (65.6–112.5) ±55.5* | 62.4 (41.7–83.2) ±50.3* |
| TIBC [µg/dL] | 240.3 (210.4–270.2) ±69.2* | 166.5 (135.2–197.8) ±75.8* |
| STS [%] | 35.8 (26.0–45.8) ±21.9 | 33.5 (25.0–42.0) ±19.7 |

GPS – Glasgow Prognostic Score; Hb – hemoglobin; Htc – hematocrit; RBC – red blood cell; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; TIBC – total iron binding capacity; STS – serum transferrin saturation; * statistically significant differences in hematological variables between patients with different GPS results.

cancer and other cancer progression. Moreover, the association between them has been investigated by several authors.[27–29] However, to the best of our knowledge, there has been no study concerning the relationship between nutritional status evaluated by simple, routinely used anthropometric parameters and the MNA test, and parameters related to red blood cells and iron status in lung cancer patients. Moreover, the study evaluated the relationship between anemia and inflammation using Glasgow Prognostic Score in this condition. These points of view could bring new insights and opportunities to the evaluation of the relationship between malnutrition and anemia.

Of all the parameters used in this study to assess nutritional status, the MNA test was found to detect the highest percentage of the group with malnutrition or risk of malnutrition. This result indicating the high sensitivity of the MNA test is in agreement with other studies, where the MNA test was recommended as a useful tool for the evaluation of nutritional status in this condition.[2,3,29] However, we did not find more than 2 studies which assess the relationship between the MNA test and hematological parameters among lung cancer patients. Gioulbasanis et al. showed that the MNA test was significantly correlated with Hb but they did not evaluate the association with other parameters related to red blood cells.[3,29] Another study clearly demonstrated a significant correlation between the MNA test and laboratory parameters indicating hematological disorders.[30] However, this research concerned elderly people living in nursing homes, not cancer patients. In our study, we found that the MNA test was correlated with Hb and Htc. Despite this, we did not observe significant differences in other hematological parameters and parameters related to iron

**Table 5.** Sensitivity, specificity, PPV and NPV of MNA and GPS* compared to blood parameters

| Hematological parameter | Sensitivity | Specificity | PPV | NPV |
|---|---|---|---|---|
| Hb [g/dL] | 23.7; 50.0 | 90.9; 44.0 | 69.2; 46.2 | 57.9; 47.8 |
| Htc [%] | 18.9; 53.1 | 91.7; 41.2 | 84.6; 63.0 | 31.9; 31.8 |
| RBC | 18.6; 52.4 | 87.2; 44.4 | 61.5; 42.3 | 49.3; 54.5 |
| Fe [µg/dL] | 17.6; 61.3 | 90.3; 46.2 | 66.7; 73.1 | 50.0; 33.3 |
| TIBC [µg/dL] | 18.3; 64.3 | 100; 64.7 | 100; 75.0 | 25.9; 52. 4 |

* The first value in every column corresponding to MNA, the second to GPS; PPV – positive predictive value; NPV – negative predictive value; MNA – Mini Nutritional Assessment; GPS – Glasgow Prognostic Score; Hb – hemoglobin; Htc – hematocrit; RBC – red blood cell; TIBC – total iron binding capacity.

status in patients with different results of the MNA test. One reason could presumably be due to high prevalence of inflammation among the subjects. Based on the Guigoz's review, it was found that the presence of inflammation may significantly change the correlations between laboratory parameters and the MNA test.[31] This observation may, in particular, clarify the lack of significant differences in TIBC and serum iron concentration between patients assessed only on the basis of their nutritional status, without evaluating the presence of inflammation, where inflammation could have presumably influenced the concentration of these parameters to a great extent. Indeed, we observed significantly lower TIBC and iron concentration in group 2 compared to the rest of the subjects, assigned depending on GPS. Statistically significant differences in these 2 parameters were observed only when the cut-off point for GPS score was set at 2 (high C-reactive protein, low albumin concentration). No meaningful differences were found when patients were distributed into 3 groups, based on GPS score. This indicates that only inflammation and malnutrition together may have a great impact on some hematological parameters.

Cancer-related anemia is closely linked to systemic inflammation and malnutrition.[28] This explains the lower TIBC and iron concentration in group 2 compared to the remaining participants. Hypoferremia, often observed in cancer patients, is induced by impaired reutilization of iron. It is caused by reduced iron release from macrophages to circulating transferrin. During the inflammatory process, cytokines, e.g., IL-6, potentiate hepcidin synthesis, causing iron sequestration in the macrophages.[32] Moreover, during inflammation, the concentration of transferrin drops in connection with the disturbed metabolism of this protein.[33] Therefore, low TIBC, which is an indirect measure of serum transferrin, and low serum iron concentration are commonly seen with anemia of chronic disease or with inflammation. Other than this, we did not find any significant differences in the remaining parameters between the groups of patients distinguished by GPS results, although there was a tendency toward lower blood parameters in the group with systemic inflammation. In fact, it is difficult to explain why lung cancer patients with systemic inflammation and malnutrition had no other hematological parameters that were significantly lower. One explanation may be that a meaningful proportion of the subjects were at an advanced stage of the disease, which had influenced to a great extent many different metabolic processes which indirectly affect erythropoiesis. For example, the caloric malnutrition often observed in patients with the advanced disease may lead to disturbed transformation of tetraiodothyronine to triiodothyronine (functional hypothyroidism), in which anemia is induced by a reduction in the synthesis of erythropoietin.[34] This example also suggests other factors, aside from inflammation and protein-malnutrition, influencing the risk of anemia in cancer patients.

Additionally, to better assess the usefulness of the MNA test and GPS in predicting the prevalence of anemia, we describe them by terms such as sensitivity, specificity, PPVs and NPVs. We found that the MNA test had high specificity and low sensitivity when compared to blood parameters. These results demonstrate that this test might be useful in blood disorders for determining if this parameter indicates malnutrition. However, as was stated by Akobeng, the high value of specificity of the MNA test cannot be used to estimate the probability of hematological disorders in an individual patient.[35] The major limitations of both the sensitivity and specificity values is that they are of no practical use to clinicians in evaluating the probability of disturbances in an individual patient. PPVs and NPVs are more useful rather in describing the probability that the test will give the correct diagnosis. In this study, the MNA test had high PPVs when compared to all measured biochemical parameters, and GPS when compared to iron concentration and TIBC. These findings suggest that the MNA test and GPS may bring additional information about anemia incidence and type of anemia in lung cancer.

Several limitations need to be acknowledged in this study. Although the majority of recruited patients had stage III and IV of the disease, the group was not fully homogenous for clinical stage; the same problem concerned the histological type of cancer. This diversity could influence the evaluated relationships.

## Conclusions

Our data demonstrates that evaluation of nutritional status with the MNA test can provide additional predictive information regarding anemia, while GPS can help to predict the type of anemia in lung cancer patients and possibly in patients with other types of cancer. However, further, more detailed studies are needed to determine these relationships.

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# Do medical students adhere to advice regarding a healthy lifestyle? A pilot study of BMI and some aspects of lifestyle in medical students in Poland

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## Abstract

**Background.** The components of lifestyle of medical students, with comprehensive reporting of their physical activity and drinking and eating behavior, are rarely evaluated. Being overweight (increased body mass index – BMI) is associated with health problems, an unhealthy lifestyle (inadequate sleep, diet and exercise) being implicated.

**Objectives.** The aim was to determine if there were discrepancies between assessments of actual lifestyle and advice regarding the principles of a healthy lifestyle.

**Material and methods.** The relationship between lifestyle and BMI was investigated in 270 medical students (158 females, 112 males) who answered a questionnaire about aspects of their lifestyle.

**Results.** The mean ±SD BMI in males (23.41 ±0.25kg/m$^2$) was significantly higher than in females (20.52 ±0.16kg/m$^2$). Many aspects of lifestyle differed significantly with gender, including sleep habits, number of meals eaten, types of food eaten (fast food, amounts of fresh fruit and vegetables, sweets, etc.) and alcohol consumption, males generally having less healthy lifestyles. After correcting the associations between BMI and lifestyle factors for gender, one main finding was a positive association between BMI and alcohol intake, BMI rising by 0.014 kg/m$^2$/g alcohol intake per week.

**Conclusions.** These results show clear differences between actual and advised lifestyle with regard to many aspects of sleep, food and fluid intake, and exercise. Most students, particularly males, had not adopted a healthy lifestyle. Possible future problems associated with this require more emphasis.

**Key words:** obesity, sleep, BMI, food intake

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# Introduction

Overweight or obesity is increasing in frequency in many countries, and is associated with several problems, including metabolic disorder and increased cardiovascular risk.[1] Obesity occurs when energy intake exceeds its output for an extended period of time. The obvious causes of this imbalance are excessive food intake and/or inadequate physical activity, but other lifestyle factors – poor diet and sleep hygiene – also exist; these have all been considered in detail.[2,3] The present study elucidates the extent to which advice relating to healthy behavior (e.g., physical activity, eating appropriate food) is related to actual diet and physical activity among medical students. The main aim is to get individuals to develop a lifestyle which will prevent them from becoming overweight and obese, concentrating on medical students, who, in the future, will be health educators. With regard to the other lifestyle factors, sleep durations of less than normal length (defined in most studies as ≤6 h) and sleep disorders have been connected with numerous metabolic changes.[4–7] Epidemiological studies have shown that insufficient sleep may increase the risk of metabolic disturbances, including insulin resistance, obesity and type 2 diabetes.[8,9] In addition, it has been argued that people whose sleep is shorter than normal have more time to eat and so increase their calorie intake by eating snacks or fast food, often high in fat, salt and carbohydrate content.[10] A poor diet may also be one that contains too little roughage.[11] The problems of obesity and the associated higher risks of metabolic and cardiovascular disorders that are found in adults can begin in childhood and be present in young adults. For example, body mass index (BMI) values indicative of being overweight or obese have been found in adolescent males aged 12–19 years, in 18–29-year-old college students and, when comparing BMI and weight gain, in individuals in the age ranges of 18–24 years and 25–30 years.[12–14]

Advice to improve sleep hygiene and diet with the aim of reducing the likelihood of becoming overweight or obese is becoming important.[15,16] Anti-obesity programs should be directed at children (or their guardians) and adolescents as well as adults, and the development of an effective program aimed at adolescents who are undergoing a critical period of weight gain might be particularly important. Directing such advice at young adults is also important because they are beginning to become independent, and so they need to develop a healthy lifestyle. For example, the Polish government offers some advice in this regard (National Health Programme 2007–2015).[17]

These are the reasons why the present pilot survey has focused on this stage of life. University students should form a good sample to investigate not only because they are becoming independent but also because they are old enough to be able to answer a questionnaire accurately (removing possible problems due to parents' mis-remembering or mis-reporting their child's habits, for example).[18–20]

The present study has examined sleep patterns, diet and physical activity among medical students at Poznan University (Poland), in order to establish if the aspects of lifestyle that might be associated with the early stages of becoming overweight or obese (as assessed by BMI) are present in young adults. It also seems reasonable to suppose that medical students are aware of the problems associated with being overweight and having a poor diet and poor sleep hygiene at least to the same extent as the population as a whole.

# Material and methods

## Subjects

Medical students who were attending Poznan Medical University between October 2012 and March 2013 were asked to take part in the survey. It was made clear that the participation was voluntary, and that not doing so or not completing the study would have no negative consequences upon the individual. Volunteers completed a consent form. The study was approved by the Ethics Committee of Poznan Medical University. A total of 270 students (158 females, 112 males), aged 19–32 years (median = 21.0 years) took part in the study. About 95% of them asked for a return of completed forms.

## Physical measurements

A random sample of the volunteers was chosen, and it was confirmed that they had reported their height and weight (used to calculate BMI) accurately. BMI is defined as the individual's body mass divided by the square of their height ($kg/m^2$).[21] Overweight was defined as a BMI > 25, and obesity as one of ≥30.[22]

## Questionnaire

The volunteers then answered a questionnaire (45 questions) on their "recent habits" (based upon a typical day), with regard to sleep, food and fluid intake, and the amount of physical activity they had had. The questionnaire was in 3 parts and took about 15 minutes to answer: 1) general questions about their age, gender, height and weight; 2) questions about their sleep patterns; and 3) questions about their dietary habits and physical activity.

The questions relating to sleep patterns asked about typical times of retiring/rising and sleep length. Those about dietary habits regarded the frequencies of eating breakfast, a mid-morning snack, lunch, a mid-afternoon snack, dinner and supper. Questions were also asked about the weekly frequencies of eating fresh fruit

or vegetables, sweets, salty foods, fast food and soft drinks; in no case was specific advice about the exact meaning of these groups given. Further questions asked about the volunteer's normal weekly intake of alcohol (beer, wine and spirits); the results were converted into a weekly consumption of alcohol (in grams). Finally, volunteers were asked how many times per week they were physically active, defining this as "engaged in moderate physical activity for at least 30 min".

Table 1. Comparison of variables measured in males and females

| Topic | Variable (mean ±SE) | Females n = 158 | Males n = 112 | p-value |
|---|---|---|---|---|
| Sleep | retiring time [decimal time] | 0.4 ±0.1 | 0.3 ±0.1 | ns |
| | rising time [decimal time] | 7.1 ±0.1 | 7.3 ±0.1 | 0.056 |
| | length of sleep [h] | 6.5 ±0.1 | 6.9 ±0.1 | 0.005 |
| Food intake | breakfast [times/day] | 0.92 ±0.02 | 0.87 ±0.03 | ns |
| | elevenses [times/day] | 0.77 ±0.04 | 0.59 ±0.05 | 0.002 |
| | lunch [times/day] | 0.97 ±0.01 | 0.98 ±0.01 | ns |
| | tea [times/day] | 0.53 ±0.05 | 0.38 ±0.05 | 0.025 |
| | dinner [times/day] | 0.91 ±0.03 | 0.95 ±0.02 | ns |
| | supper [times/day] | 0.44 ±0.09 | 0.47 ±0.04 | ns |
| | total meals [per day] | 4.35 ±0.08 | 4.11 ±0.11 | 0.067 |
| Type of food | vegetables/fruit [times/week] | 4.7 ±0.2 | 3.8 ±0.2 | 0.006 |
| | sweet food [times/week] | 3.6 ±0.2 | 2.6 ±0.2 | <0.001 |
| | salty food [times/week] | 1.05 ±0.1 | 1.06 ±0.1 | ns |
| | fast food [times/week] | 0.5 ±0.1 | 1.0 ±0.1 | <0.001 |
| | soft drins[times/week] | 0.8 ±0.1 | 1.5 ±0.2 | <0.001 |
| Alcohol intake | alcohol intake [g/week] | 34 ±3 | 66 ±5 | <0.001 |
| Exercise | physical activity [times/week] | 1.5 ±0.1 | 2.6 ±0.2 | <0.001 |

ns – statistically non significant.

Table 2. Correlation of variables with BMI

| Topic | Variable of interest | Pearson's correlation coefficient, rs | p-value |
|---|---|---|---|
| Sleep | retiring time [decimal time] | 0.07 | ns |
| | rising time [decimal time] | 0.069 | ns |
| | length of sleep [h] | 0.157 | 0.01 |
| Food intake | breakfast [times/day] | 0.006 | ns |
| | elevenses [times/day] | -0.146 | 0.05 |
| | lunch [times/day] | 0.125 | 0.05 |
| | tea [times/day] | -0.027 | ns |
| | dinner [times/day] | 0.1 | ns |
| | supper [times/day] | 0.049 | ns |
| | total meals [number/day] | -0.012 | ns |
| Type of food | vegetables/fruit [times/week] | -0.054 | ns |
| | sweet food [times/week] | -0.174 | 0.01 |
| | salty food [times/week] | -0.011 | ns |
| | fast food [times/week] | 0.189 | 0.01 |
| | soft drins[times/week] | 0.13 | ns |

## Statistics

The results are presented as mean ±SE, where SE is the standard error of the mean. The Kolmogorov-Smirnov test was used to show that the distributions did not deviate significantly from normal, allowing parametric statistical tests to be used. The equality of variances was checked with the Levene's test. Gender differences were assessed by t-tests, and the relationship between BMI and the other variables was analyzed with Pearson's correlation coefficient. Multivariate linear regression analysis was also used to assess predictors of BMI, always using gender (dichotomized as 1 = female, 2 = male) as one predictor. Statistical calculations were performed using SPSS v. 17 (IBM, Armonk, USA). Significance was taken to be $p < 0.05$, and the results where $0.10 > p > 0.05$ are described as "trends" or "marginally significant". Values given as "p = 0.000" by the statistics package were recorded as "p < 0.001".

# Results

A comparison between males and females in the variables measured is given in Table 1. The results of the correlation analysis are shown in Table 2, and of the regression analysis in Table 3.

## BMI

The mean ±SE BMI of the males (23.41 ±0.25 kg/m$^2$) was significantly higher than that of the females (20.52 ±0.16 kg/m$^2$) ($p < 0.001$). The reliable and significant effect of gender upon BMI is also clearly shown in Table 3. Five females (3.1%) and 26 males (23.2%) had a BMI of 25 kg/m$^2$ or more (overweight or obese). Since there was a significant difference in BMI between males and females, this means that the interpretation of the effect of X upon BMI in the absence of this correction (Table 2) was ambiguous and that the interpretation of the effect of any factor X, upon BMI had to be corrected for this gender effect (Table 3).

## Sleep times

Females retired at 0.4 ±0.1 h (decimal time; 00:24 h ±6 min by clock time), and males at 0.3 ±0.1 h (00:18 h ±6 min, clock time); this difference was not significant ($p = 0.48$). After taking into account any effects of gender,

Table 3. Multivariate regression analysis including gender as one predictor

| Model for BMI | | Variable of interest | | Gender | |
|---|---|---|---|---|---|
| dependent: BMI [kg/m$^2$]; independent: variable of interest, gender [1 = female, 2 = male] | p-value for the model | p-value | beta coefficient | p-value | beta coefficient |
| Retiring time, gender | <0.001 | 0.071 | +0.223 | <0.001 | +2.92 |
| Rising time, gender | <0.001 | ns | ns | <0.001 | +2.89 |
| Length of sleep, gender | <0.001 | ns | ns | <0.001 | +2.89 |
| Eating breakfast, gender | <0.001 | ns | ns | <0.001 | +2.91 |
| Eating elevenses, gender | <0.001 | ns | ns | <0.001 | +2.83 |
| Eating lunch, gender | <0.001 | 0.063 | +1.67 | <0.001 | +2.86 |
| Eating tea, gender | <0.001 | ns | ns | <0.001 | +2.92 |
| Eating dinner, gender | <0.001 | ns | ns | <0.001 | +2.86 |
| Eating supper, gender | <0.001 | ns | ns | <0.001 | +2.88 |
| Number of meals eaten, gender | <0.001 | ns | ns | <0.001 | +2.92 |
| Vegetables/fruit eaten, gender | <0.001 | ns | ns | <0.001 | +2.94 |
| Sweet food eaten, gender | <0.001 | ns | ns | <0.001 | +2.84 |
| Salty food eaten, gender | <0.001 | 0.031 | -0.236 | <0.001 | +2.91 |
| Fast food eaten, gender | <0.001 | ns | ns | <0.001 | +2.90 |
| Soft drinks taken, gender | <0.001 | 0.046 | -0.192 | <0.001 | +3.04 |
| Alcohol intake, gender* | <0.001 | 0.001 | +0.014 | <0.001 | +2.41 |
| Physical activity, gender** | <0.001 | 0.007 | +0.194 | <0.001 | +2.66 |

* The regression equation was built as follows: BMI = 17.64 + 0.014 × daily alcohol intake + 2.41 × gender. The constant value was significant ($p < 0.001$); ** The regression equation was built as follows: BMI = 17.55 + 0.194 × physical activity + 2.66 × gender. The constant value was significant ($p < 0.001$).

BMI tended to increase with later retiring times ($p = 0.071$), increasing by 0.223 kg/m$^2$/h. Males tended to rise later than females (7.3 ±0.1 h vs 7.1 ±0.1 h, decimal time; $p = 0.056$) and sleep longer (6.9 ±0.1 h vs 6.5 ±0.1 h; $p = 0.005$). However, when the effects of gender were incorporated into the regression analysis, BMI was not independently associated with either rising time or sleep length ($p > 0.05$).

## Food intake

When the meals (breakfast, mid-morning snack, lunch, mid-afternoon snack, dinner and supper) were considered individually, females ate a mid-morning and mid-afternoon snack more frequently than males (0.77 ±0.04 vs 0.59 ±0.05 times/day; $p = 0.002$ and 0.53 ±0.05 vs 0.38 ±0.05 times/day; $p = 0.025$, respectively). These differences contributed to the observation that females tended to eat more meals per day than males (4.35 ±0.08 vs 4.11 ±0.11 meals per day, $p = 0.067$). However, the number of meals eaten per day did not exert a significant, independent influence on BMI. Only the frequency of eating lunch acted as a marginally significant ($p = 0.063$) predictor of BMI when the effects of gender had been taken into account, BMI increasing by 1.67 kg/m$^2$/daily frequency.

Females ate vegetables/fruit significantly more frequently (4.7 ±0.2 vs 3.8 ±0.2 times/week; $p = 0.006$), sweets significantly more frequently (3.6 ±0.2 vs 2.6 ±0.2 times/week; $p < 0.001$), and fast food and soft drinks significantly less frequently (0.5 ±0.1 vs 1.0 ±0.1 times/week; $p < 0.001$ and 0.8 ±0.1 vs 1.5 ±0.2 times/week; $p = 0.001$, respectively). Only the frequencies of eating salty food and drinking soft drinks significantly ($p = 0.031$ and $p = 0.046$, respectively) predicted BMI when gender had been taken into account, BMI decreasing by 0.236 kg/m$^2$/daily frequency for salty food and by 0.192 kg/m$^2$/daily frequency for soft drinks.

## Alcohol intake and activity

Males drank significantly more alcohol than females (66 ±5 g/week vs 34 ±3 g/week, respectively; $p < 0.001$), and BMI was strongly and positively associated with alcohol intake when gender effects had been taken into account (a rise of 0.014 kg/m$^2$/g alcohol intake per week; $p = 0.001$). This result is shown in Table 3 and also illustrated in Fig. 1. This Figure shows that, on average, males drank more than females and also had a higher mean BMI, a result that has already been described. These results would be sufficient to account for a significant positive correlation between BMI and alcohol intake if gender was not taken into account. However, each gender independently showed a positive relationship between

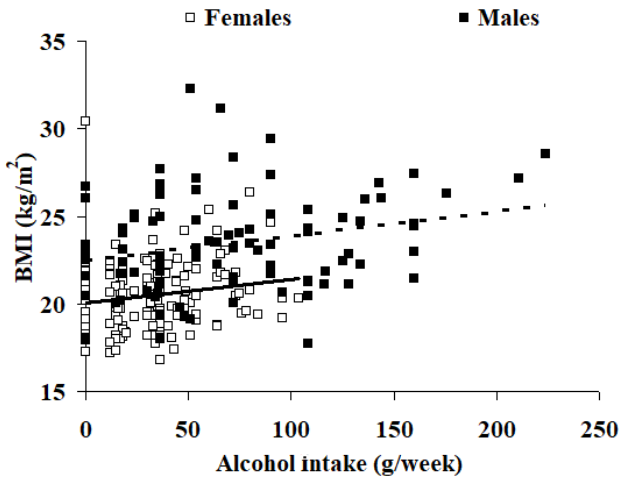


Fig. 1. Relationship between weekly intake of alcohol and BMI in males and females

Linear regression lines inserted: full line – females; dashed line – males. The regression equation is shown in Table 3.

alcohol intake and BMI, as indicated by the regression lines both having slopes that are positive and significantly different from 0.

It should be stressed that this finding, of a relationship between alcohol intake and BMI that is independent of gender effects, is in contrast with those for several other variables, discussed above, where no significant relationship between the variable and BMI was present if gender effects had been taken into account. Thus, for fast food intake, for example, males had higher mean values than females (see above and Table 1) and, coupling this finding with the fact that the males had a higher mean BMI, these results account for the significant positive correlation between fast food intake and BMI when gender had not been taken into account (Table 2); however, and by contrast with the results for alcohol, there was no significant relationship between fast food intake and BMI when gender had been taken into account (Table 3), indicating that the separate regression lines (of BMI upon fast food intake) for males and females had average slopes that did not differ significantly from 0 (data not illustrated).

Males were physically active more frequently than females (2.6 ±0.2 vs 1.5 ±0.1 times per week, respectively; $p < 0.001$), and there was a significant, positive association between physical activity and BMI ($p = 0.007$) after correction for any effect of gender, BMI rising by 0.194 kg/m$^2$/occasion of physical activity.

# Discussion

## BMI

The results of this pilot study indicate that the sample of females had a significantly lower BMI than males, and this meant that investigations of the independent effect of another factor, X, upon BMI needed to be corrected for this gender effect. Previous studies have stressed the importance of taking gender into consideration when using BMI and measuring body fat/obesity in the population, or differences in physical activity, fitness and being overweight in adolescents.[23,24] These results, which have been shown previously on many occasions, nevertheless indicate that the sample used was "normal". This can increase confidence when it comes to interpreting the new findings.

## Sleep hygiene

Even though males tended to rise later and sleep significantly longer than females (Table 1), these variables did not exert an independent effect upon BMI (Table 3); only later retiring times had a marginal effect upon BMI when gender effects had been taken into account. Therefore, the present results provide only very limited support for the view that BMI is related to sleep hygiene. Earlier studies have examined the relationship between BMI and sleep, and have reported elevated risks of weight gain or obesity among persons with short sleep durations – in both genders, particularly in women, or in men.[7,25,26] By contrast, others have found no such association.[27,28] The differences between the results of the studies might be due to the number of participants involved (studies failing to show any association tended to have smaller numbers). That is, it is possible that Type-2 errors were present in smaller studies, and that they were under-powered with regard to demonstrating the relationship between BMI and sleep hygiene. Such an explanation might also apply to the lack of significant differences, or only a marginally significant difference, found in the present study. Resolving this issue requires an increased number of participants in a study or the performance of a meta-analysis of the published data.

## Food intake

There were gender differences with regard to the frequency of eating some meals and the total number of meals eaten per day; females were more likely to eat mid-morning or mid-afternoon snacks than were males, and also tended to eat more meals per day. Eating patterns – choices involving when and where to eat, the types and amounts of foods eaten, and the circumstances leading to starting and stopping a meal or snack – can affect energy intake.[29] Individuals have been divided into "grazers" and "gorgers".[30] Grazers (those who eat small meals frequently throughout the day) may be at a metabolic advantage as compared to gorgers (who eat fewer, larger meals). Having fewer but larger meals may lead to increased obesity, possibly due to increased fat synthesis and storage following a large meal.[31] These findings would offer some explanation for why the males in the present study had a higher BMI than the females, the males being more likely to overeat. However, there are few studies specifically examining the impact of meal frequency on body composition. One approach has investigated the metabolic effects of eating a single meal of known composition at different times of the day. The results indicate that a single meal taken in the morning is associated with a better control of body mass than the same meal taken later in the day.[32] The detailed metabolic consequences of eating the same food at different times of day have still to be established, but possible differences include the amount of physical work performed during the daytime (less if there is no food intake until the evening) and endocrine responses to food intake (the insulin response to food intake being time-of-day dependent).[33] Nevertheless, in the present study, BMI was not independently predicted by the frequency with which particular meals were eaten (with the possible exception of a marginal effect of the frequency of eating lunch), nor by the total number of meals eaten per day. The present results showed gender differences with regards to the type of food eaten. The females

seemed to have a more healthy diet insofar as they ate more fresh vegetables/fruit and less fast food and soft drinks. An inverse association between vegetable and fruit intake and BMI was also found in the study by Heo et al. and in the study of Deliens et al., in which male students tended to increase their BMI more than female students.[34,35] Heo et al. also found that vegetable and fruit intake was lower in individuals who were of lower socioeconomic status, current smokers or physically inactive. These aspects have not been investigated in the present study.[34]

## Alcohol intake and activity

Gender differences were found (males drinking significantly more alcohol and performing significantly more physical activity each week) and there were also independent positive effects upon BMI of alcohol intake and the amount of physical activity performed.

These findings agree with those reported in the scientific literature. In the study by Di Milia et al., it was found that obesity was strongly associated with alcohol intake and low levels of physical activity.[36] In a study of postmenopausal women, those who were of normal body weight and who reported only moderate alcohol intake had a reduced risk of becoming overweight or obese in the future compared with those who drank more heavily.[37] In a study by Lahti-Koski et al., obesity was associated with alcohol consumption in both genders and BMI was inversely related to physical activity in women and to perceived health in men.[38] In the European population, BMI was positively associated with frequent alcohol consumption and sedentary behavior.[39]

That is, an active lifestyle coupled with restricted alcohol intake has repeatedly been found to be associated with a lower BMI. Related to this, in the Dietary Guidelines for Americans, the recommended consumption of only up to 1 "standard" drink a day for women and up to 2 "standard" drinks a day for men emphasized moderation in alcohol intake (a "standard" drink being equal to 14.0 g of pure alcohol).[40] "Heavy drinking" – more than 3 drinks on any day or more than 7 per week for women, and more than 4 drinks on any day or more than 14 per week for men – was associated with several health problems, including increased BMI levels indicative of being overweight or obese.

In summary, there is much evidence to indicate that high levels of alcohol intake lead to an increase of BMI, the observation in the present study that alcohol intake was positively associated with BMI, independent of gender differences, supporting this view. In the current sample, several participants, both males and females, drank rather more than the recommended levels; 3 females (who drank about 98 g/week) and 2 males (who drank more than 196 g/week) would be defined as "heavy drinkers". This habit might change when the students leave university, but it is important to advise them to reduce the amount they drink.

Increased physical activity was associated with increased BMI when gender effects had been taken into account. This result is in contrast to those reported by Di Milia et al., Lahti-Koski et al., and Stewart-Knox et al., where BMI decreased with the amount of physical activity.[36,38,39] The explanation of our anomalous result might be that BMI reflects body mass, and this might be due to muscle rather than body fat. As a result, many athletes, for example, would be classified as "obese", even though their increased weight (for their height) is due to the development of muscle rather than an accumulation of excess body fat. This anomaly can only be resolved by making specific measurements of body fat (skinfold thickness or whole-body impedance, for example) rather than body mass, and it is recommended that such measurements be made in future studies.

## Implications of the results

The present results enable areas where further work is needed to understand better the possible causal nexus that exists between lifestyle and BMI. This includes trying to ensure that any advice that is given is not only understood but also implemented by individuals in their daily lives. Even in this sample of highly educated young people, there is evidence that advice regarding their sleep hygiene and eating and drinking habits is not always being followed. Such advice needs to stress ways to keep BMI within recommended ranges and so reduce the risk of long-term effects associated with an increased BMI. Since in the present sample there were significant differences between females and males (females tending to live more healthy lifestyles with regard to sleep times and the intake of food and alcohol), it would appear that the need for such advice is greater in males. It has also been reported that males are less concerned about weight and a healthy lifestyle than women.[41]

Even though the results of the study showed only low prevalence of obesity and overweight amongst the students sampled, and even though health problems related to obesity and being overweight are more common in those who are middle-aged and elderly, the study by Friedenberg et al. showed that BMI at the age of 18 was strongly predictive of obesity later in adulthood.[42] Such findings stress that the provision of advice regarding these aspects of health promotion should be focused on young adults – university students, for example.

## Limitations and further work

– In the present study, height, weight and sleep duration were self-reported by the subjects. Although self-reported weight and height are highly correlated with actual height and body weight, self-reported sleep duration correlates only moderately.[43] People with sleep of short duration are more likely to over-report their sleep duration than are people with longer sleep durations; this may lead to a bias in the recorded values for sleep.[44]

– More detailed questions about sleep hours, waking activity and several aspects of food intake are required, particularly with regard to any differences that might exist between workdays and rest days. In addition, the results from questionnaires are always open to the criticism as they can be inaccurate. Keeping a daily diary of sleep and food/fluid intake during weekdays and weekends might reduce some of these problems.
– Obesity and overweight are better assessed by measuring skinfold thickness or whole-body impedance rather than calculating BMI from body height and mass, where ambiguities due to weight being due to muscle development or fat deposition exist.[45]

A new study in which at least some of these limitations are being addressed is currently under way.

## Conclusions

The present study indicated that many students, particularly males, had not adopted a healthy lifestyle. These results highlight a gap between the knowledge that students have with regard to a healthy lifestyle and their practice on a daily basis.

BMI differed between the genders and there were several gender-based differences when rising time, length of sleep, frequency of eating some meals, and some types of food eaten were considered. In addition, independently of gender effects, BMI tended to be higher when individuals went to bed later, ate lunch more frequently, ate salty foods and drank soft drinks less often, and drank more alcohol. These findings concur with those of others and provide limited support for the view that BMI is adversely affected by poor sleep hygiene and eating habits.[32,46]

These results can provide a rationale for devising simple intervention measures aimed at preventing a rise in BMI and improving long-term health but they also show that any such advice is not being acted upon as seriously as it might be, at least with regard to medical students in Poland. Clearly, the results of the present study do not say if similar problems exist in other sections of the community in Poland, and whether the situation is similar in other countries. However, the epidemiological evidence (considered in the Introduction) points to the problems being widespread.

Nevertheless, one direct implication of the present findings is that the Polish Medical Association needs to advertise its recommendations more effectively, and more effort must be made to ensure that the recommendations are implemented.[17]

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# Central aortic pressure, arterial stiffness and echocardiographic parameters of children with overweight/obesity and arterial hypertension

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## Abstract

**Background.** A non-invasive estimation of central aortic pressure and echocardiographic parameters, and appropriate interpretation thereof make it possible to determine the status of the vascular wall and myocardium. These parameters are early markers of unfavorable remodeling of the cardiovascular system.

**Objectives.** The aim of this study was to analyze the central aortic pressure and echocardiographic parameters of overweight/obese children (with or without concomitant arterial hypertension).

**Material and methods.** The study included 84 children (mean age: 15 years) – 45 with primary arterial hypertension, 39 normotensive, and 38 controls. Central aortic systolic (cSys) and diastolic (cDia) pressures, pulse wave augmentation index (Aix@75), peripheral resistance, pulse wave reflection and pulse wave velocity (PWV) were determined by means of brachial oscillometry. A number of echocardiographic parameters were recorded.

**Results.** Obese children with arterial hypertension showed the highest values of cSys, cDia and PWV, as well as interventricular septal end-diastolic thickness (IVS), left atrial diameter (LAD), left ventricular mass index (LVMI), elongation index and cardiac output (CO). Patient age, cSYS, cDIA and LAD were identified as significant predictors of PWV. The groups did not differ in terms of Aix@75, peripheral resistance and pulse wave reflection values.

**Conclusions.** Children with overweight/obesity present with elevated values of cSys, PWV, LVMI, LAD and CO. The risk of these abnormalities is further increased due to concomitant arterial hypertension.

**Key words:** pulse wave velocity, arterial stiffness, obesity, arterial hypertension, echocardiography

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## Introduction

The methods for early assessment of cardiovascular risk in children are gaining increasing interest.

The process of atherosclerosis may already begin in early childhood.[1] Obesity and arterial hypertension are established risk factors of early atherosclerosis and its cardiovascular complications.[2] The incidence of obesity and hypertension among children and adolescents is still increasing.[3] Due to their high incidence and severe systemic complications, both diseases are considered a serious social problem.[4] This substantiates research on non-invasive markers of early cardiovascular changes that could be used in patients at increased risk.

Determination of arterial blood pressure is a routine component of physical examination. Many previous studies have shown that arterial blood pressure correlates with cardiovascular risk. However, conventional measurement provides information only on blood pressure in peripheral arteries. More accurate data on vascular wall status can be obtained due to determination of central aortic pressure and pulse wave analysis.[5] Despite the availability of non-invasive methods for central aortic pressure determination, this parameter is not routinely considered in clinical practice, and the number of published results of non-invasive examination of the cardiovascular system in children with obesity and concomitant arterial hypertension is fairly limited.[5,6] Furthermore, the results of the sparse available studies dealing with the problem in question are inconclusive.[7–9] The issue whether hypertension changes central aortic pressure parameter values remains open.

The aim of this study was to analyze the central aortic pressure and echocardiographic parameters of overweight/obese children (with or without concomitant primary arterial hypertension) and to investigate the relationship between obesity and hypertension and central blood pressure and echocardiographic parameters.

## Material and methods

The study included 84 children and adolescents aged between 10 and 18 years (mean age: 15 years). The investigational group included children with overweight/obesity, among them individuals with primary arterial hypertension (n = 45) and a subset of normotensive subjects (n = 39). The control group comprised children with normal body weight and no history of arterial hypertension (n = 38) (Table 1). Children with structural cardiac abnormalities, established arrhythmia or organic etiology of arterial hypertension were excluded from the study.[6]

The following data was noted in all patients: anthropometric parameters (weight, height, waist circumference, standardized body mass index – BMI SDS), blood pressure (including central) and echocardiography.

The measurements were performed just after the diagnosis of arterial hypertension had been established before any pharmacotherapy. The anthropometric data of the study participants was referenced against the recently updated standards for Polish children, including weight percentile charts according to sex and age (Polish nationwide OLAF project).[10] Underweight was defined as BMI SDS ≤ -1, normal body weight as 1 > BMI SDS > -1, overweight as 2 > BMI SDS ≥ 1, and obesity as BMI SDS ≥ 2. The intra- and inter-observer coefficient of variation was 1.0% and 1.5% (respectively) for height, 0.8% and 1.1% for weight, 1.6% and 1.6% for waist circumference, and 2.2% and 2.8% for blood pressure.

Patients with arterial hypertension were identified on the basis of blood pressure tables for children and adolescents according to age, sex, and body height, by 3 ambulatory measurements with 24 h blood pressure monitoring confirmation.[11] All the study participants were subjected to central aortic pressure determination and echocardiographic examination.

Central aortic pressure was determined by means of brachial oscillometry with a Mobil-o-Graph device (I.E.M. GmbH, Stolberg, Germany) with inbuilt ARC-Solver algorithm (brachial waveform). Central aortic systolic (cSys) and diastolic (cDia) pressures were determined, along with pulse wave augmentation index (Aix@75), peripheral resistance, pulse wave reflection and pulse wave velocity (PWV), all adjusted to sex, age and height.[6,12,13] The measurements were taken in the morning hours, at least 30 min after the last meal. A total of 4 measurements per person were taken at 15 min intervals with the patient in a sitting position.

Echocardiographic examination was conducted with a Toshiba Istyle aplio MX SSA 780A device (Toshiba, Minato, Japan) by a single experienced cardiologist who did not know that children were included into this study. Interventricular septal end-diastolic thickness (IVS), left ventricular end-diastolic diameter (LVDd), left ventricular posterior wall thickness (PWT), left ventricular end-systolic diameter (LVDs) and left atrial diameter (LAD) were determined as proposed by Kampmann et al.[14]

**Table 1.** Median values, lower and upper quartiles (25:75) of age, height and body mass index in the analyzed groups of children

| Variable | Control group n = 38 (group I) | Obesity and hypertension n = 45 (group II) | Obesity without hypertension n = 39 (group III) | p-value |
|---|---|---|---|---|
| Age (years) | 16 (13:17) | 15 (14:16) | 16 (12:17) | ns |
| Sex | male – 20<br>female – 19 | male – 26<br>female – 19 | male – 17<br>female – 22 | ns |
| Height (cm) | 165 (159:176) | 171 (157:182) | 164 (158:170) | ns |
| BMI SDS | -0.11 (-0.67:0.39) | 2.76 (1.49:4.98) | 3.84 (2.37:5.25) | II vs I p < 0.0001<br>III vs I p < 0.0001 |

The above-mentioned data was used to calculate relative wall thickness (RWT = (IVS + PWT) : LVDd) and left ventricular ejection fraction (EF). Left ventricular mass index (LVMI) was calculated using the formula by AV-Cube: LV mass (g) = 0.8 × [1.04 × (LVDd + IVS + PWT)$^3$- LVDd$^3$] × 0.001 + 0.6. LV mass (LVM) was indexed for body height (LVMI), using height to the allometric power of 2.7 in order to minimize the contribution of body size to LVH.[15]

The 95$^{th}$ percentile for LVMI and the upper normal limit for RWT were defined according to Daniels et al.[16]

Furthermore, stroke volume (SV), heart rate (HR) and cardiac output (CO) were determined on the basis of Doppler flow analysis.[17]

The results were recorded in a Microsoft Access for Windows database and subjected to statistical analysis with STATISTICA v. 10.0 (StatSoft, Inc., Tulsa, USA) and GraphPad Prism v. 5.0 (GraphPad Software, Inc., La Jolla, USA) packages. As the data lacked normal distribution (as shown with the Kolmogorov-Smirnov and Shapiro-Wilk tests), one-way analysis of variance (ANOVA Kruskal-Wallis) and post hoc-tests were used for intergroup comparisons. The power and direction of the relationships between pairs of analyzed variables were determined on the basis of the Spearman's coefficients of rank correlation. Multivariate linear regression analysis was used to evaluate the impact of clinical parameters on basic hemodynamic values (e.g., PWV). The threshold of statistical significance for all the tests was set at $p < 0.05$. The results are presented as medians, along with lower and upper quartiles.

Table 2. Median values, lower and upper quartiles (25:75) of hemodynamic parameters determined on the basis of central aortic pressure measurement in the analyzed groups of children

| Variable | Control group n = 38 (group I) | Obesity and hypertension n = 45 (group II) | Obesity without hypertension n = 39 (group III) | p-value |
|---|---|---|---|---|
| cSys (mm Hg) | 103 (97:109) | 116 (106:121) | 110 (101:115) | II vs I $p < 0.0001$<br>II vs III $p < 0.05$<br>III vs I $p < 0.05$ |
| cDia (mm Hg) | 72 (65:77) | 79 (71:89) | 76 (69:82) | II vs I $p < 0.05$ |
| Aix@75 (%) | 19.5 (13.7:25.2) | 20.5 (13.0:27.0) | 21.0 (14.0:29.0) | ns |
| Peripheral resistance (s.mm Hg/mL) | 1.1 (1.0:1.2) | 1.2 (1.1:1.3) | 1.2 (1.1:1.3) | ns |
| Pulse wave reflection (%) | 58 (48:62) | 55 (50:62) | 58 (49:64) | ns |
| PWV (m/s) | 4.7 (4.5:4.9) | 5.2 (4.6:5.5) | 4.9 (4.6:5.6) | II vs I $p < 0.0001$<br>II vs III $p < 0.05$ |

Table 3. Median values, lower and upper quartiles (25:75) of echocardiographic parameters in the analyzed groups of children

| Variable | Control group (group I) | Obesity with hypertension (group II) | Obesity without hypertension (group III) | p-value |
|---|---|---|---|---|
| IVS (mm) | 7.9 (6.2:8.9) | 8.9 (7.6:9.7) | 8.0 (7.6:8.7) | II vs I $p < 0.05$ |
| LVDd Z-score | 1.26 (0.32:1.74) | 0.64 (-0.20:1.70) | 0.53 (-0.49:1.37) | ns |
| LAD Z-score | 0.45 (0.00:1.00) | 0.90 (0.05:1.73) | 0.85 (0.09:1.75) | II vs I $p < 0.0001$<br>III vs I $p < 0.001$ |
| LVMI > 95 pc | 0 | 18 (45%) | 5 (14%) | |
| RWT | 0.33 (0.30:0.36) | 0.34 (0.31:0.37) | 0.32 (0.30:0.34) | ns |
| RWT > 0.41 | n = 0 | n = 2 | n = 1 | |
| EF (%) | 68 (66:72) | 68 (65:72) | 69 (64:73) | ns |
| SV (mL) | 61.9 (42.3:87.9) | 82.3 (52.5:101) | 76.5 (52.9:91.4) | ns |
| HR (bpm) | 70 (56:74) | 72 (58:87) | 74 (62:87) | ns |
| CO (L/min) | 4.11 (3.44:5.44) | 6.50 (5.02:7.30) | 5.53 (4.02:6.63) | II vs I $p < 0.05$ |

## Results

The children from the investigational group did not differ from the controls in terms of age, sex and height characteristics (Table 1).

The parameters of central aortic pressure, determined by means of applanation tonometry, are presented in Table 2. Obese children with concomitant arterial hypertension showed the highest values of cSys and cDia. Obese normotensive subjects also presented with significantly higher cSys than the controls. The analyzed groups did not differ significantly in terms of such pulse wave parameters as Aix@75, peripheral resistance or pulse wave reflection. However, significantly higher values of PWV were found in obese hypertensive children when compared to both the controls and normotensive patients with obesity.

The results of the comparative analysis of echocardiographic parameters are presented in Table 3. Children and adolescents with overweight/obesity and concomitant arterial hypertension showed significantly higher values of IVS and LVMI than did

**Table 4.** Correlation coefficient of PWV, Aix@75, LVMI, LAD, and CO

| Variable | PWV (m/s) | Aix@75 (%) | LVMI (g/ht$^{2.7}$) | LAD (mm) | CO (L/min) |
|---|---|---|---|---|---|
| BMI SDS | 0.35* | -0.04 | 0.42* | 0.49* | 0.50* |
| cSys (mmHg) | 0.94* | 0.01 | 0.41* | 0.40* | 0.33 |
| cDia (mmHg) | 0.45* | 0.34* | 0.13 | 0.16 | 0.13 |
| PWV (m/s) | – | -0.12 | 0.18 | 0.39* | 0.40* |
| Aix@75 (%) | -0.12 | – | -0.13 | -0.38* | -0.16 |
| LVMI (g/ht$^{2.7}$) | 0.18 | -0.13 | – | 0.52* | 0.33 |
| LAD (mm) | 0.39* | -0.38* | 0.52* | – | 0.56* |
| CO (L/min) | 0.40* | -0.16 | 0.33 | 0.56* | – |

* $p < 0.001$.

the controls. Moreover, they presented with significantly higher CO. Normotensive children with overweight/obesity also showed higher values of LVMI and LAD when compared to the controls.

We found a number of significant correlations between the analyzed anthropometric and hemodynamic parameters (Table 4). After the analysis of these correlations, 2 regression models were developed to explain the sources of variance in cardiovascular risk markers: PWV and Aix@75. We showed that patient age, cSys, cDia and LAD explained up to 91% of variance in PWV ($p < 0.0001$). In turn, patient age and sex, HR, LV mass, LAD and cDia were identified as significant predictors of Aix@75, explaining up to 41% of variance in this parameter. Both models turned out to be statistically significant after stratification of patients according to their body weight and incidence of arterial hypertension.

## Discussion

Our findings suggest that children with overweight/obesity and hypertension present with higher values of pulse wave velocity and cardiac output. All overweight/obese children had high LVMI and LAD. Central aortic systolic pressure was higher not only in overweight/obese hypertensive children but also in the overweight/obese peripheral normotensive group. These findings may contribute to increased risk for arterial stiffness in this group of patients.

Central aortic pressure depends on stroke volume, peripheral resistance and elastic properties of arteries. Pulse wave velocity and augmentation index represent well-established markers of arterial stiffness.[7,18] Pulse wave velocity is an outcome of arterial elasticity and rigidity. Augmentation index is also a blood pressure-independent measure of arterial elasticity. Obesity is known to be associated with greater arterial stiffness and increased incidence of cardiovascular disease in adulthood.[2,19] Our findings are consistent with the results of previous studies, in which children and adolescents with overweight/obesity presented with higher values of PWV than their peers with normal body weight.[8,9,20] One previous study demonstrated a correlation between PWV and continuous metabolic syndrome score in Indian children.[21] This evidence suggests that obesity may be an independent factor affecting the status of the vascular wall. However, this association is likely disease-stage specific, as it was shown to be particularly strong in adult patients with angiographic evidence of changes in coronary arteries.[22]

In our study, children with obesity and arterial hypertension presented with significantly higher values of PWV when compared to both controls and obese normotensive subjects. There were no statistically significant differences in PWV between obese normotensives and controls. Elevated arterial blood pressure in childhood has been demonstrated to be a consistent predictor of arterial stiffness in otherwise asymptomatic young adults.[23] The presence of arterial hypertension or even prehypertension in childhood/adolescence is a predictor of cardiovascular impairment during early adulthood.[7]

We identified cSys, cDia, LAD and patient age as significant predictors of variance in PWV. An association between PWV and age was previously demonstrated in a large group of children.[24] Despite the positive correlation between PWV and BMI observed in our sample (Table 4), the latter did not prove to be a significant predictor of PWV on regression analysis. Other authors have documented associations between PWV and other markers of cardiovascular risk such as preperitoneal fat tissue thickness and waist/hip circumference.[8,22] However, we did not include these parameters in our analysis.

AS can be also quantified on the basis of augmentation index, Aix@75.[5] This parameter turned out to be correlated with the presence of obesity and coronary artery disease in adults.[22] In the case of children and adolescents, this association was more evident in patients with arterial hypertension than in obese individuals.[7,9] However, we did not find a similar relationship in our material; the only predictors of Aix@75, both in the whole group of children and in their subsets with/without overweight/obesity and arterial hypertension, were age, sex, HR, LV mass, LAD and cDia.

Central aortic pressure exerts a direct effect on left ventricular load. LV overload leads to myocardial hypertrophy and is associated with increased risk of myocardial ischemia.[25] In our study, children with overweight/obesity presented with significantly higher values of LVMI than their peers with normal body weight (Table 3). In contrast, we did not find significant differences in the LVMI values of overweight/obese patients with or without concomitant arterial hypertension. Obese children whose arterial blood pressure level did not correspond to hypertension presented with significantly higher values of central aortic systolic pressure (cSys) than did the normotensive subjects. LVMI exceeded the 95$^{th}$ percentile for this parameter, proposed by Daniels et al., in up to 45% of children with arterial hypertension and in only 14% of normotensive obese subjects (Table 3).[16] According to some authors, the LVMI of children and adolescents with primary hypertension correlates significantly with their 24 h systolic blood pressure.[26] The authors of another study found left ventricular hypertrophy (LVH) in 30% of obese adolescents at mean age <18 years, despite a low prevalence of arterial hypertension in this group.[27] Aside from higher mean ambulatory blood pressure levels, children with arterial hypertension and LVH may also present with higher mean BMI Z-scores when compared to LVH-free individuals.[28] Therefore, left ventricular hypertrophy seems to result from the influence of metabolic and hormonal factors associated with obesity, rather than from arterial hypertension itself. Previous studies of children, adolescents and young adults showed that both obesity and systolic blood pressure correlate significantly with the incidence of left ventricular hypertrophy, and showed weaker associations between LVH and other components of the metabolic syndrome.[29]

We found strong correlations between LVMI, cSys, BMI SDS and LAD (Table 4). However, similar to previous studies, we did not observe an association between LVMI and cDia.[26] Moreover, we did not demonstrate significant correlations between LVMI, PWV and Aix@75. Adult patients with pharmacologically controlled arterial hypertension were shown to present with normal left ventricular mass, which correlated significantly with PWV, but not Aix@75.[30]

The type of left ventricular remodeling may also have its unfavorable prognostic implications.[6] Children and adolescents with increased left ventricular mass show a variable degree of concentric/eccentric hypertrophy and concentric remodeling. Most of our patients with LVH presented with eccentric hypertrophy, and only sporadically did their RWT values exceed 0.41 (Table 3). According to the literature, patients with concentric hypertrophy are characterized by higher BMI Z-scores, while those with eccentric hypertrophy present with higher values of systolic blood pressure and larger left atrial diameters.[28] Due to the small number of patients with concentric hypertrophy, we did not conduct a similar comparative analysis in our sample.

Despite the lack of clinical symptoms and normal ejection fraction, LV hypertrophy is associated with development of left ventricular diastolic dysfunction.[4,7] An alteration of LV geometry leads to changes in aortic diameter and coexists with enlargement of the left atrium. Left ventricular diastolic dysfunction was observed in children and adolescents with obesity, as well as in subjects with arterial hypertension.[7,29] In our study, both groups of obese children presented with significantly higher left atrial diameters (LAD) despite the lack of significant changes in ejection fraction. Enlargement of the left atrium is known to correlate with BMI, systolic blood pressure and left ventricular mass in children and adolescents.[29] We observed such correlations in our sample, along with associations between LAD, PWV and Aix@75 (Table 4). However, it should be stressed that left atrial diameter reflects the volume of circulating blood. This was confirmed in our study, as children with overweight/obesity presented with higher values of cardiac output than their peers with normal body weight.

Our study showed that children with overweight/obesity present with elevated values of central aortic systolic pressure, pulse wave velocity, left ventricular mass index, left atrial diameter and cardiac output. The risk of these abnormalities is further increased due to concomitant arterial hypertension. The abovementioned changes may correspond to early stages of unfavorable remodeling of the cardiovascular system in young patients. This substantiates routine determination of central systolic aortic pressure, PWV and selected echocardiographic parameters (IVS, LAD) in overweight/obese children. Such attitude could be helpful in the identification of patients at increased risk of cardiovascular dysfunction and its complications, and would result in an intensification of preventive and therapeutic measures in this group.

## Limitations

A non-invasive estimation of the central aortic pressure was used.

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# Biologically active form of vitamin B1 in human peritoneal effluent

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## Abstract

**Background.** Supplementation with vitamin B1 protects the peritoneal membrane from inflammatory and oxidative insults and preserves residual kidney function in rat models of peritoneal dialysis (PD). It is assumed that an active form of vitamin B1, thiamin diphosphate (ThDP), is responsible for this protective effect. However, it has never been shown whether ThDP, a compound known not to cross cellular membranes, is actually detectable in human peritoneal effluent.

**Objectives.** This study was designed to investigate the concentration, appearance rate, and daily loss of ThDP in the peritoneal effluent of patients treated with PD.

**Material and methods.** We performed 24-hour effluent collection as well as the peritoneal equilibration test (PET) and analyzed the relation between the transport characteristics of the peritoneal membrane and appearance rate of ThDP in a cohort of 26 PD patients.

**Results.** ThDP was detectable in peritoneal effluent in humans. ThDP appearance rate was independent of the transport characteristic of peritoneal membrane, and was not associated with peritoneal transport of other small solutes.

**Conclusions.** We conclude that ThDP can be found in detectable concentrations in the peritoneal effluent in humans and is transported through the peritoneal membrane in a pattern independent of other small solutes. Our finding opens novel opportunities in further research on the protection of peritoneal membrane in humans.

**Key words:** peritoneal dialysis, thiamine, micronutrient

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## Background

Thiamin, also known as vitamin B1, is an essential nutrient for humans. Whereas it is synthesized by many plants and microorganisms, all mammals depend on its consumption with diet. Besides being a potent cofactor for numerous enzymatic activities essential in energy metabolism pathways, thiamin is involved in brain functioning and interneuronal communication.[1] Also, it plays a role in the regulation and activation of the immune system. Interestingly, via suppressing activation of necrosis factor NFκB, thiamin inhibits a release of inflammatory markers by macrophages.[2] Vitamin B1 has also been regarded to constitute anti-inflammatory factor and regulate the expression of inflammatory agents.[3] Thiamin has received increased interest recently, since the discovery that it is able to reduce peritoneal AGE accumulation, fibrosis, neovascularization, and inflammation in uremic rats. Also, it preserves the remnant kidney function and has an impact on residual renal function in peritoneal dialysis (PD).[4] These protective effects are assumed to occur due to the action of thiamin diphosphate (ThDP), a biologically active derivative of vitamin B1. It is a product of an intracellular reaction involving thiamin phosphokinase activity.[1] Interestingly, unlike thiamin monophosphate or free thiamin cation, ThDP is believed not to cross cellular membranes.[1] Hence, despite the beneficial effect of ThDP described in a PD model in rats, it is not known whether this compound can be detected in human peritoneal effluent.

Taking into account the high prevalence of inadequate vitamin B1 status, which may lead to neuropathy, autonomic dysfunction, impaired immune system reactivity, and enhanced inflammatory response, the scarcity of evidence concerning peritoneal thiamin losses may be surprising. Only few studies have assessed vitamin B1 status in PD patients.[5–10] To the best of our knowledge, only 1 measured vitamin B1 peritoneal losses.[5] However, ThDP losses have not been assessed and reported yet. Consequently, there is no data on the factors that may contribute to the magnitude of such losses, and the relation of ThDP appearance rate to the transport characteristics of the peritoneal membrane has never been addressed.

The peritoneal equilibration test (PET) is a clinical tool to assess the peritoneal membrane characteristics of individual patients in order to provide an appropriate PD prescription. The PET can be used to estimate transport properties of the peritoneal membrane. Assessment of ThDP in dialysis effluent during the PET makes it possible to analyze active thiamin compound appearance in the effluent under standardized and reproducible conditions. Also, it makes it possible to analyze the relationship between ThDP loss and the transport characteristics of the peritoneal membrane.

The aim of our research was to measure ThDP concentration in the peritoneal effluent, to assess ThDP losses related to PD, and to predict the pattern of ThDP transport, as compared to the transport of blood urea nitrogen (BUN), creatinine, and glucose in PD patients.

## Methods

Written consent was obtained from all participants before entering the study, and the local ethics committee approved the protocol. In this cross-sectional study, we recruited 26 adult participants. They comprised 16 patients treated with continuous ambulatory peritoneal dialysis (CAPD) and 10 patients treated with automated peritoneal dialysis (APD). Patients with an episode of peritonitis in the preceding 30 days, with PD catheter malfunction, ultrafiltration failure, uncontrolled blood glucose, active infection, or the ones clinically overhydrated or dehydrated were excluded from the study. On the day of the PET performance, all patients underwent estimation of dialysis adequacy (KT/V), normalized protein catabolic rate (nPCR) and calculation of glomerular filtration rate (GFR) and peritoneal clearances based on 24-hour collections of urine and dialyzate. We measured ThDP, creatinine, BUN and protein levels in a 24-hour effluent collection. The PET was performed according to standard procedure (2-liter bag of 2.3% glucose solution, effluent sampling at 0 h, 2 h, and 4 h). ThDP was measured in dialysis effluent at 2 h and 4 h. In 8 patients, a blood sample for ThDP analysis in plasma was also taken at 2 h. Plasma was separated after centrifugation at 4500 RPM and stored with samples of dialysis effluents in -80°C for further processing.

We also analyzed demographic (age, sex, body mass index (BMI), time on dialysis) and clinical (diabetes mellitus (DM) comorbidity, PD modality, ultrafiltration rate (UF), residual renal function (RRF), glomerular filtration rate, peritoneal transport characteristics, peritoneal clearance, normalized protein catabolic rate (nPCR), weekly KT/V, hemoglobin (Hb), hematocrit (Htc), and neutrophil to lymphocyte ratio (N/L)) indices, as predicted contributors that may influence ThDP concentration.

Total body water was estimated according to Watson's formula, and nPCR was calculated using Bergstrom's formula.[11,12] All laboratory measures were performed with an autoanalyzer (Modular Roche, Roche Diagnostics, Indianapolis, USA), using standard methods as follows: BUN with the spectrophotometric method, creatinine with the colorimetric kinetic test, glucose with the spectrophotometric hexokinase method, and protein with the colorimetric method. Complete blood count was performed with flow cytometry (Sysmex XE 2100D, Sysmex Corp., Kobe, Japan).

ThDP levels in effluent and plasma were detected using high performance liquid chromatography (HPLC Dionex equipped with C-18 reversed phase column Hypersil Gold, Dionex Corp., Sunnyvale, USA) with coupled detection – spectrophotometric (detector UV 340S Dionex) and coulometric (detector Coulochem II model 5019 ESA,

Thermo Fisher Scientific, Waltham, USA). The calibration curves proved to be linear; the method is characterized by a precision of 2.9%. The minimum limit of quantification (LOQ) was equal to 8.7 ng/mL; the minimum limit of detection (LOD) was equal to 2.9 mg/mL.

## Statistics

Distributions of all data were tested according to the Shapiro-Wilk test. Variables are presented as medians (95% CI) and ranges, when appropriate. Multiple regression was performed in order to find associations between ThDP concentration and appearance rates and potential contributors. Univariate correlations between the variables were calculated using the Spearman correlation coefficient. Statistical significance was considered at $p < 0.05$. Data analysis was accomplished using STATISTICA v. 12 PL (StatSoft, Inc., Tulsa, USA).

**Table 1.** Clinical and biochemical characteristics of participants

| Characteristic | Median (95% CI) n = 26 |
|---|---|
| Age (years) | 56 (50–59) |
| Gender ( M/F ) | 12/14 |
| No. of diabetics | 5 |
| Time on PD (months) | 18 (12–46) |
| PD modality (APD/CAPD) | (10/16) |
| BMI (kg/m$^2$) | 25.3 (24.1–27.9) |
| Residual renal function: GFR (mL/min/1.73 m$^2$) | 2.9 (2.8–6.1) |
| Urine output (mL/24 h) | 900 (676–1296) |
| Plasma creatinine (mg/dL) | 7.8 (7.1–9.7) |
| Plasma BUN (mg/dL) | 48.5 (44.8–60.0) |
| Plasma glucose (mg/dL) | 92 (86–106) |
| Hemoglobin (g/L) | 10.8 (10.2–11.1) |
| Hematocrit (%) | 33 (31–34) |
| Neutrocytes/lymphocytes ratio | 2.8 (2.4–3.6) |
| CrCl (mL/min/week) | 68 (69–94) |
| pCrCl (mL/min/week) | 39.5 (30.6–41.3) |
| Kt/V (weekly) | 2.1 (2.05–2.73) |
| nPCR (g/kg/24 h) | 1.20 (1.10–1.40) |
| Protein peritoneal loss (g/24 h) | 0.5 (0.4–0.7) |
| Ultrafiltration at 4 h (mL) | 450 (368–493) |
| No. of patients receiving thiamine supplements (6 mg daily) | 15 |

**Table 2.** Thiamin diphosphate in peritoneal effluent and plasma

| Variable | Median (95% CI) n = 26 | Range n = 26 |
|---|---|---|
| Peritoneal loss in 24 h (µg) | 92.6 (55.4–351.6) | 11.9–1546.7 |
| Appearance rate in effluentafter 240 min (ng/min) | 103.1 (86.7–124.6) | 38.3–176.5 |
| Concentration in 24-hour effluent collection (ng/mL) | 91.0 (58.3–330.1) | 168–1381 |
| Concentration in effluent at the 2$^{nd}$ hour of the PET (ng/mL) | 80 (70.8–115.9) | 16–222 |
| Concentration in effluent at the 4$^{th}$ hour of the PET (ng/mL) | 93 (85.5–123.9) | 38–181 |
| Concentration in plasma (ng/mL) | 17.2 (14.7–19.3)* | 13.2–22.2* |

* n = 8.

## Results

The characteristics of the study group are shown in Table 1. With regard to the PET results, the mean value of D/P (dialyzate-to-plasma ratio) of creatinine at 240 min was 0.59 ±0.1. Four participants were classified as fast, 14 as average fast/high, 7 as average slow/low, and 1 as slow transporter. Figure 1 depicts ThDP appearance rate at 240 min of the PET and ThDP loss in 24-hour effluent collection in individual participants. Both parameters varied markedly interindividually. Nevertheless, the appearance rate and daily losses of ThDP were significantly correlated (Spearman $r = 0.702$; $p < 0.05$). Also, a relationship was found between daily loss of ThDP and its concentration in effluent at the 2$^{nd}$ and 4$^{th}$ hour of the PET (Fig. 2).

The median value of ThDP daily loss is shown in Table 2. Table 2 also shows ThDP concentrations in 24-hour effluent collection, and in samples at 2 h and 4 h of the PET. In 8 patients, plasma concentrations of ThDP were also measured. In all cases, the plasma values were low, close to the lower limit of the reference range for humans.

In multivariate regression, supplementation with thiamin hydrochloride, the modality of PD treatment (CAPD vs APD), diabetic status or anuria were not associated with ThDP appearance rate and losses. ThDP losses, dialyzate concentration and appearance rates could also not be explained by age, BMI, time on PD, ultrafiltration rates, peritoneal protein loss, nPCR, weekly KT/V, total week clearances of urea, and creatinine or by residual GFR.

ThDP effluent concentrations and appearance rates were not associated with plasma ThDP, as well as plasma and effluent concentrations and appearance rates of BUN and creatinine. There was no relationship between ThDP and plasma or effluent glucose concentrations. Also, peritoneal membrane characteristics, as classified in the PET, did not influenced daily ThDP losses.

In univariate analysis, we found weak associations between ThDP effluent concentrations at 4 h of the PET and N/L index ($r = 0.397$; $p < 0.05$), peritoneal urea and creatinine clearances ($r = 0.487$ and $r = 0.429$, respectively; $p < 0.05$) and patients' weight and height ($r = -0.460$ and $r = -0.499$; $p < 0.05$).

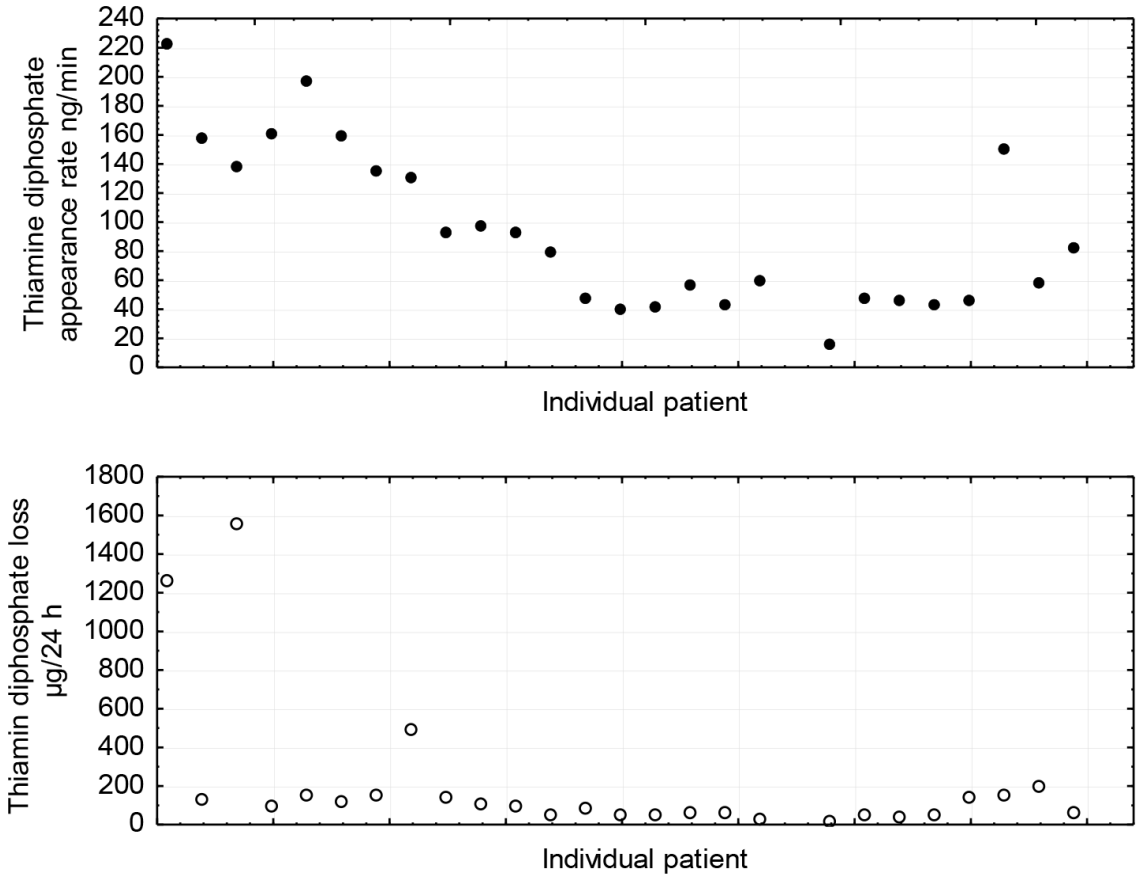


Fig. 1. Appearance rate of thiamin diphosphate (ThDP) at 240 min of the peritoneal equilibration test (PET) and daily dialyzate losses of ThDP in all individual cases (n = 26)

## Discussion

Peritoneal losses and concentration of ThDP, a biologically active form of thiamine, in human dialyzate, have not been studied so far – our study aimed to fill this gap in knowledge. In view of the recent findings of the benefits of thiamine supplementation, which seems to protect the peritoneal membrane from inflammatory and oxidative insults, knowledge of ThDP may have important clinical implications. The PET allowed us to analyze ThDP losses under standardized and reproducible conditions. The most important finding of the presented study has been an unequivocal identification of ThDP in dialysis effluents. Thus, we have not only confirmed the previous findings of Boeschoten et al. that thiamine may be lost by this route, but we have also shown that the thiamine active form appears in the peritoneal effluent.[5] ThDP losses, although modest in most cases, may exceed daily recommended intake (DRI) for vitamin B1, as is depicted in Fig. 1. Thiamine loss seems to be independent of the transport characteristics of the peritoneal membrane, but we have found significant positive associations between effluent ThDP concentrations and peritoneal clearances of urea and creatinine. Effluent BUN concentrations also correlated with those of ThDP in the 4$^{th}$ hour of the PET. Thus, thiamine transport follows to some extent the pattern of the transport of small solutes.

Plasma ThDP levels proved to be below the reference range for the method used (20–50 ng/mL), in most analyzed cases. Interestingly, plasma thiamine levels are known to respond to carbohydrate intake in humans.[13] It is plausible that glucose absorption from 2.3% dialytic solution is responsible for the low plasma ThDP in the 2$^{nd}$ hour of the PET. However, we have not found any correlation between effluent glucose concentration and plasma ThDP to support such a hypothesis.

We found a higher concentration of ThDP in the 24-hour effluent collection than the concentration in plasma. Hence, some mechanism of active transport or local ThDP synthesis must be responsible for this phenomenon. Mean ThDP D/P is well above 1, indicating other than simple gradient diffusion ways of ThDP dialyzate appearance. Neutrocyte/lymphocyte (N/L) ratio, reflecting systemic inflammatory status, was a parameter significantly associated with thiamine D/P. However, it is unclear whether it reflects the changed transport characteristics of the peritoneal membrane or rather local increased turnover of ThDP in the peritoneal cavity. This is an interesting finding that needs further investigation.

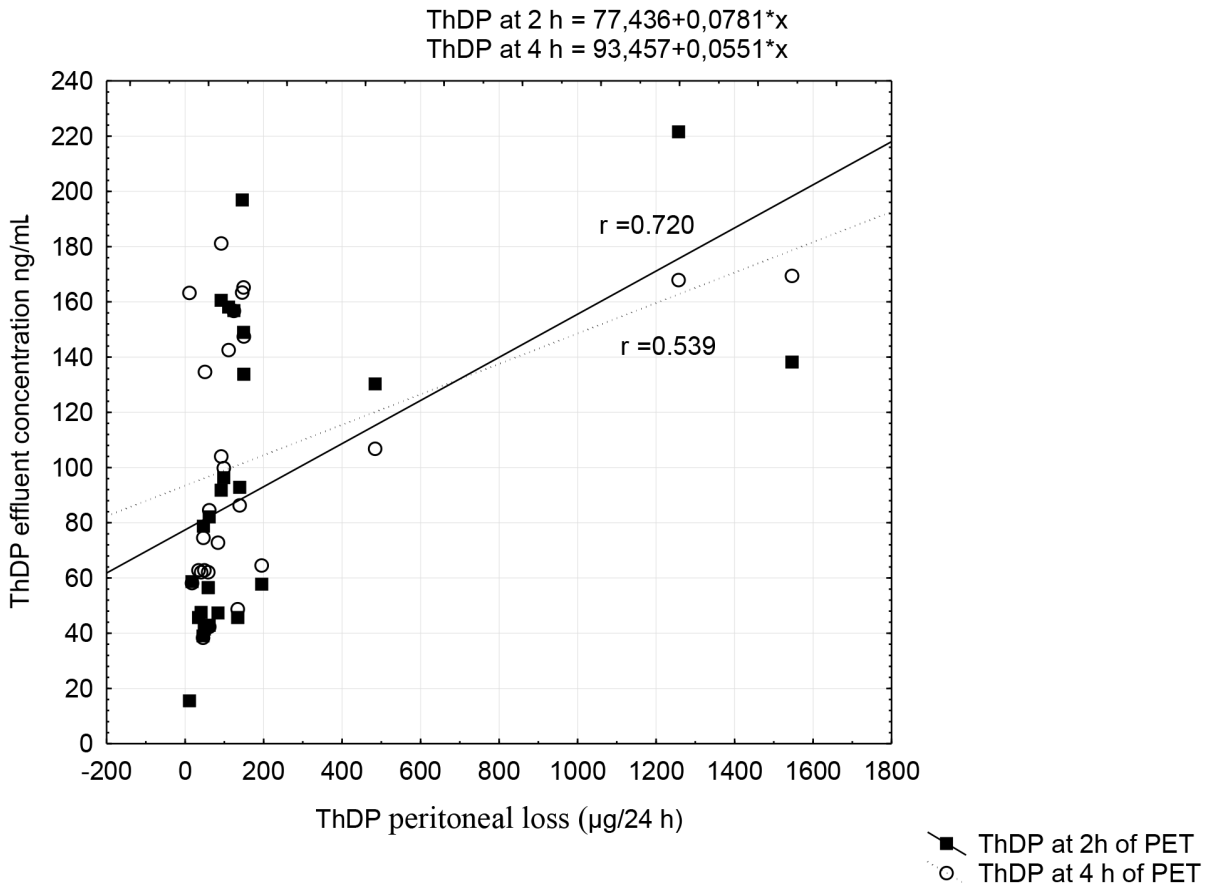


Fig. 2. Peritoneal loss and effluent concentrations of thiamin diphosphate(ThPD) at 2h and 4h of the peritoneal equilibration test (PET)

The change of glucose concentration in effluent has not influenced ThDP levels. Thus, convection seems to have little impact on ThDP peritoneal losses.

Unexpectedly, supplementation with vitamin B1 seems to have no impact on peritoneal ThDP losses. Supplement derived thiamine, which occurs in unphosphorylated compound, can be easily lost with urine.[14] Taking into account the decreased vitamin B1 ingestion due to dietary restrictions, impaired absorption, and dialysis-related losses shown in this study, the need for thiamine supplements in PD patients should be widely recognized.[15,16]

In conclusion, we have shown that the active, phosphorylated form of thiamine is present in the dialyzate effluents of PD patients. We have also shown that ThDP appearance in dialyzate is independent of the transport characteristics of the peritoneal membrane. Our findings offer novel perspectives for future research on the protection of the peritoneal membrane in humans.

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# Knowledge and selected variables as determinants of the quality of life and general health of patients with rheumatoid arthritis

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## Abstract

**Background.** Rheumatoid arthritis (RA) is an incurable disease resulting in progressive disability, which is associated with the loss of productivity and the inability to earn money, which might lead to a financial burden on the patient's family. Undoubtedly, the clinical picture of the disease and its consequences lead to the reduction of the quality of life.

**Objectives.** The aim of this study is to evaluate the influence of selected factors on the subjective assessment of the quality of life and general health of patients with RA.

**Material and methods.** The study was conducted among 270 patients with RA treated at the Department of Rheumatology and Internal Medicine. The quality of life and general health were assessed with the use of the SF-36 and the GHQ-30 questionnaires.

**Results.** In the study group, a statistically significant correlation between the results of the SF-36 and the GHQ-30 questionnaires was observed. It has been shown that the level of role limitations due to physical health problems (RP) is mostly affected by interpersonal relationships based on GHQ-30 questionnaire ($p = 0.002$), general health (GHQ-30) ($p = 0.001$) and subjective health condition (SF-36) ($p < 0.001$). In contrast, general health (GHQ-30) is positively affected by education ($p = 0.003$) and professional activity ($p = 0.001$), and negatively affected by a positive family history of RA ($p = 0.002$), frequent hospitalization ($p = 0.008$) and poor subjective health condition ($p < 0.001$).

**Conclusions.** People with poor subjective health condition are characterized by more limited activity due to physical health and lower general health condition. General health (GHQ-30) in patients with rheumatoid arthritis is influenced by education, place of residence, professional work, family history of RA and subjective health status.

**Key words:** quality of life, rheumatoid arthritis (RA), SF-36 questionnaire, General Health Questionnaire (GHQ)

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## Introduction

The quality of life is a multidimensional concept, varying over time, and takes into account an objective and subjective assessment of life in physical, psychological and social terms. One of the chronic diseases which results in the reduction of the quality of life is rheumatoid arthritis (RA), one of the most aggressive and most common rheumatic diseases. This is a condition causing chronic inflammation of the bone-joint system, and the result of this process is a permanent deformation of bones and joints along with their damage.

Rheumatoid arthritis is an incurable disease resulting in progressive disability so a social aspect of the disease is increasingly significant, especially when every 3rd person suffering from this condition is not capable of performing any paid work within 2 years from the time of diagnosis.[1–3] Progressive disability is associated with the loss of productivity, financial burden on the patient's family and the impossibility of earning money. Patients are often not prepared to change their way of life, which is the reason why it is important to make them aware of the significance of active cooperation in therapy. This applies especially to regular exercise at home, adapted to current capabilities, which helps to reduce the motor dysfunction, and the use of dietary therapy.[4]

One of the main problems of people with RA is pain,which undoubtedly is reflected in the mental condition of the patient and their social functioning. Due to the multidimensional character of the disease, it is important for the successful treatment of this patient group to improve mutual communication, engage the patient in the therapy, broaden their knowledge about the disease, which significantly influences the effects of the treatment.[5–7] The need to undertake educational activities aimed at patients with rheumatoid arthritis comes from the currently biggest challenge of contemporary medicine referring to all chronic diseases – failure in following the therapy guidelines.[8,9] Knowledge about the disease is the key to achieve the maximum effects of treatment and one of the ways to prevent the consequences of improperly treated disease.[10,11]

It is also essential to enable the patient, through counseling and retraining, to get a suitable employment opportunity, which is the basis for developing an active attitude to life and regaining independence in the later stages of the disease. It is also relevant in the process of accepting the disability and feeling satisfaction from life.[12]

The solution may be the improvement of the therapeutic relationship between the therapeutic team and the patient in order to increase the efficiency of the treatment through better knowledge about the disease, and to reduce the impact of the disease on the mental condition of the patient.[13]

## Material and methods

The study included 270 patients diagnosed with rheumatoid arthritis, treated at the Department of Rheumatology and Internal Medicine (Table 1).

The author's questionnaire, International Questionnaire SF-36 questionnaire, and the General Health Questionnaire GHQ-30 were used to collect the study data.

The author's questionnaire allowed us to gather basic information about the study group and to assess the level of knowledge about RA.[14]

Questionnaire SF-36 estimated the areas included in a wide approach to health definition, covering 8 competent scales defining physical and mental aspects of the quality of life.[15] These are:

- physical functioning – PF;
- mental health – MH;
- social functioning – SF;
- role physical – RP;
- role emotional – RE;
- vitality – VT;
- general health – GH;
- bodily pain – BP,

**Table 1.** Characteristics of the study group (data was presented as mean ±SD).

| Variable | | Characteristics of the study group | | | |
|---|---|---|---|---|---|
| | | entire group | women | men | p-value |
| Number of patients | | 270 (100%) | 195 (77.22%) | 75 (27.78%) | – |
| Age mean ±SD | | 56.9 ±15.2 | 57.6 ±15.5 | 55.11 ±14.45 | 0.23 |
| Education | basic | 28 (10.37%) | 20 (10.26%) | 8 (10.67%) | 0.001 |
| | vocational | 77 (28.52%) | 43 (22.05%) | 34 (45.33%) | |
| | secondary | 107 (39.63%) | 84 (43.08%) | 23 (30.67%) | |
| | higher | 58 (21.48%) | 48 (24.62%) | 10 (13.33%) | |
| Professional activity | work | 66 (24.44%) | 41 (21.03%) | 25 (33.33%) | 0.06 |
| | retirement pension | 72 (26.67%) | 48 (24.62%) | 24 (32%) | |
| | disability pension | 115 (42.59%) | 93 (47.69%) | 22 (29.33%) | |
| | benefit | 3 (1.11%) | 2 (1.03%) | 1 (1.33%) | |
| | unemployed | 14 (5.19%) | 11 (5.64%) | 3 (4%) | |
| Duration of the disease | 1–3 years | 71 (26.3%) | 48 (24.62%) | 23 (30.67%) | 0.006 |
| | 3–5 years | 43 (15.93%) | 23 (11.79%) | 20 (26.67%) | |
| | 5–10 years | 62 (22.96%) | 49 (25.13%) | 13 (17.33%) | |
| | >10 years | 94 (34.81%) | 75 (38.46%) | 19 (25.33%) | |
| Duration of the disease >10 years mean ±SD | | 19.2 ±7 | 18.65 ±6.5 | 21.26 ±8.51 | 0.48 |
| Disease in the family | | 121 (44.81%) | 96 (49.23%) | 25 (33.33%) | 0.02 |

Physical Component Summary (PCS) consists of PF, RP, VT, BP. Mental Component Summary (MCS) consists of MH, SF, RE, GH.

Factors of the quality of life were estimated on the basis of the system SF-36, which allowed us to estimate 8domains of life and to calculate on their grounds the level of physical and mental activities depicting the general condition of a patient. The level of physical and mental activities was calculated using the norms assumed for the USA because there are no norms created for Poland and accepted by the QualityMetric, Inc.

Five scales (PF, RP, BP, SF, RE) describe the state of a person's functioning as a lack of limitations or disability. Three of the above mentioned scales (PF, RP, BP) reflect most accurately the physical condition of a patient, but it should be noted that each of them refers to a different aspect thereof. PF measures the constraints of daily physical activity, RP includes limitations in life, while BP focuses on the pain and the resultant limitations in everyday life.

The most useful in the presentation of the mental functioning of a patient are SF and RE. Their maximum results (100 points) indicate the lack of physical and emotional problems.

Other scales such as MH, VT and GH are bipolar, i.e., they measure both the positive and the negative picture of a patient. For the MH scale, the scoring in the middle of the range (50 points) means the lack of restrictions caused by personal or emotional problems. In the case of achieving the maximum score, it indicates frequently recurring feelings of happiness and peace.

The changes in the physical and mental well-being are detected by VT and GH scales. The middle score on the VT scale means no symptoms of fatigue or exhaustion, while the maximum (100 points) indicates additionally constantly occurring feeling of energy. The middle and higher scores on the GH scale confirm general health.[14]

General Health Questionnaire (GHQ) by David Goldberg is a screening tool used to assess non-specific psychiatric morbidity. The authors of the Polish version of GHQ-30 are Krzysztof Małyszczak and Stanisław Sidorowicz.[16] The test assesses the current psychiatric condition and is used to identify the cases which are at a significant likelihood of developing disorders. The overall score of the questionnaire is influenced by individual sensibility, a way of going through and feeling during the course of disease. Therefore it is believed that using GHQ can also help to measure the condition of mental disorder and a subjective evaluation of its consequences, which is called a non-specific psychiatric morbidity. This study used a version of the questionnaire with 30 questions (GHQ-30). The maximum score a patient can get is 30 points. The threshold of 5 points provides a basis for the identification of persons with mental disorders. The study used a scoring method by Goldberg: 0–0–1–1. The questionnaire can be treated, on one hand, as a 1-dimensional tool; on the other hand, it would be reasonable to isolate specific factors, which are called: anxiety and depression, interpersonal relations and general functioning.

The statistical analysis was performed using the program STATISTICA v. 10 PL with the application of Student's t-test and $\chi^2$ test. The level of significance was set at $p = 0.05$.

The analysis of the impact of independent variables (covariates) on the level of quality of life in patients with rheumatoid arthritis was conducted through a multiple regression analysis. The model takes into account the following factors:

- X1: age;
- X2: gender (1 – woman; 0 – man);
- X3: place of residence (0 – village; 1 – city);
- X4: vocational education (0 – no; 1 – yes);
- X5: secondary education (0 – no; 1 – yes);
- X6: higher education (0 – no; 1 – yes);
- X7: professional activity (0 – no; 1 – yes);
- X8: professional activity – disability pension (0 – no; 1 – yes);
- X9: professional activity – pension (0 – no; 1 – yes);
- X10: professional activity – unemployed (0 – no; 1 – yes);
- X11: duration of disease 1–3 years (0 – no; 1 – yes);
- X12: duration of disease 5–10 years (0 – no; 1 – yes);
- X13: duration of disease >10 years (0 – no; 1 – yes);
- X14: disease in the family (0 – no; 1 – yes);
- X15: preventing procedures – regular medication (0 – no; 1 – yes);
- X16: preventing procedures – gymnastics (0 – no; 1 – yes);
- X17: lack of preventing procedures – smoking cigarettes (0 – no; 1 – yes);
- X18: preventing procedures – healthy diet (0 – no; 1 – yes);
- X19: medical consultation once a year (0 – no; 1 – yes);
- X20: medical consultation twice a year (0 – no; 1 – yes);
- X21: medical consultation more than twice a year (0 – no; 1 – yes);
- X22: hospitalization once a year (0 – no; 1 – yes);
- X23: hospitalization twice a year (0 – no; 1 – yes);
- X24: sources of knowledge – doctor (0 – no; 1 – yes);
- X25: sources of knowledge – physiotherapist (0 – no; 1 – yes);
- X26: sources of knowledge – nurse (0 – no; 1 – yes);
- X27: sources of knowledge – books (0 – no; 1 – yes);
- X28: sources of knowledge – medical journals (0 – no; 1 – yes);
- X29: sources of knowledge – journals (0 – no; 1 – yes);
- X30: sources of knowledge – television (0 – no; 1 – yes);
- X31: sources of knowledge – internet (0 – no; 1 – yes);

- X32: sources of knowledge – colleagues (0 – no; 1 – yes);
- X33: subjective estimation of the level of knowledge (0–10);
- X34: difficulties in education – lack of time (0 – no; 1 – yes);
- X35: difficulties in education – lack of interest (0 – no; 1 – yes);
- X36: difficulties in education – lack of financial possibilities (0 – no; 1 – yes);
- X37: difficulties in education – lack of place (0 – no; 1 – yes);
- X38: difficulties in education – poor preparation (0 – no; 1 – yes);
- X39: subjective health condition – good (0 – no; 1 – yes);
- X40: subjective health condition – not bad (0 – no; 1 – yes);
- X41: subjective estimation of health – bad (0 – no; 1 – yes);
- X42: state of knowledge – symptoms of disease (point);
- X43: state of knowledge – rehabilitation and pharmacological treatment (point);
- X44: state of knowledge – prevention and leisure (point);
- X45: state of knowledge – general state of knowledge (point);
- X46: a feeling that life is not worth living (1 – not at all; 2 – not more than usual; 3 – a bit more than usual; 4 – a lot more than usual);
- X47: symptoms of anxiety and depression GHQ-30 (points);
- X48: interpersonal relations GHQ-30 (points);
- X49: overall functioning GHQ-30 (points);
- X50: overall result of the health GHQ-30 (points).

## Results

The analysis of the study material showed that there were no statistically significant differences in particular domains of the SF-36 questionnaire in terms of gender (Table 2).

A multiple regression analysis of the impact of independent variables on the quality of life in patients with RA has yielded the following results:

- the level of physical functioning (PF) is positively affected by active professional work ($p = 0.002$), higher education ($p = 0.004$) and the acquisition of knowledge about the disease from friends ($p = 0.01$), and is negatively affected by age ($p = 0.001$), lack of education ($p = 0.004$), low subjective state of knowledge about the disease ($p = 0.004$) and poor subjective health (SF-36) ($p < 0.001$) (Table 3);
- limited activity due to physical health (RP) is mostly influenced by the interpersonal relationships (GHQ-30) ($p = 0.002$), the general level of health (GHQ-30) ($p = 0.001$) and subjective health condition (SF-36) ($p < 0.001$);
- the level of pain (BP) depends on interpersonal relationships (GHQ-30) ($p = 0.019$), the general health (GHQ-30) ($p < 0.001$) and subjective health status (SF-36) ($p = 0.004$);
- the general perception of health (GH) is positively influenced by the acquisition of knowledge from nurses ($p = 0.02$), and is negatively affected by age ($p = 0.005$) and the presence of RA history in the family ($p = 0.001$) (Table 4);
- the level of vitality (VT) is positively influenced by the acquisition of knowledge from a physiothera-

**Table 2.** The analysis of factors related to quality of life – SF-36

| Factors related to quality of life – SF-36 | Study group | | | |
|---|---|---|---|---|
| | entire group | women | men | p-value |
| Number of patients | 270 (100%) | 195 (77.22%) | 75 (27.78%) | |
| Subjective health condition<br>perfect<br>very good<br>good<br>not bad<br>bad | <br>0<br>2 (0.74%)<br>34 (12.59%)<br>114 (42.22%)<br>120 (44.44%) | <br>0<br>2 (1.03%)<br>25 (12.82%)<br>76 (38.97%)<br>92 (47.18%) | <br>0<br>0<br>9 (12%)<br>38 (50.67%)<br>28 (37.33%) | 0.29 |
| Subjective health state [points (0–100)] mean ±SD | 18.86 ±20.75 | 18.43 ±21.35 | 19.99 ±19.2 | 0.58 |
| Physical functioning (PF) [points] mean ±SD | 33.69 ±25 | 31.85 ±24.73 | 38.47 ±25.24 | 0.051 |
| Limited activity due to role physical (RP) [points] mean ±SD | 9.44 ±25.84 | 9.23 ±25.35 | 10 ±27.26 | 0.83 |
| Bodily pain (BP) [points] mean ±SD | 26.18 ±18.9 | 26.84 ±19.84 | 25.01 ±16.25 | 0.53 |
| General health (GH) [points] mean ±SD | 19.23 ±13.33 | 19.05 ±13.86 | 19.71 ±11.91 | 0.72 |
| Vitality (VT) [points] mean ±SD | 35.31 ±11.34 | 34.95 ±10.48 | 36.27 ±13.36 | 0.39 |
| Social functioning (SF) [points] mean ±SD | 44.77 ±25.09 | 44.62 ±25.76 | 45.17 ±23.42 | 0.87 |
| Limited activity due to role emotional (RE) [points] mean ±SD | 53.95 ±48.59 | 51.45 ±48.72 | 60.44 ±47.99 | 0.17 |
| Mental health (MH) [points] mean ±SD | 51.29 ±16.9 | 51.2 ±17.11 | 51.52 ±16.43 | 0.89 |
| Physical component summary (PCS) [points] mean ±SD | 24.18 ±7.52 | 24.01 ±7.71 | 24.63 ±7.03 | 0.54 |
| Mental component summary (MCS) [points] mean ±SD | 42.66 ±10.35 | 42.42 ±10.5 | 43.28 ±9.99 | 0.55 |

pist ($p < 0.001$), a medical journal ($p = 0.004$) or the Internet ($p = 0.005$), whereas it is negatively affected by a low level of health (GHQ-30) ($p < 0.001$);
- the level of social functioning (SF) is positively influenced by good subjective health condition (SF-36 ($p = 0.002$), whereas it is negatively affected by a feeling that life is not worth living (GHQ-30) ($p = 0.027$) and a low level of health (GHQ-30) ($p = 0.00$) (Table 5);
- role emotional (RE) depends on the frequency of medical consultations (more than twice a year) ($p = 0.003$);
- the level of mental health (MH) is positively influenced by a low level of symptoms of anxiety and depression (GHQ-30) ($p = 0.014$), whereas it is negatively affected by a positive family history ($p = 0.007$) and poor subjective health (SF-36) ($p = 0.005$);
- the level of PCS is positively influenced by declared very good health condition (SF-36) ($p = 0.037$), whereas it is negatively affected by the age factor ($p < 0.001$);
- the level of MCS is positively influenced by frequent medical consultations ($p = 0.004$), whereas it is negatively affected by a positive family history of RA ($p = 0.009$), poor subjective health ($p = 0.015$) and a low level of health according to GHQ-30 ($p < 0.001$).

The analysis of the study material obtained from the GHQ-30 questionnaire showed that particular domains did not show any statistically significant differences in terms of gender (Table 6).

A multiple regression analysis of the impact of independent variables on the health levels in RA patients has yielded the following results:

- symptoms of anxiety and depression are negatively affected by the occurrence of disease (RA) in the family, rare medical consultations and a feeling that life is not worth living;
- interpersonal relationships are positively influenced by higher education ($p = 0.036$), place of residence – a city ($p = 0.009$) and a shorter duration of disease – 1–3 years ($p = 0.001$), and are negatively affected by a positive family history of RA ($p = 0.026$) and a feeling that life is not worth living ($p < 0.001$);
- general functioning is positively influenced by a higher education ($p = 0.002$) and reading books about the disease ($p = 0.0011$), whereas it is negatively affected by age ($p = 0.004$), the presence of the disease in the family ($p = 0.012$) and a feeling that life is not worth living ($p < 0.001$);
- general health (GHQ-30) is positively affected by education ($p = 0.003$), place of residence – a city ($p = 0.013$), active professional work ($p = 0.001$), whereas it is negatively affected by a positive family history of RA ($p = 0.002$), frequent stays in the hospital ($p = 0.008$) and poor subjective health status ($p < 0.001$) (Table 7).

**Table 3.** Summing up of changeable dependable regression concerning physical functioning (R2 = 0.594)

| Factors | β | b | p-value |
|---|---|---|---|
| Age | -0.28 | -0.47 | <0.001 |
| Vocational education | 0.19 | 10.77 | 0.004 |
| Secondary education | 0.17 | 8.49 | 0.021 |
| Higher education | 0.19 | 11.62 | 0.004 |
| Professional activity – work | 0.14 | 8.01 | 0.002 |
| Source of knowledge – friends | 0.11 | 7.51 | 0.01 |
| Subjective evaluation of the knowledge level | -0.12 | -1.72 | 0.004 |
| General functioning (GHQ-30) | -0.12 | -1.10 | 0.014 |
| Subjective health condition – not bad | -0.29 | -14.79 | <0.001 |
| Subjective health condition – bad | -0.59 | -29.70 | <0.001 |

The designated method of least squares model of physical functioning takes the following form: $PF = 80.88 - 0.47 \times X1 + 10.77 \times X4 + 8.49 \times X5 + 11.62 \times X6 + 8.01 \times X7 + 7.51 \times X32 - 1.72 \times X33 - 14.79 \times X40 - 29.7 \times X41 - 1.1 \times X49 \pm 16.23$; R2 = 0.594; β – variable importance factor; b – variable coefficient (y = bx + c); p – level of significance; R2 – relevance factor; c – standard error of estimate (y = bx ±c).

**Table 4.** Summing up of changeable dependable regression concerning general health perception (R2 = 0.443)

| Factors | β | b | p-value |
|---|---|---|---|
| Age | -0.14 | -0.13 | 0.005 |
| Disease in the family | -0.15 | -4.12 | 0.001 |
| Source of knowledge – nurse | 0.11 | 4.96 | 0.02 |
| Difficulties in obtaining education – lack of time | -0.14 | -4.10 | 0.006 |
| Difficulties in education – lack of interest | 0.10 | 2.92 | 0.042 |
| Subjective health condition – not bad | -0.52 | -14.10 | <0.001 |
| Subjective health condition – bad | -0.70 | -18.82 | <0.001 |

The designated method of least squares model of general health perception takes the following form: $GH = 46.44 - 0.13 \times X1 - 4.12 \times X14 + 4.96 \times X26 - 4.1 \times X34 + 2.92 \times X35 - 14.1 \times X40 - 18.82 \times X41 - 0.17 \times X50 \pm 10.1$; R2 = 0.443; β – variable importance factor; b – variable coefficient (y = bx + c); p – level of significance; R2 – relevance factor; c – standard error of estimate (y = bx ±c).

**Table 5.** Summing up of changeable dependable regression concerning social functioning (R2 = 0.49)

| Factors | β | b | p-value |
|---|---|---|---|
| Sources of knowledge – books | -0.11 | -5.97 | 0.017 |
| Sources of knowledge – medical papers | 0.09 | 7.69 | 0.045 |
| Feeling that life is not worth living | -0.12 | -3.73 | 0.027 |
| Interpersonal relationships (GHQ-30) | 0.32 | 4.41 | 0.004 |
| General health (GHQ-30) | -0.80 | -2.00 | 0.000 |
| Subjective health condition – not bad | -0.15 | -7.77 | 0.028 |
| Subjective health condition – bad | -0.24 | -11.96 | 0.002 |
| Subjective health condition – not bad | 0.10 | 1.72 | 0.035 |

The designated method of least squares model of social functioning takes the following form: $SF = 72.41 - 5.97 \times X27 + 7.69 \times X28 - 7.77 \times X40 - 11.96 \times X41 + 1.72 \times X43 - 3.73 \times X46 + 4.41 \times X48 - 2 \times X50 \pm 18.05$; R2 = 0.49; β – variable importance factor; b – variable coefficient (y = bx + c); p – level of significance; R2 – relevance factor; c – standard error of estimate (y = bx ±c).

## Discussion

A crucial element in the treatment and rehabilitation of patients with rheumatoid arthritis should be the analysis of quality of life. Due to the fact that it is a chronic, progressive and still incurable disease, care should be taken to improve the viability of patients by means of a thorough examination of the factors that impair their quality of life. In addition, continuously developing knowledge on the quality of life of patients with RA should help to eliminate the factors that have the most negative impact on the various spheres of life of such patients.

Our study confirmed that patients suffering from RA are characterized by low quality of life both in the domain of PCS and in the domain of MCS.[17–24] The lower number of points in the PCS domain in comparison to the MCS domain may indicate that RA affects more the physical than the mental sphere as indicated by other authors.[21,23,24]

It was also shown that the quality of life of patients with RA depends on many factors of physical, mental and social functioning sphere.[17,19–21,23–31]

Studies conducted by many authors have shown that poorer functioning of the respondents in the physical sphere is conditioned by the duration of the disease.[18,24,26,28,30] Studies by Baloglu et al. and Wisłowska et al. did not confirm this dependence.[20,31] In our study, the worse results in the PF domain and the PCS scale were related to age of the respondents, which has also been indicated by other authors.[20,23,31] It was also shown that people who subjectively assessed their health as poor had a lower perception of the quality of their lives in the domains of physical activity (PF, RP and BP) as well.

The literature highlights the fact that quality of life is modified by patients' convictions about their health, which is reflected in the results of our own study, where people with poor subjective health condition were characterized by greater limitations of activity due to physical health and lower general health (GHQ-30).[30]

The studies by van Vilsteren et al. concluded that patients suffering from RA with low quality of life are characterized by decreased productivity.[26] Our study showed that economic activity has a positive effect on the general health level according to GHQ-30.

Factors such as higher education and living in a city also had a positive effect on general health (GHQ-30).

The results obtained from the SF-36 questionnaire and GHQ-30 revealed the occurrence of symptoms such as anxiety and depression and a decreased quality of life in the MCS domain. Rheumatoid arthritis is undoubtedly a disease which leaves permanent consequences of a psychological nature, especially in the emotional area, and affects quality of life.[17,20,29] There are many premises suggesting that personal predispositions of a patient, their competence and approach to the disease may have a considerable influence on the further course of the disease and its prognoses. According to Abu Al-Fadl et al. and Maiden et al., during the treatment of patients with RA, more attention should be paid to the identification of symptoms of depression because the emotional condition affects the quality of life of patients and treatment outcomes.[17,32]

Frequent contact with the members of the therapeutic team and obtaining accurate information positively affected the selected domains (GH, VT, RE, MCS) in RA patients, which was presented for the first time in our study and proves the need for providing informational and emotional support by the members of the therapeutic team.

It was also shown that interpersonal relationships affected activity limitations due to physical health (RP), the level of pain (BP) and the level of social functioning (SF). This means that people with satisfying relationships with others less frequently experienced limitations in the sphere of physical activity and experienced less pain, and better functioned in the society.

Mäkeläinen et al. attempted to find an answer to the question if there is a dependence between the level of knowledge in 252 patients with rheumatoid arthritis and their physical functioning.[33] The studies did not reveal a correlation between the level of knowledge of patients and their physical functioning in everyday life. The autors' own study showed, however, that the level of knowledge about the disease affected the SF domain in the SF-36 questionnaire.

The evaluation of quality of life has an enormous significance in economic analyses. Financing health care, costs of treatment, its effectiveness are the leading subjects. We need to find an answer to the question if the treatment means applied for this purpose correlate with the effects, patient satisfaction and the level of quality of life.[34]

The quality of life of patients suffering from rheu-

Table 6. The analysis of data obtained from the General Health Questionnaire – GHQ-30

| Variable | Study group | | | |
|---|---|---|---|---|
| | all group | women | men | p-value |
| Number of patients | 270 (100%) | 195 (77.22%) | 75 (27.78%) | |
| Anxiety and depression symptoms [points] mean ±SD | 5.16 ±4.36 | 5.41 ±4.31 | 4.51 ±4.46 | 0.13 |
| Interpersonal relationships [points] mean ±SD | 1.51 ±1.79 | 1.59 ±1.80 | 1.33 ±1.77 | 0.29 |
| General functioning [points] mean ±SD | 3.58 ±2.66 | 3.68 ±2.68 | 3.32 ±2.62 | 0.32 |
| General health [points] mean ±SD | 12.57 ±9.95 | 13.07 ±9.89 | 11.27 ±10.04 | 0.18 |
| Feeling that life is not worth living<br>not at all<br>not more than usual<br>a bit more than usual<br>a lot more than usual | <br>127 (47.04%)<br>103 (38.15%)<br>27 (10%)<br>13 (4.81%) | <br>92 (47.18%)<br>71 (36.41%)<br>21 (10.77%)<br>11 (5.64%) | <br>35 (46.67%)<br>32 (42.67%)<br>6 (8%)<br>2 (2.67%) | 0.58 |

Table 7. Summary of changeable dependable regression concerning general health (GHQ-30) (R2 = 0.41)

| Factors | β | b | p-value |
|---|---|---|---|
| Place of residence | -0.13 | -3.05 | 0.013 |
| Secondary education | -0.17 | -3.38 | 0.002 |
| Higher education | -0.17 | -4.08 | 0.003 |
| Professional activity – work | -0.19 | -4.27 | 0.001 |
| Professional activity – disability pension | -0.17 | -3.76 | 0.002 |
| History of RA in the family | 0.15 | 3.01 | 0.002 |
| Hospitalization twice a year | 0.13 | 3.65 | 0.008 |
| Source of knowledge – books | -0.11 | -2.34 | 0.031 |
| Subjective health condition – good | -0.34 | -10.29 | <0.001 |
| Subjective health condition – not bad | -0.41 | -8.10 | <0.001 |

The designated method of least squares model of general health (GHQ-30) takes the following form: GH = 22.79 – 3.05 × X3 – 3.38 × X5 – 4.08 × X6 – 4.27 × X7 – 3.76 × X8 + 3.01 × X14 + 3.65 × X23 – 2.34 × X27 – 10.29 × X39 – 8.1 × X40 ±7.76; R2 = 0.41; β – variable importance factor; b – variable coefficient (y = bx + c); p – level of significance; R2 – relevance factor; c – standard error of estimate (y = bx ±c).

matoid arthritis is determined by psycho-physical activities and many other factors. It is promising that there are more and more studies on the quality of life in rheumatoid diseases. The conducted research and the results of our own study showed that the International Questionnaire – SF-36 and the General Health Questionnaire GHQ-30 are good tools to assess the quality of life in patients with rheumatoid arthritis and help to identify therapeutic strategies aimed at minimizing the deficits in the area of emotional support and education in patients with RA.[23,31,35]

# Conclusions

Physical activity of patients with rheumatoid arthritis depends on their age and subjective health condition. General health (GHQ-30) in patients with rheumatoid arthritis is influenced by education, place of residence, professional work, family history of RA and subjective health status. Frequent contact and accurate information obtained from the members of the therapeutic team has a positive effect on the selected domains of quality of life in patients with RA. The SF-36 scale and the GHQ-30 questionnaire may be used at the time of hospitalizations at the rheumatoid ward as methods of detecting patients with possible risk of emotional disorders and disorders in social functioning.

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# Effects of vector ultrasonic system debridement and conventional instrumentation on the levels of TNF-α in gingival crevicular fluid of patients with chronic periodontitis

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None declared

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## Abstract

**Background.** Tumor necrosis factor alpha (TNF-α) is an inflammatory mediator whose levels are increased in the gingival crevicular fluid and blood serum in the case of chronic periodontitis.

**Objectives.** The aim of this study was to determine the effect of vector ultrasonic system (VUS) on the levels of TNF-α in gingival crevicular fluid (GCF) and the clinical parameters in patients with chronic periodontitis.

**Material and methods.** The study protocol was conducted using split-mouth design in 30 patients with chronic periodontitis. VUS and scaling and root planing (S/RP) were applied separately to 2 quadrants, including the upper and the lower jaws. At baseline and after 6 months, clinical parameters including plaque index (PI), gingival index (GI), probing depth (PD), clinical attachment level (CAL) were recorded, and concentrations of TNF-α in GCF were determined by enzyme-linked immunosorbent assay (ELISA). Intergroup comparisons were evaluated by the independent Students' t-test, and the Pearson correlation was used to determine the relationship between parameters. The level of significance was set at 5%.

**Results.** Both treatment modalities provided statistically significant improvements in clinical periodontal parameters and TNF-α levels after 6 months ($p < 0.05$). Also, there were no significant correlations between the TNF-α levels in GCF and the clinical parameters in both treatment group at baseline and at the end of 6 months period ($p > 0.05$).

**Conclusions.** The use of the vector ultrasonic system in the non-surgical treatment of chronic periodontitis presents beneficial improvements for the clinical attachment level and the probing pocket depth as well as TNF-α levels in GCF.

**Key words:** non-surgical periodontal debridement, tumor necrosis factor alpha, chronic periodontitis

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# Introduction

Chronic periodontitis (CP) is a multifactorial inflammatory disease affecting the supporting tissues of teeth and is associated with loss of gingival attachment, destruction of the alveolar bone and periodontal ligament, leading to eventual tooth loss. Immune-inflammatory response has an important role in the course of chronic periodontitis.[1] Immune-inflammatory products appear in gingival crevicular fluid (GCF) and saliva, and these markers carry diagnostic information related to periodontal diseases. GCF contains oral bacteria, enzymes, leukocytes, the structure cells of periodontium, and complex-structured substances that expressed from serum. The presence of pro-inflammatory cytokines in GCF, especially interleukin-1 (IL-1), interleukin-6 (IL-6), and tumor necrosis factor alpha (TNF-α), may be an indicator of the activity of the periodontal disease.[2,3]

TNF-α, which is an inflammatory cytokine belonging to the TNF family, has been reported to play important roles in bone resorption and the inhibition of bone formation. In animal models, it has been reported that the level of TNF-α was increased in cases of periodontitis.[4]

Hand instrumentation is known as conventional periodontal therapy, and is considered the gold standard for the non-surgical treatment of periodontal diseases. Based on conventional periodontal treatment which includes scaling and root planing (S/RP), numerous studies have reported favorable developments in both the clinical and microbial parameters.[5,6] In spite of the successful clinical outcomes, hand instrumentation has several disadvantages, including being time-consuming and exhausting for patients as well as clinicians.[7]

Vector ultrasonic system (VUS) (Dürr Dental, Bietigheim-Bissingen, Germany) is used non-surgically for the procedures of subgingival debridement. VUS generates vibrations at a frequency of 25 kHz and has metal and fibre tips which are used on the buccal, lingual, and interdental surfaces, and in the furcation area. This instrument comprises a ring-shaped resonant body vibrated by an ultrasonic drive. The energy of vertical vibration converted by the resonating ring of the device is transmitted from the working tip to the root surface and the periodontal tissues by means of the hydroxyapatite-contained suspension and water. Thus, the root surfaces of teeth are hydrodynamically cleaned rather than coming into direct contact with the working tips.[8–10] A study has indicated that the effects of the new vector device are the decrease of infection and a significant acceleration of the tissue healing process in peri-implantitis cases.[11] Moreover, in several studies, there was a significant decrease in the probing depth and bleeding upon probing and an increase of clinical attachment gain in patients with severe periodontitis treated with both VUS and hand instruments.[12–14]

Numerous studies have demonstrated decreased levels of TNF-α following the non-surgical periodontal treatment performed with the hand instruments.[15,16] In the literature, there are no reports associated with the effects of vector ultrasonic system on the concentration of inflammatory mediators in patients with periodontal diseases. In light of this data, the aim of this study was to determine the effect of VUS on the levels of TNF-α in gingival crevicular fluid of patients with chronic periodontitis.

# Material and methods

## Patient selection

In the study, 30 patients with CP (12 females and 18 males, aged 27–66 years) were selected from among individuals who applied to the Clinic of Periodontology, Faculty of Dentistry, Dicle University (Diyarbakır, Turkey). All participants signed an informed consent form before beginning the study. The study was approved by the Ethical Committee of Dicle University and conducted in accordance with the Helsinki Declaration of 1975, as revised in 1983.[17] Inclusion criteria for patient selection were: a) no systemic diseases; b) no use of antibiotics and/or anti-inflammatory drugs within 6 months prior to the treatment; c) no smoking; d) no treatment of periodontitis within 12 months prior to the treatment; e) no pregnancy and no lactation in females; and f) the presence of bone loss as detected radiographically. Patients who had at least 14 teeth and at least 2 teeth with ≥5 mm probing depth in each quadrant were included in the study.

## Study groups

Our study was performed according to split-mouth design. Study groups were as follows:

- I – scaling and root planing (S/RP Group) by hand instruments: randomly to the selected 2 quadrants in upper and lower jaws;
- II – vector ultrasonic system (VUS Group): to the remaining 2 quadrants in upper and lower jaws (used hydroxyapatite-particled suspension for irrigation).

## Study design and clinical parameters

Following oral hygiene instruction, the routine clinical periodontal indexes (plaque index (PI), gingival index (GI), probing depth (PD), and clinical attachment level (CAL) measurements) were obtained from all patients at baseline. Before treatment, GCF samples were collected from the approximal site of a single-rooted tooth at least 5 mm or more probing depth. Teeth that had any fixed prosthesis, endodontic-periodontal lesion, filling materials, or caries were excluded for the GCF sampling. The measurement of clinical parameters and GCF samplings were repeated after 6 months by 1 blinded examiner.

## Measurement of clinical parameters

Silness and Löe Plaque Index (PI) and Löe and Silness Gingival Index (GI) were taken from all existing teeth out of the 3rd molars in each patient.[18,19] Probing depth measurement, which is the distance from gingival margin to the bottom of the gingival sulcus, was obtained from the mesio-buccal, mid-buccal, disto-buccal, mesio-lingual, mid-lingual, and disto-lingual surfaces of each tooth. Also, the clinical attachment level was measured from the 6 surfaces in the same manner as the probing depth, but its value was the distance between the cemento-enamel junction and the bottom of the gingival sulcus. Clinical measurements such as PD and CAL were obtained using a periodontal probe (PCPUNC 15® Hu-Friedy, Chicago, USA).

## Non-surgical periodontal treatment

On the 1st day of treatment, S/RP was performed using the Gracey curettes (Hu Friedy, Chicago, USA) under local anesthesia in the 2 quadrants as mentioned above until the visible and detectable deposits on the root surfaces of teeth no longer remained. Twenty-four hours after the 1st appointment, the remaining 2 quadrants were treated using VUS (Dürr Dental, Bietigheim-Bissingen, Germany). Seven LED lights and metal Paro Probe tips were used for the VUS group.

The premature contacts were removed and false restorations, e.g., fillings and fixed partial prosthesis, were corrected. Because the medication may positively affect the healing of periodontal tissues, any antibiotic and antimicrobial drugs were not prescribed. All the treatment procedures were performed by the same surgeons.

## Collection of GCF samples

Two absorbent paper strips (Periopaper, Amityville, USA) were used to collect the GCF from 2 quadrants of each patient in this study. Before the collection of GCF, the sites were isolated with cotton rolls and gently dried using an air syringe. To collect GCF samples, periopaper strips were placed into the gingival sulcus for 30 s according to the shallow intra-crevicular technique.[20] Then, the periopaper strips were delivered into the eppendorf tubes filled with 200 µL of phosphate buffer saline (pH = 7.4). They were preserved at -80°C until the evaluation of TNF-α levels.

## Determination of TNF-α levels

Sandwich enzyme immunoassay of ELISA (Human TNF-alpha Platinum ELISA-Bender Medsystems, GmBH, Vienna, Austria) was used to evaluate the concentration of TNF-α in the gingival crevicular fluid. The test was performed according to kit instructions. A total of 50 mL from each standard fluid or patient sample was added to each well in duplicate, and 50 µL of the sample diluent was added to each well. Then 50 µL of the biotinylated antibody reagent was added to each well and incubated for 1 h at room temperature, 20–25°C. Plates were then washed 4 times with a washing solution. Then, 100 µL of TMB substrate solution was added and incubated for 10 min at room temperature, in the dark. In order to stop the reaction, 100 µL of stop solution was added to each well. Absorbances were measured on an automated ELISA plate reader (Dynex, DSX, Chantily, USA) set at 450 nm wavelength. The standard curve was generated by plotting the average absorbance obtained for each standard concentration on the vertical (Y) axis vs the corresponding TNF-α concentration on the horizontal (X) axis. The amount of TNF-α in each sample was determined with this curve as pg/µL.

## Statistical analysis

Normal distribution and homogeneity of the data were verified with the Kolmogorov-Smirnov test and the Levene's test, and Repeated Measures ANOVA test was performed for pairwise comparisons. For intergroup comparisons, the Student's t-test for dependent and independent samples was used to analyze the data. Pearson's correlation was used to determine the relationship between parameters. Statistical analyses were performed using the SPSS v. 15.0 for Windows (SPSS, Inc., Chicago, USA). The level of significance was set at 5%.

# Results

At baseline, this study began with 35 participants. But 3 patients did not return for the follow-up visits and 2 patients took medication that was excluded by the study, resulting in total of 5 patients being excluded from the study. Therefore, the study was pursued with 30 patients throughout the 6-month follow-up period. Gender, age range, and mean values belonging to the subjects are shown in Table 1.

The clinical parameters of the full-mouth and the sampling sites include PI, GI, PD, and CAL, significantly decreased in the 2 groups. Moreover, the levels of TNF-α

Table 1. Demographic characteristics of the patient population

| Gender | n | Age range | | mean ±SD |
|---|---|---|---|---|
| | | min | max | |
| Females | 12 | 27 | 48 | 34.66 ±5.38 |
| Males | 18 | 32 | 66 | 42.61 ±8.99 |
| Total | 30 | 27 | 66 | 39.43 ±8.61 |

n – number of subjects; min – minimum; max – maximum; SD – standard deviation.

obtained from the sampling sites also decreased depending on the improvement of clinical parameters (Tables 2, 3).

Based on improvements in PD and gain in CAL for the full-mouth and sampling sites, there were significant differences in the 6 months compared to the baseline measurements for the 2 groups. When the groups were compared to each other, these parameters had no statistically significant differences. The average amounts of PD reduction and CAL gain at 6-month time point is presented in Table 4.

The correlation between the levels of TNF-α with the clinical parameters of both the full-mouth and sampling sites was analyzed. There was no statistically significant correlation between TNF-α levels and PI, GI, PD, and CAL (Tables 5, 6).

**Table 2.** Comparison of full-mouth clinical parameters and TNF-α levels belonging to S/RP and VUS group at baseline and after 6 months between and within the groups

| Parameters/ treatment groups | Baseline (mean ±SD) | 6 months (mean ±SD) | p-value |
|---|---|---|---|
| PI | | | |
| VUS | 1.95 ±0.57 | 0.41 ±0.23 | <0.001 |
| S/RP | 2.01 ±0.52 | 0.44 ±0.22 | <0.001 |
| p-value | 0.699 | 0.584 | |
| GI | | | |
| VUS | 1.72 ±0.37 | 0.28 ±0.20 | <0.001 |
| S/RP | 1.74 ±0.35 | 0.27 ±0.16 | <0.001 |
| p-value | 0.820 | 0.827 | |
| PD | | | |
| VUS | 3.62 ±0.71 | 2.30 ±0.32 | <0.001 |
| S/RP | 3.74 ±0.55 | 2.27 ±0.23 | <0.001 |
| p-value | 0.464 | 0.693 | |
| CAL | | | |
| VUS | 3.96 ±0.80 | 3.07 ±0.62 | <0.001 |
| S/RP | 4.07 ±0.65 | 3.06 ±0.65 | <0.001 |
| p-value | 0.560 | 0.922 | |
| TNF-α | | | |
| VUS | 3.65 ±0.42 | 3.04 ±0.20 | <0.001 |
| S/RP | 3.75 ±0.56 | 3.01 ±0.16 | <0.001 |
| p-value | 0.437 | 0.353 | |

PI – plaque index; GI – gingival index; PD – probing depth; CAL – clinical attachment level; TNF-α – tumor necrosis factor alpha; VUS – vector ultrasonic system; S/RP – scaling and root planning; SD – standard deviation.

**Table 3.** Comparison of clinical parameters relating to sampling sites at baseline and after 6 months between and within the groups

| Parameters/ treatment groups | Baseline (mean ±SD) | 6 months (mean ±SD) | p-value |
|---|---|---|---|
| PI | | | |
| VUS | 2.17 ±0.53 | 0.30 ±0.46 | <0.001 |
| S/RP | 2.20 ±0.40 | 0.37 ±0.49 | <0.001 |
| p-value | 0.861 | 0.585 | |
| GI | | | |
| VUS | 1.77 ±0.50 | 0.40 ±0.62 | <0.001 |
| S/RP | 1.97 ±0.41 | 0.40 ±0.62 | <0.001 |
| p-value | 0.943 | 1.00 | |
| PD | | | |
| VUS | 6.30 ±1.60 | 2.93 ±0.11 | <0.001 |
| S/RP | 6.43 ±1.31 | 2.87 ±0.68 | <0.001 |
| p-value | 0.502 | 0.716 | |
| CAL | | | |
| VUS | 6.37 ±1.58 | 3.47 ±0.54 | <0.001 |
| S/RP | 6.63 ±1.62 | 3.57 ±1.71 | <0.001 |
| p-value | 0.442 | 0.927 | |

PI – plaque index; GI – gingival index; PD – probing depth; CAL – clinical attachment level; VUS – vector ultrasonic system; S/RP– scaling and root planning; SD – standard deviation.

## Discussion

The causes of chronic periodontitis may be microbial plaque biofilm, food debris, and/or dental calculus accumulating on the surface of teeth. The mechanical removal of these deposits, which is performed by hand and power-driven instruments, is essential for the treatment of chronic periodontitis.[21] Several studies showed that hand instrumentation could promote microbial and clinical periodontal parameters. The efficiency of VUS for the removal of microbial biofilms has been demonstrated to be as good as conventional periodontal treatment in a study performed by some investigators.[22] The elimination of predisposing factors, including calculus, dental stains, false restorations, and similar factors for the retention of dental plaque in both the VUS and S/RP groups showed to facilitate the effective brushing of the patients. The findings of our study related to dental plaque scores were in accordance with various other studies in which different methods were applied for the non-surgical periodontal treatment.[13,22,23]

During inflammation, observable changes occur in the gingival tissues. To determine the extent of these gingival changes subjectively, different gingival indexes are used, such as the Löe and Silness Gingival index.[19] With respect to our gingival index scores, it was determined that the S/RP and VUS groups had statistically significant improvements in theirs gingival tissues at the end of 6 months, but there was a similarity between the 2 groups in gingival and dental plaque scores. These results comply with those of several other studies.[22,24]

It was observed that there was a statistically significant reduction in the mean values of PD from the baseline to the 6$^{th}$ month in the S/RP and VUS groups. In the comparison of the groups, the mean values of PD in the S/RP group declined from 3.74 ±0.55 mm to 2.27 ±0.23 mm. Similarly, the VUS group demonstrated a reduction of mean PD values from 3.62 ±0.71 mm to 2.30 ±0.32 mm. In the split-mouth design study performed by Christgau et al. who examined the efficiency of VUS and S/RP in a group of 20 patients with chronic periodontitis, similar differences were observed between the 2 groups at the end of 6-month period.[22] In another study,

the results showed that mean PD changed in the VUS group from 4.5 ±0.5 mm to 3.7 ±1.2 mm and in the SRP group from 4.5 ±0.3 mm to 3.4 ±1.1 mm.[14] In the study conducted by Guentsch et al., a decrease of probing depth was observed from 5.20 ±0.70 mm to 2.40 ±0.57 mm in the VUS group, and from 5.12 ±0.60 mm to 2.33 ±0.32 mm in the S/RP group.[25] These decreases were statistically significant at the end of 6-month period in comparison to the baseline. In the present study, the results associated with PD reduction were similar to those of the above-mentioned studies. Especially, the PD reductions related to the sampling sites were more pronounced.

The difference between the average values of CAL at baseline and 6 months is interpreted as a gain in attachment level. The full-mouth mean attachment gain was statistically significant at the 6$^{th}$ month as compared to baseline both in the VUS and in the S/RP, 0.88 ±0.66 mm and 1.01 ±0.63 mm, respectively ($p < 0.05$). There were no statistically significant differences between the 2 groups ($p > 0.05$). Based on the sampling sites, mean CAL values were reduced from 6.37 ±1.58 mm at baseline to 3.47 ±0.54 mm at 6 months in the VUS group, whereas in the S/RP group they were reduced from 6.63 ±1.62 mm to 3.57 ±1.71 mm. The measurement of CAL gains obtained at the end of 6-month period were 2.90 ±1.24 mm in the VUS group and 3.06 ±1.31 mm in the S/RP group. These changes were statistically significant at the 6$^{th}$ month for the 2 groups. Numerous studies demonstrated significant improvements in terms of clinical attachment levels which are similar to those of our study. In a study, especially at deep sites (>5 mm probing depth), the S/RP group showed a CAL gain of 0.7 ±0.4 mm, while the VUS group showed a CAL gain of 0.6 ±0.4 mm at the end of 6-month period postoperatively. The findings of CAL gain in this study showed that the non-surgical periodontal treatment carried out with the hand instruments and the VUS may result in significant improvements.[10,14,26] Some studies showed better results in terms of full-mouth CAL gain when compared with our study.[12,27] These disparities in the gain of attachment level are based on differences in the initial probing pocket depth. As the probing pocket depth increases without gingival recession, the clinical attachment gain increases after proper periodontal therapy.[10]

Although some periodontal parameters including plaque index, gingival index, bleeding on probing, probing pocket depth, clinical attachment level, alveolar bone loss, etc. might provide useful information about the severity of periodontal disease, these parameters are not the indicators of the activity of the disease. Therefore, different methodologies have been used, such as biochemical and immunologic diagnostic tests, which analyze the levels of numerous inflammatory mediators in GCF samples.[28] In particular, the increase of TNF-α levels in GCF is a sign of periodontal inflammation.[29] Gamonal et al. detected an increase in the levels of TNF-α in the GCF of patients with chronic periodontitis as compared to healthy subjects.[30]

In the present study, a decrease of GCF-TNF-α levels was observed at the end of 6-month period, when compared to baseline ($p < 0.05$). However, at the same time, there were no statistically significant

**Table 4.** Mean values of PD reduction and CAL gain from baseline to 6th month

| Parameters | Full-mouth | | | Sampling sites | | |
|---|---|---|---|---|---|---|
| | S/RP (mean ±SD) | VUS (mean ±SD) | p-value | S/RP (mean ±SD) | VUS (mean ±SD) | p-value |
| PD | 1.46 ±0.49 | 1.31 ±0.52 | 0.261 | 3.56 ±1.35 | 3.36 ±1.21 | 0.572 |
| CALg | 1.01 ±0.63 | 0.88 ±0.66 | 0.448 | 3.06 ±1.31 | 2.90 ±1.24 | 0.671 |

PD – probing depth; CALg – clinical attachment level gain; VUS – vector ultrasonic system; S/RP – scaling and root planning; SD – standard deviation.

**Table 5.** Correlations between full-mouth clinical parameters and TNF-α levels at different time points

| Parameters | Baseline | | | | 6 months | | | |
|---|---|---|---|---|---|---|---|---|
| | S/RP | | VUS | | S/RP | | VUS | |
| | r | p | r | p | r | p | r | p |
| PI-TNF-α | -0.151 | 0.427 | -0.027 | 0.888 | -0.304 | 0.102 | 0.232 | 0.218 |
| GI-TNF-α | -0.125 | 0.511 | 0.079 | 0.678 | -0.153 | 0.421 | 0.174 | 0.359 |
| PD-TNF-α | -0.141 | 0.475 | 0.145 | 0.445 | -0.110 | 0.563 | 0.337 | 0.069 |
| CAL-TNF-α | -0.222 | 0.237 | 0.068 | 0.721 | 0.191 | 0.313 | 0.145 | 0.446 |

PI – plaque index; GI – gingival index; PD – probing depth; CAL – clinical attachment level; TNF-α – tumor necrosis factor alpha; VUS – vector ultrasonic system; S/RP – scaling and root planning; significance level p < 005; r – Pearson's correlation coefficient.

**Table 6.** Correlations between clinical parameters of sampling sites and TNF-α levels at baseline and after 6 months

| Parameters | Baseline | | | | 6 months | | | |
|---|---|---|---|---|---|---|---|---|
| | S/RP | | VUS | | S/RP | | VUS | |
| | r | p | r | p | r | p | r | p |
| PI-TNF-α | 0.125 | 0.510 | 0.043 | 0.822 | -0.204 | 0.280 | 0.265 | 0.157 |
| GI-TNF-α | -0.016 | 0.934 | -0.113 | 0.553 | -0.025 | 0.897 | 0.059 | 0.757 |
| PD-TNF-α | 0.083 | 0.664 | -0.008 | 0.966 | -0.067 | 0.725 | 0.238 | 0.205 |
| CAL-TNF-α | 0.085 | 0.654 | 0.071 | 0.709 | 0.171 | 0.368 | 0.253 | 0.177 |

PI – plaque index; GI – gingival index; PD – probing depth; CAL – clinical attachment level; TNF-α – tumor necrosis factor alpha; VUS – vector ultrasonic system; S/RP – scaling and root planning; significance level p < 005; r – Pearson's correlation coefficient.

differences between the groups ($p > 0.05$). Unfortunately, because there were no similar reports using the method of our study in the literature, we could not compare the results related to TNF-α levels in the VUS group. There was no correlation between GCF-TNF-α levels and the clinical periodontal parameters, neither at baseline nor after 6 months for the 2 groups. In the study, which performed a non-surgical treatment of chronic periodontitis by S/RP, Erdemir et al. did not observe any correlation at the 6-month follow-up.[31] These findings support the view that local expressions of inflammatory mediators vary from site to site and from subject to subject.[32]

In conclusion, the use of the vector ultrasonic system for non-surgical periodontal treatment presents beneficial improvements in the clinical attachment level and the probing pocket depth as well as TNF-α levels. Although no significant differences were found in the 2 groups, the decrease of TNF-α levels in the S/RP group was a slightly better than in the VUS group. To reach a definitive judgment on the relationship between the levels of TNF-α and treatment type, we believe that further studies are needed.

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# Polymorphism of Gly39Glu (c.116G>A) hMSH6 is associated with sporadic colorectal cancer development in the Polish population: Preliminary results

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## Abstract

**Background.** Colorectal cancer (CRC) remains a major source of cancer-related mortality, accounting for 10% of all cancer-related deaths. DNA mismatch repair mechanism (MMR) responsible for correcting errors generated during DNA replication and its deficiency is associated with both hereditary and sporadic CRC. Single-nucleotide polymorphisms (SNPs) are the most common forms of genetic variation, and it has been shown that the SNPs in *MMR* genes may modify CRC risk.

**Objectives.** The aim of the study was to determine the relationship between gene polymorphism Glu39Gly (c.116G>A) of the *hMSH6* gene and the modulation of the risk of sporadic colorectal cancer in the Polish population.

**Material and methods.** A total of 128 patients with resectable colorectal carcinoma as well as 189 sex-, age-, and ethnicity-matched control subjects without cancer history were enrolled in this study. Patients with a family history of CRC or inflammatory bowel diseases were excluded from this study. The DNA was isolated from peripheral blood lymphocytes of enrolled patients, and gene polymorphisms were analyzed by restriction fragment length polymorphism-polymerase chain reaction (RFLP-PCR).

**Results.** We observed that the genotype G/A variant of Glu39Gly (c.116G>A) genotype is associated with an increased risk of colorectal cancer (OR 1.65; 95% CI: 1.01–2.69; $p = 0.44$). The presence of A allele was also significantly higher in patients with CRC (OR 1.57; 95% CI: 1.04–2.38; $p = 0.032$). When comparing the prevalence of genotypes with clinical staging, genotype G/A and A allele were significantly less frequent in stage III–IV than in I (OR 0.3409; 95% CI: 0.124–0.939; $p = 0.0375$, and OR 0.4462; 95% CI: 0.201–0.991; $p = 0.044$, respectively).

**Conclusions.** These findings suggest that hMSH6 Glu39Gly polymorphism is associated with the risk of developing colorectal cancer in the Polish population, probably due to a defective mismatch repair system. The presence of G/A genotype and A allele is, however, associated with less advanced disease.

**Key words:** colorectal cancer, SNP, *hMSH6*, DNA MMR

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## Introduction

Colorectal cancer (CRC) is the most common gastrointestinal malignancy. Only in the USA, around 13,449 new cases are expected to be diagnosed with colorectal malignancy with the estimated mortality of about 49,190 in 2016.[1] In the past decade, there has been a significant general decrease in the incidence of CRC associated mortality observed in Western countries.[2] On the other hand, incidence rates are increasing among adults younger than 50 years of age and in certain countries with historically low prevalence of CRC, e.g., Eastern Europe.[1] Although often viewed as a single disease, CRC more accurately represents a constellation of heterogeneous subtypes that result from different combinations of genetic events and epigenetic alterations.[3] Although the majority of CRCs are the results of chromosomal instability, approx. 15% of cancers develop via an alternative pathway characterized by defective function of the DNA mismatch repair (MMR) system.[4] The MMR system recognizes and corrects mismatched bases and small insertion-deletion loops generated during DNA replication. Highly conserved series of proteins, including MSH2, MSH6, MSH3, MLH1, PMS2, PCNA, and EXO1 are involved in the MMR system, which operates functionally, forming 2 heterodimers: hMSH2-hMSH6 and hMSH2-hMSH3. The recognition of mismatches and insertion-deletion loops are carried by these 2 heterodimers, respectively. This process is coordinated by another heterodimer formed out of MLH1 and PMS2. Inefficient function of the MMR system leads to the accumulation of mutations, resulting from the inability to repair single nucleotide DNA mismatches, particularly in highly repeated sequences known as microsatellite instability (MSI). Germline mutations in *MMR* genes or hypermethylation of the MLH1 promoter lead to a deficient MMR system (MMR-D) and a high degree of MSI in genome, resulting in the development of a variety of human cancers, e.g., endometrial or gastric cancer. MMR-D accounts for 15% of all CRCs, with 3% being associated with hereditary non-polyposis colorectal carcinoma (HNPCC), and the remaining 12% arise sporadically.[5] Although the prevalence could vary between populations, it is clear that the MSI phenotype represents a clinically meaningful proportion of CRCs. On the other hand, lots of CRCs are sporadic and have a proficient MMR system with low-frequency MSI. It is, however, widely hypothesized that the heritable nature of CRC might be associated with the co-inheritance of multiple low-risk variants that may also further interact with environmental factors.[6,7] This hypothesis was supported by the identification of single-nucleotide polymorphisms (SNPs) localized in different genomic regions that influence the risk of CRC.[8] SNPs of *MMR* genes may alter the gene and protein expression pattern, thus modifying its efficacy and therefore the risk of developing CRC. The risk of CRC associated with each of the variants is individually low, but the combined effect of these variants could significantly contribute to disease development, especially given the high prevalence of these variants in the general population.[9] MSH6 Gly39Glu (116G>A) polymorphism (rs1042821) first described by Nicolaides, et al. has been investigated for the role in its development of both HNPCC and sporadic CRCs.[10] The inactivating mutations of MSH6 in yeast and human tumor cell lines are associated with an impaired ability to repair single-base mispairs and small insertion-deletion loops but not large insertion-deletion loops.[11] This indicates that hMSH6 mutations are prone to be associated with a MSI-L phenotype than a MSI-H phenotype, which is more typical to sporadic CRC. MSI-L colorectal tumors have been proposed as possible candidates for *hMSH6* mutations by Wu.[12] Berndt and Campbell reported an association between MSH6 Gly39Glu (116G>A) polymorphism and the development of rectal and colon cancer.[13,14] However, the results of further studies were conflicting.[11,15] These reports prompted us to investigate a common polymorphism in the *MSH6* gene of MMR system and their role in the susceptibility to sporadic colorectal cancer in the population of our region.

## Material and methods

Test DNA was isolated from peripheral blood samples collected from 128 unrelated patients with confirmed CRC. Only patients with a resectable disease were enrolled into the study. Blood samples for the analysis were taken on the day of admission in both groups of patients. A detailed characteristic of the patients with the CRC is shown in Table 1. The control group consisted of 189 patients hospitalized in the same surgical ward without any medical history of cancer, inflammatory diseases and diabetes. The reason for hospitalization was mostly benign proctological disease or hernia. The analyzed and control groups members were age- and sex-matched.

### Detection of *hMSH6* gene mutation

The DNA for genotyping was isolated from the blood samples of CRC patients using a commercial kit QIAamp DNA Blood Mini Kit for isolating high-molecular-weight DNA (Qiagen). Detection of Gly39Glu (c.116G>A ) polymorphisms of the *hMSH6* gene was carried out by RFLP-PCR analysis. Primers used for amplifications of the analyzed region are shown in Table 2. PCR products were generated using, in each reaction, a total volume of 20 µL containing 10 pM of each primer, 1 U Taq DNA polymerase (Qiagen), 0.2 mm of each dNTP, 1.5 mM $MgCl_2$, and 50 mM KCl, 20 mM Tris-HCl (pH = 8.4), and 50 ng of genomic DNA. PCR amplification was performed in MultiGene TC9600-G termocycler (Labnet International, Inc., Edison, USA) under the following conditions:

**Table 1.** Primers used for RFLP-PCR

| Gene | Direction | Sequence |
|---|---|---|
| MSH6 Gly39Glu (116G>A) polymorphism (rs1042821) | forward | 5′-GCG CTG AGT GAT GCC AAC AAG-3′ |
| | reverse | 5′-CAG CAG GCG CTA CCG ATC TC-3′ |

initial denaturation at 1 cycle at 95°C for 5 min and amplification of 35 cycles (30 s at 95°C, 30 s at 55°C, and 30 s at 72°C), followed by a final cycle of 7 min at 72°C; 8 µl of PCR products was diluted in 2 µl of denaturation buffer (95% formamide, 0.05% bromophenol blue, and 0.05% xylene cyanol) and was separated on a 1.5% polyacrylamid gel at 10 V/cm for 1 h at room temperature. The final products were visualized by ethidium bromide staining.

## Statistical analysis

The statistical analysis was conducted using STATISTICA software v. 13 PL (StatSoft, Inc.). The resulting number of each genotype was compared with the expected value based on the Hardy-Weinberg equilibrium. The significance of differences between the frequencies of alleles and genotypes between the groups was assessed using the $\chi^2$ test and the Fisher exact test. Odds ratio (OR) with corresponding confidence interval 95% (CI 95%) were calculated during multivariate regression analysis.

# Results

Distributions of genotypes and alleles in the CRC and control group are presented in Table 3. G allele has been used in our study as a reference in calculations. According to the Hardy-Weinberg $\chi^2$ analysis, the distribution of genotypes and alleles in CRC group and the control group was not consistent with H-W equilibrium ($\chi^2 = 0.07$, $p = 0.786$ and $\chi^2 = 0.34$, $p = 0.558$, respectively). No significant difference in alleles or genotypes distribution was observed between age and sex groups (<60, >60, females, males). Risk of CRC was approximately 2-fold higher for the G/A genotype (OR 1.57; 95% CI: 1.04–2.38; p = 0.032) and A allele (OR 1.65; 95% CI: 1.01–2.69; p = 0.44). When comparing the prevalence of genotypes with clinical staging, genotype G/A and A allele were significantly less frequent in stage III–IV than in I (OR 0.3409; 95% CI: 0.124–0.939; p = 0. 0375, and OR 0.4462; 95% CI: 0.201–0.991; p = 0.044, respectively) (Table 4).

**Table 2.** Clinical and pathological characteristics of the analyzed group of patients with colorectal cancer

| Characteristic | Patients n (%) |
|---|---|
| Gender | |
| male | 54 (42) |
| female | 74 (58) |
| Age | |
| <60 years | 40 (31) |
| >60 years | 88 (69) |
| Localization | |
| colon | 65 (51) |
| rectum | 63 (49) |
| Histology | |
| adenocarcinoma tubulare | 61 (47) |
| adenocarcinoma muccosinum | 55 (44) |
| adenocarcinoma planoepitheliale | 12 (9) |
| Grading | |
| 1 | 21 (17) |
| 2 | 100 (78) |
| 3 | 7 (5) |
| TNM Classification | |
| I and II | 62(48) |
| III and IV | 66 (52) |

**Table 3.** Genotypes and alleles distribution of Gly39Glu (c. 116G>A) polymorphism of *hMSH6* gene in patients with colorectal cancer and in control group

| Genotype Allele | CRC patients (n = 128) number (frequency) | Control group (n = 189) number (frequency) | OR (CI 95%) | p-value |
|---|---|---|---|---|
| additive model | | | | |
| G/G | 80 (0.63) | 137 (0.72) | 1 Ref. | |
| G/A | 44 (0.34) | 49 (0.26) | 1.65 (1.01–2.69) | 0.044 |
| A/A | 4 (0.03) | 3 (0.02) | 2.34 (0.51–10.73) | 0.230 |
| G | 204 (0.8) | 323 (0.85) | Ref. | |
| A | 52 (0.21) | 55 (0.15) | 1.57 (1.04–2.38) | 0.032 |

Table 4. Genotypes and alleles distribution of Gly39Glu (c. 116G>A) polymorphism of *hMSH6* gene in patients with colorectal cancer according to staging by American Joint Committee on Cancer

| Genotypes/ alleles | Patients n = 128 | | | II° vs I° | | III° vs I° | | III° vs II° | |
|---|---|---|---|---|---|---|---|---|---|
| | I° (T1–2N0) | II° (T3–4N0) | III° i IV° (T1–4 N1–2 M0 i M1) | OR (95% CI) | p-value | OR (95% CI) | p-value | OR (95% CI) | p-value |
| G/G | 9 | 25 | 46 | 1 (ref) | | 1 (ref) | | 1 (ref) | |
| G/A | 11 | 15 | 18 | 0.5245 (0.1805–1.5236) | 0.185 | 0.3409 (0.1238– 0.9391) | 0.0375 | 1.472 (0.6495–3.336) | 0.403 |
| A/A | 1 | 1 | 2 | 0.5 (0.0297–8.4166) | 1 | 0.625 (0.0538–7.2605) | 1 | 1.25 (0.1097–14.2383) | 1 |
| G | 29 | 65 | 110 | 1 (ref) | | 1 (ref) | | 1 (ref) | |
| A | 13 | 17 | 22 | 0.5834 (0.605–1.3574) | 0.538 | 0.4462 (0.2008–0.9914) | 0.044 | 0.7647 (0.3785–1.545) | 0.469 |

## Discussion

The existence of a link between human cancers and the MMR determines the relevance of investigations of the DNA mismatch repair system. The inheritance of variations in *MMR* genes may influence individual susceptibility to the development of colorectal cancer. While the presence of mutation in *hMLH1* and *hMSH2*, leading to MSI-H CRCs is well described, this involves mostly tumors developing in HNPCC.[7,16] For sporadic CRCs, where MSI-L or MSI stable genotype is observed, it was suggested that impairment in MMR system efficiency may arise from other genes, whose alternations lead to the complete loss of function of heterodimers.[17,18] That is why *hMSH6* was investigated for their possible role in tumor neogenesis in sporadic CRCs. *hMSH6* form complex with *hMSH2*, that recognizes single-base mispairs and small (i.e., single-base) insertion-deletion loops.[19] Mutations in MSH6 result in a partial loss of mismatch repair.[20] Human tumor cell lines with MSH6 mutations exhibit MSI primarily in mononucleotide and not in dinucleotide repeats, which suggests its association rather with MSI-L CRCs. Parc, et al. identified 16 hMSH6 alterations.[11] Fifteen of these were germ-line changes and 1 was a somatic change. Only 2 of the 15 germ-line alterations led to a change in the primary sequence of the hMSH6 protein: Gly39Glu and Leu395Val, both of which are reported to be polymorphisms.[10,21] By now, few studies investigated the association between the MSH6 Gly39Glu variant and the risk of sporadic colorectal cancer.[13,14,21] Berndt, et al. reported that homozygosity for the MSH6 39Glu variant was associated with a more than triple risk of rectal cancer. However, no association was found between the MSH6 variant and colorectal cancer overall. Since the number of rectal cases in this study was small, the authors recommended interpreting the observed association for rectal cancer with caution.[13] Campbell, et al. analyzed whether MSH6 (Gly39Glu) polymorphisms were associated with the risk of colon cancer in the data from 1,609 colon cancer cases and 1,972 controls. Male participants heterozygous (G/A) or homozygous (A/A) for the MSH6 variant were at 27% increased risk of colon cancer, while no associations were observed among females. On the other hand, further association was not observed with MSI-negative status of CRCs. Additionally, this study observed effect modification between MMR variants and lifestyle factors that contribute to higher CRC risk overall.[14]

This is the first report concerning polymorphism Gly-39Glu and the risk of colorectal cancer in the Polish population. In our study, GG genotype was more prevalent than the AA genotype. The presence of the MSH6 39Glu variant allele (A) was associated with the increased risk of colon cancer (OR 1.57; 95% CI: 1.04–2.38; p = 0.032) in the analyzed population. In contrast to the previous study, the homozygosity for A allele did not lead to a higher risk of CRC, but was observed in the cases of heterozygosity (OR 1.65; 95% CI: 1.01–2.69; p = 0.44). Similar results concerning higher concentration of hMSH6 A allele in CRC cases were obtained in the study by Kolender (23% in colorectal cancer cases as compared to 15% in controls), although no formal statistical comparisons were made.[21] The presence of G/A genotype and A allele were more frequently detected in early stage tumors, which can suggest the possible MSI-H phenotype. Though MSI status was not checked in our study, the linkage between hMSH6 mutation and MSI status is now a matter of wide debate. The lack of a clear association with MSI positive or negative status raises many questions concerning the role of mutations in this gene on the development of MSI-L or MSI-H CRC, or broadly speaking in NHPCC and sporadic colorectal cancers. In comparisons to *hMLH1* or *hMLH2*, the observed mutations in *hMSH6* do not cause lesser gene expression in healthy and cancerous tissues.[22] Plaschke reported that only 3 out of 146 patients with sporadic cancer exhibited abnormal *hMSH6* expression, with 2 having germ-line mutations. However, possible missense mutations may be present that impair gene function without substantially affecting protein expression or resulting in the MSI-H phenotype.[15] Wu et al. emphasized that an MSI-low phenotype cannot be considered an exclusion criterion for mutation testing of *MMR*

genes in general.[12] Still, we can suspect that a fair amount of sporadic cancers can arise in HNPCC-like families, in which, though not evident, the MSI-H phenotype and other MMR genes mutations may be present. The possible answers may also involve epigenetic or somatic changes whose impact on carcinogenesis is intensified by the presence of environmental factors such as smoking or a Western diet. This idea of investigating the interactions between environmental exposures and MMR polymorphisms is strengthened by findings of increased oxidative stress and DNA damage resulting from tobacco smoking as well as from a Western diet, alcohol and obesity.[23–26] However, for a better explanation of these phenomena further research is warranted.

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# Relationship between resistin and IL-23 levels in follicular fluid in infertile patients with endometriosis undergoing IVF-ET

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## Abstract

**Background.** Endometriosis (EM) interferes with the reproductive process and affects the success rate of in vitro fertilization (IVF). Inflammatory cytokines are suggested to play a role in infertility in patients with EM.

**Objectives.** In this study, we aimed to investigate the relationship between resistin and interleukin 23 (IL-23) levels in follicular fluid (FF) and serum together with the severity of endometriosis and in vitro fertilization/embryo transfer (IVF-ET) outcome.

**Material and methods.** Samples from 116 infertile women were studied using enzyme-linked immunosorbent assay (ELISA). The study group consisted of 76 infertile patients diagnosed with EM (40 with stages I–II and 36 with stages III–IV) undergoing IVF-ET. The control group included 40 women with tubal factor infertility. FF and serum samples were collected on the day of follicle aspiration and hCG administration, respectively.

**Results.** The serum and FF resistin levels were significantly higher in the EM group than in the control group (p-value <0.05). The FF resistin and IL-23 levels were significantly higher in EM stages III–IV than in stages I–II (p-value <0.05), and the serum resistin and IL-23 levels were also significantly (p-value <0.01) higher in stages III–IV than in stages I–II. The $E_2$ level on the day of hCG administration and the implantation rate were both significantly lower in the EM group than in the control group. However, there were no differences in the Gn duration and dose, and the cleavage, implantation and clinical pregnancy rates between the 2 groups.

**Conclusions.** Our results suggest that patients with EM exhibit increased resistin level in FF and serum. Advanced EM may contribute to infertility via decreased embryo implantation rates because of inflammation and immune rejection. No influence was observed on pregnancy outcomes after IVF-ET.

**Key words:** endometriosis, IVF-ET, resistin, IL-23, pregnancy outcome

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# Introduction

Inflammatory cytokines are suggested to play a role in infertility in patients with endometriosis (EM). Previously, a meta-analysis carried out on the effects of endometriosis on in vitro fertilization/embryo transfer (IVF/ET) outcomes concluded that EM interferes with all aspects of the reproductive process, and the success rate of in vitro fertilization (IVF) in EM patients was almost half compared to women without EM.[1] However, the mechanism by which EM affects fertility in IVF remains unclear. Human follicular fluid forms the microenvironment of the developing oocyte and has an important influence on oocyte quality, embryo development, and implantation.[2] In this study, we measured the follicular fluid (FF) and serum concentrations of resistin and interleukin 23 (IL-23) in EM and control patients and in different stages of EM, and retrospectively assessed IVF-ET outcomes in order to understand the possible impact of EM on IVF-ET outcomes.

# Material and methods

## Patients

This study was approved by the Ethics Committee on Human-Assisted Reproduction of Fuzhou General Hospital, and informed consent was obtained from all the study participants.

This was a randomized controlled retrospective database-search study of a total of 116 infertile patients from January 2012 to December 2012. The patients were 22 to 38 years old (mean age 30.67 ±4.32). The study group consisted of 76 women diagnosed with EM (40 with stages I–II and 36 with stages III–IV) with no other factor than EM causing infertility. The control group consisted of 40 women with tubal factor infertility. A total of 25, 15, 19, and 17 women in the study group had stages I, II, III, and IV EM, respectively. The EM was confirmed by laparoscopy, and all the patients were treated surgically. The patients in the EM group were scored according to the revised American Fertility Society (AFS) classification (1997).[3] Based on the observation of ectopic endometrial parts in laparoscopic surgery, the number, size, degree of adhesion rate, and scores of stages I–IV EM patients were 1–5, 6–15, 16–40, and >40, respectively. Clinical and laboratory data was analyzed.

## Inclusion criteria

Patients were recruited to the study based on the following inclusion criteria: (1) women under the age of 38 years; (2) those with ≥10 punctured follicles or retrieved oocytes; (3) those who were receiving IVF-ET for the first time; (4) underwent transfer of 1–2 embryos; and (5) had not received drugs affecting the function of the hypothalamus-pituitary-ovarian axis for at least 3 months before the study.

## Exclusion criteria

The following exclusion criteria were applied: (1) women above the age of 38 years; (2) those with basic FSH (bovine follicle-stimulating hormone, bFSH) levels >11.0 mIU/m; (3) patients with autoimmune disease, liver disease, diabetes, or malignant tumors; (4) reproductive organ dysplasia; (5) acute pelvic inflammation and other changes confirmed by laparoscopy; and (6) inadequate data for analysis.

## Controlled ovarian stimulation

Pituitary-ovarian suppression was achieved by administration of the standard "long protocol" gonadotropin-releasing hormone analogue (GnRH) agonist with triptorelin acetate (Decapeptyl, Ferring, St. Prex, Switzerland) starting in the midluteal phase of the preceding cycle. When complete pituitary desensitization was confirmed by low plasma $E_2$ levels ≤30 pg/mL, LH level ≤2 mIU/mL, E2 <30 pg/mL and endometrial thickness <5 mm, the dosage was decided according to the age, basal endocrine, anti-Müllerian hormone (AMH), antral follicle count (AFC), and body mass index (BMI).

Ovarian stimulation was initiated by administration of recombinant FSH (Gonal-F, Serono, Switzerland; or Puregon, Schering-Plough, USA). The stimulated cycles were monitored by daily transvaginal ultrasonography (TVS) and intermittent assessment of the serum $E_2$ levels. The daily gonadotrophin dose was customized based on an individual dose-response scheme. Final maturation of oocytes was induced by injection of 250 µg of recombinant human chorionic gonadotrophin (hCG; Ovidrel, Serono, Switzerland) when the serum $E_2$ level was ≥150 pg/dominant follicle and the 2 leading follicles reached a mean diameter of 18 mm. The oocytes were retrieved transvaginally 34–36 h after hCG administration. Usually less than 2 best-quality embryos were transferred on day 3 after oocyte retrieval, and any extra good-quality embryos were cryopreserved for subsequent frozen-embryo transfer (FET) cycles.

## Collection of serum and follicular fluid

Serum samples were collected on the day of hCG trigger. Ovarian follicular fluid samples were collected during oocyte retrieval by follicle aspiration using transvaginal puncture assisted by ultrasonic guidance. All visible follicles were aspirated separately. Samples of yellow, clear FF were centrifuged at 1500 rpm for 10 min. The clear supernatants were stored immediately at -80°C. Only material from the first 2 or 3 aspirated follicles was used in order to avoid contamination with blood. No contaminated samples were used for further analysis.

## Quantification of resistin and IL-23

Resistin and IL-23 ELISA kits were purchased from Xin Bo Sheng Biotechnology Co. The operation was carried out according to the kit instructions.

## High-quality embryos and pregnancy outcome

Good-quality embryos were identified as 2 pn, with at least 7 blastomeres and ≤20% fragmentation. One or 2 embryos were chosen for transfer to the uterus under vaginal ultrasound guidance. The serum hCG was measured 2 weeks after embryo transfer to diagnose pregnancy, and the pregnancy was clinically confirmed by ultrasound at the 4$^{th}$ week after ET. Transplantation was canceled in the case of uterine cavity effusion, endometrial thickness <7 mm, $E_2$ on hCG day >5000 pmol/L, and/or oocyte number >15.

## Statistical analysis

All results are expressed as mean ±SE. All data was analyzed using Statistical Package for Social Sciences (SPSS) v. 16.0. Student's t-test (for 2 groups) and analysis of variance (ANOVA; for more than 2 groups) were used for statistical analysis. The clinical pregnancy rates of the different groups were analyzed using the Student's t-test. P-values of <0.05 were considered significant.

# Results

## Resistin and IL-23 levels in FF and serum in the 2 groups

Table 1 presents the FF and serum resistin and IL-23 levels in both groups. The resistin levels in the FF and serum were significantly higher in the EM group than in the control group ($p < 0.05$). The FF and serum IL-23 levels showed an increasing trend in the EM group compared to the control group, but this difference was not statistically significant ($p > 0.05$).

**Table 1.** Comparison of follicular fluid and serum resistin and IL-23 levels between patients in the endometriosis and control groups

| Indication | Cases | Resistin (pg/mL) | | IL-23 (pg/mL) | |
|---|---|---|---|---|---|
| | | follicular fluid | serum | follicular fluid | serum |
| EM | 76 | 140.66 ±16.14 | 33.44 ±28.26 | 18.03 ±11.68 | 18.37 ±12.16 |
| Control | 40 | 42.66 ±12.32 | 13.20 ±6.09 | 16.38 ±2.17 | 16.38 ±2.17 |
| t | – | 2.58 | 2.462 | 0.487 | 0.564 |
| P | – | 0.012 | 0.016 | 0.628 | 0.574 |

## FF and serum resistin and IL-23 levels in different stages of EM

Table 2 presents the FF and serum resistin and IL-23 levels in different stages of EM (groups I–II and III–IV). The resistin and IL-23 levels in the FF was significantly higher in EM stages III–IV than in EM stages I–II ($p < 0.05$). The resistin and IL-23 levels in the serum were also significantly higher in EM stages III–IV than in stages I–II ($p < 0.01$).

**Table 2.** Comparison of follicular fluid and serum resistin and IL-23 levels of patients with different stages of EM (I–II and III–IV)

| Indication | Cases | Resistin (pg/mL) | | IL-23 (pg/mL) | |
|---|---|---|---|---|---|
| | | follicular fluid | serum | follicular fluid | serum |
| I, II | 40 | 54.64 ±8.64 | 14.04 ±6.21 | 13.56 ±1.11 | 13.48 ±1.08 |
| III, IV | 36 | 130.88 ±21.81 | 52.86 ±28.40 | 22.99 ±15.58 | 23.26 ±15.80 |
| t | – | 8.909 | -8.231 | 3.822 | -3.806 |
| P | – | 0.012 | 0.000 | 0.000 | 0.000 |

## General information

Tables 3 and 4 present the general data and clinical COS outcomes of the patients in the 2 groups. The $E_2$ level on the day of hCG administration and the implantation rate were significantly lower in the EM group than in the control group ($p < 0.05$). However, the downregulation duration, Gn dose, Gn duration, rate of fertilization, cleavage and clinical pregnancy, ovarian hyperstimulation syndrome (OHSS) and abortion showed no statistical significance between the 2 groups.

**Table 3.** General data and clinical control ovarian stimulation outcomes of the patients in the 2 groups ($\bar{x} \pm s$)

| Indicators | EM (n = 76) | Control (n = 40) | p-value |
|---|---|---|---|
| Age (years) | 31.24 ±4.46 | 30.37 ±3.53 | 0.218 |
| Infertility duration | 5.02 ±3.42 | 4.89 ±2.60 | 0.769 |
| Body mass index | 22.34 ±3.26 | 23.35 ±4.18 | 0.376 |
| Down-regulation duration (d) | 18.12 ±4.26 | 19.4 ±6.22 | 0.258 |
| Gn duration (d) | 10.82 ±1.33 | 11.51 ±1.08 | 0.130 |
| Gn dosage (Amp) | 33.88 ±8.34 | 35.12 ±11.02 | 0.404 |
| hCG injection day<br>endometrial thickness (mm)<br>E2 (pg/mL) | <br>11.14 ±2.42<br>2239.68 ±788.9 | <br>11.43 ±2.40<br>2556.80 ±1050.76 | <br>0.483<br>0.037 |

## Discussion

Endometriosis is a chronic disease characterized by the presence of endometrium-like tissue outside the uterus, most commonly on the ovary. It affects 10–15% of women of reproductive age.[4] Previous studies have reported that 30–40% of infertile women have endometriosis, and these women are 20 times more likely to have EM than fertile women.[5,6]

EM is a complex genetic diseases whose pathogenesis is closely related to heredity, immunity, hormones, and environment.[7,8] The mechanism by which EM causes infertility is yet to be understood, and it may be due to the ectopic lesions caused by EM on oocytes and embryos and other harmful endometrial factors that may affect the quality of embryos and implantation. There is increasing evidence suggesting that grow rate of embryos drop in patients with EM.[8]

Resistin is known as a fat tissue-specific factor and is closely related to the inflammatory response. Yi et al. determined that the mean concentration of PF resistin was significantly higher in women with endometriosis compared to the controls.[9] The resistin levels increase during acute inflammation and have been found to be positively associated with inflammatory factors such as C-reactive protein (CRP).[10,11] A meta-analysis of the resistin levels in 6636 patients reported that the resistin levels were higher in women than in men.[12]

IL-23 is a type of inflammatory cytokine that is known to participate in autoimmune diseases by promoting inflammation.[13,14] Ours is the first study to explore the mechanism of EM fertility by determining the relationship between FF and serum resistin and IL-23 expression levels and the IVF/ET pregnancy outcome in EM patients.

The FF and serum resistin levels were significantly higher in the EM group than in the control group ($p < 0.05$). The FF and serum IL-23 levels in the EM group showed an increasing trend compared to the control group, although this difference was not significant ($p > 0.05$).

Chen et al. studied the resistin levels in patients with and without and they suggested that the serum resistin levels might be a good predictor of ovarian response in infertile women without polycystic ovary syndrome (PCOS) during IVF.[15] Further studies should investigate the role of serum resistin in response to inflammation caused by EM or chronic pelvic infection, both of which are major causes of female infertility. The findings of this study suggest a potent role of resistin in endometriosis. Further studies are needed to elucidate the biological implications of resistin in EM. The role of serum resistin in response to the inflammation caused by endometriosis or chronic pelvic infection should be examined in closer detail.

We also determined that EM was associated with increased levels of follicular IL-23. This result was not surprising. Impaired follicular fluid microenvironment characterized by elevated inflammatory cytokines may be the cause for poor oocyte quality, which could lead to poor IVF outcomes in patients with endometriosis.[14,15]

The FF resistin and IL-23 levels were significantly higher ($p < 0.05$) in patients with severe EM (stages III and IV) than in patients with milder EM (stages I–II). Similarly, the serum resistin and IL-23 levels were significantly higher ($p < 0.01$) in patients with severe EM (stages III and IV) than in patients with milder EM (stages I–II). Andreoli et al. measured the IL-23 levels in the serum and peritoneal fluid of women with minimal or mild EM and compared them with the corresponding levels in control subjects without endometriosis.[16] Higher IL-23 levels were encountered in the peritoneal fluid of women with EM, suggesting a possible role of this cytokine in infertility in these women.

Many retrospective studies have investigated the IVF/ET outcomes in women with EM.[1,17–19] Harkki et al. described that the Gn duration and Gn dose increased while the $E_2$ level decreased in patients with EM.[17] We observed similar results in the $E_2$ level in EM patients on the day of hCG administration (2239.68 ±788.9 pg/mL vs 2556.80 ±1050.76 pg/mL; $p > 0.05$), which was lower than the corresponding level in the control group. Kuivasaari et al. reported that the oocyte recovery, implantation, and clinical pregnancy rates were significantly lower in EM patients than in control subjects, which was similar to our findings: we observed a lower implantation rate in EM patients.[18]

In a meta-analysis, Barnhart et al. found that the implantation and clinical pregnancy rates were lower in the EM group than in the control group.[1] Similar to these findings, we found that

Table 4. Comparison of IVF/ET outcomes between patients in the 2 groups ($\bar{x}$ ±s)

| Indicators | EM (n = 76) | Control (n = 40) | p-value |
|---|---|---|---|
| Fertilization rate (%) | 88.76 ±22.00 | 88.05 ±18.9 | 0.856 |
| Cleavage rate (%) | 98.86 ±12.33 | 97.28 ±6.08 | 0.286 |
| Good-quality embryos rate (%) | 70.86 ±38.11 | 72.72 ±24.30 | 0.703 |
| No. of embryos transferred | 1.99 ±0.22 | 1.99 ±0.11 | 0.250 |
| Implantation rate (%) | 18.33 (22/120) | 31.43 (22/70) | $p < 0.05^a$ |
| Clinical pregnancy rate (%) | 33.87% (21/62 | 52.78 (19/36) | $p > 0.05^b$ |
| OHSS rate (%) | 1.61 (1/62) | 0 (0/36) | 0.63 |
| Abortion rate (%) | 19.04% (4/21) | 10.53 (2/19) | $p < 0.05^c$ |

[a] $\chi^2 = 4.26$; [b] $\chi^2 = 3.37$; [c] $\chi^2 = 0.57$.

the implantation rate in the EM group (18.33%, 22/120) was significantly lower ($p < 0.05$) than that in the control group (31.43%, 22/70), suggesting that the higher resistin and IL-23 levels may cause embryo implantation failure. Pouly et al. found that the number of recovered oocytes was lower in the group of patients with EM than in the control group.[19]

Neeta et al. compared the ovarian stimulation characteristics between women with EM and tubal infertility.[20] The number of oocytes retrieved and the fertilization rate were significantly lower in the EM group than in the tubal group, but there was no significant difference in the percentage of metaphase II (M2) oocytes, cleavage rate, and percentage of grade 1 embryos formed between the 2 groups. Moreover, the mean number of embryos transferred did not differ between the 2 groups. The clinical pregnancy rate between the 2 groups was comparable.

The duration of GnRH-a downregulation, Gn dose, Gn duration, rate of fertilization, rate of cleavage, clinical pregnancy rate, OHSS, and abortion rate between the EM group and control group during IVF/ET therapy were compared, and the differences were not statistically significant. The clinical pregnancy rate in the EM and control groups were 33.87% (21/62) and 52.78% (19/36), respectively, and although this difference was not of statistical significance ($p > 0.05$), the EM group had a lower implantation rate.

Finally, EM is the cause of infertility where multiple factors are involved, and the high expression levels of resistin and IL-23 lead to inflammation and play an important role in the formation of EM; thus, inhibition of inflammation and improving the IVF-ET implantation rate and clinical pregnancy rate may be effective methods in treating EM.

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# Intestinal epithelial barrier: The target for pathogenic *Escherichia coli*

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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

Diarrheagenic *Escherichia coli* strains are included in 9 pathotypes (pathovars) that present different virulence factors responsible for the patomechanism of infections they cause. As all other intestinal pathogens, *E. coli* exerts a significant effect on intestinal epithelium. To initiate the infection, these microorganisms have evolved countless strategies to subvert the epithelial barrier and efficiently colonize the intestinal epithelium. The barrier function of the intestinal epithelium is achieved by the presence of a tight junction protein network surrounding individual cells around their circumference that links neighboring cells and seals the intracellular space. Pathogenic *E. coli* strains may impair intestinal epithelial barrier in 3 different pathways: (i) through a direct effect of their virulence factors on tight junctions proteins, (ii) by disrupting host cell actin cytoskeleton that indirectly damages epithelial barrier, and (iii) via stimulation of the secretion of proinflammatory cytokines that directly disrupt epithelial tight junctions or trigger neutrophils migration through intestinal epithelium, thus disrupting the intestinal barrier. Most pathogenic *E. coli* generates all these 3 pathways concomitantly upon interaction with intestinal epithelium.

**Key words:** *Escherichia coli*, intestinal barrier, tight junctions

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## The intestinal epithelial barrier

The epithelium lining the gastrointestinal tract makes up the largest interface between the external and internal milieu in human organism. This single columnar cell layer provides a selectively permeable barrier that allows nutrients, water, and electrolytes to be absorbed, but simultaneously prevents the passage of luminal antigens like toxins and microbial flora to the lamina propria and to the bloodstream.[1,2] Furthermore, armed with the secretory immunoglobulin A (sIgA), Paneth cells that produce antibacterial peptides as well as immune cells, i.e., dendritic cells, mast cells, macrophages, and B and T cells localized beneath the epithelial cells in lymphoid follicles, the intestinal epithelium, serve as a first line of the host's innate immune defense.[3,4]

The barrier function of the intestinal epithelium is achieved by the presence of a protein network surrounding individual cells around their circumference that links adjacent cells and seals the intracellular space.[1] The protein connections between adjacent cells are composed of 2 complexes including the apical junction complex (AJC) that forms 2 separate zones, i.e., tight junction (TJ) and the adherens junction (AJ), and desmosomes localized at the basolateral membranes, which support epithelial stability (Fig. 1). Both TJ and AJ form 2 extracellular loops in the paracellular space that interlock the same structures of the neighboring cell. At the other end, TJ and AJ are anchored directly to the F-actin cytoskeleton (Fig 1). The main role of AJC is to polarize epithelial cells by separating the apical membrane above the AJC that faces the intestinal lumen and the basolateral membrane below AJC which is in contact with the lamina propria.[1,5]

TJ is the most apical complex connecting and sealing the intercellular space between adjacent cells. This highly specialized network of proteins is composed of key transmembrane barrier proteins, such as occludin, claudins and junctional adhesion molecules (JAMs) linked to the peripheral membrane (scaffolding) proteins (e.g., zonula occludens (ZO), afadin) that are in turn connected with actin and microtubules by linkers, e.g., cingulin and non-muscle myosin. Myosin and actin, collectively referred to as actomyosin, form a ring encircling the cell at the level of AJC (Fig. 2).[6]

Claudins are the family of at least 24 important proteins essential for barrier function that form paracellular ion-selective channels across TJs. They have 4 transmembrane domains that form 2 loops in the extracellular space specifying their ion-selective channel functions. These extracellular loops of claudins of neighboring cells appearing opposite to each other are linked together forming TJ strands. The largest 1st loop is considered to be critical for determining the paracellular tightness and selective ion permeability, whereas the 2nd one has a holding function between opposing cell membranes, although it may also narrow the paracellular cleft.[7] The cytoplasmic C-terminus of claudins is linked to the peripheral membrane adaptor ZO proteins of the cytoplasmic plaque (i.e., ZO-1, -2 and -3 proteins). ZO proteins link TJs to the actin and microtubule cytoskeleton (Fig. 2). Different claudin isoforms are expressed simultaneously in all epithelial tissues, and exhibit distinct expression patterns specific to the tissue and cell types.[6,8,9]

Occludin also forms 2 loops in the extracellular space with N- and C-terminal domains anchored in the host cell cytosol that are important in the sealing of the TJ. Phosphorylation of long C-terminal domain via cellular

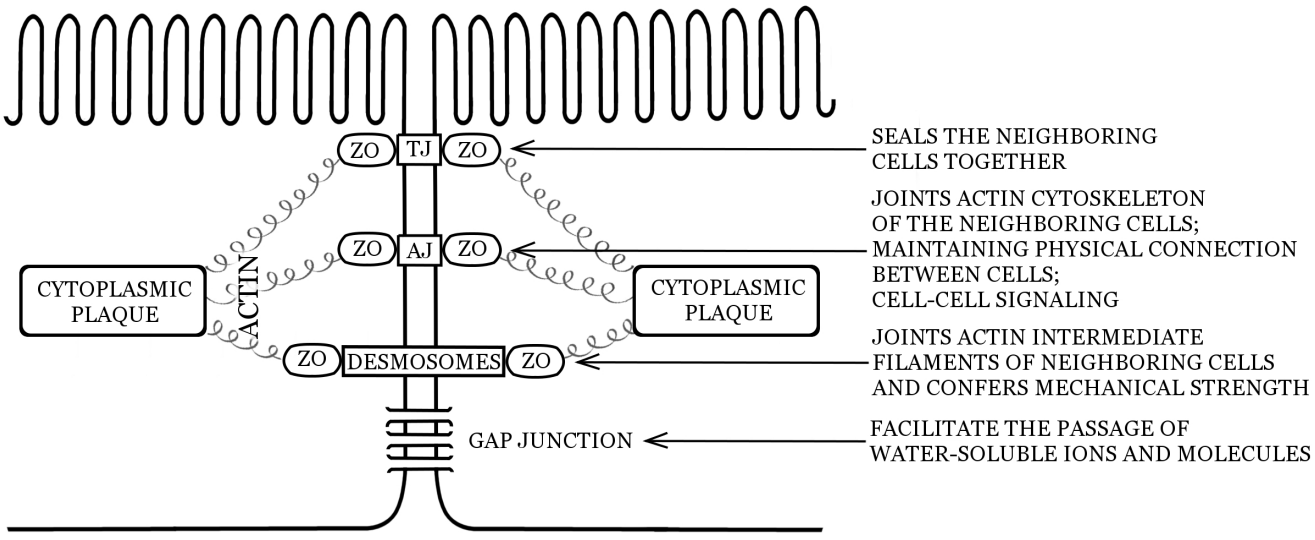


**Fig. 1.** Main types of cell junctions

TJ – tight junction; AJ – adherens junction (adhesion belt); ZO – zonula occludens.

kinases or phosphatases regulates occludin's localization in the TJ and influences occludin-ZO-1 interactions, and thus barrier function maintenance. Moreover, occludin interacts with claudins and JAM-A.[10] By multiple interaction with many signaling molecules, occludin play a role in signal transduction.[8]

JAMs transmembrane glycoproteins belong to an immunoglobulin (Ig) superfamily and play a role in regulating epithelial cell polarity as well as in leukocytes–epithelial or endothelial cells interactions.[8,11] There are 3 JAMs isoforms, i.e., JAM-A, -B, and -C, that can form homophilic and heterophilic interactions at the adhesion points via PDZ-binding domain at their cytoplasmic portion. PDZ domain is a common structural domain for binding to a short motif at C-terminal domain of other protein. JAM-A together with E-cadherin and ZO-1 localizes in a spot-like adherens junctions at early cell–cell fusion point. As a result, contacting cells start to polarize via formation of TJs and AJs.[11,12]

Cytosolic TJ plaque proteins comprise 2 main categories: (i) peripheral proteins (e.g., ZO-1, -2, -3, cingulin) that organize transmembrane proteins and link them to other cytoplasmic proteins as well as to actin cytoskeleton; and (ii) signaling proteins involved in TJs assembly.[10]

AJ through its interactions with F-actin network is responsible for the maintenance of the physical connection between neighboring cells and is thus important in cell–cell signaling. AJ consists of transmembrane proteins (cadherins) and adaptor proteins. Cadherins are calcium-dependent cell adhesion molecules that are divided into classical cadherins, e.g., E-cadherin, N-cadherin, P-cadherin, desmosomal cadherins, protocadherins, and non-conventional cadherins.[13] Highly conserved cytoplasmic domains of classical cadherins interact with cytoplasmic proteins, i.e., β-catenin that in turn bind to α-catenin, which is linked to actin-binding proteins.[14] Thus, all these cytoplasmic components of AJ play an important role in the strength and stability of cell–cell contact and epithelial polarity (Fig. 3).[13,15]

Desmosomes are intercellular junctions that make focal connections between intermediate keratin filaments of neighboring cells. Due to their characteristic structure, desmosomes confer tensile strength and resilience to cells. Morphologically, desmosomes are highly organized, and they consist of a central core region between opposing cells and 2 identical cytoplasmic plaques that are associated with the cytoskeleton network. As previously clarified, all desmosomes are composed of desmoplakin, plakoglobin, at least 1 isoform each of plakophilin and the desmosomal cadherins: desmocollin and desmoglein (Fig. 4).[16] Additionally, various accessory proteins are involved in desmosomal adhesion.[17]

Under physiological conditions, FJ, TJ, AJ and desmosomes link adjacent cells and cooperate together to maintain the intestinal epithelial barrier function. However, direct contact of intestinal epithelial cells and their junctional complexes with luminal content exposes the epithelium to a number of pathogenic microorganisms which gain access to the intestines with food and water.

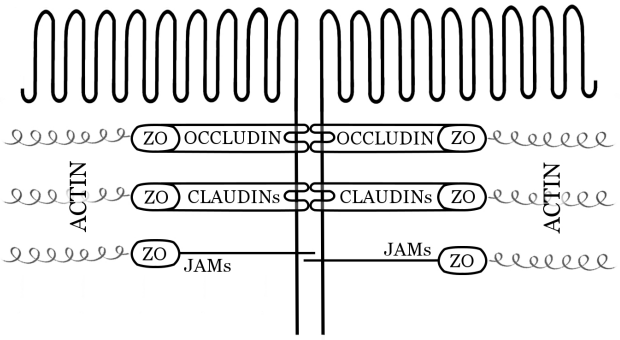


Fig. 2. The simplified architecture of tight junction (TJ)

ZO – zonula occludens; JAMs – junctional adhesion molecules.

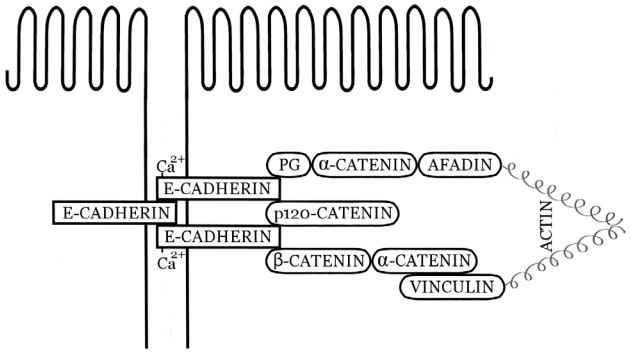


Fig. 3. The simplified architecture of adhernes junction (AJ)

PG – plakoglobin; AJs are located below TJs in the apical part of the basolateral membrane and comprise $Ca^{2+}$ – dependent adhesion molecules cadherins. Cadherins attach to catenins and then the complex is linked to actin filaments via vinculin. p120 catenin interacts with juxtamembrane domains of cadherins participating in the cell–cell adhesion.

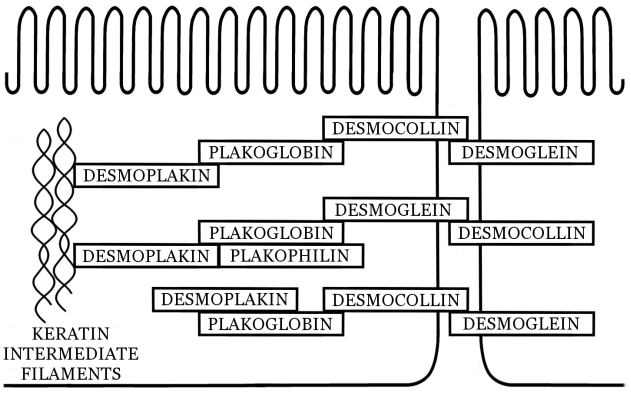


Fig. 4. A simplified schematic diagram of desmosomes

## How pathogenic *Escherichia coli* induces intestinal epithelial barrier disturbances

Intestinal pathogens have evolved countless strategies to interfere with junctional complexes and to cross the epithelial layer. Over the recent years, the association between the dysfunction of the intestinal barrier and the development of Crohn's disease, ulcerative colitis, and microbial infections has been discovered.[18] One of the best-known pathogens responsible for gastrointestinal tract infections or combined with idiopathic intestinal diseases, e.g., inflammatory bowel disease or bowel cancers, are pathogenic *Escherichia coli* (*E. coli*) strains.

Diarrheagenic *E. coli* strains belong to 9 pathotypes, each one of which has a characteristic set of virulence factors responsible for different patomechanism of infections they cause. These pathotypes include enteropathogenic *E. coli* (EPEC), enterohemorrhagic *E. coli* (EHEC), shiga toxin-producing *E. coli* (STEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), enteroaggregative *E. coli* (EAEC), diffusely-adhering *E. coli* (DAEC), cell-detaching *E. coli* (CDEC), and adherent-invasive *E. coli* (AIEC). All these pathotypes can impair intestinal barrier integrity upon infection.

Intestinal epithelial barrier disruption is mediated by pathogenic *E. coli* in many different ways, e.g., upon adhesion, via toxins or effector proteins delivered directly to the host cells, or is induced by cytokines stimulated upon infection with these pathogens.[19]

## Enteropathogenic *E. coli* and enterohemorrhagic *E. coli* – Host cell actin cytoskeleton modulators

Enteropathogenic *E. coli* is a common cause of gastroenteritis in infants, whereas enterohemorrhagic *E. coli* causes bloody diarrhea in humans that may progress to hemolytic urinary syndrome (HUS). Both EPEC and EHEC, as well as *Citrobacter rodentium* and rabbit EPEC (REPEC), are pathogenic for animals, and they all share a common patomechanism involving the production of characteristic histopathological lesions A/E (attaching and effacing) in the host's intestinal epithelium and are, therefore, collectively named A/E pathogens. The adherence and tight attachment of A/E pathogens to enterocytes induces a cascade of morphological and structural alterations in the epithelial cells involving localized destruction and collapse (effacement) of brush border microvilli. The development of the A/E lesions is also combined with the accumulation of filamentous actin that together with α-actinin, ezrin, talin, and myosin form a pedestal-like structures beneath the adhering bacteria.[19]

Decreased transepithelial electrical resistance (TEER) of polarized epithelial cells, indicating decreased epithelial permeability that accompanies infections caused by A/E pathogens initiated research on the influence of A/E pathogens on TJs. These studies demonstrated that A/E pathogens subvert the host's intestinal permeability barrier in many different pathways, but intimate contact of the pathogen with the host cell is crucial in the process.

The process is initiated immediately after the attachment of A/E pathogen to the enterocyte by the injection of effector proteins through syringe-like apparatus, i.e., a type III secretion system (TTSS) directly to the host cell cytozol. Translocated intimin receptor (Tir) delivered through TTSS to the apical cytoplasmic membrane of enterocytes is one of the most important effectors modulating actin cytoskeleton of the host cells. Interaction of Tir inserted to the host cell cytoplasmic membrane, by means of the adhesion molecule intimin in the outer membrane of A/E pathogens, triggers Tir phosphorylation by host tyrosine kinases, e.g., Src family kinase c-Fyn, followed by the recruitment of the adaptor signaling protein Nck, which in turn recruits neuronal Wiskott-Aldrich Syndrome protein (N-WASP) and the actin-relating protein Arp2/3 complex.[19,20] N-WASP is a member of the WASP family of proteins that signal to the host cell cytoskeleton through the Arp2/3 complex, nucleating new actin filaments and cross linking existing filaments into actin networks. Activation of N-WASP-Arp2/3 complex mediates actin pedestals formation, i.e., histopathological lesions A/E beneath the adhering bacterium. Moreover, it has been shown that Tir of EPEC can also bind to the host cell multidomain protein IQGAP1, a well-known regulator of the cy-

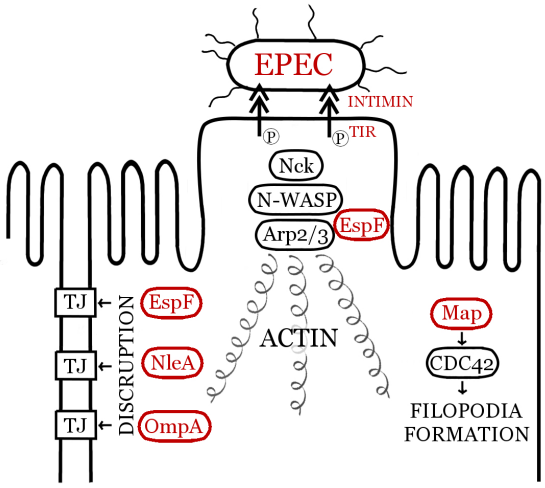


Fig. 5. The impact of EPEC on epithelial barrier

Adherence of enteropathogenic *E. coli* (EPEC) to the small bowel enterocytes induces the development of characteristic actin-rich pedestals beneath adherent bacteria which is associated with the collapse of microvilli.

toskeleton, involved in Rho family GTPases Rac1/Cdc42 and $Ca^{2+}$/calmodulin signaling, and actin polymerization.[19–21] On the other hand, Brown et al. also demonstrated that although cells lacking protein IQGAP1 have significantly attenuated actin polymerization in response to EPEC attachment, the knockout of IQGAP1 did not abrogate pedestal formation.[20] Peralta-Ramirez et al. in their study of REPEC strain demonstrated that EspF protein secreted by A/E pathogens via TTSS to a host cell is involved in the regulation of actin polymerization by binding to a complex of proteins, e.g., N-WASP and Arp2/3, but also to ZO-1, ZO-2 proteins at the TJs. Furthermore, EspF caused claudin, occludin, ZO-1 and ZO-2 proteins redistribution and their recruitment into the actin pedestals.[22] These authors suggested that EspF may cause local actin depolymerization and thus EspF-induced TJ disruption.

The disturbances in the host cell actin cytoskeleton induce the contraction of actin filaments attached to the TJs pulling the junction opened.[23] Similar results were demonstrated by Muza-Moons et al., who confirmed the crucial role of EspF protein in TJs disruption.[24] In their study, T84 epithelial cells infected with EPEC progressively lost interaction of occludin and claudin-1 with the cytosolic plaque protein ZO-1. Mutation of the gene encoding EspF prevented the disruption of TJs. Alto et al. have shown that EspF localizes to membrane trafficking organelles and nucleates a multiprotein signaling complex consisting of eukaryotic sorting nexin 9 (SNX9) and N-WASP involved in multiple host cellular regulatory pathways, e.g., membrane trafficking, actin-remodeling, and the overall epithelial homeostasis.[25] Their study indicated that EspF is necessary, although not sufficient, to regulate TJ architecture during EPEC infection. On the other hand, Malladi et al. have shown that other virulence factors of EPEC may contribute to intestinal barrier disruption.[26] These authors demonstrated that outer membrane proteins (OMPs) of EPEC activate protein kinase C (PKC), which is associated with phosphorylation of cadherins and leads to the dissociation of the cadherin/β-catenin complex from AJ. Additionally, 2 other proteins of EPEC, i.e., mitochondrial associated protein (Map) and NleA (EspI) cause barrier disruption by perturbing TJs. It has been shown that Map is a guanidine-nucleotide exchanging factor (GEF) for CDC42, a small GTPase of the Rho family that regulates actin dynamics through binding to N-WASP. NleA disrupts COPII, a coat protein complex involved in trafficking membrane proteins from endoplasmic reticulum, thus blocking the delivery of new TJs proteins (Fig. 5).[27]

All these results suggest that A/E pathogens via TTSS secreted effector proteins but also other virulence factors may utilize many pathways impairing barrier integrity to promote effective colonization of intestinal epithelium. In turn, disturbances in epithelial permeability contribute to the development of secretory diarrhea and thus provide the exit and the way of spreading for A/E pathogens to new hosts.

## Enteroaggregative *E. coli*

Enteroaggregative *E. coli* strains differ from other pathogenic *E. coli* characteristic aggregative adherence pattern to epithelial cells (Fig. 6).

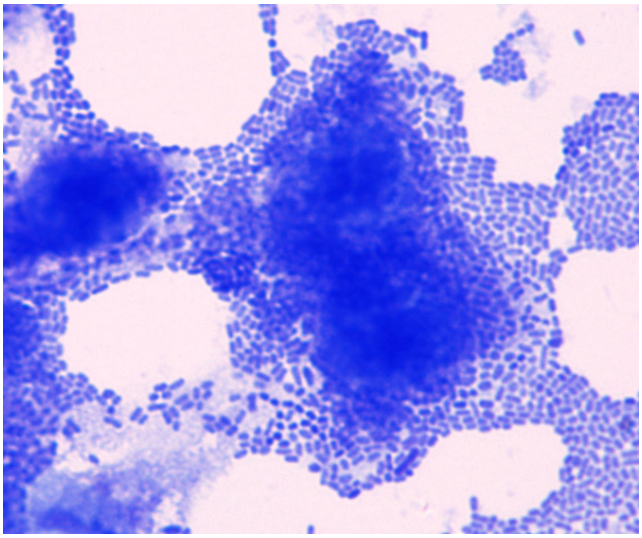

Fig. 6. Aggregative adherence of EAEC to HEp-2 cells

In the picture there are visible epithelial cells totally covered with adherent bacteria. Giemsa stain, magnification ×100.

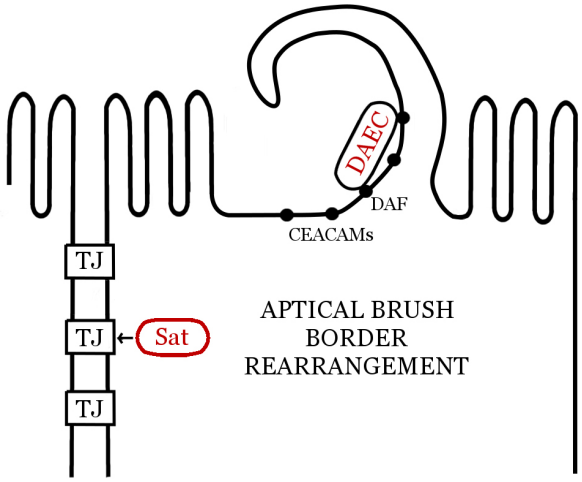


Fig. 7. Epithelial barrier disruption by DAEC

CEACAMs – carcinoembryonic antigens; DAF – decay accelerating factor; both CEACAMs and DAF serve as anchoring molecules on the surface of intestinal epithelium for binding diffusely adhering *E. coli* (DAEC). Effector proteins of DAEC induce specific apical brush border rearrangement leading to the formation of a long finger-like edgings that wrap the adherent bacteria.

These pathogenic *E. coli* strains are responsible for acute and chronic diarrhea among children and adults all over the world.[28] Strauman et al. demonstrated on in vivo intestinal cell model that EAEC O42 and JM221 reference strains induced decrease in epithelial TEER via aberrant localization of the tight junction proteins occludin, ZO-1, and claudin-1.[28] They also found that the effect was induced by aggregative adherence fimbriae I and II (AAF/I and AAF/II) specific to EAEC pathotype as afimbrial mutant strains did not impair the TJ integrity. Involvement of claudin-1 is a common theme in the enteric infections, as several intestinal pathogens, including EHEC and *Salmonella enterica* serovar Typhimurium, cause a redistribution of claudin-1 in addition to disrupting paracellular permeability.[28] Moreover, EAEC can disrupt intestinal epithelial integrity via the action of Pet, a serine protease autotransporter enterotoxin, that cleaves the actin-binding protein α-fodrin within the cytoskeleton, leading to cell rounding and detachment from the substratum.[29] Pet enterotoxin is a member of serine protease autotransporter proteins of *Enterobacteriaceae* (SPATE) that have multiple effects on host cell cytoskeleton and cell–cell connections.

## Diffusely adhering *E. coli*

Diffusely adhering *E. coli* strains presenting Afa/Dr fimbrial and afimbrial adhesins produce Sat toxin, another member of serine protease autotransporters, which causes an increase in paracellular permeability and the rearrangement of the tight junction proteins of ZO-1, ZO-2, ZO-3, occludin, and claudin-1 that are linked to the cytoskeleton and play a pivotal role in the TJ architecture.[19,30,31] Another serine protease of SPATE family, i.e., EspC secreted by EPEC through TTSS, contributes indirectly to the alteration of epithelial barrier via the destruction of the actin cytoskeleton. Similarly to Pet enterotoxin of EAEC, EspC cleaves α-fodrin (spectrin) that links actin filaments with the host cell plasma membrane, and thus maintains the stability of cellular cytoskeleton and its mechanical properties. Loss of α-fodrin disrupts the structural link between actin cytoskeleton and plasma membrane, leading to the collapse of cytoskeleton and TJs disruption with resulting influx of luminal antigens into the submucosa (Fig. 7).[19]

Irrespective of the Sat, Peiffer et al. demonstrated that the reference Afa/Dr DEAC C1845 strain causes selective lesions in the intestinal epithelial barrier producing increased paracellular permeability to mannitol but no change in the paracellular passage of nonionic molecules which have higher molecular masses.[31] This phenomenon was accompanied by a dramatic alteration in the distribution of TJ-associated occludin and ZO-1 protein, and was independent of apical cytoskeleton rearrangements produced by the strain. However, the bacterial factor contributing to the alteration of TJ proteins is still unknown.[31] Apart from the impact of DEAC on host cell junctions, the adherence of these strains to the intestinal epithelial cells produces prominent brush border

**Table 1.** The influence of pathogenic *E. coli* on the intestinal epithelial barrier

| *E. coli* pathotype | Virulence factor | Effect on epithelial barrier |
|---|---|---|
| AIEC | type 1 pili | up-regulation of CEACAM6 associated with abnormal expression of claudin-2, ZO-1, occludin, and E-cadherin; disorganization of actin cytoskeleton |
| EPEC/EHEC | EspF effector protein | redistribution of claudin, occludin, ZO-1, and ZO-2 leading to TJ disassembly |
| | Map protein | reduced host cell proteins trafficking; TJs disassembly |
| | NleA (EspI) protein | inhibition of host cell protein trafficking and blocking the delivery of a new TJs proteins |
| | EspC protein | cleavage of α-fodrin linking actin filaments with cell membrane, leading to the cytoskeleton collapse and TJs disruption |
| | outer membrane protein A (OmpA) | activation of protein kinase C (PKC) and dissociation of cadherin/β-catenin complex from AJ |
| ETEC | STb enterotoxin | redistribution of ZO-1, claudin-1, and occludin proteins |
| DAEC | Sat serine protease | rearrangement of ZO-1, ZO-2, ZO-3, and claudin and disrupting TJs architecture |
| | unknown factor | altered distribution of occludin and ZO-1 protein |
| | Afa/Dr fimbriae | rearrangement of apical cytoskeleton proteins: F-actin, villin, α-actin, ezrin, tropomyosin |
| EAEC | aggregative adherence fimbriae (type AAFI – III) | decreased TEER, aberrant localization of occludin, ZO-1 and claudin-1 |
| | Pet serine protease | cleaves the actin-binding protein α-fodrin |
| EIEC | Ipa and IcsA effector proteins | recruitment of actin-nucleating complex Arp2/3 and N-WASP |

injury and rearrangement in apical cytoskeleton proteins, i.e., F-actin, villin, α-actinin, ezrin, and tropomyosin, thus playing an important role in the organization and maintenance of the brush border integrity.[19]

## Enteroinvasive *E. coli*

Rearrangement of the host cell action cytoskeleton, along with the disruption of intestinal epithelial barrier integrity, also accompanies infections caused by Enteroinvasive *E. coli*. The pathotype causes an invasive colitis that occasionally presents as dysentery. Through the TTSS, these pathogens secrete multiple effector proteins, i.e., IpaA, IpaB, IpaC, and IpaD, that are responsible for the EIEC entering into colonocytes, the lysis of the endocytic vacuole and phagosome escaping into host cell cytoplasm, and then cytoskeleton rearrangements necessary for intracellular spread of these organisms.[32] Many effector proteins of EIEC interact with small Rho GTPases, i.e., Rac1 and Cdc42, which recruit the actin-nucleating complex Arp2/3. In turn, the IcsA, an outer membrane protein of EIEC delivered via TTSS into host cell membrane, interacts with N-WASP at 1 bacterial pole, allowing for EIEC to move through the epithelial cell cytoplasm and spread to the adjacent cells.[19]

## Enterotoxigenic *E. coli*

Enterotoxigenic *E. coli*, endemic in most developing countries, is an important cause of watery diarrhea among children and travelers. ETEC characterizes the production of heat-stable (ST) enterotoxins a (STa) and b (STb), and heat-labile (LT) enterotoxins I (LTI) and II (LTII) that disrupt intestinal epithelial cells secretory activity, resulting in diarrhea.[27] Nassour et al. have demonstrated that STb enterotoxin induces intestinal epithelial barrier dysfunction through changes in the tight junction protein claudin-1.[33] Mukiza et al. have demonstrated that STb internalized by T84 epithelial cells caused a significant reduction of TEER and an increase in paracellular permeability, which was associated with the alteration of F-actin stress fibers.[34] Changes in F-actin dissolution and fragmentation were related to the redistribution and/or fragmentation of ZO-1, claudin-1, and occludin proteins.

## Crohn's associated adherent – Adherent-invasive *E. coli* (AIEC)

The pathogenesis of Crohn's disease, although still unclear, seems to be multifactorial and related to genetic predisposition and to a dysregulated immune response to altered host intestinal microflora. Increased intestinal permeability observed in patients with Crohn's disease promotes the exposition of intestinal epithelium to luminal content and triggers chronic inflammatory response to intestinal microflora. Adherent-invasive *E. coli* is the pathotype, comprising *E. coli* strains, which characterizes the ability to adhere to and invade epithelial cells and capability to survive and multiply within macrophages.[35] Although there are several reports presenting evidence that AIEC may be the pathogen triggering Crohn's disease, presently, they are considered to be a pathobiont which intensifies the preexisting inflammation rather than induce this form of inflammatory bowel disease. Nevertheless, it has been shown that AIEC binds via type 1 pili to carcinoembryonic antigen CEACAM6 overexpressed on the epithelial cells of patients with Crohn's disease, enhancing further the expression of CEACAM6. The expression of CEACAM6 is additionally increased by the proinflammatory cytokines, i.e., tumor necrosis factor alpha (TNFα) and interferon gamma (IFNγ) produced by macrophages infected with AIEC and lymphocytes.[35] Denizot et al. demonstrated on a CEABAC10 transgenic mouse model expressing human CEACAMs that barrier disruption precedes a relapse of Crohn's disease in asymptomatic patients.[36] These authors suggested that up-regulated CEACAMs expression on epithelial cells interfere with other adhesion molecules located between lateral membranes and may disrupt epithelial cells architecture, leading to the development of colitis. Moreover, they also showed that the infection of the CEABAC10 mice led to a 3-fold increase in intestinal permeability and to the disruption of mucosal integrity via interaction of type 1 pili of AIEC with CEACAM6. Interestingly, the process was associated with abnormal expression of claudin-2, disorganization of actin cytoskeleton, and mislocalization of ZO-1 and E-cadherin.[36,37] Similar results were presented by Wine et al., who demonstrated that an infection of polarized T84 cells with AIEC caused a reduction in TEER and increased dextran flux, both accompanied by the redistribution of the TJ adaptor protein ZO-1.[38] Moreover, it has been demonstrated that AIEC induces increased expression of claudin-2 and decreased expression of occludin.[39] Although the direct impact of AIEC infection on polarized epithelial cells induces alterations in epithelial permeability, it is noteworthy that AIEC may induce intestinal barrier disruption indirectly by eliciting strong immune response and release proinflammatory cytokines, a well-known TJs proteins modulator. Infection of intestinal epithelium with AIEC results in the secretion of an array of cytokines, i.e., interleukin-1β (IL-1β), IL-6, TNFα, that may impair intestinal barrier integrity. Similarly to AIEC, other *E. coli* pathotypes trigger transepithelial migration of dendritic cells and neutrophils as well as an expression and upregulation of monocyte chemotactic protein 1 (MCP-1), macrophage inflammatory protein 3 (MIP3α), and a number of chemokines, i.e., IL-8, GROα, apart from mentioned above.[40]

## Summary

Although the direct impact of pathogenic *E. coli* on TJs proteins has been demonstrated in several manuscripts, it seems that the main target for these pathogens is the F-actin cytoskeleton of intestinal epithelial cells. Enterocytes presenting a number of glycoproteins provide the adherence sites for pathogenic *E. coli*, thus protecting them from removal via peristalsis. As successful pathogens, pathogenic *E. coli* must provide themselves a niche to replicate sufficiently. EIEC and AIEC pathotypes can invade and replicate within enterocytes and intestinal macrophages, whereas other remain on the surface of enterocytes which is associated with the necessity to interact with the host immune response. Direct contact of adhering *E. coli* with the host cells surface triggers the activation of the immune response that can be utilized by some pathogens. Neutrophils migration on the surface of intestinal epithelium upon infection with EIEC is associated with the opening of TJs, facilitating the access of EIEC to the basolateral surfaces and their invasion into enterocytes. A similar strategy can be used by AIEC to reach macrophages in the lamina propria. EAEC pathotype has the unusual ability to form a specific biofilm on the surface of intestinal epithelium that makes these pathogens difficult to remove via peristalsis, but also protecting them from hostile intestinal environment as well as immune system cells. DAEC strains induce the host cell apical cytoskeleton disassembly and the formation of elongated microvilli that surround the attached *E. coli*, protecting them from phagocytes. Similarly, EPEC induces actin cytoskeleton rearrangements and formation of pedestals reaching up to the adhering *E. coli* above the surface of enterocytes, and thus protecting them from immune cells. The main targets in the intestinal epithelial barrier for pathogenic groups of *E. coli* are summarized in Table 1.

In any case, drastic cytoskeleton changes affect the epithelial barrier and have an influence on the redistribution of TJs proteins.

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# The role of pancreatic polypeptide in pancreatic diseases

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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

The aim of this study was to review the diagnostic significance of pancreatic polypeptide (PP) in pancreatic diseases. PP may play a significant role in monitoring the development of the disease and the patient's healing process, particularly after the removal of a portion of the pancreas. Determining PP in acute pancreatitis is quite controversial. At the 1st stage of severe pancreatic damage, there is excessive PP release followed by its fall. In patients with chronic pancreatitis, a significant decrease in PP secretion was found in the presence of a food stimulant. In this case, PP could be a good marker for determining the stage of pancreatitis. Pancreatic polypeptide also functions as a hepatic glucose regulator. PP increases hepatic insulin sensitivity, resulting in reduced hepatic glucose production. Therefore, impaired hepatic insulin sensitivity in chronic pancreatitis is abrogated after the PP administration. Endocrine pancreatic tumors initially grow without specific symptoms. In contrast, they are almost always correlated with elevated serum pancreatic polypeptide. Therefore, the level of PP may be a good diagnostic parameter confirming the presence of pancreatic cancer. Depending on the type of disease, the polypeptide concentration can be increased or decreased, evidencing the disease progress or regression.

**Key words:** pancreatic polypeptide, acute pancreatitis, chronic pancreatitis, diabetes

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# Introduction

The clinical significance of pancreatic polypeptide (PP) is closely related to the function of this hormone in the body. Due to its localization in the pancreas, it appears to be a useful diagnostic parameter in acute and chronic pancreatitis (AP, CP).[1,2]

Playing its role in the body, PP reduces energy demand, thereby decreasing the amount of food intake. This observation contributed to the development of research on the treatment of obesity.[3] Moreover, studies using the immunohistochemical technique showed an increase in the number and change of PP cell location in the pancreatic parenchymal tissue sections obtained from tobacco smoking patients with chronic pancreatitis and concomitant diabetes. Long lasting CP may lead to the development of insulin-dependent diabetes. PP determination can be a useful diagnostic parameter of diabetes development in the course of chronic pancreatitis.[1,2] The aim of the review was to provide the current stage of knowledge on PP and its function in the body, in physiological and pathological conditions of the pancreas.

# Discussion

## Pancreatic polypeptide

Pancreatic polypeptide is a hormone consisting of 36 amino acids with a molecular weight of approx. 4227 Da. Its precursor is a protein composed of 95 amino acid residues called pancreatic prepropolipeptyde. The C-terminus of pancreatic polypeptide is tyrosine.[4,5] PP belongs to the peptide family, which includes neuropeptide Y (NPY) and peptide YY (PYY). Five different affinity subtypes of NPY-, PYY- and PP-binding receptors were identified.[5–8] They include receptors Y1, Y2, Y4, Y5, and Y6. Pancreatic polypeptide has the greatest affinity with the Y4 receptor, which is a heptahelical receptor that binds the G-protein, thereby inhibiting the action of adenylatecyclase and influencing the phospholipase C activation.[7] In human plasma, PP is represented in at least 4 forms: 1-36PP, 3-36PP, the other 2 forms have not been fully described yet. The identification was performed using the HPLC chromatography technique.[9] PP circulates in the blood mainly as a dimer. PP half-life in vivo is 6–7 min, and it is excreted in the active form predominantly by the kidneys. The normal PP concentration in human plasma ranges from 40 to 80 pmol/L. PP release from cells is a rapid process, and in the case of certain mammals and humans, this phenomenon is biphasic. A certain role in the secretion of PP into the bloodstream is played by bloating intestines, cholecystokinin release (to a lesser extent, also other intestinal hormones) as well as direct digestible nutrients. However, the primary incentive taking part in PP cell stimulation to secrete this hormone is the release of acetylcholine from cholinergic fibers extending to the pancreas. Hypoglycemia, during which the muscarinic acetylcholine receptors and, to a lesser extent, adrenergic receptors are activated, also has an influence on PP release.[10] PP is released into the plasma during after-meal stimulation, in particular in the case of meals containing protein and fat. Its physiological role includes gastric stimulation inhibition, gastric acid secretion stimulation, delayed gastric emptying (Fig. 1). This hormone inhibits the exocrine pancreatic secretion and insulin secretion in the organ, as well as stimulates the contraction of the gallbladder. These effects slow down the digestive process and the process of absorption of nutrients into circulation, preventing the glucose concentration in the blood from increasing after a meal.[8] PP secretion remains at a low level when fasting but its concentration increases at all stages of digestion. PP secretion depends on the part of the gastrointestinal tract. A strong stimulus affecting PP release in the cephalic phase decreases the concentration of blood glucose and hypoglycemia-induced insulin. A key role in the mechanism of PP release is played by an intact vagus nerve and the cholinergic nerves. Vagotomy and muscarinic receptor antagonist treatment inhibit PP secretion.[8,11]

## The significance of pancreatic polypeptide in obesity

Previous studies have shown that PP appears to be involved in regulating food intake and energy balance.[8,11] Transgenic mice overexpressing PP gained less weight because of decreased food intake and this was accompanied by decreased fat mass. Studies on the impact of peripheral PP action on food intake were also carried out on mice with the obesity gene. PP concentration was observed in the plasma of healthy and obese mice. No increase in the hormone levels in the plasma of obese subjects was observed, despite increased PP content in the pancreas of tested animals. These results indicate that PP cell hyperplasia occurs in obese mice due to lower PP levels in the plasma and because of reduced tissue sensitivity to the hormone.[12] The congenital human obesity (Prader-Willi syndrome) is characterized, similarly as in the case of experimental animals, by PP cell hyperplasia, obesity, hyperglycemia and hyperinsulinemia. The problem of obesity in this case has its source in the lack of adequate PP secretion stimulation. Intravenous PP infusion in obese mice leads to increased secretion of this polypeptide and reduction of food intake by tested objects. These observations suggest that PP circulating in the blood stream affects feeding behavior. PP administered to obese mice by injection twice a day for 2 weeks reduces their weight growth. PP can modulate the vagovagal reflexes as well as the autonomous regulation of gastrointestinal function. The hormone changes the function of these neurons, leading in effect to decreased food intake.

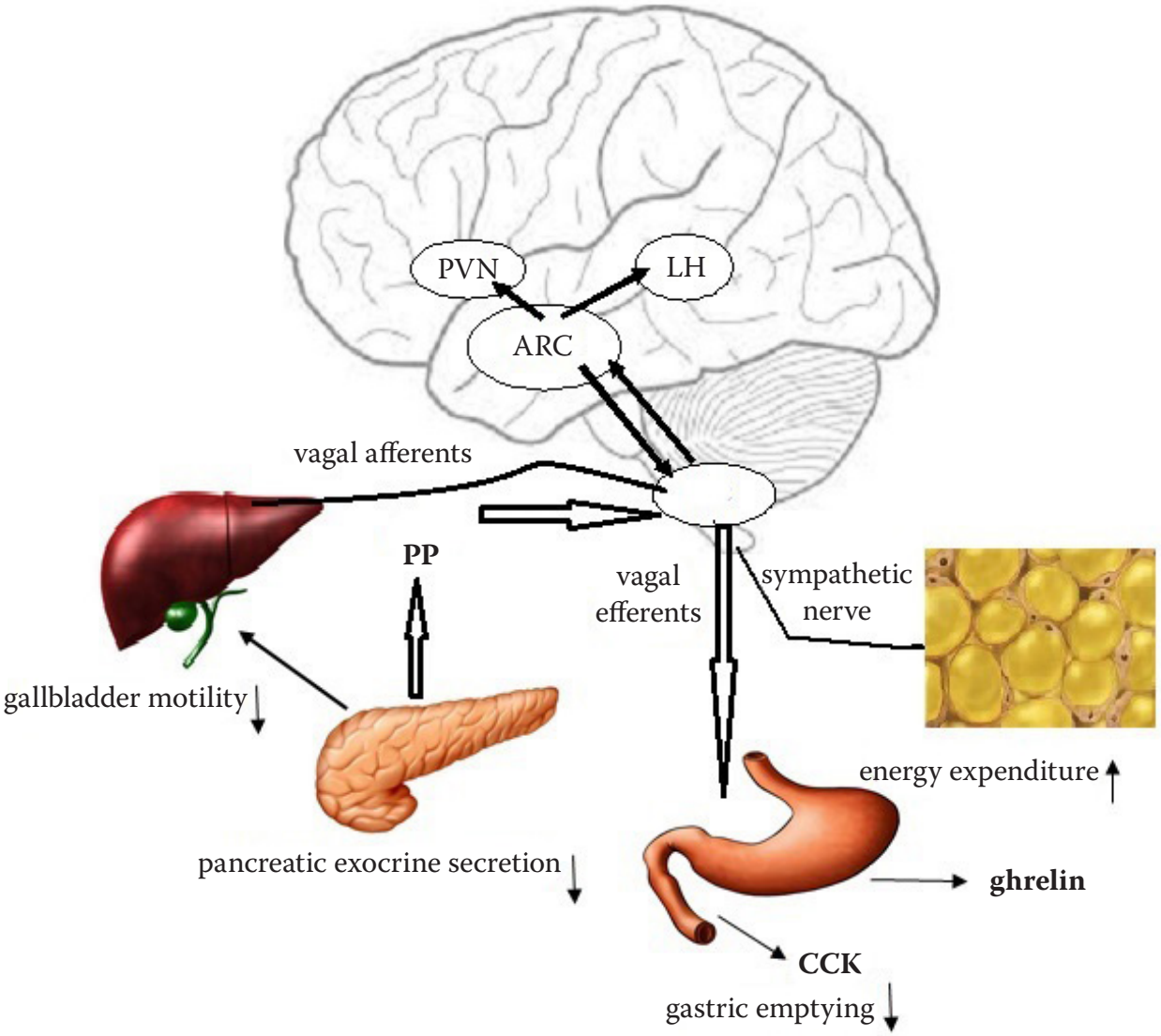


Fig. 1. Regulatory functions of pancreatic polypeptide: inhibition of pancreatic exocrine secretion, gallbladder motility and gastric emptying

PP acts through blood circulation and vagal afferents, and the signal is transmitted to the dorsal vagal complex (DVC) and the hypothalamus (ARC). PP decreases food intake partly through stimulation of cholecystokinin (CKK) and inhibition of ghrelin, and increases energy expenditure through stimulation of the sympathetic nervous system innervating brown adipose tissue. ARC – arcuate nucleus of the hypothalamus; LH – lateral hypothalamic area; PVN – paraventricular nucleus of the hypothalamus.[7]

PP secreted by food stimulation becomes a signal to inhibit further eating and participates in the control of meal sizes by changing gastric emptying during meals.[11,13]

Intravenous peripheral administration of PP to genetically obese ob/ob mice induced a state of negative energy balance because of decreased food intake and increased energy expenditure.[14]

Recent studies have shown that the satiety effects of PP involve an impact on the Y4 receptors in hypothalamic nuclei, which affects the pathway distinct from the one mediating the functions of PYY.[12,15] Research suggests that pancreatic polypeptide, the preferential Y4 receptor agonist, regulates the energy balance of the body. When administered intravenously or intraperitoneally into mice, it accelerates the metabolism rate, reducing at the same time hyperglycemia, insulin resistance and hyperlipidemia in obese mice. The 20-fold higher concentration of the hormone in the plasma of transgenic mice, when compared to healthy subjects, reduces food intake and, consequently, body weight and the adipose tissue content in these rodents.[16] PP impacts food intake reduction via the Y4 receptor localized in the brainstem and the hypothalamus. Recent work suggests that PP is also the primary ligand for the Y6 receptors, which also regulate energy homeostasis.[17]

The above PP effect is abolished when the vagus nerve has been cut in rodents. This observation suggests that PP has an impact on the process of food intake inhibition by the vagus nerve. It was noted that PP might also affect the process of food intake stimulation. This phenomenon is dependent on the peripheral control of PP release. Differences in the impact of PP on feeding behavior may result from the stimulation of different receptors. However, this phenomenon requires further studies to investigate specific mechanisms. There is a decrease in serum

PP concentration after meals, and it is proportionate to the increasing body weight. Other studies have shown elevated PP levels after meals in patients with anorexia.[5] In the case of patients with Prader-Willi syndrome, it was noted that basic PP release was reduced increasingly in relation to older age and larger body weight.[3,13]

PP inhibitory effect on the feeding process was demonstrated in a number of experimental studies. In the case of mice with acute and chronic pancreatitis, a decrease in the food intake was observed. Obese mice with leptin deficiency were noted to lose weight, improve insulin resistance and reduce hyperlipidemia after intraperitoneal PP administration. Furthermore, transgenic mice with elevated serum PP levels had reduced body weight and showed a decreased demand for food when compared to wild type strains.[16] Intravenous PP administration helps maintain normal weight in the case of human subjects by reducing food intake by 25% in 24 h.[18] Furthermore, the administration of suitable PP doses 2 times a day to patients with Prader-Willi syndrome caused a 12% lower demand for food.[18] Low serum levels of PP have been observed in obese people and intravenous infusions of PP reduced food intake, and PP secretion was thought to be primarily under vagal control, although other factors have also been shown to alter serum PP concentrations.[12,19–21]

## The importance of pancreatic polypeptide in acute and chronic pancreatitis

Both acute pancreatitis and chronic pancreatitis lead to the disturbance of endocrine and exocrine secretions of the organ. High incidence of these diseases leads to severe complications, and researchers are seeking diagnostic markers allowing early detection. One of the useful parameters in diagnosing pancreatic disease appears to be pancreatic polypeptide.

In most cases, acute pancreatitis is a fairly mild and self-limiting process. The morphology of this disease corresponds to the inflammatory type interstitial edema. The mild form of acute pancreatitis is associated with mild disorders in other organs. AP may have the necrohemorrhagic form (5–20% of cases), in the course of which an uncontrolled development of inflammation may appear. The pathomechanism of the disease consists in the premature activation of trypsinogen by intracellular lysosomal enzymes in the granules of pancreatic lobules of secretory cells, leading to the destruction of the organ.[22] The diagnostic role of PP in patients with acute pancreatitis is not clearly identified. In animal experiments, there was a significant PP concentration level increase in the first 6 h after acute pancreatitis onset.[23] In another experiment, it was demonstrated that PP concentration rises significantly during the first 96 h after the onset of acute hemorrhagic pancreatitis.

In a pilot study conducted on patients with acute pancreatitis, an increase in PP concentration in the plasma was noted; however, subsequent studies did not confirm the correlation. The observed value of the average hormone concentration in the plasma of patients with mild to moderate acute pancreatitis was similar to the mean PP concentration in the plasma of healthy subjects.[24]

In clinical conditions, this AP marker has diagnostic value when its concentration is and remains elevated for a longer time during the disease. In experimental studies on an animal model, PP concentration measurement satisfies the diagnostic marker criteria, while in studies on human subjects, this correlation does not occur. Therefore, it was concluded that the PP measurement during fasting did not have diagnostic significance in the course of acute pancreatitis. Differences in the studies may be explained by the fact that in the case of animal experiments, PP measurements started upon acute pancreatitis onset, while in the case of human subjects, the hormone measurements, even after 72 h following the onset of disease symptoms, were still considered to be done in the initial phase of the disease. One should also take into account the acute pancreatitis pathophysiology in experimental and clinical cases. It is not fully explained yet to what extent the experimental models reflect acute pancreatitis in humans. Most studies did not differentiate between mild, moderate and severe disease types.[1]

Chronic pancreatitis is a heterogeneous syndrome of clinical symptoms, which include: recurrent, prolonged hypogastric pain, anorexia, vomiting, nausea, fatty food intolerance, persistent diarrhea, foul-smelling stool, and progressive weight loss. In the morphological image of the pancreas, there are prevailing focal changes or diffuse necrosis of the glandular tissue with segmental or diffuse fibrosis, the latter being a progressive and irreversible process. As the disease progresses, exocrine and endocrine insufficiency of the organ develops.[22]

Several years ago, it was found in multiple studies that the determination of PP concentrations in serum, together with immunoreactive trypsin and isoamylase activity, may be useful in chronic pancreatitis diagnosis.[1] To determine particular disease subtypes from mild, through moderate to severe, PP concentration was measured in patients with chronic pancreatitis.[25] During the research, lower PP concentration and greatly reduced secretion of the hormone after appropriate stimulation were noted. In addition, other studies have shown that alcohol-abusing patients with chronic pancreatitis have a low fasting PP concentration. The disadvantage of using PP level assessment in the diagnosis of chronic pancreatitis is a significant impact of age on the hormone level in the fasting state. Some researchers believe that PP level assessment has little diagnostic value in the case of patients with mild to moderate chronic pancreatitis, due to its low sensitivity.[1] It was found that other hormone secretion (meal, pentagastrin, cholecystokinin) is an important element when assessing the PP level in patients with chronic pancreatitis. In order to clarify

the role of PP in the course of chronic pancreatitis, a prospective study should be performed on a healthy control group and on a group of patients with other gastrointestinal disorders.[1] It is well documented that a persistently reduced PP level in the course of CP corresponds to the degree of pancreatic islet damage, and is also an indication of exocrine and endocrine insufficiency of the pancreas (Fig. 2).[26]

## The significance of PP in diabetes associated with chronic pancreatitis

Pancreatic diabetes may also be associated with a deficiency of pancreatic islet cell hormones: insulin, glucagon, and PP. PP cells are located at the periphery of Langerhans islets and are distributed between the vesicles and mucous membrane of the pancreatic duct epithelium (Fig. 2). It is probable that insulin-producing beta cells located in the "center" of the pancreatic islets are protected against damage by surrounding PP cells.[26,27] Accordingly, decreased PP production may be the first sign of cell dysfunction in the islets of the pancreas. Important clinical manifestations of hormonal disturbances occur late in the course of diabetes. Therefore, there is a role of PP as an early marker of hormonal insufficiency. PP may be used as a prognostic factor for chronic pancreatitis.[1] In another group of studies, it was confirmed that the absence of the beta-cell function in the course of chronic pancreatitis is associated with significant PP cell function impairment and severe exocrine pancreatic insufficiency.[28] The role of PP in the regulation of insulin secretion after pancreatitis is not fully clarified because changes in glucose tolerance are associated with abnormal PP levels. The assumption that PP functions as a hormone influencing the glucose level in blood has already been made.[26,29] However, in the animal model, acute PP infusion failed to improve glucose tolerance and did not augment insulin release, but there was an effect of PP on hepatic sensitivity to insulin by decreasing hepatic glucose production.[29]

Impaired hepatic insulin sensitivity in chronic pancreatitis was observed after PP administration. Therefore, the hepatic insulin resistance could be a reversible effect of PP deficiency as well as of reduced stimulation of PP receptors on hepatocytes. These processes can be PP-specific, but there is a possibility that, due to high pancreatic polypeptide homology with the Y and PYY neuropeptides, the latter are involved in the modulating action of insulin in the course of hepatic glucose release. It is suggested that PP has a key role in glucose homeostasis. Furthermore, it can serve as an early diagnostic and prognostic marker for chronic pancreatitis in diabetes.[1]

PP infusions were also able to improve glycemic profiles both in patients and in animals with chronic pancreatitis.[30] Adult patients with type 1 diabetes appear to have elevated levels of pancreatic polypeptide. This suggests

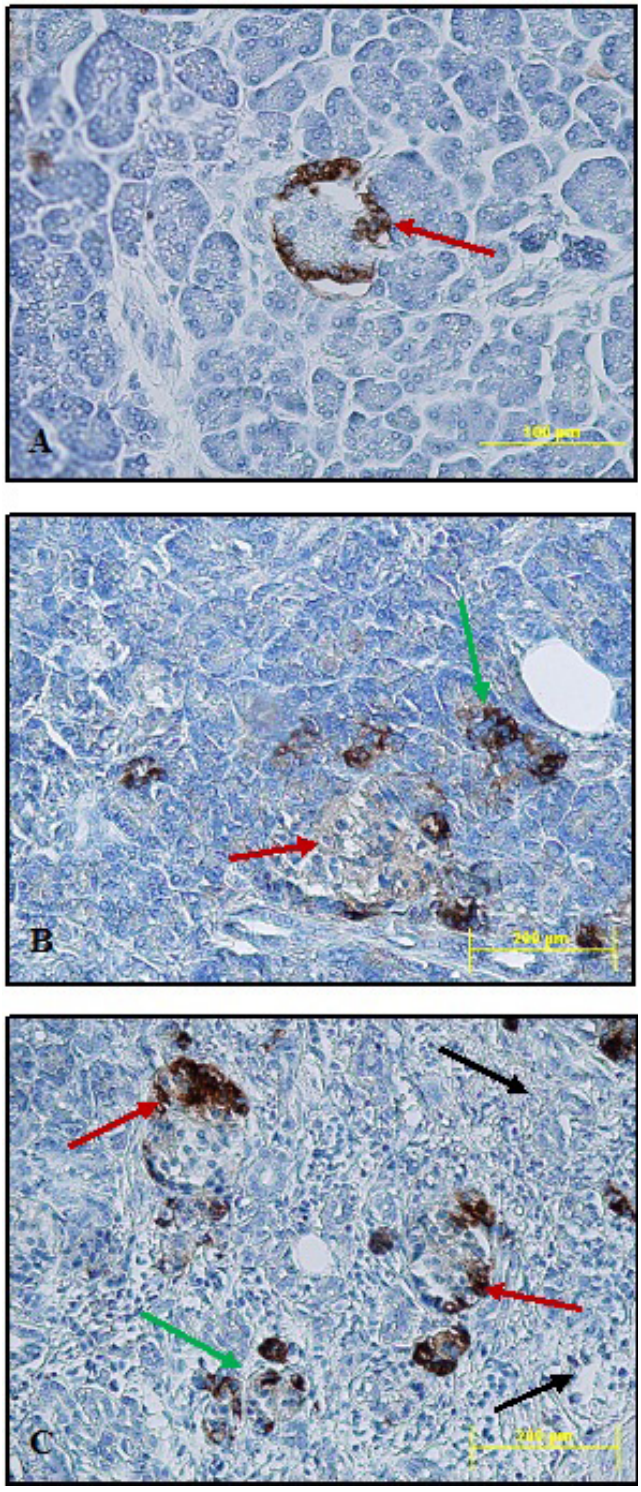


Fig. 2. Immunohistochemical localization of pancreatic polypeptide in the tissue of the pancreas

A – normal pancreas (a strong immunohistochemical reaction in a single cell of PP at the periphery islets of Langerhans); B – chronic pancreatitis (a weak immunohistochemical reaction for PP in the cells at the periphery islets of Langerhans, a moderate to strong immunohistochemical reaction for PP in cells between acinar cells); C – chronic pancreatitis with diabetes (a very strong immunohistochemical reaction in cells of PP in the whole area of islets of Langerhans, a strong immunohistochemical reaction PP in a single cell secreting pancreatic polypeptide in the part of the exocrine pancreas, reduction of stromal tissue in the process of fibrosis). Arrows: red – pancreatic islets; green – cells PP between acinar cells; black – fibrous lesions in the sublayer. Photograph – own study.

an association between serum PP levels and insulin sensitivity.[31] The exact mechanism of their action is currently under debate.

PP treatment led to increased insulin secretion from isolated human islets, most likely because it decreased the secretion of somatostatin, a known inhibitor of insulin secretion. Kim et al. present evidence for the functional significance of increased PP secretion within pancreatic islets. It decreases somatostatin secretion, which has an inhibitory effect on insulin secretion.[7] Elevated PP levels in type 2 diabetes are likely to represent a compensatory response in an effort to restore normal glycemic level by decreasing somatostatin secretion within islets.[32] Gut-islet interactions have been well studied because incretin hormones (GIP, GLP-1), released from scattered enteric endocrine cells, regulate glucose-mediated insulin secretion. Somatostatin inhibits the release of many gastrointestinal endocrine hormones but the levels of all these actually increase in the circulation after eating. Hence, the suppression of somatostatin postcranially in the gut is a vital necessity. Gut-islet interactions via hormones are bidirectional.[7] Additionally, somatostatin is known to inhibit pancreatic exocrine secretion, hepatic bile secretion and gallbladder emptying.[7,30,33] These 3 effects need to be disinhibited after eating. PP seems to be responsible for via local inhibition of somatostatin secretion.[7]

The immunohistochemical technique allows for the identification of morphological abnormalities in the Langerhans islet cells in the case of patients with diabetes. Regularly, PP cells are arranged at the periphery of islets. The percentage and the distribution of PP cells increases significantly during the course of diabetes.[26]

Researchers from the University of Prague conducted a study on the level of hormones secreted into the gastrointestinal tract in young adults with type 1 diabetes. The highlighted group of metabolically active hormones secreted by pancreatic islet cells comprised of: amylin, ghrelin, GLP-1, GIP, leptin, PYY polypeptide, insulin, and pancreatic polypeptide. Studies have shown a consistent correlation of the measured levels of concentration of these peptide hormones with glycemia, with control metabolic markers as well as anthropometric measurements of patients. Serum PP levels showed no statistically significant difference between the study and control groups. The value of PP was 139.00 ±96.38 pg/mL with the median 111.18 pg/mL.[31] No data concerning PP levels in patients with type 1 diabetes are available in the international literature; however, the presence of PP cell hyperplasia in young adults with type 1 diabetes has been documented. PP concentrations in the study group chosen by the researchers showed some upwards tendency, yet they are not statistically significant. The elevated PP level in patients with type 1 diabetes is associated with hyperglycemia. At the same time, PP increases hepatic insulin sensitivity and reduces hepatic glucose production.[31,34] The study showed the PP action in a positive relation with amylin and a negative correlation with insulin. The researchers suggest a positive role of PP as a drug medication for patients with type I diabetes.[31]

A group of scientists from the Michigan and Minnesota Universities conducted a study on the secretion of glucagon, catecholamines and PP in patients diagnosed with type 1 diabetes, after pancreatic transplantation. The stimulus which triggered PP release was the supply of insulin, which induced a state of hypoglycemia, and stimulation with arginine. The study group consisted of 38 patients with diabetes, after an organ transplant, as well as 54 patients with diabetes, without a transplant. The control group consisted of 26 non-diabetic healthy patients. No significant differences in PP secretion between the test groups and the control group were found. However, PP secretion in both groups of patients with diabetes was lower compared to the control group. The researchers suggest that similar PP secretion results among patients from the control group can be found only in patients with native pancreas. It is suggested that after a series of PP monitoring studies such patients may provide valuable information concerning pancreatic transplants in the future.[32] PP might thus become an important glucose metabolism marker.[34,35]

In type 2 diabetic subjects, PP cells secrete excess of PP and plasma PP levels are significantly elevated in the postprandial state, compared to non-diabetic subjects.[32] Loss of pancreatic parenchyma following the resection of this organ causes extreme disturbances in the glucose homeostasis, similar to those in pancreatic diabetes. This form of glucose intolerance is different from other forms of diabetes owing to more frequent episodes of iatrogenic hypoglycemia. The improvement of postoperative care and a possibility of an early disease diagnosis greatly extend the length and quality of life of patients after pancreatic resection. The pathophysiology of pancreatic diabetes is associated with the deficiency of pancreatic hormones and changes in response of the liver and other organs to lower than normal hormone levels. PP deficiency is associated with hyperglycemia resulting from an unrestrained hepatic glucose production. It is a characteristic symptom of diabetes caused by pancreatic resection. Influencing PP release may mitigate the difficulties with glucose concentrations. PP may probably also improve liver cell sensitivity to insulin, thus beneficially influencing the glycemic control system.[36]

## Pancreatic polypeptide as a tumor marker

The first case of a tumor secreting only PP (PP-oma) by Bordi was registered in 1978. The pancreatic tumor was accidentally discovered during surgery of a duodenal ulcer. Immunohistochemical studies of the tumor showed a very large amount of PP immunoreactive cells. The serum concentration in these patients was an important indicator that this patient had a 10 times higher concentration

of PP in the blood. In addition to the excessive secretion of the peptide, there were no clinical and laboratory signs of tumor development.[37] PP-oma gives some atypical symptoms, e.g., abdominal pain, itchy rash, weight loss and watery diarrhea.[38]

Pancreatic polypeptide secreting tumors are usually benign adenomas.[37,38] They can also transform into malignant tumors with metastatic potential to adjacent lymph nodes and the liver, which can be potentially fatal. However, most PP-omas are benign tumors with a prevalent location in the pancreatic head. The recommended treatment for these patients is surgical removal of the tumor. In some cases, a total pancreatectomy with lymphadenectomy is required. After this treatment the level of PP in the blood must be monitored due to the possible PP cell hyperplasia.[37,38]

Pancreatic polypeptide-producing cells in small amounts often occur in endocrine tumors of the pancreas. Most commonly they occur in glucagonomas, but also in ininsulinomas, gastrinomas, and VIP-omas. In these tumors, immunoreactive PP cells may be discretely scattered throughout the tissue or form focal aggregates.[37] In patients with endocrine tumors of the pancreas, the serum PP level is considerably increased compared to the corresponding age control group. This parameter can only be a confirmation of the diagnosis, since the correct concentration of PP does not exclude the presence of pancreatic tumors. Therefore, a preoperative measurement of the hormone level has little clinical value. On the other hand, PP level is a valid parameter for monitoring the course of the disease. The increase in the concentration of the basic PP after a resection may be the result of incomplete removal of the tumor cells. This may also be indicative of PP cell hyperplasia, but not of their cancer growth.[38]

In patients with multiple endocrine neoplasia type 1 (MEN1), there are 2 characteristic changes. The first is the microadenomatosis of islet cells of the pancreas, while the other, less frequent changes take the form of large single endocrine tumors. Numerous adenomas are scattered along the entire pancreatic gland. The number of microadenomas may exceed 100, and the diameter of a single adenoma can sometimes be greater than 0.5 cm. These changes are functionally asymptomatic and considered benign, both clinically and histologically. Large tumors are sporadic in MEN1 and occur most frequently as background microadenomatosis of islet cells. Most likely, *MEN1* gene inactivation (chromosome 11q13) is responsible for the development of pancreatic endocrine tumors secreting pancreatic polypeptide.[37,39]

Mutch et al. attempted to define the relationship between the level of fasting PP and the presence of radiographically detectable pancreatic endocrine tumors in patients with MEN1. The results indicated a greatly increased concentration of pancreatic polypeptide (PP levels above 3 times higher compared to the corresponding age control group). These values were 95% sensitive and 88% specific for islet cell tumors detected radiologically. The relationship, however, serves as a screening test because normal PP concentration does not exclude the presence of a tumor.[39] Standardization of the test, in which a stimulus that stimulates the secretion of PP is food, allows for the confirmation of pancreatic endocrine tumors in 75% of young patients with MEN1, in which the tumor is still asymptomatic.[37] In turn, the test in which the stimulus stimulates the secretion of pancreatic polypeptide secretin does not exhibit the diagnostic sensitivity. Elevated concentrations of PP in patients with MEN1 syndrome is clinically very important because it is almost always associated with the presence of pancreatic islet cell tumor, which can be surgically removed. After the removal of the tumor, the level of PP can be a good prognostic indicator for monitoring patients.[39] Excessive secretion of PP is also observed in endocrine tumors localized in the gastrointestinal tract and related organs. PP cell growth was also seen in tumors of the thyroid, lungs and ovaries.

However, non-pancreatic tumors secreting pancreatic polypeptide are rare. It was found that pancreatic polypeptide and related peptides with PP were found in 62% of cases of carcinoid rectum. Rectal carcinoid, secreting pancreatic polypeptide, behaves similarly to other peptide secreting tumors. Usually, they take the form of small polyps located in the submucosa, revealing a benign character. When the tumor diameter exceeds 2 cm, it tends toward malignancy. Therefore, it may metastasize to the lymph and blood, placing itself in the wall of the intestine, liver and lymph nodes.[37]

## Conclusions

Pancreatic polypeptide is a hormone secreted by PP cells, located at the periphery of the pancreatic Langerhans islets. The physiological roles of this hormone include what follows: stomach stimulation inhibition, delayed gastric emptying, pancreatic exocrine function inhibition, and inhibition of insulin secretion and hepatic glucose production. As part of its role in the functioning of the body, PP reduces the demand for energy, thereby decreasing the amount of food intake. This observation has led to the development of research concerning the treatment of obesity. It turned out that PP regulates the body energy balance. An intravenous or intraperitoneal PP administration decreased metabolic rate in obese mice. In addition, PP resulted in the animal body weight loss, hyperlipidemia and insulin resistance. The clinical significance of PP can also be observed in the course of acute and chronic pancreatitis. In acute pancreatitis, due to the rapid progression of pancreatic islet destruction, PP concentration is initially high, but then it decreases. The dynamics of changes in hormone concentrations may be an indication of the disease phase. However, in the course of CP, PP concentration is maintained

at a low level. It is proportional to the degree of islet damage and points to exocrine and endocrine secretion failure of the pancreas. Long lasting chronic pancreatitis may develop into insulin-dependent diabetes. PP cells located at the periphery of the Langerhans islets play a protective role with regard to the beta cells located in the islet center. Therefore, reduced PP levels may be the first signal of pancreatic endocrine cell failure. In addition, PP increases hepatic insulin sensitivity by reducing hepatic glucose production. It has been suggested that PP plays a role in the inflammation process of the pancreas; therefore, determining the PP concentration in the serum may be a useful diagnostic parameter of the diabetes development in the course of chronic pancreatitis.

Elevated concentrations of pancreatic polypeptide are often observed in patients with endocrine tumors of the pancreas. Almost always there is a high level of PP in patients with MEN1. However, in these conditions high levels of PP can only confirm the diagnosis because normal concentration of PP does not exclude the presence of a tumor. On the other hand, the level of PP can be a good parameter to monitor diagnostic patient after the removal of the tumor. The increase in the basic concentration of PP after the operation may be the result of incomplete removal of the tumor cells. It may also indicate a PP cell hyperplasia.

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# Brain death-associated pathological events and therapeutic options

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## Abstract

**Background.** At present, organ transplantation is the most efficient treatment of end-stage failure of various organs, including the heart, lungs, pancreas, intestines, kidney, and liver. Despite the efforts to use organs from living donors or from donors after circulatory death, most of the organs are recovered from brain dead (BD) donors.

**Methods.** The Medline and Web of Science databases were searched without time limit on November 2015 using the terms "brain dead donor" and "deceased donor" in conjunction with "transplantation", "graft", "organ", "hemodynamic", "hormonal", or "management". The search was limited to the English, Polish and Spanish literature. Articles that did not address the topics were excluded and the full text of the remaining articles was reviewed.

**Results.** It is well established that brain death is associated with a cascade of hemodynamic, inflammatory, and immunologic events that affect the outcome of transplanted organs. Proper management of the potential organ donor may help increase the supply of organs for transplantation. However, because there is a lack of good quality evidence, it is difficult to establish specific BD donor management guidelines.

**Conclusions.** In this paper we present a review of studies and literature concerning the detrimental impact of donor brain death on graft function. We present pathologic changes that take place after brain death, their influence on graft quality and therapeutic solutions to enhance transplanted organ function.

**Key words:** transplantation, brain dead donor, deceased donor, organ management

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## Introduction

At present, organ transplantation is the most efficient treatment of end-stage failure of various organs, including the heart, lungs, pancreas, intestines, kidney, and liver. Despite the efforts to use organs from living donors or from donors after circulatory death, most of the organs are recovered from brain dead (BD) donors. Persistent donor organ shortage causes extended waiting lists, and a substantial percentage of patients die while waiting for an organ. As a result, a move toward accepting suboptimal donors is increasing.

Despite improvements in organ preservation, reduction of cold ischemia time, better organ allocation and tailored recipient pharmacotherapy, the outcomes reached with organs from living donors are far superior when compared to grafts procured from deceased donors.[1]

## Methods

The Medline and Web of Science databases were searched without a time limit on November 2015 using the terms "brain dead donor" and "deceased donor" in conjunction with "transplantation", "graft", "organ", "hemodynamic", "hormonal", or "management". Boolean operators (NOT, AND, OR) were also used in succession to narrow and broaden the search. Autoalerts in Medline were also run, and reference lists of original articles, review articles, and book chapters were searched for further eligible articles. The search was limited to the English, Polish and Spanish literature. Articles that did not address the topics were excluded, and the full text of the remaining articles was reviewed.

In this paper we present a review of studies and literature concerning the detrimental impact of donor brain death on graft function. We present pathologic changes that take place after brain death, their influence on graft quality, and therapeutic solutions to enhance transplanted organ function.

## Discussion

The function of grafts from BD donor's graft function is affected by various factors. Characteristics of a donor, such as gender, age, race, serum creatinine before harvesting, history of comorbidities (e.g., hypertension, diabetes, HCV status), and the cause of death all impact organ function. Additionally, because of logistical reasons, cold ischemia time is usually longer in deceased donor transplantation. Deceased donors are frequently older than living donors; nevertheless, it has been shown that survival rates of living donor organs are better in every age category, compared to BD's organs.[2] What is more, average HLA matching is usually worse in living donation. This suggests that weak survival of grafts from BD donors cannot be solely caused by donor characteristics and differences in immunogenicity.

By definition, BD is the irreversible loss of brain and brain stem function. It is frequently caused by a cerebral hemorrhage, hypoxia, or/and metabolic dysregulation. According to the American Academy of Neurology 2010 guidelines, the BD definition is composed of the following criteria: unawareness of and unresponsiveness to external stimuli, no spontaneous movements or breathing and absence of brainstem reflexes. Attention is needed for states such as high cervical cord injury, drug intoxication, hypothermia, fulminant Guillain-Barré syndrome, and hypotension. In some cases, ancillary tests such as EEG, transcranial Duplex-ultrasound, digital subtraction cerebral angiography or nuclear cerebral blood flow scanning are required to confirm BD.[3]

As a result of BD, cascade of hemodynamic (hypotension, arrhythmias), hormonal (diabetes insipidus, hypoglycemia), pulmonary (pulmonary edema, ventilator-induced lung injury), metabolic, and inflammatory changes is unleashed. This all leads to impaired graft function and accelerated immunogenicity, and is associated with impaired patient as well as graft survival rates.

## Hemodynamic changes

After cerebral hemorrhage or injury, the increased intracranial pressure (ICP) causes damage to the cerebrum tissue, leading to a great stimulation of the parasympathetic system and to decreased systemic blood pressure. Progressive elevation of ICP results in herniation of the brain stem through the foramen magnum, which is related to arterial compression and occlusion with advancing brain ischemia. When the pons becomes ischemic, the sympathetic nervous system is activated. Simultaneous sympathetic and parasympathetic activation causes the Cushing reflex – the phenomenon composed of multiple disturbances in the physiology, such as bradycardia, hypertension, and an irregular breathing pattern.

After the vagal cardiomotor nucleus has become ischemic, parasympathetic stimulation decreases. Further, uncontrolled systematic and myocardial catecholamine release causes an increase in the heart rate and leads to vasoconstriction with increased vascular resistance and blood pressure. This so-called "catecholamine storm" is considered to be the ultimate effort to restore cerebral perfusion. During that process, serum catecholamine levels are 100–1000-fold higher compared to normal values, and the scale of catecholamine release is related to the severity of a brain injury. The catecholamine-induced vascular resistance can reach levels fourfold higher than normal values. This causes a significant drop in organ perfusion and leads to ischemic damage of potential grafts. An increase in pulmonary vascular resistance leads

to escalated right ventricular pressure and dysfunction. Differences in pulmonary and systemic vascular resistance and blood flow lead to pooling of blood in the lungs and neurogenic pulmonary edema.

Finally, due to ischemia of the spinal cord, sympathetic stimulation decreases, leading to a decline in blood pressure, heart rate, cardiac output, and finally, general hypoperfusion.[4]

## Hormonal changes

The hormonal changes after BD are generally related to the hypothalamus and to anterior and posterior pituitary gland failure. Following BD, the hypothalamus and pituitary ischemia, as well as the critical illness stress response, produce hormonal production alternations. Endocrine changes vary in timing and severity. It has been demonstrated in animal models with BD that circulating catecholamine levels increase during the first 15 min after BD. Afterwards, epinephrine and norepinephrine concentrations decline, but dopamine concentrations increase up to 90 min after BD. Adrenocorticotropic hormone (ACTH), cortisol and vasopressin levels decrease significantly by 45 min after BD.[5] Following the onset of BD, plasma levels of free 3,3',5-triiodo-L-thyronine (T3) gradually decrease, whereas the levels of thyroid stimulating hormone, free thyroxine, reverse T3, and cortisol are variable.[6,7]

## Treatment

Because of the growing demand for organs, there is an increasing necessity to optimize donation potential in BD patients. An optimized management of BD patients can increase the amount of harvested grafts.

The main goals include the maintenance of proper body temperature, adequate oxygenation, sufficient circulating volume, cardiovascular stability, and adequate urine output.

In the past, easily remembered series of goals was the "rule of 100" – systolic arterial pressure greater than 100 mm Hg, urine output greater than 100 mL/h, PaO2 greater than 100 mm Hg, and hemoglobin concentration of 100 g\L. Later, new blood sugar 100% normal goal was added.[8]

## Cardiovascular support and fluid management

### Fluids

The first step in managing a donor with vasoplegia and hypotension is to preserve an adequate intravascular volume. There is a lack of reliable evidence proving which type of fluid is better in the management of a BD donor. The choice of fluid and administration rate should result from previous therapy, incidence of diabetes insipidus polyuria, and considerations of the effects of excessive fluids on the respiratory system.

It was shown that restrictive fluid management can prevent fluid overload and lung neurogenic edema, and can increase the rate of lung grafts available for transplant supports. Restrictive fluid management may provide adequate perfusion to vital organ systems even with a CVP < 6 mm Hg, yet, according to actual Eurotransplant guidelines, CVP should be maintained between 6 and 10 mm Hg.[9]

In case of active bleeding, hemoglobin level below 9.6 g/dL or hematocrit level below 20%, blood product replacement should also be utilized.

Previously, it has been suggested that the use of crystalloids, in contrast to colloid solutions, may be deleterious to lung grafts because of fluid overload; however, the available randomized control trials (RCTs) do not support these findings.[10] One study suggested that hydroxyethylstarch (HES) impairs immediate renal function in kidney-transplant recipients. Another study did not find any differences between liver donor groups when HES was used in comparison with crystalloid solutions.[11,12] Nevertheless, these results may be related to the use of older HES formulations.[13]

### Cardiovascular support

After the BD event and following "catecholamine storm", there is a constant reduction in circulating catecholamines levels. Various guidelines support the administration of inotropes or vasopressors to stabilize potential deceased heart-beating donors. However, due to the absence of high quality evidence, the consensus on the specific agent or combination therapy is lacking. According to Eurotransplant guidelines, regardless of the exact treatment strategy, a mean arterial pressure greater than 90 mm Hg, urine output greater than 1 mL/kg/h and pulmonary capillary wedge pressure of 10–15 mm Hg ought to be sustained.

It has been previously shown that dopamine or norepinephrine BD donor treatment is an independent beneficial factor in renal transplant outcome.[14]

More recently, it has been proven in RCTs that donor treatment with dopamine causes a reduction in dialysis requirement after kidney transplantation. In another study, authors stated that dopamine treatment clearly improves renal histology. However, no clinically significant impact on graft or patient survival was noted.[15,16]

Furthermore, in a recent meta-analysis of septic shock patients, it was shown that dopamine administration is associated with greater mortality and a higher incidence of arrhythmic events compared to norepinephrine therapy.[17] On that basis it can be concluded that norepinephrine is safer when compared with dopamine.

However, in a small study by Stoica et al., authors stated that the use of high doses of norepinephrine in BD donors is associated with increased cardiac graft dysfunction and higher mortality in recipients.[18]

In other retrospective analysis of 936 patients, it was shown that neither norepinephrine nor dopamine pre-treatment of potential heart donors showed superior overall survival. What is more, in a sub-population of long-term survivors, norepinephrine pre-treatment was associated with better survival in a rather small cohort of heart transplant recipients.[19]

Nevertheless, high doses of catecholamines can reduce renal and hepatic perfusion. Additionally, it was suggested from animal studies that elongated catecholamine administration could lead to a reduction in the myocardial beta-adrenergic receptors expression density and may potentially influence heart graft function.[20] According to actual Eurotransplant guidelines, catecholamines use in the BD donor management should be avoided whenever possible. However, catecholamines may exert a beneficial effect when administered after organ recovery. It was demonstrated that during adult liver transplantation surgery, the administration of either 10 μg of epinephrine or 100 μg of phenylephrine at the reperfusion time is an efficient method for reducing the occurrence of postreperfusion syndrome and the need for vasoactive support.[21]

# Hormonal management

Hormonal substitution therapy is a subject of intense debate. It has been shown in a retrospective analysis of more than 10,000 consecutive donors that aggressive pharmacologic therapy results in more organs suitable for transplantation.[22] Nevertheless, other authors do not to confirm these observations.[23]

At present, despite low-quality evidence, various guidelines recommend hormonal substitution therapy that include the administration of thyroid hormones (T3 or T4), corticosteroids, vasopressin (ADH), and insulin.

## Thyroid hormones T3/T4

It has been suggested that diminished thyroid hormone concentration after BD causes hemodynamic instability, leading to a decline of myocardial energy stores and a change from aerobic to anaerobic metabolism.[24]

Experimental studies have demonstrated enhanced cardiac function after thyroid hormonal therapy in animal model. Various retrospective analyses state that thyroid hormonal administration may improve cardiac function and increase the number of organs transplanted per donor.[23] It was shown that T3 administration before the induction of BD decreases liver cell injury and apoptosis in animal model.[25] Recent retrospective analysis of 66,629 donors showed that T3/T4 therapy results in more transplantable organs, yet with no detriment to post-transplantation graft survival.[26]

Two meta-analyses of thyroid hormone effectiveness were published lately. The authors of both studies stated that the majority of available studies were of low quality and were heterogeneous in nature. The most interesting finding was that the all nonrandomized reports concluded that thyroid hormone therapy was beneficial. On the contrary, every randomized controlled studies stated that there was no clinical benefit, such as post-transplant function improvement, circulating troponin levels reduction or hemodynamic stability progress. Additionally, the analysis of the 4 placebo-controlled studies failed to identify any benefit of thyroid hormone on donor cardiac index or vasoactive drug requirements.[23,27,28]

## Corticosteroids

BD results in hypothalamic–pituitary–adrenal axis disruption. Although cortisol secretion was observed in some potential donors to be reduced, other authors state that cortisol levels after BD can be normal or high.

It is hypothesized that corticosteroids supplementation may increase hemodynamic stability, reduce inflammatory response and, therefore, improve clinical outcomes in brain dead donors.

In the recently published systematic review, authors shown no clear clinical benefit based on RTCs from the administration of corticosteroids to potential donors. On the contrary, observational studies have shown advantageous outcomes of the administration of corticosteroids on hemodynamic and oxygenation parameters. Moreover, most of observational studies found better outcomes in organ recovery when corticosteroids were used.[29] However, the majority of studies evaluated only methylprednisolone, and the quality of the included studies was poor, with high risk of bias identified in the majority.

Other new, multicenter, prospective study showed that hydrocortisone administration during the resuscitation of a brain-dead donor is associated with decreased vasopressor doses need to maintain a stable hemodynamic state. The authors state that, despite no observed benefits of the steroid administration on primary function recovery of transplanted grafts, the administration of glucocorticoids should be a part of the resuscitation management of deceased donors with hemodynamic instability.[30]

Concerns relate also to steroid doses. One recent study compared the use of low-dose hydrocortisone with high-dose MP. Authors demonstrated that lower-dose corticosteroid protocol did not worsen donor pulmonary or cardiac function, and insulin requirements and glycemic control were improved.[31]

## Vasopressin

Main vasopressin (ADH) physiological effect, i.e., water retention, is achieved by increased urine osmolarity and decreased water excretion. Diabetes insipidus (DI) (diuresis >5 mL/kg/BW/h, urine specific gravity <1005) caused by ADH deficit applies to 80% of BD donors. It is caused by the rapid depletion of ADH secretion because of pituary gland ischemic failure and results in intensified diuresis, followed by hypovolemia, hyperosmolarity, and hypernatremia. Additionally, the downregulation of aquaporin-2 channels caused by BD aggravates the decline of hemodynamic stability.[32]

To maintain adequate blood pressure, hormonal replacement therapy with vasopressin is advised.[33] Low-dose vasopressin therapy, when compared to fluid volume replacement, improves blood pressure, decreases inotrope requirements and preserves levels of myocardial high energy phosphates, and allows for the reduction of catecholamine supply.[4] Administration of only crystalloid solutions results in the exacerbation of neurogenic pulmonary edema and frustrates pulmonary procurement. In therapy, both vasopressin and desmopressin can be used. Intravenous, subcutaneous, intramuscular or intranasal administration of desmopresin results in V2 receptors activation. ADH infusion results in both the V1 and V2 receptors activation and, therefore, either vasoconstriction and water retention. Some authors suggest the use of terlipressin, a synthetic analog of vasopressin characterized by greater selectivity for the V1 receptor than vasopressin.[34]

In a recent meta-analysis, the authors demonstrated that the use of desmopressin is safe and useful to limit the harmful effects of profuse polyuria; however, it was not associated with better kidney graft outcomes.[23]

In other study, it was demonstrated that vasopressin compromised both the systemic and superior mesenteric artery blood flow. It was also associated with inadequate oxygen delivery. These adverse effects were not observed with dopamine.[35]

Nevertheless, it was shown that chronic V2-receptor stimulation was independently associated with a decrease in the calculated glomerular filtration rate over a median follow-up time of 3.6 years after kidney transplantation. However, it is suspected that chronic V2-receptor stimulation increases renal plasma flow and induces hyperfiltration, which in turn causes renal hypertrophy and proteinuria.[36]

## Insulin

Hyperglycemia and insulin resistance are common in potential BD donors. Additionally, hyperglycemia in often aggravated because of common steroid administration.

It was shown that higher average glucose values and greater variability in glucose concentrations were associated with worse post-transplant renal function.[37] Therefore, it is advised to aggressively treat hyperglycemia episodes targeting control levels between 120 and 180 mg/dL. Hyperglycemia management enhances the hemodynamic performance of myocardium, reduces myocardial injury, and diminishes inotrope requirements by optimizing substrate utilization, reducing toxic circulating free fatty acids, direct inotropic and anti-apoptotic effects of insulin, as well as the potential to improving the calcium handling and beta-adrenergic properties of the myocyte.[4] Intensive insulin therapy in intensive care unit (ICU) patients is also associated with the prevention of newly acquired kidney injury, accelerated weaning from mechanical ventilation and the reduction of the inflammatory response.[38]

# Mechanical ventilation

The best quality evidence in the management of BD organ donor refers to mechanical ventilation. Lungs in BD patients may be damaged because ventilator-induced injury, trauma, aspiration pneumonitis, and fat emboli. Formerly, high tidal volumes and low positive end-expiratory pressure (PEEP) were used in ICUs for lung recruitment. Recently, it has been suggested that lung injury from BD donors is similar to the injury seen in acute lung injury and acute respiratory distress syndrome**.** Therefore, modern acute lung injury ventilator strategy was adapted also in the case of BD patients. Protective ventilatory strategy (peak pressure <35 mm Hg, tidal volumes of 6–8 mL/kg, PEEP of 5–10 cm $H_2O$, apnea tests performed by using continuous positive airway pressure, closed circuit for airway suction) increased the number of eligible and harvested lungs compared to a conventional strategy. Moreover, avoiding high-inspired oxygen concentrations may decrease the risk of bronchiolitis obliterans syndrome in lung recipients.[39]

# Temperature

One of the consequences of BD is body temperature dysregulation.[40] Actual practice includes active warming to maintain a donor's body temperature higher than 35°C. In a 5-year retrospective analysis, it has been shown that hypothermia (temperature <36°C) was associated with a significant decline in the eligibility for organ donation. Patients suffering hypothermia were less likely to donate solid organs, and when they did, they donated fewer organs per donor. Furthermore, patients in hypothermia had higher fresh frozen plasma, vasopressin, and dopamine requirements.[41]

On the other hand, some authors hypothesize that rapid cooling with ice-cold fluid just before organ harvesting may slow down organ metabolism and oxygen consumption, improve tolerance to ischemia, and improve organ viability in some patients.[42]

In a recent prospective, randomized, controlled trial on 394 donors, it was shown that a noninvasive temperature management protocol aimed at achieving mild hypothermia (34–35°C) decreased the rate of delayed graft function. This effect was particularly visible in kidney recipients from the highest-risk donors.[43]

Table 1. Brain dead donors management goals

| Parameter | Values |
|---|---|
| Temperature | 34–36°C |
| Ventilation parameters | peak pressure <35 mm Hg<br>tidal volumes of 6–8 mL/kg<br>PEEP of 5–10 cm $H_2O$ |
| Hemoglobin and hematocrit levels | hemoglobin >9.6 g/dL<br>hematocrit >20% |
| Hemodynamic parameters | CVP: 6–10 mm Hg<br>mean arterial pressure >90 mm Hg<br>urine output >1 mL/kg/h<br>pulmonary capillary wedge pressure: 10–15 mm Hg |
| Blood glucose level | 120–180 mg/dL |

# New insights

## Erythropoietin/carbomylated erythropoietin

Erythropoietin (EPO) was shown to have neuroprotective activity after brain ischemia by diminishing brain cell apoptosis and necrosis, reducing brain edema, and decreasing the expression of several proinflammatory genes. What is more, EPO stimulates the proliferation of cardiomyocytes and decreases myocardial infarction size. It also helps to reduce systemic inflammation and to preserve endothelial integrity. In the kidney, EPO leads to increased heat shock protein expression, decreases apoptosis after ischemia/reperfusion, and decreases infiltration of polymorphonuclear cells. It was proven that kidney function decreases almost by 50% after brain death, but may be fully restored after treatment with EPO. However, the benefits of erythropoietin are accompanied by unwanted overstimulation of the bone marrow, inducing a prothrombotic state. Because of that, carbamylated erythropoietin (CEPO), a modified derivative of EPO that is free from erythropoietic properties, was synthesized. It was shown in animal model that CEPO can be used as renoprotective agent for clinical intervention during donor management and before retrieval, followed by ischemia/reperfusion injury.[44,45]

## Anti-thymocyte immunoglobulin

Anti-thymocyte immunoglobulin (ATG) is a purified fraction of IgG from the rabbit serum immunized against human thymocytes, and is administered for the prevention of post-transplant rejection.

It was shown in an animal model that ATG administration to potential BD donors may ameliorate renal injury and, therefore, improve graft function.

Animals treated with ATG showed a significant decrease in acute tubular necrosis score and creatinine values, a significant increase in IL-10 expression, and a significantly lower in situ expression of monocyte chemotactic protein 1.[46] In another study, authors found statistically significant attenuation of histological damage in lungs, as well as a tendency of attenuation in heart graft and small bowel when ATG was administered to donors.[47]

## Exendin-4

Exendin-4 (Ex-4), a glucagon-like peptide-1 (GLP1) analogue, holds anti-inflammatory and cytoprotective properties. It was demonstrated in a recent study that Ex-4 administration to brain dead rats reduces BD-induced liver damage. In their earlier study, authors also showed that donor treatment with Ex-4 increases viability and function of pancreatic islets after isolation.

The authors state that Ex-4 in the clinical practice may promote not only liver, but also pancreatic islet transplantation outcomes.[48,49]

## Vagus nerve stimulation

It is a well-known fact that the vagus nerve supplies parasympathetic fibers to many visceral organs. Additionally, it is involved in the cholinergic anti-inflammatory pathway, also known as inflammatory reflex. Activation of vagus nerve leads to acetylcholine release, which binds to nicotinic cholinergic receptor found on macrophages. It results in macrophage activation inhibition and decrease of TNF synthesis. The cholinergic anti-inflammatory reflex is considered putative therapeutic target. Experimental studies have shown the efficacy of cholinergic agonists in sepsis, septic shock, and ischemia reperfusion injury models.

The vagus nerve electrostimulation may be also a way to improve transplantation results in renal recipients. It was shown in animal model, that vagal stimulation of BD donors improved survival of the recipients, improved renal function in the recipients, and significantly reduced intimal arteritis, tubulitis and chronic tubulopathy in the grafts.[50,51]

# Gaseous modulators

## Nitric oxide

Supportive BD donor management such as ventilation or administration of vasoactive agents may have a small ability to preserve end-organ oxygen delivery, which is primarily a function of local tissue perfusion. Local perfusion

is in part self-regulated through NO bioactivity, and BD-induced interruption of endocrine NO bioactivity may result in the tissue ischemia and organ damage. It was shown lately that inhalation of BD swine with the S-nitrosylating agent ethyl nitrite (ENO) was an effective method to attenuate brain death-induced reductions in S-nitrosohemoglobin (SNO-Hb) concentrations, to maintain tissue oxygenation, and to reduce tissue injury, inflammation, and organ damage.[52] It was also demonstrated that NO inhalation may be helpful in the improvement of hypoxemia caused by neurogenic pulmonary edema (NPE) in BD organ donors.[53]

Another compound, tetrahydrobiopterin (H4B), is an essential co-factor for all NO synthases isoforms. It was observed in multiple studies in animal models that H4B BD treatment ameliorates ischemia-reperfusion injury (IRI) and microcirculation derangements and significantly improves recipient survival following various organs transplantations.[54]

## Hydrogen sulfide

Hydrogen sulfide ($H_2S$) is involved in various homeostatic functions, such as blood pressure control, electrolyte balance and apoptosis, and regulates various pathological mechanisms, including oxidative stress and inflammation. It is suggested that $H_2S$ has a protective effect against ischemia-reperfusion injury, and $H_2S$ therapy can present a promising approach in organ protection.[55]

It was shown in an animal model that during reperfusion, lungs pretreated with inhaled $H_2S$ exhibited better oxygenation, ventilation, lower pulmonary artery pressures, and lower reactive oxygen species levels.[56]

It was also shown that $H_2S$ treatment during cold storage of kidneys and livers effectively mitigates organ IRI improving allograft function and survival, and decreases allograft injury.[57,58]

## Carbon nanoxide

Carbon monoxide (CO) is endogenously synthesized in human cells in heme metabolic pathways. It is hypothesized that CO protects against cellular injury by relaxing the blood vessels, inhibiting platelet aggregation and derepressing fibrinolysis. What is more, CO reduces IRI and inflammation responses, inhibits endothelial and epithelial cells apoptosis, and diminishes smooth muscles, fibroblasts and T lymphocytes proliferation. There is a significant amount of preclinical data to support the hypothesis that CO donors, organs or recipients' treatment can prevent graft function deterioration.[59,60]

# Conclusions

Nowadays, the majority of organs for transplantation originate from BD donors. It is well established that brain death is associated with a cascade of hemodynamic, inflammatory, and immunologic events that affect the outcome of transplanted organs. Proper management of the potential organ donor may help increase the supply of organs for transplantation. We presented the current therapeutic approach and a number of new concepts, which in the future will reduce the gap between the demand and supply of organs.

However, because of a lack of good quality evidence, it is difficult to establish specific BD donor management guidelines. Moreover, well-designed research is needed to understand the mechanisms of injury and repair during massive cerebral injury and to identify optimal donor management strategies.

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