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Contents

Original papers

Reviews

Human cytomegalovirus promotes the activation of TGF-β1 in human umbilical vein endothelial cells by MMP-2 after endothelial mesenchymal transition

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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. Human cytomegalovirus (HCMV) infection is one of the risk factors of cardiovascular disease; the most important pathological change is the change of vascular endothelial cell (VEC) function, but its mechanism is still unclear. Transforming growth factor β1 (TGF-β1) is an important cytokine associated with fibrosis; it can induce the occurrence of endothelial mesenchymal transition (EndMT) in VECs, which means endothelial cells acquire the characteristics and phenotypes of mesenchymal cells and secrete molecules associated with the deposition and remodeling of the extracellular matrix. Many in vivo and in vitro studies have shown that HCMV infection promotes the secretion and activation of TGF-β1.

Objectives. This study aims to observe the changes of endothelial cells after HCMV infection and EndMT occurrence induced by TGF-β1 and to explore the possible mechanism of HCMV infection in the pathogenesis of cardiovascular disease.

Material and methods. Immunofluorescence staining, reverse transcription polymerase chain reaction (RT-PCR), enzyme-linked immunosorbent assay (ELISA), and immunoprecipitation methods were used in this study to analyze the changes in morphology and gene expression.

Results. We found that EndMT-related morphological and gene expression changes occurred in human umbilical vein endothelial cells (HUVECs) infected and uninfected with HCMV after treatment with TGF-β1. Human umbilical vein endothelial cells infected with HCMV, which are treated with TGF-β1, can activate the extracellular potential TGF-β1 by activating matrix metalloproteinase 2 (MMP-2).

Conclusions. Our findings provide a molecular basis for the association between HCMV infection, TGF-β1 and cardiovascular disease.

Key words: TGF-β1, cardiovascular disease, MMP-2, human cytomegalovirus (HCMV), endothelial mesenchymal transition

Cite as
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Introduction

Human cytomegalovirus (HCMV) belongs to the β-herpesvirus family and is a double-stranded DNA virus. Human cytomegalovirus infection is very common all over the world, and the virus will remain in the body for the rest of one's life once infected. It has been found that HCMV is associated with a variety of cardiovascular diseases such as atherosclerosis (AS), coronary heart disease and hypertension.[1–6] Human cytomegalovirus infection is also related to the dysfunction of vascular endothelial cells (VECs).[7–9] Antiviral drugs, including ganciclovir, can reduce the risk of a heart transplant-associated AS.[10] Human cytomegalovirus infection can also aggravate AS.[11–14] However, the mechanism of HCMV infection in cardiovascular disease is still unclear.

Vascular endothelial cells are an important regulatory factor that can participate in a variety of physiological and pathological processes of cardiovascular disease. Recent studies have shown that endothelial dysfunction and its associated cardiovascular diseases can be induced through a process known as endothelial mesenchymal transition (EndMT).[15] In the process of EndMT, endothelial cells (ECs) lose specific endothelial markers, such as vascular endothelial cadherin (VE-cadherin) and leukocyte differentiation antigen 31 (CD31), obtain the mesenchymal cell markers alpha smooth muscle actin (α-SMA), fibroblast specific protein 1 (keratin) and fibronectin, and acquire migratory, invasive and proliferative phenotypes. EndMT-derived fibroblasts are increased in advanced atherosclerotic plaques, and there is a clear association between EndMT and the progression of AS.[15–17] Endothelial mesenchymal transition is involved in the process of AS plaque formation and promotes the expression of cardiac fibroblasts and collagen. It is one of the major factors of myocardial fibrosis.[18] The occurrence of EndMT is regulated by many cytokines; transforming growth factor β1 (TGF-β1) is the key cytokine for the development of EndMT and an important cytokine in vascular pathophysiology.[19] Increased TGF-β1 can promote the occurrence of cardiovascular diseases, such as pulmonary hypertension (PAH) and AS, and blocking the expression of TGF-β1 can inhibit the PAH process and the formation of unstable AS plaques.[20]

Cytomegalovirus infection can increase the expression of TGF-β1, but TGF-β1 is always in an inactive latent state.[21] The activation factors of latent TGF-β1 mainly contain protease (plasmin), metalloproteinase (MMPs), thrombospondin (TSP-1), $\alpha_v\beta_6$, and $\alpha_v\beta_8$.[22] In the HCMV-infected placenta, HCMV induces TGF-β1 and collagen IV expression by $\alpha_v\beta_6$.[23] Thus, HCMV-infected VECs may be involved in the pathogenesis of AS by inducing the expression or activation of TGF-β1. In this study, we observed the changes of endothelial cells after HCMV infection and EndMT induced by TGF-β1, and we explored the possible mechanism of HCMV infection in the pathogenesis of cardiovascular disease.

Material and methods

Cells and virus

Human umbilical vein endothelial cells (HUVECs), human embryonic lung fibroblasts (HELF) and mink lung epithelium cells were purchased from ATCC (Manassas, USA). Human umbilical vein endothelial cells were cultured with human endothelial serum-free medium containing bFGF (20 ng/mL), EFG (10 ng/mL) and human plasma fibronectin (10 μg/mL) (Life Technologies, Carlsbad, USA) at 37°C. Human umbilical vein endothelial cells were randomly divided into the HCMV TR-infected group, uninfected group, HCMV-infected +raTGF-β1 treatment group, and raTGF-β1 treatment group. Human embryonic lung fibroblasts and mink lung epithelium cells were cultured with Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (Life Technologies) at 37°C. Mink lung epithelium cells were used to detect TGF-β1 activity.

The HCMV TR virus strain was cultured in HELF cells. The supernatant of the viral culture was centrifuged at 4°C, 16,000 × g for 2 h, and the virus was resuspended with human endothelial serum-free medium (SFM) and stored at −80°C. The virus was placed in a crosslinked chamber (Bio-Rad, Hercules, USA) and irradiated with 150 mJ UV rays to inactivate the virus. The titer of the virus was titrated in HELF cells by detection of early antigen fluorescent foci (DEAFF).

Reagents

Recombinant human active TGF-β1 (raTGF-β1), Quantikine human TGF-β1 enzyme-linked immunosorbent assay (ELISA) kit and TGF-β1 blocking antibodies were purchased from R&D Systems (Minneapolis, USA); Luciferase assay kit and β-galactosidase detection kit were purchased from Promega Corp. (Madison, USA); RNeasy kit was purchased from Qiagen (Valencia, USA); RT^2 First Strand Kit, SuperArray Human Extracellular Matrix PCR Array and human matrix metalloproteinase 2 (MMP-2) shRNA kit were purchased from SA Biosciences (Frederick, USA); GM6001, rabbit anti-MMP-2 antibody (AB19167), rabbit anti-MT3-MMP antibody (AB853) and mouse anti-αvβ6 blocking antibody (MAB2077Z) were purchased from Merck Millipore (Billerica, USA); anti-GFP antibody, AlexaFluor conjugated phalloidin and secondary antibodies, and SuperScript III kit were purchased from Invitrogen (Carlsbad, USA); Cytogam was purchased from CSL Behring (King of Prussia, USA); Nucleofector device and transfection kit V were purchased from Amaxa (Gaithersburg, USA); RT-PCR primers were purchased from Applied Biosystems (Waltham, USA); fibronectin (Hs.01549976_m1), MMP-9(Hs.00957562_m1), ADAMTS1 (Hs.00199608_m1), TGF-β1 (Hs.00932734_m1), collagen 5A1 (Hs.00609088_m1), MMP-2 (Hs.01548733_m1), thrombospondin-1 (Hs.00170236_m1), 18S RNA (part

#4333760-0904029), mouse anti-MMP-2, mouse anti-TIMP-2, and rabbit anti-MT1-MMP polyclonal antisera were purchased from Abcam (Cambridge, UK); mouse anti HCMV, IE1 and p52 monoclonal antibodies were provided by Dr. Gan (Anhui Medical University, Hefei, China).

Detection of virus titer

Human embryonic lung fibroblasts and HUVECs were inoculated in 6-well plates; HCMV was inoculated in the cells according to multiplicity of infection (MOI) = 1 and the cells were cultured at 37°C for 1 h. They were washed with phosphate-buffered saline (PBS) 3 times, and culture medium was added into them; the supernatant and cells were collected daily for 6 days and then stored in −80°C. The cells were resuspended with 0.5 mL of PBS, and the titer was detected using DEAFF after serial dilution. The titer of the supernatant was also detected using DEAFF after serial dilution.

Immunofluorescence staining

Human cytomegalovirus TR virus was inoculated in the HELF and HUVECs in 24-well plates on coverslips according to MOI = 1 with or without recombinant active TGF-β1 (raTGF-β1, 15 ng/mL) treatment for 6 days. The cells were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. They were incubated at 4°C overnight after appropriately diluted primary antibodies were added. Then, they were washed and incubated at room temperature for 1 h with AlexaFluor 488- or AlexaFluor 594-labeled secondary antibodies. Then, they were incubated at room temperature for 15 min with AlexaFluor 488-labeled phalloidin and 4′,6-diamidino-2-phenylindole (DAPI), and the slides were mounted after washing and observed under a fluorescence microscope (Olympus Fluoview BX51, Center Valley, USA).

Western blotting analysis

The cells were harvested and lysed with radioimmunoprecipitation assay (RIPA) lysis buffer (PBS containing 1% NP40, 0.1% SDS, 5 mM EDTA, 0.5% sodium deoxycholate, 1 mM sodium orthovanadate, and protease inhibitors) on ice for 30 min with shaking at 12,000 rpm/min. Total cellular protein was collected, and the concentration was determined with a BCA Kit. Equal amounts of protein (25 μg/well) were separated using 10% SDS-PAGE electrophoresis. Then, it was electrotransferred to the polyvinylidene fluoride (PVDF) membrane. After the transfer, the PVDF membrane was rinsed with TBS for 10–15 min, placed in TBS/T blocking buffer containing 5% (w/v) skimmed milk powder and shaken at room temperature for 2 h. It was incubated at 4°C overnight after adding primary antibodies at the appropriate dilution. Then, the membrane was washed with TBST 3 times (for 5 min each time). The membrane was incubated at 37°C for 1 h with HRP labeled secondary antibody (1:10,000), diluted with TBST containing 0.05% (w/v) skimmed milk powder. The gel was developed with ECL (Perkin-Elmer Inc., Waltham, USA) for 5 min. The protein bands were quantified using an Imagequant LAS4000 (GE Healthcare, Tokyo, Japan).

RNA extraction and RT-PCR

Total RNA was extracted from different groups using the RNeasy kit according to the manufacturer's manual. Their concentration and purity were detected with an Agilent 2100 Bioanalyzer (Agilent Technologies Inc., Santa Clara, USA). A total of 1 μg of RNA was subjected to reverse transcription using the RT^2 First Strand Kit. Reverse transcription PCR was performed using the FastStart Universal SYBR Green Master (Thermo Fisher Scientific, Waltham, USA). Real-time polymerase chain reaction (RT-PCR) primers were purchased from Applied Biosystems. Reaction parameters were 95°C for 45 s, 95°C for 5 s and 60°C for 30 s, with 40 cycles. The relative expression of genes was calculated using 18S RNA as control. The expression levels of TIMP-2, MT1-MMP and MT3-MMP microRNA (mRNA) in different treated HUVECs were detected using RT-PCR methods; they were performed using Platinum™ Taq Green Hot Start DNA Polymerase (Life Technologies) according to the instructions. The primers were as follows: TIMP-2 (F:5'-GAGCGAGAAGGAGGTGGATTCCGGG-3'; R: 5'-ATGTCAAGAAACTCCTGCTTCGGGGG-3'); MT1-MMP (F:5′-GGATACCCAATGCCCATTGGCCA-3′; R: 5′-CATTGGGCATCCAGAAGAGAGC-3′); MT3-MMP (F: 5′-ACAGTCTGCGGAACGGAGCAG-3′; R: 5′-GTCAATTGTGTTTCTGTCCAC-3′); β-actin (F:5′-TGAGGATGTCACGGTTCCAG-3′; R: 5′-GTCACCTTCACCGTTCCAGT-3′).

TGF-β1 content detection

Human umbilical vein endothelial cells were seeded in a 24-well plate (2 × 10^{4}cells/well) and 6-well plate (1 × 10^{5}cells/well), and EndMT in cells was induced after they were treated with raTGF-β1 for 48 h. Then, HCMV was inoculated in the cells according to MOI = 1, and HUVECs without EndMT or HCMV infection were used as the control. Human umbilical vein endothelial cells were infected according to MOI = 2, 4, 6, 8, and 10 to analyze HCMV infection dose; then, they were cultured at 37°C for 1 h and then washed 3 times, and the raTGF-β1 was removed. Then, they were cultured with fresh medium for 24 h, and the TGF-β1 content in the culture supernatant was detected. The activation of luciferase was determined according to reference.[24] The activated TGF-β1 and total TGF-β1 in the cell culture supernatant was quantified using the Quantikine human TGF-β1 ELISA kit and an Ebioscience ELISA kit according to the kit instructions, respectively.

ELISA assay

Transforming growth factor β1 neutralizing antibody (0.5 μg/mL, 1 μg/mL, 2 μg/mL, and 4 μg/mL) was added to HUVECs and they were treated with raTGF-β1 (final concentration 0.6 nM) for 48 h. Human cytomegalovirus TR virus was inoculated in the cells according to MOI = 1, and they were cultured at 37°C for 1 h. Then, the cells were washed 3 times and cultured with fresh medium for 24 h. The TGF-β1 content in the culture supernatant was detected using bioassay of the reporter gene in mink lung epithelial cells. GM6001 (0.5 nM), aprotinin (200 mg/mL), anti-thrombospondin-1 (25 ng/ml) and anti-$\alpha_v\beta_6$ (10 mg/mL) were added to them before raTGF-β1 treatment for 1 h to analyze the effect of different inhibitors on the activation of TGF-β1.

Immunoprecipitation with MMP-2 antibody

Human umbilical vein endothelial cells were infected with HCMV or treated with raTGF-β1 according to the above method. The cells were harvested using centrifugation and lysed with pre-cooled RIPA lysis buffer containing protease inhibitor. Matrix metalloproteinase 2 antibody was added to the cells, which were incubated at 4°C overnight. Then, the protein A-agarose was added and incubated with them, and they were lysed with pre-cooled RIPA lysis buffer. The cells were resuspended, SDS-PAGE loading buffer was added to them, and the resulting solution was boiled. Electrophoresis was carried out with 8% SDS-PAGE gel. TIMP-2, MT1-MMP and MT3-MMP were detected in the cell lysate before and after immunoprecipitation using the western blot method.

MMP-2 shRNA transfection

Matrix metalloproteinase 2 shRNA and its control plasmids were transfected into HUVECs using the Amaxa Cell Line Nucleofector Kit. The cells were treated with raTGF-β1 when a clear green fluorescence was observed in the cells under fluorescence microscopy, and HCMV was inoculated according to MOI = 1. The supernatant and cells were collected with centrifugation. The TGF-β1 content in the supernatant was detected using a bioassay of the reporter gene in mink lung epithelial cells. Matrix metalloproteinase 2, GFP and β-actin expression levels in the precipitate were detected using western blot and RT-PCR methods.

Statistical analysis

The data was expressed as the mean values ± standard error of the mean (SEM). All analyses were conducted using Prism v. 5.0 software (GraphPad, San Diego, USA). To compare 2 groups, we used the Student's t-test. To compare more than 2 groups, we used one-way analysis of variance (ANOVA) or two-way ANOVA. P-values <0.05 were considered an indicator of a significant difference.

Results

HCMV can proliferate in HUVECs but is not affected by raTGF-β1

A logarithmic increase in the number of progeny viruses was observed in the supernatants and HELF cells, which indicated that HELF supports the proliferative infection HCMV TR (Fig. 1A). Human umbilical vein endothelial cells also support the proliferative infection of HCMV TR, but the proliferation curve is linear; the progeny virus proliferation was detected only in cells (Fig. 1B). The proliferation curves of HCMV in raTGF-β1 treated and untreated HUVECs were not different (Fig. 1C), which was different from the results of HELF cells. Indirect immunofluorescence results showed that HCMV p52 could be expressed in the nucleus of HUVECs, and the distribution was similar to that in HELF (Fig. 1D). Human cytomegalovirus p52 is a coenzyme of DNA polymerase, and p52 expression indicates that HCMV enters proliferative infection.

HCMV can infect HUVECs with EndMT induced by TGF-β1

Indirect immunofluorescence results showed that the cytoskeletal structure of HUVECs infected with HCMV was oval, which was the same as that of the control group. However, the cytoskeleton changed obviously after they were treated with raTGF-β1, and the elongated mesenchymal phenotype appeared to form a parallel actin stress fiber, while the oval endothelial morphology disappeared. These results showed that HCMV infection can cause EndMT-like morphological changes in HUVECs in the presence of raTGF-β1 (Fig. 2A).

Western blot results showed that VE-cadherin was expressed in the HCMV-infected group and the control group, but α-SMA was not expressed in these groups, which is the phenotype of endothelial cells. However, α-SMA was expressed in the raTGF-β1 treatment group and the HCMV and raTGF-β1 co-treatment group, while VE-cadherin was not expressed in these groups, which matches the phenotype of mesenchymal cells. In all raTGF-β1 treatment groups, p-SMAD2 could be detected, but p-SMAD2 was not expressed in the HCMV infection alone group. This suggested that TGF-β1 induced EndMT in HCMV-infected HUVECs also through SMAD2 signaling pathways. Human cytomegalovirus infection could induce the expression of α-SMA and p-SMAD2 without VE-cadherin expression in the presence of raTGF-β1; these changes were

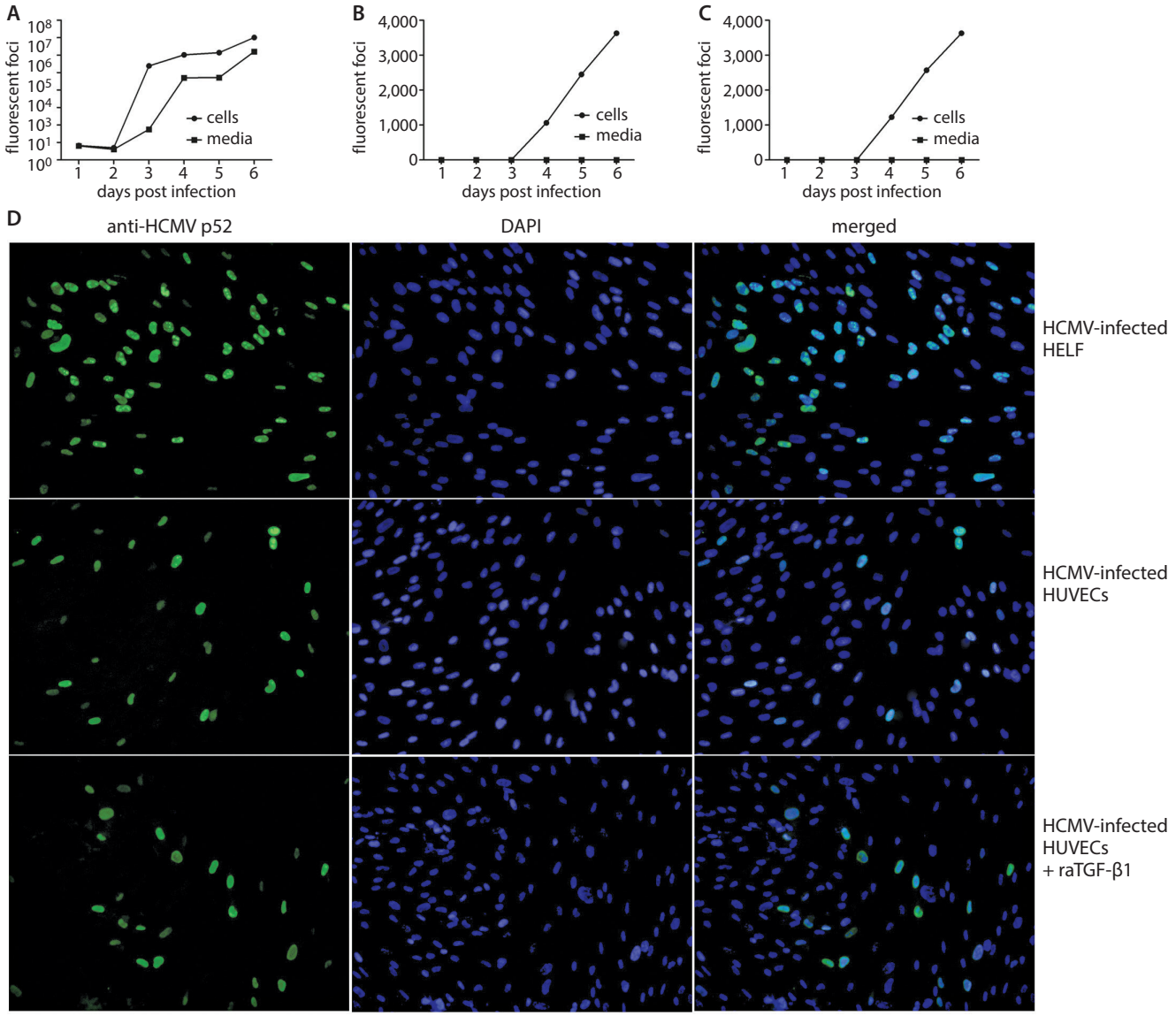

Fig. 1. The proliferation of HCMV in HUVECs

A – the titer of HCMV in HCMV-infected HELF cells and their culture supernatant; B – the titer of HCMV in HCMV-infected HUVECs and their culture supernatant; C – the titer of HCMV in raTGF-β1-treated and HCMV-infected HUVECs and their culture supernatant; D – indirect immunofluorescence results of HCMV p52 expression in HELF cells, HUVECs and raTGF-β1-treated HUVECs.

consistent with the phenotypic changes of HUVECs with EndMT (Fig. 2B).

Reverse transcription PCR results showed that the mRNA expression changes of fibrosis matrix protein in HCMV-infected HUVECs were less than 10 times compared with those of the control group, which suggested that there were no significant changes in cell phenotypes; this was consistent with morphological observations. In contrast, the mRNA expression levels of fibronectin, MMP-9 and α_v integrin were significantly increased in the raTGF-β1 treatment groups. The expression level of TIMP-2 in the HCMV-infected group was higher than that in the raTGF-β1 treatment group and the HCMV and raTGF-β1 co-treatment group. The expression levels of ADAMTS1, TGF-β1, β-catenin, collagens, MMPs, TIMP-1, and thrombospondins in the HCMV and raTGF-β1 co-treatment group were higher than that of the HCMV-infected group and the raTGF-β1 treatment group. These results suggested that HCMV infection upregulates the expression of many fibrosis molecules in HUVECs with EndMT induced by raTGF-β1 (Fig. 2C).

HCMV could induce the activation of TGF-β1 in HUVECs with EndMT

The luciferase reporter gene assay and ELISA assay showed similar results. The luciferase reporter gene assay was more sensitive. The results showed that only a small amount of activated TGF-β1 could be detected in the culture supernatant of HCMV TR–/raTGF-β1– HUVECs and HCMV TR+/raTGF-β1– HUVECs, which suggested that HCMV infection did not induce the upregulation

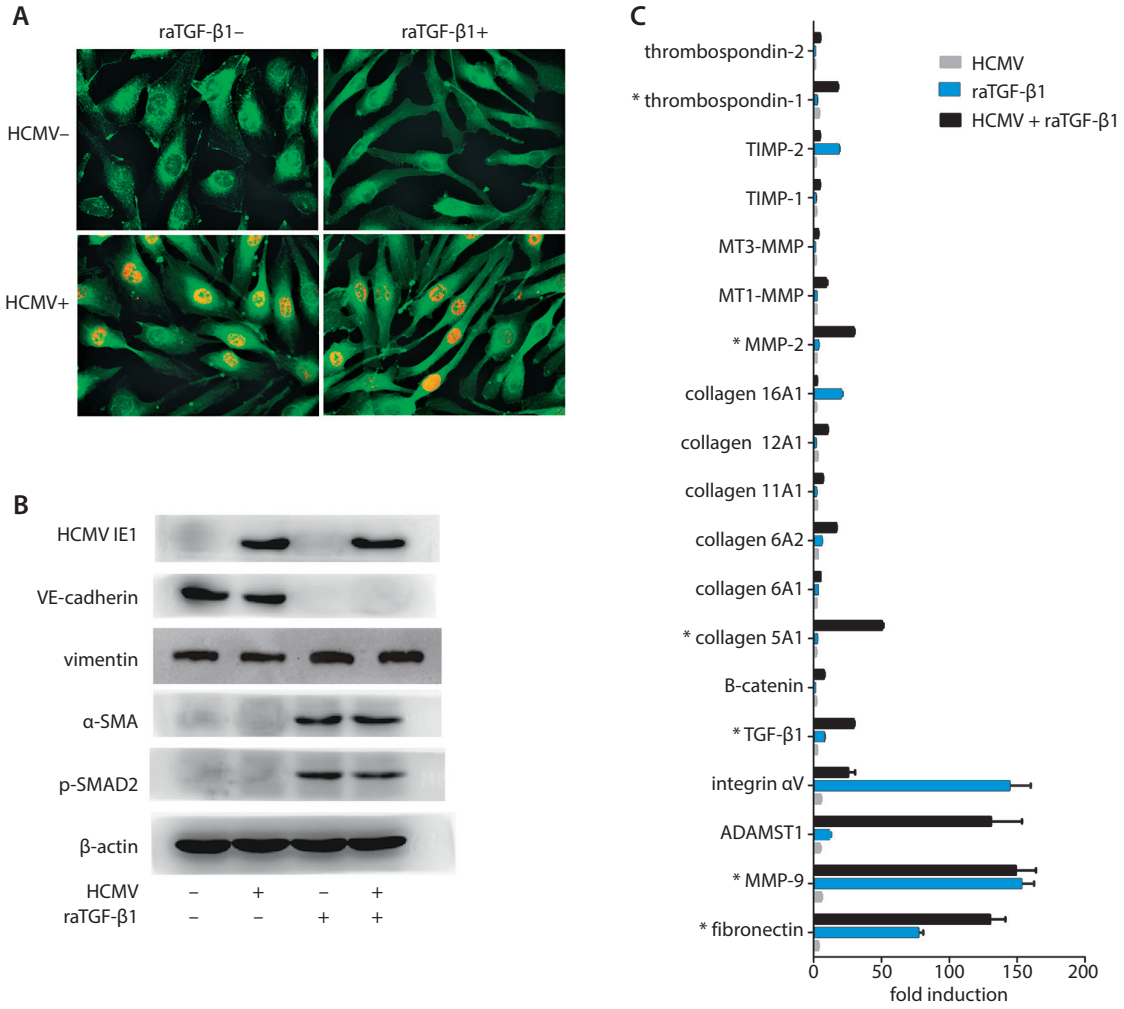

Fig. 2. HCMV could infect HUVECs with EndMT induced by TGF-β1

A – indirect immunofluorescent staining results; B – western blotting results of HCMV IE1, VE-cadherin, vimentin, and α-SMA; C – RT-PCR results of extracellular matrix-related genes expression

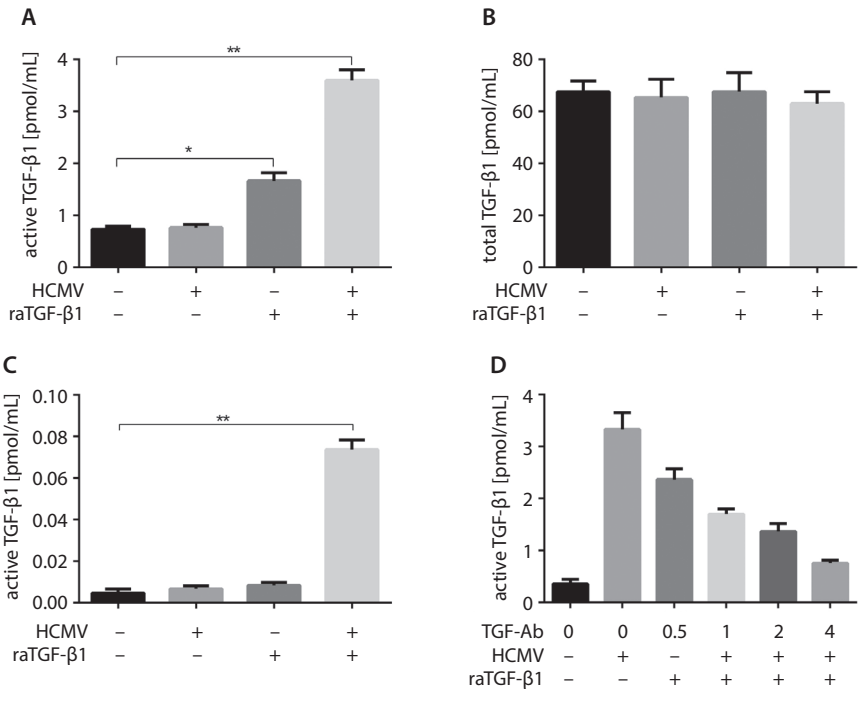

Fig. 3. HCMV could induce the activation of TGF-β1 in HUVECs with EndMT

A – the content of activated TGF-β1 in supernatant of cell culture detected using luciferase reporter gene assay; B – the content of total TGF-β1 in supernatant of cell culture detected using Quantikine human TGF-β1 ELISA kit; C – the content of activated TGF-β1 in supernatant of cell culture detected using ELISA assay; D – TGF-β1 antibody can inhibit the activation of TGF-β1

of TGF-β1 activation in HUVECs (Fig. 3A,C). The content of activated TGF-β1 in the culture supernatant was similar in HCMV TR–/raTGF-β1+, HCMV TR–/raTGF-β1– and HCMV TR+/raTGF-β1– HUVECs. These results suggested that activated TGF-β1 could not be upregulated in HUVECs with EndMT induced by exogenous raTGF-β1 (Fig. 3A,C). The content of activated TGF-β1 in culture supernatant significantly increased in HCMV TR+/raTGF-β1+ HUVECs. These results showed that only HCMV infection or raTGF-β1 treatment could not induce new activation of TGF-β1 in HUVECs; the new activated TGF-β1 could be produced when HUVECs with EndMT were infected with HCMV (Fig. 3B). The content of activated TGF-β1 gradually decreased in the supernatant of cell culture with the increase of blocking antibodies (Fig. 3D).

The amount of newly synthesized activated TGF-β1 was positively correlated with the infective dose of TGF-β1 and HCMV

As shown in Fig. 4A, the amount of activated TGF-β1 in the supernatant increased with the increase of the raTGF-β1 amount, but there was no significant change in total TGF-β1 among different groups, which suggested that HCMV-infected HUVECs with EndMT could stimulate the cells to secrete activated TGF-β1 in an autocrine manner. Figure 4B shows that the content of activated TGF-β1 in the supernatant of cell culture increased with the increase of HCMV MOI. Ultraviolet inactivated HCMV did not upregulate the amount of activated TGF-β1 (Fig. 4C). These results suggested that the binding of HCMV and HUVECs with EndMT did not result in the production of activated TGF-β1, and replication and proliferation of viruses are required in cells.

MMP-2 is involved in the upregulation and activation of TGF-β1 in VECs with EndMT induced by HCMV infection

We found that the activated TGF-β1 decreased by 60% when the HUVECs were treated with GM6001 ($p < 0.01$), and the activated TGF-β1 decreased by 9% when the HUVECs were treated with aprotinin ($p > 0.05$). Other blocking agents had no effect on the production of activated TGF-β1. These results suggested that MMP and serine proteases may be involved in the upregulation of activated TGF-β1 in HUVECs with EndMT infected by HCMV (Fig. 5A). Immunoprecipitation results showed that MMP-2 was bound with TIMP-2 and MT3-MMP, but not with MT1-MMP. These results suggested that HCMV infection may activate MMP-2. Matrix metalloproteinase 2 formed a membrane associated complex with TIMP-2 and MT3-MMP in HUVECs with EndMT, which promoted the formation and secretion of TGF-β1 (Fig. 5C,E). Reverse transcription PCR and western blotting results showed that the mRNA and protein expression of MMP-2 decreased significantly after short hairpin RNA (shRNA) transfection (Fig. 5B,D), and the amount of activated TGF-β1 in the cell supernatant was also significantly reduced (Fig. 5F). These results indicated that HCMV infection induced the activation of TGF-β1 by activating MMP-2 in HUVECs with EndMT cells.

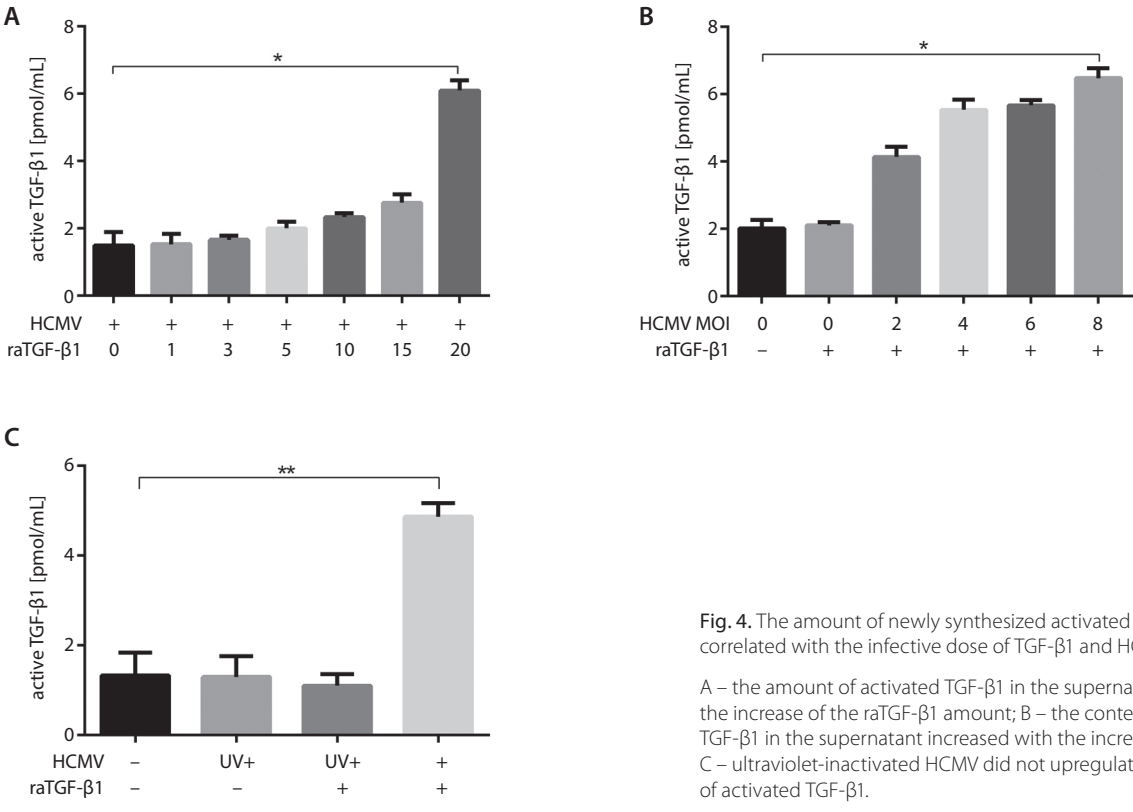

Fig. 4. The amount of newly synthesized activated TGF-β1 was positively correlated with the infective dose of TGF-β1 and HCMV

A – the amount of activated TGF-β1 in the supernatant increased with the increase of the raTGF-β1 amount; B – the content of activated TGF-β1 in the supernatant increased with the increase of HCMV MOI; C – ultraviolet-inactivated HCMV did not upregulate the amount of activated TGF-β1.

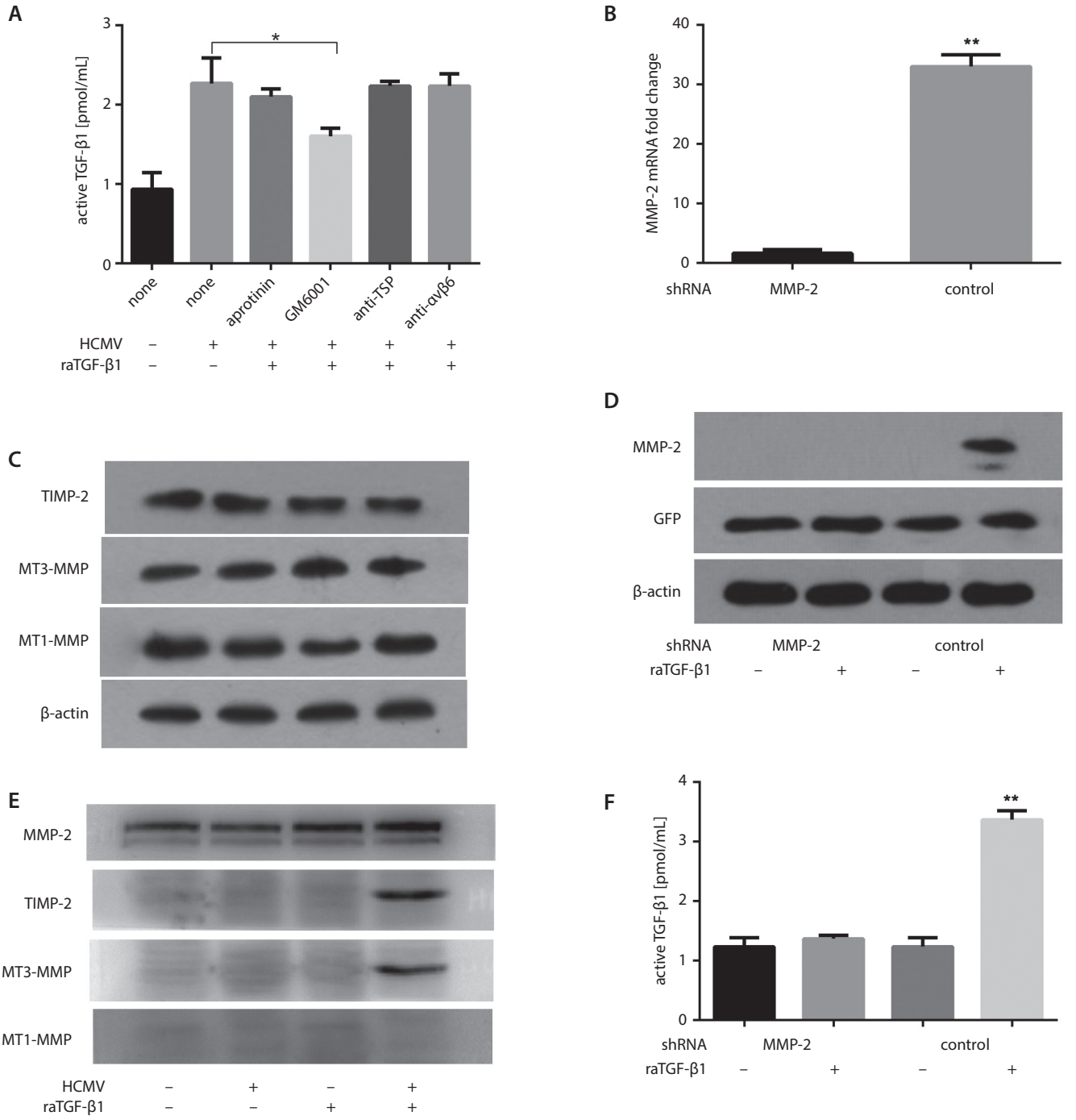

Fig. 5. MMP-2 is involved in the upregulation and activation of TGF-β1 in HUVECs with EndMT induced by HCMV infection

A – the effect of different blocking agents on the production of activated TGF-β1, GM6001 could significantly inhibit the activation of TGF-β1; B – RT-PCR results showed that MMP-2 mRNA expression was downregulated after shRNA transfection; C – western blotting and RT-PCR results of TIMP-2, MT3-MMP and MT1-MMP proteins; D – western blotting results of MMP-2 after shRNA transfection; E – immunoprecipitation results showed that MMP-2, TIMP-2 and MT3-MMP could form complexes; F – transfection of HUVECs with shRNA vector inhibited the activation of TGF-β1 induced by HCMV.

Discussion

Endothelial mesenchymal transition participates in many physiological and pathological processes of the cardiovascular system, and endothelial cells are involved in the formation of cardiac valves through EndMT in heart development.[25] Endothelial mesenchymal transition is also involved in the formation of embryonic vascular systems.[26] It is shown that EndMT is related to myocardial fibrosis; the production and accumulation of collagen and cardiac fibroblasts are related to the process of EndMT in patients with myocardial fibrosis.[27] Endothelial mesenchymal transition can promote perivascular fibrosis in diabetic mice.[28] Chen et al. found that TGF-β-induced EndMT could promote plaque

growth and increase plaque thickness by increasing the deposition of extracellular matrix and enhancing the expression of adhesion molecules such as VCAM-1 and ICAM-1 in endothelial cells.[18] Pulmonary arterial hypertension (PHA) is a chronic disease caused by accumulation of fibroblasts and the thickening or narrowing of the arterial wall.[29] Recent studies have demonstrated the presence of EndMT in the pulmonary arteries of hypertension, and the accumulation of transformed cells produced by EndMT increases blood pressure and results in cardiac dysfunction and heart failure.[30] Studies have confirmed that TGF-β1 is involved in the pathogenesis of myocardial fibrosis, AS and PHA. Human cytomegalovirus infection may promote the expression of TGF-β1 or increase its activity, and it may also be associated with cardiovascular disease.

In this study, we found that HCMV could infect HUVECs. Human cytomegalovirus infection did not affect the expression level of TGF-β1 in HUVECs; however, the autocrine of activated TGF-β1 in HUVECs could be enhanced by MMP cascade reaction and promote the EndMT of HUVECs. These could promote the development of cardiovascular disease. The autocrine phenomenon helps us understand why elevated levels of TGF-β1 are observed in patients with cardiovascular disease or animal models after infection with HCMV; it also helps promote the pathological process of fibrosis in vivo.[19] It was shown that MMPs were involved in fibrosis-related cardiovascular diseases by degrading the basement membrane, enhancing cell motility, activating cell growth factors, and regulating cell adhesion molecules.[31] It is believed that the regulation of MMP activity will become a new potential therapeutic method for cardiovascular disease. However, the effect of MMPs on cardiovascular disease with HCMV infection has not been further studied. In this study, we found that the trimer formed by MMP-2, MT3-MMP and TIMP-2 can promote the activation of TGF-β1 in HUVECs infected by HCMV, and this may be the mechanism of TGF-β1 activation induced by HCMV infection in VECs.

Epidermal growth factor (EGF) can enhance the migration phenotype of HK-2 cells induced by TGF-β1, and synergistically increase the expression of MMP-9.[32] Interestingly, we found that HCMV infection caused fibrosis in HUVECs after EndMT; HUVECs with endothelial phenotype infected by HCMV could not significantly increase the expression of TGF-β1. This suggests that in normal circumstances, HCMV infection of VECs does not cause the appearance of fibroblasts, which is also consistent with the presence of no cardiovascular changes in asymptomatic patients with HCMV infection.

The effect of HCMV on the extracellular matrix and fibrosis has been verified in heart and kidney transplantation models of infected cytomegalovirus rats.[33,34] Cytomegalovirus infection can upregulate the expression of a large number of molecules associated with fibrosis and angiogenesis, and HCMV infection could also activate TGF-β1 by the integrin mediated pathway. These mechanisms suggest that HCMV infection of the placenta can alter the extracellular matrix, allow HCMV transplacental translocation, and promote congenital infection. Our results support the possibility that HCMV infection may alter the extracellular matrix in inflammatory conditions. Therefore, under the condition of decreased immune status with local inflammation and EMT caused by TGF-β1 in the host cells, latent infection of HCMV may be involved in the progression of vascular fibrosis by reactivation. These conditions may exist simultaneously in cardiac transplantation or cardiac bypass surgery, but HCMV infection is not necessary for fibrosis in cardiovascular disease, because the presence of TGF-β1 can cause related diseases.

Conclusions

In summary, this study showed that HCMV could infect HUVECs with EndMT induced by TGF-β1. Human cytomegalovirus infection could enhance the autocrine release of TGF-β1 in VECs by an MMP cascade reaction. These findings suggested that HCMV-infected arterial endothelial cells may contribute to fibrosis by activating TGF-β1, which is involved in cardiovascular disease.

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CHA_2DS_2-VASc score and fibrinogen concentration in patients with atrial fibrillation

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Abstract

Background. Assessment of thromboembolic risk is crucial in choosing appropriate treatment in atrial fibrillation (AF). Current guidelines recommend basing the decision on the CHA_2DS_2-VASc score. However, the score is based only on clinical parameters and therefore its relationship with laboratory-assessed coagulation status might not always be objective.

Objectives. The aim of this study was to assess if the CHA_2DS_2-VASc score is associated with blood parameters in AF patients.

Material and methods. Patients with continuous AF prequalified for catheter ablation were enrolled into the study and had CHA_2DS_2-VASc calculated and blood taken for coagulation parameters.

Results. The study population comprised of 266 patients (65.0% males; age 57.6 ±10.1 years). Patients were divided into those with CHA_2DS_2-VASc score 0, and those with ≥1 points, respectively requiring and not requiring anticoagulation treatment. The group with CHA_2DS_2-VASc = 0 (12% of patients) compared to those with CHA_2DS_2-VASc ≥ 1 had a significantly lower fibrinogen concentration (285.6 ±82.0 vs 322.6 ±76.4 mg/dL; $p = 0.02$). Partial thromboplastin time was not significantly different between groups ($p > 0.05$). Differences were noticed in parameters concerning red blood cells. Lower risk patients had a lower red blood cell count (4.9 ±0.4 vs 5.1 ±6.0 10^6/μL); $p = 0.03$), higher hemoglobin concentration (14.9 ±1.0 vs 14.3 ±1.4 g/dL; $p = 0.04$), and higher hematocrit (43.5 ±2.6 vs 41.7 ±4.7%; $p = 0.001$). It was observed that along with the increase in CHA_2DS_2-VASc score mean fibrinogen concentration increased (p-value for trend = 0.04).

Conclusions. In summary, a higher CHA_2DS_2-VASc score is independently associated with an increase in fibrinogen concentration. Further research is needed to assess the value of fibrinogen in thromboembolic risk assessment.

Key words: fibrinogen, atrial fibrillation, thromboembolic risk, CHA_2DS_2-VASc score

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Introduction

Atrial fibrillation (AF) is one of the most common types of cardiac arrhythmia. The current estimate of the prevalence of AF in the developed world is approx. 1.5–2% of the general population, but the part of the population affected by AF is steadily increasing.[1,2] For people 40 years of age and older, a lifetime risk for developing of AF is approx. 25%.[3] The presence of arrhythmia is associated with an increased long-term risk of heart failure, pulmonary embolism and stroke, and all-cause mortality.[1,4,5] It is estimated that approx. 1/5 of all strokes are attributable to AF; further, the risk of pulmonary embolism is assessed to be 80% higher in those with AF compared with those without the arrhythmia.[5,6] It explains why the management of AF focuses on preventing thromboembolism, regarding equally relevant to managing heart rate/rhythm.[7]

Current guidelines recommend estimating thromboembolic risk individually for every patient and planning the anticoagulant treatment according to the risk.[1] Both current recommended prognostic scores $CHADS_2$ and CHA_2DS_2-VASc are based on basic and easy to obtain clinical data, including the presence of congestive heart failure, hypertension, diabetes mellitus or vascular disease, age, sex, and history of stroke. Point values obtained in the scores inform us about approximately how high the annual stroke risk is and, in consequence, about indications for anticoagulant treatment.[8,9] However, current scores do not include laboratory parameters, while biomarkers of inflammation, coagulation or myocardial injury may help refine the risk estimated by the scores.

Proposed mechanisms linking inflammation and the pro-thrombotic state in AF include endothelial activation, increased platelet activation and increased expression of fibrinogen.[10] Increased levels of plasma fibrinogen are associated with an increased risk of ischemic heart disease and stroke, and may promote disease by increasing fibrin formation and platelet aggregation.[11]

The aim of this study was to assess if the CHA_2DS_2-VASc score currently used for thromboembolic risk assessment is associated with laboratory parameters in AF patients.

Material and methods

Study population

The study was designed and performed in accordance with the Declaration of Helsinki, and the study protocol was approved by the University Ethics Committee. Continuous patients with confirmed diagnosis of AF, prequalified for catheter ablation by means of pulmonary vein isolation (PVI), were prospectively enrolled into the study between 2011 and 2013. Written informed consent for study participation was obtained from every enrolled patient. Lack of consent prior to the enrollment or later resulted in exclusion from the study. Also, patients with myocardial infarction or decompensation of heart failure within 6 months prior to study entry and with estimated life expectancy less than 6 months were excluded from the cohort. The criteria for inclusion were as follows: age 18–75 years, persistent AF defined in accordance with the definitions of the European Society of Cardiology,[1] and qualification for ablation of AF made prior to the study initiation. After applying the criteria, the study included 266 patients.

Diagnosis of atrial fibrillation and qualification for ablation

Diagnosis of AF was based on the European Society of Cardiology Guidelines.[1,12] Diagnosis of arrhythmia was confirmed when 12-lead ECG or 24-hour ECG Holter monitoring documented at least 1 episode of AF defined as 30 s or more of irregular ventricular response with fibrillation wave and without P-waves. Every case was verified individually by 2 expert cardiologists. Figure 1 shows samples of ECG tracing of patients' AF. All patients included in the study were qualified for AF ablation prior to enrollment in the study by qualified specialist according to ECG guidelines criteria.[1,12] Briefly, qualified patients had symptomatic AF and symptomatic recurrences of AF on antiarrhythmic drug therapy, or ablation was considered as an alternative to antiarrhythmic drug therapy, considering patient choice, benefit and risk ratio.

Assessment of thromboembolic risk

According to the current scoring guidelines, all patients were assessed with $CHADS_2$ and CHA_2DS_2-VASc scores.[1] In the $CHADS_2$ score, 1 point was assigned for the history of congestive heart failure, arterial hypertension, age ≥75 years, and diabetes mellitus, while 2 points for the history of stroke or transient ischemic attack (TIA). In the CHA_2DS_2-VASc score, 1 point was assigned for each of the following: the history of congestive heart failure, arterial hypertension, diabetes mellitus, vascular disease, age between 65 and 74 years and female sex, whereas 2 points were given for age ≥75 years and history of the stroke or TIA or thromboembolism. All patients were interviewed for the presence of abovementioned factors, history and conditions or taking drugs applicable or de novo diagnosis.

Biochemical measurements

From each patient, a 10 mL blood sample for coagulation parameters and blood morphology assessment was drawn in vacuum tubes with sodium citrate after 12 h of fasting. The samples were immediately centrifuged for 20 min at 2,000 g. The plasma was aliquoted and stored at –70°C until analyzed. Plasma fibrinogen levels were measured

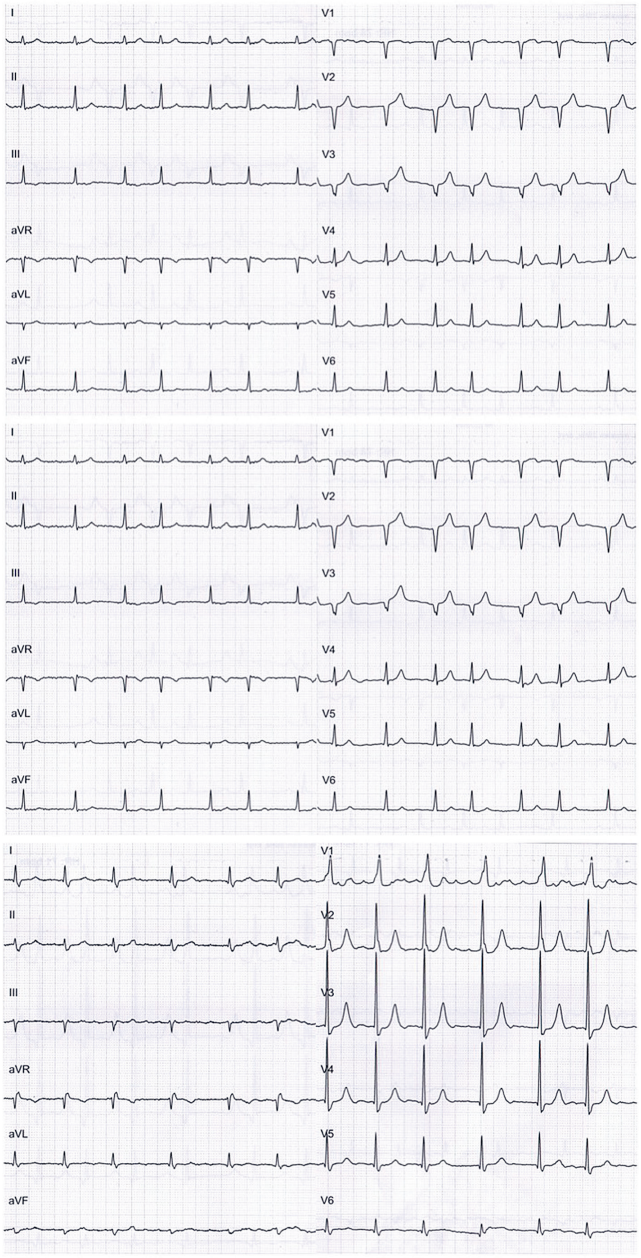

Fig. 1. Samples of atrial fibrillation ECG recorded in the study population

according to the von Clauss method, which is indirect, based on the thrombin clotting time.[13] The assay was performed according to recommendations of the manufacturer. The assay was calibrated against human plasma standard. Other laboratory parameters were obtained and assessed with regard to applicable laboratory methods and current guidelines.

Statistical analysis

Continuous data is presented as the mean ± standard deviation (SD) and was compared using the Mann–Whitney test or Student's t-test. Categorical variables were compared using either the χ^2 or Fisher's exact tests. A p-value of less than 0.05 was considered statistically significant, whereas the confidence intervals (CI) were 95%. Statistical processing of data was made using SPSS v. 21 software (IBM Corp., Armonk, USA).

Results

The study population comprised of 266 continuous patients; 35.0% were females. Mean age of the study population was 57.6 ±10.1 years. Arterial hypertension was present in 198 (74%) patients and diabetes mellitus in 27 (10.2%) patients. Twenty-nine patients (10.9%) suffered from vascular disease and 4 (1.5%) were afflicted with heart failure. Twenty-four patients (9%) had a history of stroke or TIA. The population characteristics and details of the patients' blood test results are presented in Table 1.

According to the current European guidelines, patients were divided into those with CHA_2DS_2-VASc score 0

Table 1. Baseline characteristics of the study population

Parameter	Value
ALT [U/L]	43.2 ±25.0
AST [U/L]	35.0 ±18.5
APTT [s]	35.0 ±8.8
Fibrinogen [mg/dL]	318.7 ±77.7
Glucose [mg/dL]	99.8 ±21.2
Protrombin time [s]	17.4 ±11.0
Creatinine [mg/dL]	1.0 ±0.4
Urea [mg/dL]	39.7 ±10.4
White blood cells [10^3/μL]	7.3 ±1.7
Red blood cells [10^6/μL]	5.1 ±5.6
Hemoglobin [g/dL]	14.4 ±1.3
Hematocrite [%]	42.0 ±4.5
MCV [fl]	89.0 ±5.8
MCH [pg]	31.5 ±14.2
MCHC [g/dL]	34.2 ±1.1
Platelets [10^3/μL]	219.9 ±45.2
Potassium [mmol/L]	4.5 ±0.4
Sodium [mmol/L]	141.4 ±2.7
TSH [μIU/mL]	2.1 ±2.3
CHA_2DS_2-VASc score components	
Chronic heart failure	4 (1.5%)
Hypertension	198 (74%)
Diabetes mellitus	27 (10.2%)
Female	97 (36.5%)
History of stroke or thromboembolism	24 (9.0%)
Vascular disease	29 (10.9%)

ALT – alanine transaminase; AST – aspartate transaminase; INR – international normalized ratio; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; TSH – thyroid-stimulating hormone. Values are presented as mean ± standard deviation (SD) or n (%).

(31 patients) and those with ≥1 points (235 patients), respectively requiring and not requiring anticoagulation treatment. Analysis of blood parameters revealed that the group with CHA_2DS_2-VASc = 0 (12% of patients) compared to those with CHA_2DS_2-VASc ≥1 had a significantly lower fibrinogen concentration (285.6 ±82.0 vs 322.6 ±76.4 mg/dL; p = 0.02) and shorter prothrombin time (13.6 vs 17.9 s; p = 0.01). Partial thromboplastin time and platelet count were not significantly different between the groups (p > 0.05). Differences were noticed also in the parameters concerning red blood cells. Patients with lower thromboembolic risk had a lower red blood cell count (4.9± 0.4 vs 5.1 ±0.6 10^6/μL; p = 0.03), higher hemoglobin (14.9 ±1.0 vs 14.3 ±1.4 g/dL; p = 0.04) and higher hematocrit (43.5 ±2.6 vs 41.7 ±4.7%; p = 0.001) (Table 2). No differences were seen also in transaminase enzymes, renal parameters, thyroid-stimulating hormone, glucose, as well as potassium and sodium level.

After dividing patients into 6 categories associated with results in CHA_2DS_2-VASc score: CHA_2DS_2-VASc = 0, CHA_2DS_2-VASc = 1, CHA_2DS_2-VASc = 2, CHA_2DS_2-VASc = 3, CHA_2DS_2-VASc = 4, and CHA_2DS_2-VASc ≥5, we observed that along with the increase in CHA_2DS_2-VASc scores, mean fibrinogen concentration inreased (285.6 ±82.1 vs 307.6 ±82.6 vs 327.8 ±74.5 vs 332.9 ±58.6 vs 339.5 ±64.1 vs 349.3 ±77.1 mg/dL; p-value for trend = 0.04) (Fig. 2). On the other hand, patients with higher thromboembolic risk had lower mean hemoglobin concentrations (14.9 ±1.0 vs 14.8 ±1.2 vs 14.1 ±1.3 vs 14.0 ±1.3 vs 13.7 ±1.7 vs 13.9 ±1.2 g/dL; p-value for trend ≤0.001) (Fig. 3). It has not been demonstrated that platelet count depends on the CHA_2DS_2-VASc score (Table 3).

Discussion

Our study has revealed that fibrinogen concentration is associated with CHA_2DS_2-VASc score results. It has been observed that patients with different scores also have different results in the fibrinogen level. The more points

Table 2. Characteristics of patients with CHA_2DS_2-VASc = 0 vs CHA_2DS_2-VASc ≥ 1

Parameter	CHA_2DS_2-VASc = 0 (n = 31)	CHA_2DS_2-VASc ≥1 (n = 235)	p-value
ALT [U/L]	37.3 ±15.7	44.0 ±26.0	0.36
AST [U/L]	34.7 ±9.2	35.0 ±8.9	0.81
APTT [s]	30.9 ±13.5	35.6 ±19.1	0.14
Fibrinogen [mg/dL]	285.6 ±82.1	322.6 ±76.4	**0.02**
Glucose [mg/dL]	93.0 ±12.4	100.8 ±22.0	0.05
Protrombin time [s]	13.6 ±9.0	17.9 ±11.2	**0.01**
Creatinine [mg/dL]	1.0 ±0.2	1.0 ±0.4	0.75
Urea [mg/dL]	39.0 ±8.3	39.7 ±10.7	0.66
White blood cells [10^3/μL]	7.3 ±1.6	7.3 ±1.8	0.90
Red blood cells [10^6/μL]	4.9 ±0.4	5.1 ±6.0	**0.03**
Hemoglobin [g/dL]	15.0 ±1.0	14.3 ±1.4	**0.02**
Hematocrite [%]	43.5 ±2.6	41.7 ±4.7	**0.01**
MCV [fl]	89.6 ±4.2	88.9 ±6.0	0.77
MCH [pg]	31.0 ±1.6	31.6 ±15.2	0.35
MCHC [g/dL]	34.4 ±1.0	34.2 ±1.1	0.36
Platelets [10^3/μL]	223.0 ±37.7	219.8 ±46.1	0.59
Potassium [mmol/L]	4.6 ±0.3	4.5 ±0.4	0.17
Sodium [mmol/L]	141.0 ±2.5	141.4 ±2.8	0.10
TSH [μIU/mL]	2.5 ±2.6	2.1 ±2.3	0.32
CHA_2DS_2-VASc score components			
Chronic heart failure	0 (0.0%)	4 (1.7%)	0.61
Hypertension	0 (0.0%)	198 (84.0%)	**<0.0001**
Diabetes mellitus	0 (0.0%)	27 (11.5%)	**0.03**
Female sex	0 (0.0%)	97 (41.3%)	**<0.0001**
History of stroke of thromboembolic disease	0 (0.0%)	24 (10.2%)	**0.04**
Vascular disease	0 (0.0%)	29 (12.3%)	**0.03**

ALT – alanine transaminase; AST – aspartate transaminase; INR – international normalized ratio; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; MCHC – mean corpuscular hemoglobin concentration; TSH – thyroid-stimulating hormone; values are presented as mean ± standard deviation (SD) or n (%); p-values in bold indicate statistical significance (p < 0.05).

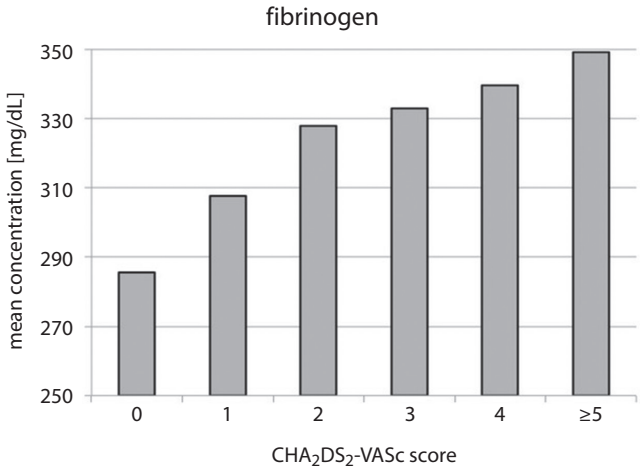

Fig. 2. Comparison of mean concentration of fibrinogen in different thromboembolic risk strata, according to CHA_2DS_2-VASc score

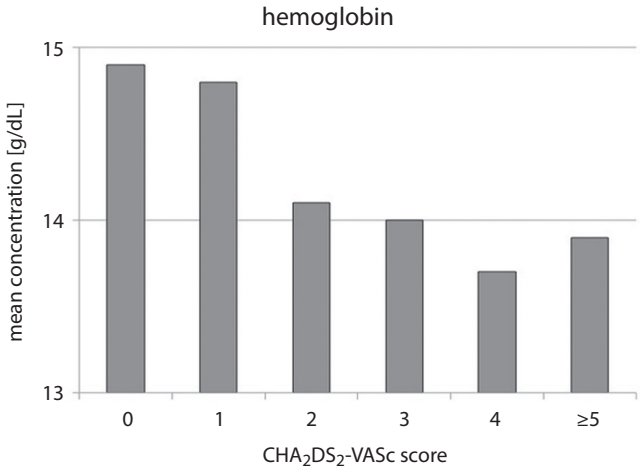

Fig. 3. Comparison of mean concentration of hemoglobin in different thromboembolic risk strata, according to CHA_2DS_2-VASc score

in the CHA_2DS_2-VASc score, the higher fibrinogen concentration, which independently proves its clinical utility, especially in a relatively young population assigned to pulmonary vein isolation. To the best of our knowledge, this particular association has not been thoroughly investigated thus far, although the relationship of hemostatic plasma parameters and risk of stroke has been proved.[26,27,34]

The CHA_2DS_2-VASc score is a simple scheme to assess cardiovascular risk among patients with AF and the legitimacy of using anticoagulation treatment. It includes clinical parameters that are all proven to be independent predictors of a pro-thrombotic state. The score is an independent predictor of mortality.[1,14] The CHA_2DS_2-VASc score is used in order to plan anticoagulation therapy. If a patient gets 0 points, no anticoagulant is needed; otherwise, the treatment should be introduced.[1]

The CHA_2DS_2-VASc score is seen to predict the thromboembolic and stroke risk in different patient populations.[15] Nevertheless, it is based only on clinical parameters and does not include biochemical and even some clinical data.[16–20] In the current study, we showed that despite its flaws, the CHA_2DS_2-VASc score has a direct reflection in altered coagulation parameters. One of the most important biochemical factors of stroke is fibrinogen concentration.

Fibrinogen is one of the plasma proteins which is converted to fibrin by thrombin and then forms a clot. Fibrinogen is synthesized in the liver by hepatocytes and then is secreted into circulation; therefore, it plays an important role in platelet aggregation. It is also a biomarker of inflammation.[21,22]

It has been proved that higher concentration of fibrinogen is associated with risk of cardiovascular disease.[22,23] Appiah et al. in the ARIC study have examined patients between 1993 and 1995 in order to assess the relationship between fibrinogen and cardiovascular disease endpoint. Results showed that the fibrinogen concentration correlates positively with heart failure, peripheral artery disease and cardiovascular deaths. In their opinion, fibrinogen leads to atherosclerosis by inducing inflammation.[24]

Fibrinogen concentration is said to be an important factor of stroke episodes among patients with cardiovascular disease.[25] Fibrinogen concentration is strongly associated with thromboembolic risk. Furthermore, increased hemostatic markers have been observed in AF patients; however, the mechanism taking part in the pathogenesis of AF is multifactorial.[26,27]

Tables 3. Comparison of selected laboratory results in different thromboembolic risk strata, according to CHA_2DS_2-VASc score

Parameters	CHA_2DS_2-VASc = 0 (n = 31)	CHA_2DS_2-VASc = 1 (n = 104)	CHA_2DS_2-VASc = 2 (n = 56)	CHA_2DS_2-VASc = 3 (n = 42)	CHA_2DS_2-VASc = 4 (n = 13)	CHA_2DS_2-VASc ≥ 5 (n = 20)	p-value for trend
Fibrinogen [mg/dL]	285.6 ±82.1	307.6 ±82.6	327.8 ±74.5	332.9 ±58.6	339.5 ±64.1	349.3 ±77.1	**0.04**
Protrombin time [s]	13.7 ±9.2	16.5 ±10.0	16.5 ±10.2	17.5 ±7.6	20.4 ±16.3	26.6 ±16.6	0.29
Hematocrite [%]	43.4 ±2.6	42.7 ±5.5	41.4 ±3.7	41.2 ±4.1	39.9 ±4.2	40.9 ±3.2	0.08
Hemoglobin [g/dL]	14.9 ±1.0	14.8 ±1.2	14.1 ±1.3	14.0 ±1.3	13.7 ±1.7	13.9 ±1.2	**<0.001**
Creatinine [mg/dL]	1.0 ±0.2	1.0 ±0.2	0.9 ±0.2	1.1 ±0.8	1.0 ±0.2	1.0 ±0.4	0.40
MCV [fl]	89.7 ±4.3	88.7 ±7.6	88.4 ±4.3	89.0 ±4.1	90.8 ±4.0	89.4 ±6.0	0.79
Platelets [10^3/μL]	222.3 ±38.2	214.1 ±45.7	226.4 ±50.0	224.0 ±52.3	208.4 ±33.0	225.5 ±30.4	0.46

MCV – mean corpuscular volume. Values are presented as mean ± standard deviation (SD) or n (%); p-values in bold indicate statistical significance ($p < 0.05$).

Recently, several blood biomarkers have been identified to be helpful in diagnosis, outcomes and prognosis of AF, e.g., d-dimer, which is strongly related to fibrinogen concentration. It has been said that blood biomarkers can play an important role in predicting the development of AF and its complications (especially stroke episodes). Unfortunately, fibrinogen concentration has not been included as one of the parameters of risk-assessment scores.[28] Moreover, specific treatment can promote coagulation disturbance.[29]

It has not been discovered yet how AF contributes to thromboembolism and stroke episodes. Several hypotheses of thrombogenesis in AF patients have been published. The most probable mechanism is associated with Virchow's triad for thrombogenesis, inflammation factors, growth factors, and anatomical abnormalities, which contribute to hypercoagulable state in this arrhythmia.[30] A recent meta-analysis showed that the levels of coagulation, fibrinolytic and endothelial markers are significantly higher in AF patients than in patients with sinus rhythm.[31] However, the level to which this elevation is important and associated with prognosis has not been assessed yet. Moreover, the concentration seems not to be affected by anticoagulation treatment.[32] Assessment of the role of fibrinogen in AF patients is also important in light of recent findings, showing that fibrinogen concentration can be predictive of other cardiovascular disease, including coronary artery disease severity.[23,33]

Another parameter that was associated with higher thromboembolic risk in the CHA_2DS_2-VASc score is lower hemoglobin concentration and lower hematocrit. This phenomenon may be associated with the fact that, due to the overlapping factors, a higher CHA_2DS_2-VASc score is usually observed in patients with higher HAS-BLED. This may be associated with elevated bleeding risk, also subclinical, resulting in lower hemoglobin concentration and lower hematocrit.[34]

We suggest the implementation of fibrinogen concentration as an additional laboratory parameter to clinical variables in the CHA_2DS_2-VASc score. Still, this relationship requires further research.

A major limitation of this study is that laboratory parameters were assessed with relation to the risk scores only. An exploration of long-term follow-up with clinical endpoints (stroke, peripheral embolism, death) would be much more valuable.

Conclusions

Higher CHA_2DS_2-VASc score is independently associated with increased fibrinogen concentration. This finding may be a link between CHA_2DS_2-VASc and thromboembolic complications. Further research is necessary to assess if fibrinogen – an easy to obtain laboratory parameter – can add additional value to CHA_2DS_2-VASc as a predictor of higher thromboembolic risk.

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Association of XPD Asp312Asn polymorphism and response to oxaliplatin-based first-line chemotherapy and survival in patients with metastatic colorectal cancer

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Abstract

Background. Identification of biomarkers predicting a response to chemotherapeutic drugs would greatly ease the selection of personalized therapy. The protein xeroderma pigmentosum group D (XPD) functions in nucleotide excision repair (NER) to remove DNA cross-links and in the regulation of transcription. The potential role of the Asp312Asn polymorphism in predicting the response to chemotherapy has not been established.

Objectives. This prospective study was designed to determine the role of the XPD Asp312Asn polymorphism in predicting the response to oxaliplatin-based first-line chemotherapy and survival in patients with metastatic colorectal cancer.

Material and methods. A total of 106 patients treated with 2 cycles of either FOLFOX4 (n = 72) or XELOX (n = 34) regimen as the chemotherapy were enrolled. The genotype of XPD Asp312Asn polymorphism was analyzed using TaqMan probe-based real-time polymerase chain reaction (PCR). Logistic regression was applied to predict the response to treatment protocols. Cox regression models were applied to predict overall survival.

Results. The overall response to chemotherapy was 57.6% (61/106). FOLFOX4 and XELOX regimens demonstrated comparable efficacy. The XPD Asp312Asn polymorphism was not associated with the response to either FOLFOX4 or XELOX regimen in univariate and in multivariate logistic regression analyses. Levels of carcinoembryonic antigen (CEA) ≥5 ng/mL and female gender were associated with a lack of response to FOLFOX4, but not to XELOX regimen. In a multivariate survival analysis, XPD Asp312Asn AA genotype, lack of response to chemotherapy, CEA ≥ 5 ng/mL, and age ≥65 were significantly associated with worse overall survival.

Conclusions. The XPD Asp312Asn polymorphism is associated with overall survival, but it is not a biomarker in predicting the response to oxaliplatin-based first-line chemotherapy in patients with metastatic colorectal cancer.

Key words: colorectal cancer, polymorphism, oxaliplatin, survival, XPD

Introduction

Colorectal cancer is one of the most frequently diagnosed malignancies and a leading cause of cancer deaths worldwide.[1,2] Distant metastasis is the main cause of death for colorectal cancer patients. It is notable that approx. 15–25% of colorectal cancer patients have been identified with distant metastases at the time of initial diagnosis.[3] Moreover, the incidence of metastatic colorectal cancer continues to increase.[4] Investigations into improved treatment regimens may lead to findings that can be applied towards extending the survival rates for patients. Oxaliplatin-based chemotherapy, with or without molecular targeting agents, is the current primary treatment for metastatic colorectal cancer patients. Oxaliplatin promotes the formation of intra-strand adducts that block DNA replication and transcription, leading to apoptosis.[5] Due to cancer heterogeneity, metastatic colorectal cancer patients demonstrate different sensitivity to chemotherapy.[6] A portion of patients unresponsive to chemotherapy will not benefit from this treatment and some will suffer from severe side effects. It is of clinical significance to identify biomarkers that predict the response to oxaliplatin-based chemotherapy. Routine clinicopathological factors may be insufficient to predict sensitivity to treatment and susceptibility to side effects that correspond to specific regimens used in chemotherapies.[7] In contrast, many genetic factors demonstrate the potential to predict responses to different chemotherapies and provide aid in the selection of the best regimen.[8–11]

The xeroderma pigmentosum group D (*XPD*) gene, also known as excision repair cross complementing group 2 (ERCC2), is located on chromosome 19q13.3. The protein encoded by this gene functions in nucleotide excision repair (NER) to remove DNA cross-links, and in the regulation of transcription. Asp312Asn (rs1799793) and Lys751Gln (rs13181) are 2 common polymorphisms found in the *XPD* gene. Several studies have reported that the XPD Lys751Gln polymorphism is a predictor for the response to chemotherapies, progression-free survival and overall survival in colorectal cancer patients.[12–14] In contrast, the potential role of the XPD Asp312Asn polymorphism in predicting the response to chemotherapy has not been established.[14] The purpose of this study was to determine whether the XPD Asp312Asn polymorphism was a predictor for the response to oxaliplatin-based first-line chemotherapy and survival in patients with metastatic colorectal cancer.

Material and methods

From January of 2013 to June of 2016, a total of 185 patients with metastatic colorectal cancer were diagnosed and treated in 2 district hospitals. Criteria used to select patients were the following: pathological diagnosis of colorectal cancer at stage IV without a previous palliative treatment, neoadjuvant chemotherapy or oxaliplatin-based chemotherapy within the past 6 months; life expectancy over 3 months; normal liver and kidney functions; age of 18 or older; Eastern Cooperative Oncology Group Scale (ECOG) score ≤2; and tumor size measurable to evaluate response to the drug treatment. The procedure for patient selection is shown in the flowchart (Fig. 1).

In addition, all enrolled patients agreed to receive one of 2 oxaliplatin-based regimens as the first-line chemotherapy. Modified FOLFOX4 regimen consisted of: oxaliplatin 130 mg/m^2 intravenously (iv.) in 3 h on day l; calcium folinate (CF) 130 mg/m^2 iv. in 2 h on days 1~5; fluorouracil (5-FU) 300 mg iv. in 4 h on days 1~5; repeated every 3 weeks. XELOX regimen consisted of: oxaliplatin 130 mg/m^2 iv. in 2 h on day 1; oral capecitabine 250 mg/m^2 2 times a day continued for 2 weeks; repeated every 3 weeks. Patients selected the regimen with the help of doctors. Only patients who had completed 2 cycles of chemotherapy were recruited for this study. Patients who failed to finish 2 cycles of the oxaliplatin-based chemotherapy would receive other treatments and therefore were excluded from the study. This study was approved by the Medical Ethics Committee of our hospital. Informed consent from all patients was obtained.

The efficacy of chemotherapy was evaluated based on the results of computed tomography (CT) scans and of other imaging approaches. Using the World Health Organization (WHO) standard, the response to chemotherapy was divided into 4 categories: complete response (CR), partial response (PR), stable disease (SD), and progressive disease (PD). The CR, PR, and SD patients were categorized as responders, whereas PD patients were considered non-responders.

Approximately 4 mL of venous blood was collected with the anticoagulant ethylenediaminetetraacetic acid (EDTA) from each patient after chemotherapy. Qiagen Blood DNA mini kit (Qiagen, Hilden, Germany) was used to extract DNA from whole blood cells. A TaqMan probe-based real-time polymerase chain reaction (PCR) approach was used to genotype the XPD Asp312Asn polymorphism. Primers and probes (C_3145050_10) were designed and synthesized by Applied Biosystems (Waltham, USA). Twenty nanograms of genomic DNA was used for each PCR reaction. The PCR reactions were performed using an ABI7900 real-time PCR instrument (Applied Biosystems). The genotyping results were analyzed using ABI SDS v. 2.3 software (Applied Biosystems).

Patient follow-up

After chemotherapy, follow-up with patients occurred once every 3 months in the first 2 years, every 6 months thereafter during the first 5 years and once a year after

the 5th year. The follow-up was conducted by either outpatient or inpatient review or by telephone. Overall survival was defined as the period from the date the chemotherapy started to the date of death or last follow-up visit.

Statistical analysis

In order to assume random mating, genotypic distribution of single nucleotide polymorphism (SNP). XPD Asp312Asn were checked for the Hardy–Weinberg equilibrium using the χ^2 test. The difference in clinicopathological features between the FOLFOX4 and XELOX groups were examined with the χ^2 test. Logistic regression analysis was applied to examine the predictor effect of the XPD Asp312Asn polymorphism and clinicopathological factors in response to chemotherapy. Cox regression models were used to determine the effect of the XPD Asp312Asn polymorphism and clinicopathological factors on overall survival. The Kaplan–Meier method with log-rank test was used for generating survival curves. A p-value <0.05 was considered statistically significant. All statistical analyses were performed using SAS v. 9.3 software (SAS Institute Inc., Cary, USA).

Results

A total of 106 consecutive patients were enrolled in this study. Among these patients, 63 were males and 43 were females, with a median age of 52 years (range: 22–81 years). Seventy-two patients were treated with FOLFOX4 regimen and 34 patients were treated with XELOX regimen. The baseline features of patients with metastatic colorectal cancer are presented in Table 1. Except for carcinoembryonic antigen (CEA) levels, patients in both groups had similar baseline clinicopathological features. The FOLFOX4 group had a significantly higher proportion of patients with CEA levels ≥5 ng/mL than the XELOX group (81.9% vs 58.8%; p = 0.0108).

Our data revealed that 49 colorectal cancer patients (46.2%) had the XPD Asp312Asn genotype of G/G, 42 patients (39.6%) had G/A and 15 patients (14.2%) had A/A (Table 1). The genotypic distribution was in line with the Hardy–Weinberg equilibrium (p = 0.2301).

After 2 cycles of chemotherapy, none of the patients in either group achieved CR. Thirty-two (30.2%), 29 (27.4%) and 45 (42.4%) patients achieved PR, SD and PD, respectively. The overall response rate was 57.6% (61/106). A trend of higher response rate was observed for patients in the XELOX group compared with those in the FOLFOX4 group (70.6% and 51.4%, respectively; p = 0.0650).

Both univariate and multivariate logistic regression analyses revealed that the XPD Asp312Asn polymorphism was not a predictor for response to either FOLFOX4 or XELOX regimen (Table S1). Carcinoembryonic antigen levels and the number of metastatic sites were predictors of response

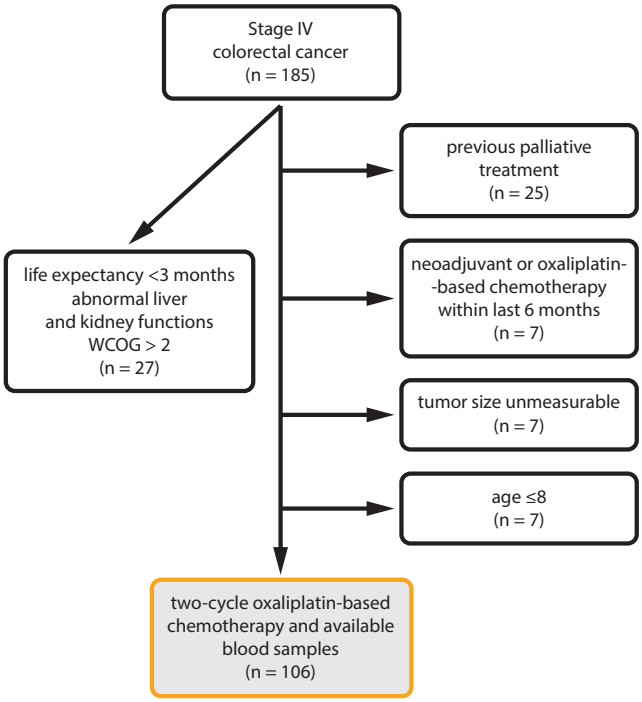

Fig. 1. Flowchart of patient selection

Table 1. Baseline features of metastatic colorectal cancer patients

Variables	Overall (n = 106) (%)	FOLFOX4 (n = 72) (%)	XELOX (n = 34) (%)	p-value
Age [years]				
<65	87 (82.1)	57 (55.6)	30 (67.7)	0.2559
≥65	19 (17.9)	15 (44.4)	4 (32.3)	
Gender				
male	63 (59.4)	40 (61.0)	23 (57.5)	0.2366
female	43 (40.6)	32 (39.0)	11 (42.5)	
ECOG				
0~1	59 (55.7)	40 (55.6)	19 (55.9)	0.9748
2	47 (44.3)	32 (44.4)	15 (44.1)	
Tumor location				
left colon	17 (16.0)	8 (11.1)	9 (26.5)	0.1180
right colon	15 (14.2)	10 (13.9)	5 (14.7)	
rectum	74 (69.8)	54 (75.0)	20 (58.8)	
Histology				
adenocarcinoma	76 (71.7)	59 (81.9)	27 (79.4)	0.7557
others[#]	20 (18.9)	13 (18.1)	7 (20.6)	
Number of metastatic sites				
1	51 (48.1)	36 (50.0)	15 (44.1)	0.5715
≥2	55 (51.9)	36 (50.0)	19 (55.9)	
CEA [ng/mL]				
<5	27 (25.5)	13 (18.1)	14 (41.2)	0.0108
≥5	79 (74.5)	59 (81.9)	20 (58.8)	
Genotype of XPD Asp312Asn				
G/G	49 (46.2)	32 (44.4)	17 (50.0)	0.5519
G/A	42 (39.6)	28 (38.9)	14 (41.2)	
A/A	15 (14.2)	12 (16.7)	3 (8.8)	

[#] – including 18 cases of mucinous adenocarcinoma, 1 case of signet ring cell adenocarcinoma and 1 case of undifferentiated carcinoma.
ECOG – Eastern Cooperative Oncology Group Scale; CEA – carcinoembryonic antigen; XPD – xeroderma pigmentosum group D.

to the FOLFOX4 regimen in univariate logistic regression analysis, whereas gender and CEA levels were significantly associated with response to the FOLFOX4 regimen in multivariate analysis (Table S1). No clinicopathological factors were significantly associated with the response to the XELOX regimen in either univariate or multivariate analysis (Table S1).

The role of the XPD Asp312Asn polymorphism in predicting the response to the oxaliplatin-based first-line chemotherapy was also examined in all patients (Table S2). Neither univariate nor multivariate logistic regression analysis revealed a possible role for the XPD Asp312Asn polymorphism in predicting the response to chemotherapy. Multivariate analysis showed that blood CEA levels ($p = 0.0129$), the number of metastatic sites ($p = 0.0493$) and gender ($p = 0.0369$) were predictors of the response to oxaliplatin-based chemotherapy in colorectal cancer patients (Table S2).

Univariate survival analysis showed that the XPD Asp312Asn polymorphism was not associated with overall survival for metastatic colorectal cancer patients treated with either FOLFOX4 or XELOX regimen. Age, tumor location and efficacy of chemotherapy were significantly associated with overall survival in patients treated with either regimen (Table 2). Survival curves for age, CEA levels and efficacy of chemotherapy are presented in Fig. 2A–F. Multivariate survival analysis showed that the XPD Asp312Asn polymorphism, efficacy of chemotherapy, CEA

Table S1. Factors in predicting response to FOLFOX4 or XELOX regimens

Variables	FOLFOX4						XELOX			
	univariate				multivariate		univariate			
	responsive (%)	PD (%)	OD (95% CI)*	p-value	OD (95% CI)	p-value	responsive (%)	PD (%)	OD (95% CI)	p-value
Age										
<65	30 (52.6)	27 (47.4)	1				22 (73.3)	8 (26.7)		
≥65	7 (46.7)	8 (53.3)	1.27 (0.41–3.97)	0.6814			2 (50.0)	2 (50.0)	2.75 (0.33–22.92)	0.3499
Gender										
male	24 (60.0)	16 (40.0)	1		1		17 (73.9)	6 (26.1)		
female	13 (40.6)	19 (59.4)	2.19 (0.85–5.66)	0.1044	2.89 (1.03–8.08)	0.0437	7 (63.6)	4 (36.4)	1.62 (0.35–7.56)	0.5400
ECOG										
0~1	22 (55.0)	18 (45.0)	1	0.4931			12 (63.2)	7 (36.8)		0.2906
2	15 (46.9)	17 (53.1)	1.39 (0.55–3.52)				12 (80.0)	3 (20.0)	0.43 (0.09–2.06)	
Tumor location										
rectum	27 (50)	27 (50)	1				15 (75.0)	5 (25.0)	1	
left colon	5 (62.5)	3 (37.5)	0.6 (0.13–2.76)	0.5122			7 (77.8)	2 (22.2)	0.86 (0.13–5.56)	0.8716
right colon	5 (50)	5 (50)	1.00 (0.26–3.86)	1.000			2 (40.0)	3 (60.0)	4.5 (0.58–35.15)	0.1515
Histology										
adenocarcinoma	31 (52.5)	28 (47.5)	1				19 (70.4)	8 (29.6)	1	
others	6 (46.2)	7 (53.8)	1.29 (0.39–4.31)	0.6772			5 (71.4)	2 (28.6)	0.95 (0.15–5.96)	0.9566
Number of metastatic sites										
1	23 (63.9)	13 (36.1)		0.0353			12 (80.0)	3 (20.0)		0.2906
≥2	14 (38.9)	22 (61.1)	2.78 (1.07–7.22)				12 (63.2)	7 (36.8)	2.33 (0.48–11.23)	
CEA levels [ng/mL]										
<5	10 (76.9)	3 (23.1)	1		1		11 (78.6)	3 (21.4)		0.3966
≥5	27 (45.8)	32 (54.2)	3.95 (0.99–15.8)	0.0484	5.38 (1.24–23.41)	0.0249	13 (65.0)	7 (35.0)	1.97 (0.41–9.52)	
Genotypes										
G/G	17 (53.1)	12 (46.9)	0.79 (0.25–2.52)	0.5000			12 (70.6)	5 (29.4)	#	0.9483
G/A	15 (53.6)	13 (46.4)	0.62 (0.16–2.43)	0.4917			9 (64.3)	5 (35.8)	#	0.9510
A/A	5 (41.8)	7 (58.3)	1				3 (100.0)	0 (0)	1	
G/A+A/A	20 (50.0)	20 (50.0)	0.63 (0.18–2.19)	0.4628			12 (70.6)	5 (20.4)	1.0 (0.23–4.37)	1.000
G/G+G/A	32 (53.3)	28 (46.7)	0.88 (0.35–2.24)	0.7922			21 (67.4)	10 (32.3)	#	0.9679

>1000.0 (<0.001–>999); OR – odds ratio; CI – confidence interval; responsive – referred to be partial response (PR) and stable disease (SD); PD – progressive disease.

levels, tumor location, and age were significantly associated with overall survival in patients treated with FOLFOX4 regimen. The XPD Asp312Asn polymorphism, efficacy of chemotherapy and CEA levels were associated with overall survival in patients treated with the XELOX regimen (Table 3).

Survival analysis was performed on all patients treated with either FOLFOX4 or XELOX regimen. Univariate analysis showed that age, tumor location, CEA levels, and efficacy of chemotherapy were significantly associated with overall survival (Table 4). Multivariate survival analysis showed that the A/A genotype of XPD Asp312Asn, lack of response to chemotherapy, CEA ≥ 5 ng/mL, and age ≥65 were significantly associated with reduced overall survival in metastatic colorectal cancer patients (Table 4).

Discussion

This study examined the genotypes of the XPD Asp312Asn polymorphism in metastatic colorectal cancer patients who received oxaliplatin-based first-line chemotherapy. Our data revealed that the XPD Asp312Asn polymorphism was not a predictor for response to either the FOLFOX4 or XELOX regimen. The A/A genotype of the XPD Asp312Asn polymorphism, CEA levels ≥5 ng/mL and lack response to chemotherapy were significantly associated with worse overall survival of patients treated with FOLFOX4 or XELOX regimen.

Studies have shown that efficacy of chemotherapeutic drugs may be substantially influenced by specific genetic polymorphisms.[15,16] This study examined the potential role for the XPD Asp312Asn polymorphism in predicting response to oxaliplatin based first-line chemotherapy

Table S2. Factors in predicting response to oxaliplatin-based chemotherapy in all patients

Variables	Efficacy of therapy		Univariate		Multivariate	
	responsive (%)	PD (%)	OR (95% CI)	p-value	OR (95% CI)	p-value
Age						
<65	52 (59.8)*	35 (402.0)				
≥65	9 (47.4)	10 (52.6)	1.65 (0.61–4.48)	0.3246		
Gender						
male	41 (65.1)	22 (34.9)				
female	20 (46.5)	23 (53.5)	2.14 (0.97–4.73)	0.0593	2.49 (1.05–5.87)	0.0369
ECOG						
0~1	34 (57.6)	25 (42.4)				
2	27 (57.5)	20 (42.5)	1.01 (0.46–2.19)	0.9851		
Number of metastatic sites						
1	35 (68.6)	16 (31.4)				
≥2	26 (47.3)	20 (52.7)	2.44 (1.10–5.40)	0.0277	2.31 (1.00–5.31)	0.0493
Tumor location						
left colon	12 (70.6)	5 (26.4)	0.55 (0.18–1.71)	0.2997		
right colon	7 (46.7)	8 (53.3)	1.5 (0.49–4.57)	0.4755		
rectum	42 (56.8)	32 (43.2)	1			
Histology						
adenocarcinoma	50 (58.1)	36 (41.9)	0.88 (0.33–2.34)	0.7981		
others	11 (55.0)	9 (45.0)	1			
CEA [ng/mL]						
<5	21 (77.8)	6 (22.2)				
≥5	42 (50.6)	39 (49.4)	3.41 (1.24–9.36)	0.0171	3.89 (1.33–11.35)	0.0129
Genotypes of XPD Asp312Asn						
G/G	29 (59.2)	20 (40.8)	0.79 (0.25–2.52)	0.6884		
G/A	24 (57.1)	18 (42.9)	0.86 (0.26–2.80)	0.7986		
A/A	8 (53.3)	7 (46.7)				
G/A+A/A	32 (56.1)	25 (43.9)	1.13 (0.52–2.46)	0.7520		
G/G+G/A	53 (58.2)	35 (41.8)	0.82 (0.27–2.45)	0.7219		

OR – odds ratio; CI – confidence interval; PD – progressive disease; responsive – referred to be partial response (PR) or stable disease (SD).

Table 2. Univariate analysis of factors in predicting overall survival in metastatic colorectal cancer patients

Variables	FOLFOX4			XELOX		
	HR	95% CI	p-value	HR	95% CI	p-value
Gender						
male	0.87	0.54–1.39	0.5598	0.82	0.39–1.72	0.6033
female	1	–	–	1	–	–
Age						
<65	0.32	0.18–0.59	0.0003	0.10	0.03–0.47	0.0021
≥65	1	–	–	1	–	–
ECOG						
0~1	1.28	0.79–2.06	0.3142	1.04	0.52–2.11	0.9060
2	1	–	–	1	–	–
Tumor location						
left colon	1	–	–	2.05	0.85–4.92	0.1101
right colon	4.19	1.35–12.97	0.0130	3.54	1.19–10.60	0.0235
rectum	3.83	1.45–10.14	0.0068	1	–	–
Histology						
adenocarcinoma	0.93	0.51–1.69	0.7998	0.87	0.37–2.04	0.0966
others	1	–	–	1	–	–
Number of metastatic sites						
1	75	0.47–1.22	0.2460	0.93	0.47–1.86	0.8384
≥2	1	–	–	1	–	–
CEA [ng/mL]						
<5	0.59	0.32–1.09	0.0898	0.50	0.24–1.03	0.0605
≥5	1	–	–	1	–	–
Efficacy						
responsive	0.18	0.10–0.32	<0.0001	0.07	0.02–0.23	<0.0001
PD	1	–	–	1	–	–
Genotypes of XPD Asp312Asn						
G/G	1	–	–	1	–	–
G/A	1.13	0.68–1.89	0.6439	1.47	0.68–3.17	0.3218
A/A	1.50	0.77–2.94	0.2348	1.91	0.52–6.96	0.3288
G/A+A/A	1.22	0.76–1.96	0.4094	1.53	0.73–3.20	0.2585
G/G+G/A	1.42	0.76–2.65	0.2713	1.56	0.46–5.30	0.4731

HR – hazard ratio; CI – confidence interval; PD – progressive disease; responsive – referred to be partial response (PR) or stable disease (SD); ECOG – Eastern Cooperative Oncology Group Scale; CEA – carcinoembryonic antigen; XPD – xeroderma pigmentosum group D.

and survival in metastatic colorectal cancer patients. Our data demonstrated that the XPD Asp312Asn polymorphism was not a biomarker to predict response to either the FOLFOX4 or XELOX regimen for first-line chemotherapy. However, the multivariate survival analysis indicated that the XPD Asp312Asn polymorphism was a predictor for overall survival in metastatic colorectal cancer patients treated with either FOLFOX4 or XELOX regimen. The predictive role of the XPD Asp312Asn polymorphism in survival was reported in lung cancer patients treated with platinum chemotherapy.[17,18] In contrast to some of our findings, the XPD Asp312Asn polymorphism was not a predictor for response to 5-fluorouracil/oxaliplatin and overall survival in patients with metastatic colorectal cancer,[14] and for the response to cisplatin-based chemotherapy, event-free survival and overall survival in patients with osteosarcoma.[19] Previous studies also indicated that the XPD Asp312Asn polymorphism was not a predictor of colorectal cancer risk.[20,21] More studies are warranted to clarify the role of the XPD Asp312Asn polymorphism in predicting the response to other oxaliplatin-based regimens and to verify its role in predicting overall survival in metastatic colorectal cancer patients.

Oxaliplatin is the first platinum-based anticancer drug for the treatment of colorectal cancer.[22] The platinum in these drugs promotes the formation of bulky adducts between 2 adjacent guanine residues through inter-strand and intra-strand links that block DNA replication and transcription

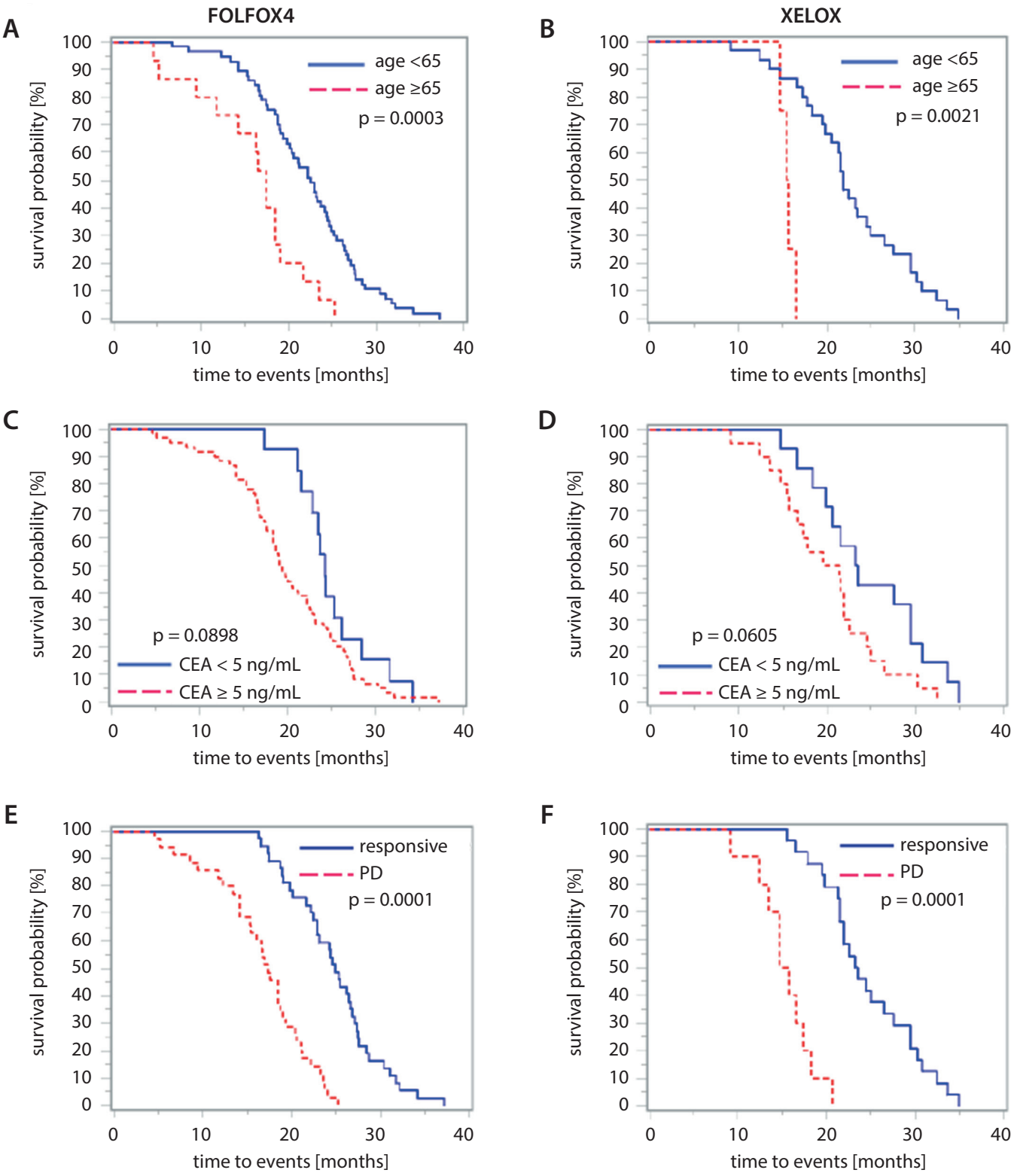

Fig. 2. Overall survival curves of patients with metastatic colorectal cancer who underwent oxaliplatin-based chemotherapy. Survival curves for the FOLFOX4 group by (A) age, (C) CEA levels and (E) efficacy of chemotherapy; and for the XELOX group by (B) age, (D) CEA levels and (F) efficacy of chemotherapy

and thus induce death of cancer cells.[5] The NER pathway is responsible for repairing DNA damage caused by these drugs[23] and the XPD protein is a component of this pathway that plays a critical role in removing DNA lesions. It has been reported that the XPD polymorphism Asp312Asn is associated with reduced DNA repair activity.[24,25] More studies are needed to clarify whether possible loss of function in this XPD variant accounts for the different survival rates in patients with metastatic colorectal cancers after oxaliplatin-based treatments.

Table 3. Multivariate analysis of factors in predicting overall survival in patients with metastatic colorectal cancer

Variables	FOLFOX4			XELOX		
	HR	95% CI	p-value	HR	95% CI	p-value
Age						
<65	0.16	0.08–0.33	<0.0001	–	–	–
≥65	1	–	–	–	–	–
Tumor location						
rectum	1.24	0.61–2.54	0.5492	–	–	–
left colon	0.25	0.07–0.81	0.0210	–	–	–
right colon	1	–	–	–	–	–
CEA [ng/mL]						
<5	0.36	0.18–0.72	0.0040	0.40	0.16–0.94	0.0363
≥5	1	–	–	1	–	–
Efficacy						
responsive	0.19	0.10–0.35	<0.0001	0.04	0.01–0.15	<0.0001
PD	1	–	–	1	–	–
Genotypes of XPD Asp312Asn						
G/G	1	–	–	1	–	–
G/A	2.32	1.12–4.81	0.0232	1.02	0.42–2.50	0.0782
A/A	2.30	1.29–4.10	0.0046	4.22	1.02–17.55	0.0473

HR – hazard ratio; CI – confidence interval; PD – progressive disease; responsive – referred to be partial response (PR) or stable disease (SD); CEA – carcinoembryonic antigen; XPD – xeroderma pigmentosum group D.

Oxaliplatin-based chemotherapy can offer a significant survival advantage for some patients with advanced colorectal cancer.[26,27] Combined with bevacizumab, oxaliplatin-based regimens, including FOLFOX4, mFOLFOX6 and XELOX, demonstrated similar efficacy in tumor response and progression-free survival in patients with metastatic colorectal cancer.[28] The comparable clinical outcomes between FOLFOX4 and XELOX regimen as the first-line treatment of colorectal cancer were reported in previous studies.[29,30] XELOX regimen was reported to be more cost-effective than FOLFOX4 in the treatment of colorectal cancer patients in China.[31] Metastatic colorectal patients who showed early objective tumor response to oxaliplatin-based regimens with bevacizumab had significantly better progression-free survival than those who presented no response to this regimen.[28] A recent study reported that metastatic colorectal patients whose best response to adjuvant chemotherapy was only SD still had prolonged overall survival.[32] Consistent with these findings, our study found that patients responsive to oxaliplatin-based chemotherapy showed significantly improved overall survival.

Carcinoembryonic antigen is a commonly used and cost-effective biomarker for colon cancer diagnosis and monitoring cancer progression. Data from a very recent study of 2,035 surgically-treated colorectal cancer patients showed that the pre/post-CEA ratio is associated with lymphatic and distant metastasis, tumor stage, differentiation, and overall survival.[33] Carcinoembryonic antigen monitoring was found to result in better survival outcomes than detection by self-report in patients with recurrent colorectal cancer.[34] This study revealed that blood CEA levels ≥5 ng/mL prior to initiation of oxaliplatin-based chemotherapy were significantly associated with poorer response to chemotherapy and worse overall survival in patients with metastatic colorectal cancer. Findings presented here also support the use of CEA as a biomarker in predicting response to chemotherapy and prognosis of metastatic colorectal cancer patients.[35,36]

Limitations of our study include a small sample size. Only 1 polymorphism in the *XPD* gene and 2 oxaliplatin-based chemotherapeutic regimens were examined in this study. Carcinoembryonic antigen levels were only measured once before the start of chemotherapy and were not dynamically monitored during or after chemotherapy. The side effects of the chemotherapy regimens were not evaluated. In addition, any treatments received after the oxaliplatin-based chemotherapy and progression-free survival were not recorded.

Conclusions

Results of this study indicate that the XPD Asp312Asn polymorphism is associated with overall survival, but it is not a biomarker in predicting response to oxaliplatin-based first-line chemotherapy in patients with metastatic colorectal cancer. Future studies with a larger sample size are needed to validate the current findings and to identify more biomarkers to guide personalized medicine.

Table 4. Relationship between variables and overall survival of all patients

Variables	Univariate			Multivariate		
	HR	95% CI	p-value	HR	95% CI	p-value
Gender						
male	0.86	0.58–1.27	0.4400	–	–	–
female	1	–	–	–	–	–
Age						
<65	0.28	0.17–0.48	<0.0001	0.21	0.12–0.39	<0.0001
≥65	1	–	–	1	–	–
ECOG						
0~1	1.17	0.79–1.73	0.4444	–	–	–
2	1	–	–	–	–	–
Tumor location						
left colon	1	–	–	–	–	–
right colon	2.26	1.09–4.70	0.0283	–	–	–
rectum	1.46	0.84–2.55	0.1759	–	–	–
Histology						
adenocarcinoma	0.93	0.57–1.51	0.7857	–	–	–
others[#]	1	–	–	–	–	–
Number of metastatic sites						
1	0.81	0.55–1.19	0.2735	–	–	–
≥2	1	–	–	–	–	–
CEA [ng/mL]						
<5	0.56	0.36–0.87	0.0097	0.44	0.27–0.71	0.0009
≥5	1	–	–	1	–	–
Chemotherapeutic regimens						
FOLFOX4	1	–	–	–	–	–
XELOX	1.14	0.76–1.72	0.5332	–	–	–
Efficacy						
responsive	1	–	–	1	–	–
PD	5.76	3.57–9.14	<0.0001	6.25	3.72–10.53	<0.0001
Genotypes of XPD Asp312Asn						
G/G	1	–	–	1	–	–
G/A	1.26	0.83–1.91	0.2793	1.51	0.98–2.34	0.0610
A/A	1.65	0.92–2.97	0.0938	2.43	1.31–4.53	0.0049
G/A+A/A	1.34	0.91–1.98	0.1395	–	–	–
G/G+G/A	1.45	0.86–2.58	0.1581	–	–	–

– including 18 cases of mucinous adenocarcinoma, 1 case of signet ring cell adenocarcinoma and 1 case of undifferentiated carcinoma; HR – hazard ratio; CI – confidence interval; PD – progressive disease; responsive – referred to be partial response (PR) or stable disease (SD); ECOG – Eastern Cooperative Oncology Group Scale; CEA – carcinoembryonic antigen; XPD – xeroderma pigmentosum group D.

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Differential diagnosis of leukoplakia versus lichen planus of the oral mucosa based on digital texture analysis in intraoral photography

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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. A noninvasive, accurate and quick diagnosis is very important to general practitioners and specialists who care for the health of patients' oral cavity mucosa. The main enemies are precancerous lesions: leukoplakia and lichen planus (LP).

Objectives. The aim of this study was to attempt to formulate a differential diagnosis for leukoplakia vs LP in the oral mucosa based on digital texture analysis in intraoral macrophotography.

Material and methods. The study was comprised of 21 patients affected by leukoplakia, 21 affected by LP and 21 healthy volunteers. Intraoral photography of all participants was taken perpendicularly to the buccal mucosa. To achieve the maximum possible contrast, a high-pass filter was applied and level tools were then used to equalize the histograms of the images. After that, the images were converted into 8-bit grayscale. Two features of run length matrix and 2 of co-occurrence matrix were used for texture analysis. Analysis of variance (ANOVA) was used to check for differences. Factor analysis (FA) and classification with artificial neural network (ANN) were performed.

Results. The results revealed a simple possible differentiation of both types of precancerous lesions from normal mucosa ($p < 0.05$). Factor analysis and ANN can help in differentiating the 3 study groups from one another.

Conclusions. Differential diagnosis of leukoplakia and LP in the oral mucosa based on digital texture analysis in intraoral macrophotography is possible. It can be used to develop smartphone applications and can be also a helpful tool for general dentists to define the clinical problem before a consultation with a specialist.

Key words: lichen planus, leukoplakia, texture analysis, oral mucosa pathology

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Introduction

Cancer of the oral mucosa is often preceded by premalignant lesions. Leukoplakia and lichen planus (LP) in the oral mucosa are known to pose an increased risk of malignant transformation (5% and 2%, respectively). The etiology varies in these disorders, but both present as white lesions in the mucous membrane.[1,2] The World Health Organization (WHO) definition of leukoplakia from 2005 characterizes it as "a white plaque of questionable risk having excluded (other) known diseases or disorders that carry no increased risk for cancer".[3] The etiology of leukoplakia is multifactorial. The most important risk factors are cigarette smoking, alcohol consumption, poor oral hygiene, electrogalvanic currents (due to various metals in the oral cavity, i.e., gold, amalgam and nickel), and irritation caused by food. Lichen planus is a relatively common chronic inflammatory mucocutaneous disease. It is believed to result from an abnormal T-cell-mediated immune response in which basal epithelial cells are recognized as foreign because of changes in the antigenicity of their cell surface.[4] Both antigen-specific and non-specific mechanisms are thought to be involved in the pathogenesis of oral LP.[2]

Due to the increased risk of malignant transformation, it is important to diagnose and distinguish between oral leukoplakia and LP early on. The main feature of leukoplakia and LP is hyperkeratosis of the epithelium. Both of these lesions have a very irregular shape. The heterogenic form of leukoplakia is clinically similar to the erosive form of LP, so biopsy and histopathological examination are still the golden standard during the diagnosis process.

The clinical perspective focuses on the importance of a noninvasive, accurate and timely diagnosis. Such attempts have been made in dermatology and oral surgery, leading to the conclusion that manual segmentation by general practitioners is feasible in the described computer-aided diagnostic system for classifying benign and malignant skin lesions.[5,6] To date, no studies have been published on that topic for oral mucosa diagnosis and automated image segmentation.

Texture analysis is used during computed tomography (CT) analysis in the case of bone and soft tissue lesions, but there are no publications about the application of texture analysis in the differential diagnosis of leukoplakia vs LP in the oral mucosa.[7,8]

The aim of this study is to attempt to formulate a differential diagnosis of leukoplakia vs LP in the oral mucosa based on digital texture analysis in intraoral macrophotography.

Material and methods

Patients

Twenty-one patients affected by leukoplakia (11 females and 10 males) and 21 affected by LP (16 females and 5 males) were included in this study. All lesions were histopathologically verified (with standard hematoxylin and eosin (H&E) staining) on specimens taken from pathological oral mucosa under local anesthesia. The control group consisted of 21 healthy volunteers. The mean age of the study group was 58 years.

Intraoral photography of normal oral mucosa, LP and leukoplakia were taken with a Canon EOS 500D digital camera (Canon, Ōta, Tokyo, Japan) with a 13 mm macro ring and a 50 mm lens at f1.8 (Canon), and a YN-14EX ring flashlight (Yongnuo Photographic Equipment, Shenzhen, China).

All procedures were conducted after obtaining the approval of the Ethics Committee of Wrocław Medical University, Poland (approval No. KB-367/2014).

Image preprocessing

All of the graphical operations were performed in GIMP v. 2.10.8 (GNU Image Manipulation Program; www.gimp.org). In the center of the lesion, a square 300 × 300 pixels in size was selected. These selected portions were cropped from the original photos. To achieve the maximum possible contrast, a high-pass filter was applied and level tools were then used to equalize the histograms of the images. After that, the images were converted into 8-bit grayscale. The files were saved in TIFF format without any compression algorithms. All graphical operations are presented in Fig. 1, while Fig. 2 shows the clinical photographic material of leukoplakia, LP and normal mucosa.

Texture analysis

If $p(i,j)$ is the number of times when there is a run of length j with a gray level of i, Ng is the number of gray levels and Nr is the number of runs,[9] then definitions of the parameters of the run-length matrix $p(i,j)$ are given below.

Long run emphasis inverse moments (LngREmph):

$$LngREmph = \left(\sum_{i=1}^{N_g}\sum_{j=1}^{N_r} j^2 p(i,j)\right) \Big/ C$$

Short run emphasis inverse moments (ShrtREmph):

$$ShrtREmph = \left(\sum_{i=1}^{N_g}\sum_{j=1}^{N_r} \frac{p(i,j)}{j^2}\right) \Big/ C$$

The coefficient C:

$$C = \sum_{i=1}^{N_g}\sum_{j=1}^{N_r} p(i,j)$$

The second-order histogram is known as the co-occurrence matrix $hd\theta\ (i,j)$.[10] When divided by the total number of neighboring pixels $R(d,\theta)$ in region of interest (ROI), this matrix becomes an estimate of the joint probability – $pd\theta\ (i,j)$ – of 2 pixels, a distance d apart along a given

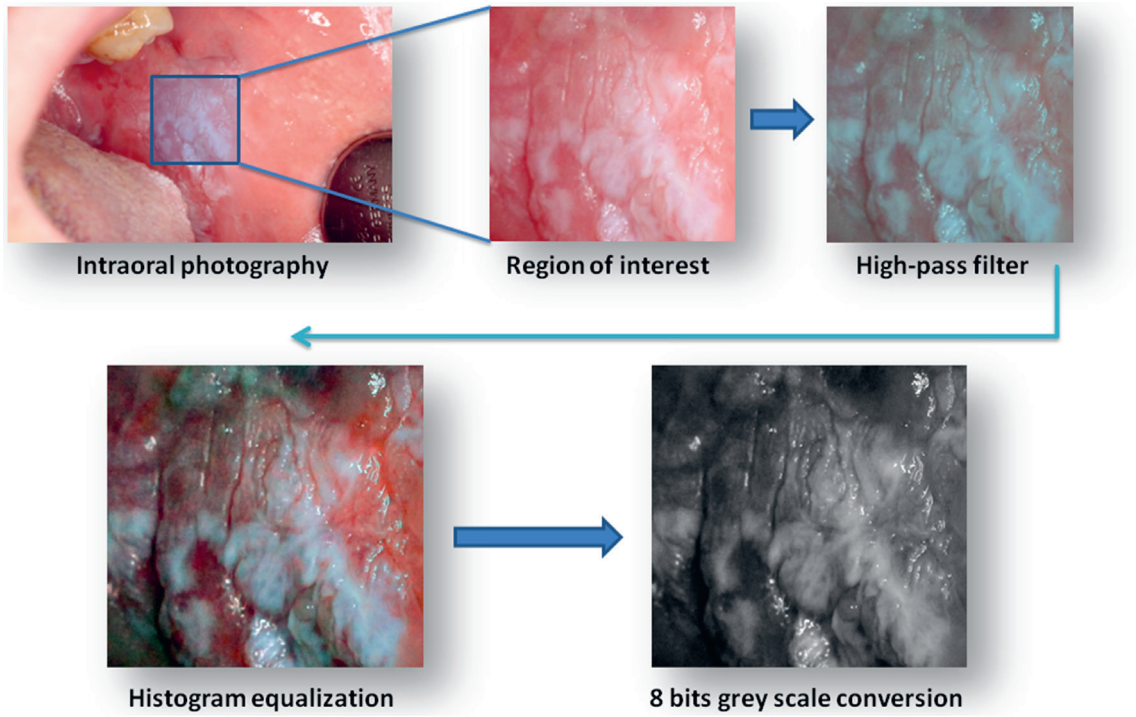

Fig. 1. Graphical operations performed despite a simple white appearance under visible light

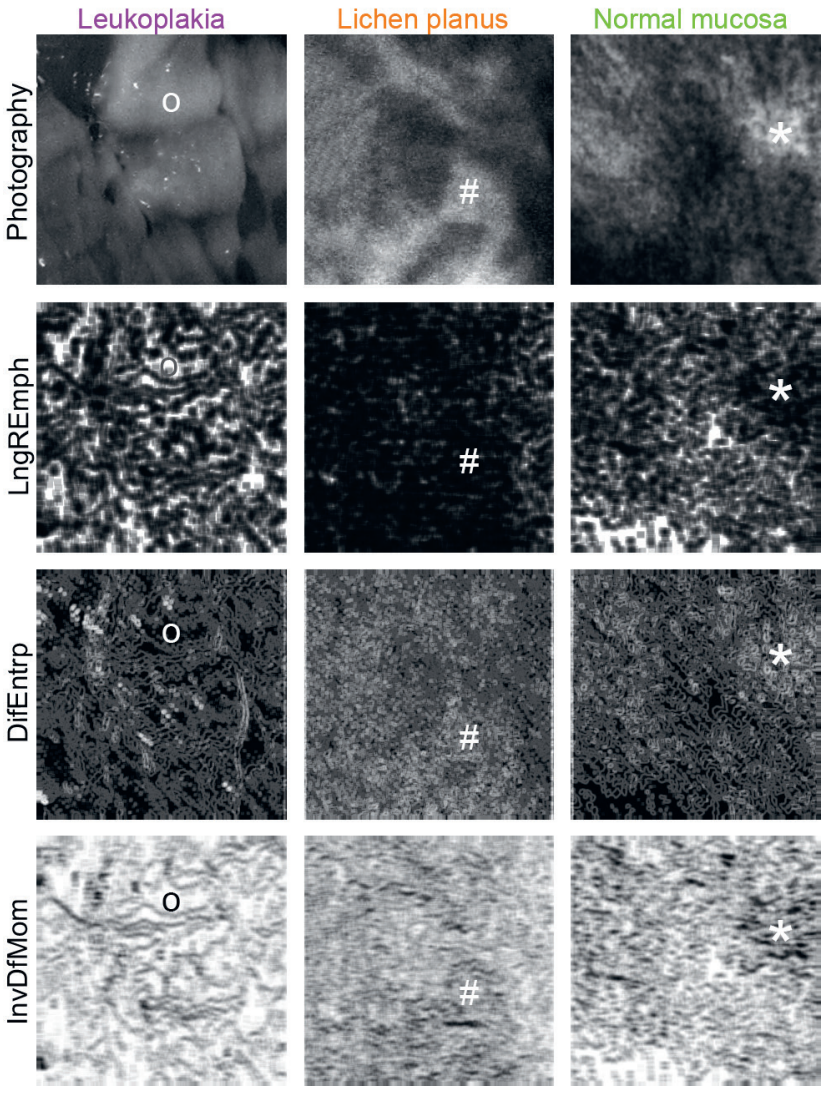

Fig. 2. Clinical photographic material of leukoplakia, lichen planus (LP) and normal mucosa

Examples of white regions visible in the intra-oral photographs are marked with O, # and *. LngREmph – long run emphasis inverse moments map describing white areas which present a high value of this texture parameter, i.e., many long lines of pixels in the same shade of grey. DifEntrp – difference entropy map indicating the area (white) where the original image presents a very chaotic/fine texture (*). As the pathology develops, the entropy decreases (less intense, i.e., a darker appearance on the map): a minor decrease in LP (#) and a major decrease in leukoplakia (O). InvDfMom – inverse difference moment map describing white areas which present a monotonic texture, contrary to black areas where the original image has a rich texture, i.e., a fine structure; that fine structure is lost in leukoplakia and LP. Note: texture analysis of the observed white regions in normal mucosa photography (*) reveals different InvDfMom results (less intense) than white regions in leukoplakia (O) and LP.

direction θ, having the particular (co-occurring) values i and j. Formally, given the function $f(x,y)$ with a set of Ng discrete intensity levels, the matrix $hd\theta\,(i,j)$ is defined such that its (i,j)th entry is equal to the number of times that

$$f(x_1, y_1) = i \text{ and } f(x_2, y_2) = j$$

where $(x_2, y_2) = (x_1, y_1) + (\mathrm{d}\cos\theta, \mathrm{d}\sin\theta)$.

This yields a square matrix whose dimension is equal to the number of intensity levels in the image, for each distance where d = 5 pixels and orientation with angles θ = 0°, 45°, 90°, and 135° (such angles are considered and then their average is calculated to combine spatial information into single number). Reducing the number of intensity levels (through quantization) helps to remove noise, with some loss of textural information (as low as 4-bit in this case). The co-occurrence matrix-derived parameters are defined by the equations that follow, where $px(i)$ and $py(j)$ are the marginal distributions.

Difference entropy (DifEntrp):

$$DifEntrp = -\sum_{i=1}^{N_g} p_{x-y}(i)\log\left(p_{x-y}(i)\right)$$

Inverse difference moment (InvDfMom):

$$InvDfMom = \sum_{i=1}^{N_g}\sum_{j=1}^{N_g}\frac{1}{1+(i-j)^2}p(i,j)$$

The calculations were performed in Mazda v. 4.6 software (Lodz University of Technology, Poland) on selected features.[11–15] The differences among the 3 study groups (normal mucosa, LP and leukoplakia) were checked using one-way analysis of variance (ANOVA). Next, in order to obtain the single factor which would account for most of the variability among the 2 variables (difference entropy and inverse difference moment) factor analysis (FA) was done. The principal component method was used. Thus, the initial communality estimates were set to assume that all of the variability in the data is due to this common factor. This factor was named the co-occurrence factor due to inner information extracted from 2 co-occurrence matrix features.

The efficacy of automated diagnosis was checked using a neural network Bayesian classifier (Fig. 3). This procedure used a probabilistic neural network (PNN) to classify cases into different diagnoses, based on 3 input variables: long run emphasis inverse moments, short run emphasis inverse moments and the co-occurrence factor. All of the statistical analyses were performed with Statgraphics Centurion XVI software (StatPoint, The Plains, USA).

Results

No differences between LP and leukoplakia were found in any of the investigated features. Both lesions presented very significant differences from the reference oral mucosa in difference entropy and inverse difference moment (Fig. 4). The results are presented in Table 1.

In the factor analysis one factor was extracted (given name: co-occurence factor), since only 1 factor had

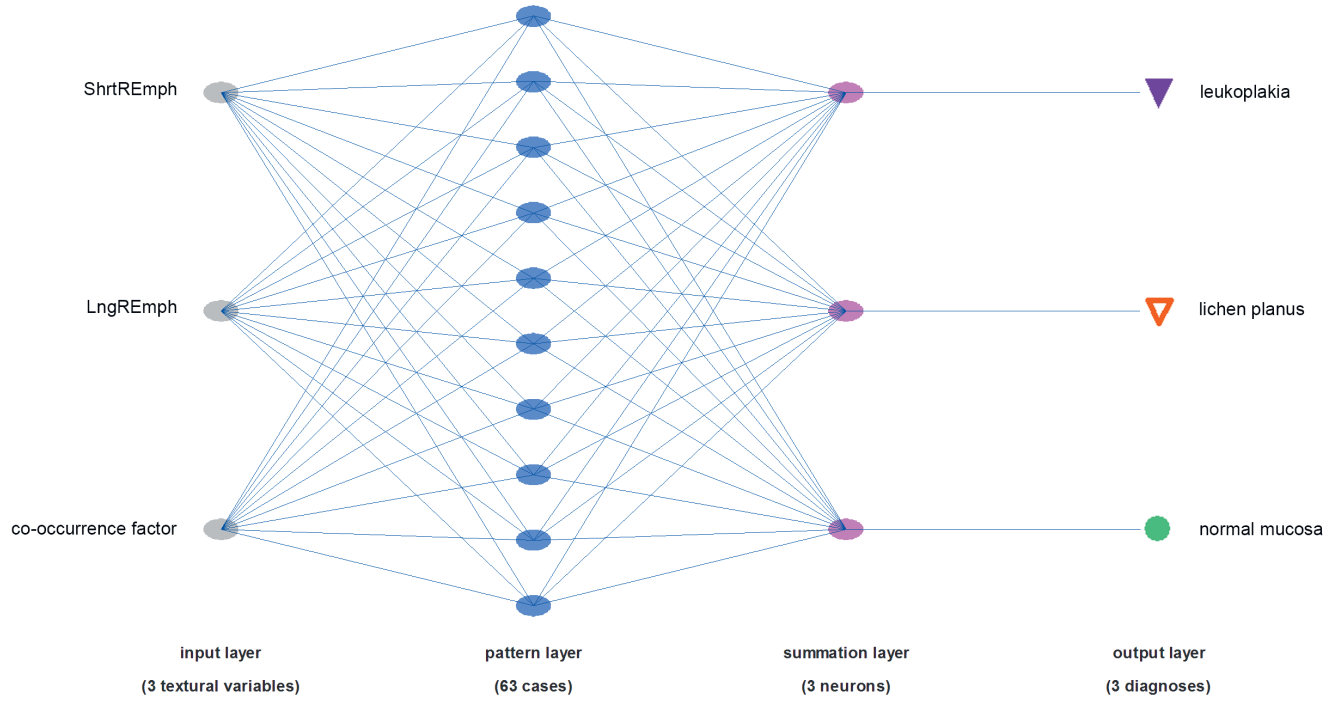

Fig. 3. Schemes of the artificial neural network (ANN) used for automated differential diagnosis of leukoplakia, lichen planus (LP) or normal mucosa in intra-oral photographic imagery. Input data include run length matrix features (ShrtREmph and LngREmph) and co-occurrence matrix features (1 factor extracted from 2 strong textural features: difference entropy (DifEntrp) and inverse difference moment (InvDfMom); the factor name is named the co-occurrence factor)

Table 1. Summary statistics of textural features in normal oral mucosa, lichen planus and leukoplakia lesions. The 2 pathological lesions cannot be diagnosed differentially from one another

Textural feature	Normal mucosa	Lichen planus	Leukoplakia lesion	ANOVA difference of both lesions vs normal oral mucosa
Long run emphasis inverse moment (LngREmph)	10.84 ±13.86*	18.11 ±18.32*	13.84 ±9.42*	p = 0.2636
Short run emphasis inverse moment (ShrtREmph)	0.61 ±0.19*	0.58 ±0.09	0.56 ±0.09	p = 0.3845
Difference entropy (DifEntrp)	0.62 ±0.10	0.53 ±0.07	0.50 ±0.07	p < 0.0001
Inverse difference moment (InvDfMom)	0.53 ±0.08	0.64 ±0.07	0.64 ±0.06	p < 0.0001
Co-occurrence factor#	0.09 ±0.17	−0.11 ±0.14	−0.14 ±0.13	p < 0.0001

No differences were found between lichen planus and leukoplakia; ANOVA – analysis of variation; * lack of normal distribution; # factor composed of difference entropy and inverse difference moment.

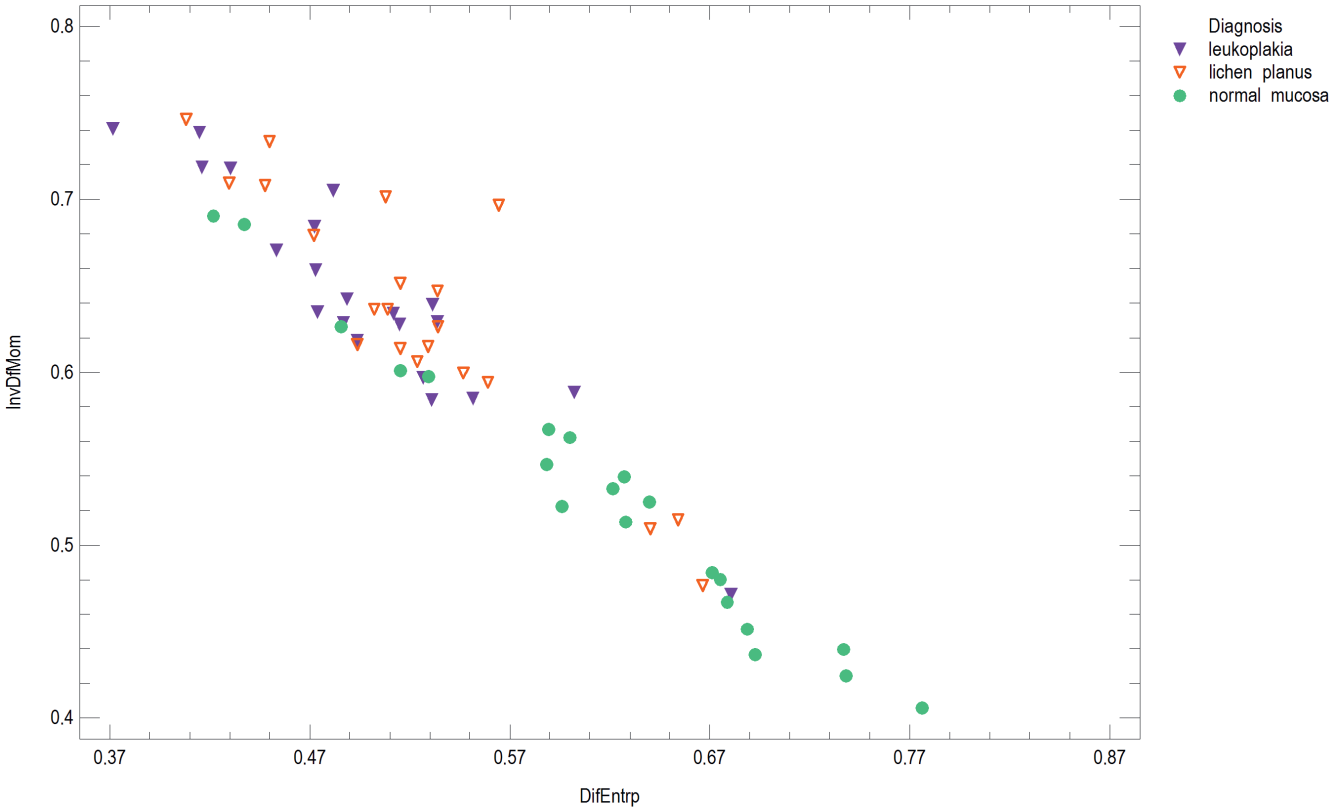

Fig. 4. The co-occurrence matrix parameters used in this study were difference entropy (DifEntrp) and inverse difference moment (InvDfMom). Both parameters clearly differentiate pathological lesions from normal mucosa ($p < 0.0001$), but cannot help in distinguishing leukoplakia from lichen planus (LP) (Table 1)

an eigenvalue greater than 1.0. This factor accounted for 97.9% of the variability of difference entropy and inverse difference moment (InvDfMom) in the original data. Both textural features can be used very reliably ($p < 0.0001$) for diagnosing leukoplakia and LP of the oral mucosa. The equation of the calculated factor is:

$$\text{Co} - \textit{occurrence factor} = \\ = 0.989253\ \textit{DifEntrop} - 0.989253\ \textit{InvDfMom},$$

where the values of the variables of the equation are standardized by subtracting their means and dividing by their standard deviations (SDs). It also shows the estimated communalities, which can be interpreted as estimating the proportion of variability in each variable which is attributable to the extracted factor. Both types of lesions showed a highly significant difference from the reference mucosa as far as this factor was concerned (Table 1).

Out of the 63 cases in the training set, 61.9% were correctly classified using the neural network (Table 2). The reference oral mucosa was correctly classified in 90% of cases, LP in 38% and leukoplakia in 57%. Based on the PNN, a correlation between short run emphasis inverse moment (ShrtREmph) and the co-occurrence factor was found (Fig. 5). Generally speaking, negative values of the co-occurrence factor combined with values of ShrtREmph higher than 0.3 describe a pathological lesion. When the ShrtREmph is 0.3–0.8 and the co-occurrence factor is –0.2, the lesion in the image should be classified as leukoplakia.

Table 2. Results of probabilistic neural network (PNN) classifying cases into different diagnoses (normal oral mucosa, lichen planus and leukoplakia), based on 3 input variables: long run emphasis inverse moments, short run emphasis inverse moments and co-occurrence factor. Percent of training cases correctly classified: 61.9%

Actual diagnosis	Group size	Predicted leukoplakia	Predicted lichen planus	Predicted normal mucosa
Leukoplakia	21	12 (57.14%)	7 (33.33%)	2 (9.52%)
Lichen planus	21	10 (47.62%)	8 (38.10%)	3 (14.29%)
Normal mucosa	21	1 (4.76%)	1 (4.76%)	19 (90.48%)

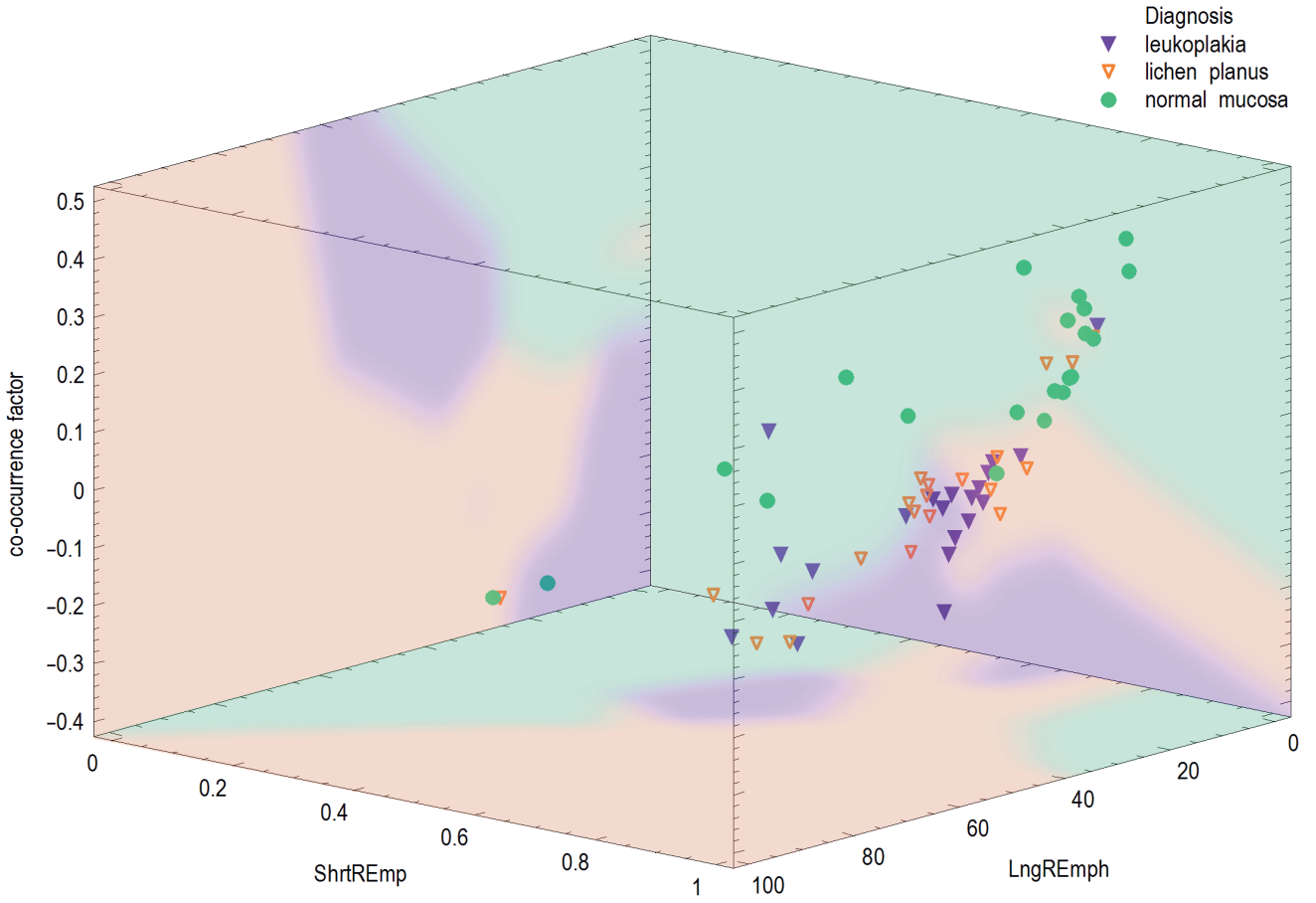

Fig. 5. The artificial neural network (ANN) is helpful in attempting to differentiate normal mucosa from pathological lesions and leukoplakia from lichen planus (LP). The points in the plot (inside the cube) represent real clinical cases classified by the ANN based on texture analysis. Two-dimensional neural network predictions are shown on the walls of the cube (there are no sharp borders due to the application of fuzzy logic in the neural network mathematical engine): the purple surface area represents a diagnosis of leukoplakia, the orange surface area of LP and the green surface area of normal mucosa. Negative values of the co-occurrence factor combined with values higher than 0.3 of short run emphasis inverse moments (ShrtREmph) predicts a pathological lesion. When ShrtREmph is 0.3–0.8 together with a value of less than –0.2 of the co-occurrence factor, the image should be considered to show leukoplakia. A positive value of the co-occurrence factor together with a low value of long run emphasis inverse moments (LngREmph) — approx. 0–40 – indicates normal oral mucosa. Lichen planus on that plane (left side wall) is presented when a higher value of LngREmph is noted. The extreme values of ShrtREmph (especially the lower extreme) combined with low values of LngREmph suggests normal mucosa (bottom of the cube). When the LngREmph value increases, a diagnosis of a pathological lesion should be strongly considered (generally LP pathology when LngREmph >50)

The sensitivity for leukoplakia detection was 57%, for LP detection it was 38% and for normal mucosa detection 94%. The specificity of leukoplakia detection was 74%, of LP 81% and of normal mucosa 88%.

Discussion

Many parameters have been proposed to describe the microstructures found in medical scans. Fractal dimensions have been applied in the investigation of oral mucosa pathology, while run-length and co-occurrence matrices have been used in osteoporosis studies to describe bone before oral surgery.[14,16–19] Due to the lack of publications on pathological oral mucosa lesion diagnosis with texture analysis, the 4 features previously used in bone healing research were introduced into this study.[20,21]

In leukoplakia, the normal, fine differences in brightness disappear and develop homogeneous white plates (Fig. 1). In LP, that process is similar, but the value of InvDfMom is lower (closer to normal mucosa) due to the pathological structure of the lesion, i.e., a mesh rather than a plate

(Fig. 3). It seems that InvDfMom is an appropriate measure of pathological lesion creation in the oral mucosa which can be detected even in visible light. Obviously, automated differential diagnosis between LP and leukoplakia is still a challenge. Advanced mathematical techniques (PNN or FA) are only somewhat helpful.

Nowadays, digital light image analysis can be used to recognize a pathological lesion in the oral mucosa as presented above. When negative values of the co-occurrence factor are found along with higher values of ShrtREmph in imagery of the oral mucosa, then the physician should suspect a precancerous lesion in the area (Fig. 4).

This study indicates the need of search for alternatives to ensure proper access to healthcare and in partnership with non-specialized doctors from different macroregions of whole country. This research demonstrates the importance of using telemedicine, since it is a diagnostic method that allows for the early detection of oral pre-malignancy lesions, thus decreasing the number of unnecessary referrals to general dentists.[22] Therefore, it helps to reduce not only wait times for face-to-face consultations, but also the costs associated with this process.

Many non-invasive systems such as ViziLite®, ViziLite®PLUS, Velscope®, and Identafi® are available to detect precancerous lesions.[23] These systems are based on fluorescence or autofluorescence of suspicious lesions. Jain et al. confirmed that a method of ViziLite®PLUS examination was most effective in cases of leukoplakia in assessing the size, borders and shape of the lesion, followed by toluidine blue and incandescent light examinations. Methods using toluidine blue and ViziLite®PLUS examination demonstrated a sensitivity of 100% and a specificity of 97.3%.[24] Pallagatti et al. used toluidine blue to detect suspicious lesion staining in in vivo dysplastic cells. Toluidine blue is an acidophilic dye that selectively stains acidic tissue components such as DNA and RNA. Dysplastic lesions and in situ carcinomas contain much more DNA and RNA than the normal surrounding epithelium, so the use of in vivo toluidine blue staining may indicate premalignant or malignant lesions.[25]

Lalla et al. detected oral epithelial dysplasia using reflectance spectroscopy (Identafi, DentalEZ). Their results show that a system using violet light offered a sensitivity of 12.5% and a specificity of 85.4% for detecting oral epithelial dysplasia.[26] Sambandham et al. applied a ViziLite® system to leukoplakia diagnosis. Their study shows that the sensitivity and specificity of ViziLite® are about 77.3% and 27.8%, respectively.[27] McIntosh et al. used a Microlux/DL system (AdDent Inc, Danbury, USA) for leukoplakia diagnosis and reported a sensitivity of 77.8% and a specificity of 70.7%.[28] Ibrahim et al. found that the sensitivity was 100% and the specificity was 32.4% in the visualization of suspicious premalignant lesions using Microlux/DL.[29] In our study, the sensitivity of leukoplakia detection was 57%, which was lower than in the abovementioned results, but the specificity of 74% was significantly higher than the ViziLite® application by Sambandham et al.

Honsi et al. used image cytometry to determine DNA ploidy in LP. The most common degree of DNA ploidy in LP lesions was diploidy. Comparing the 2 groups (χ^2 test of association, $p = 0.021$), they demonstrated that diploidy was associated with the reticular clinical form of LP, while aneuploidy was associated with the atrophic-erosive clinical form of oral LP.[30]

All of the abovementioned diagnostic methods are helpful in detecting LP and leukoplakia, but none of them is able to distinguish between these 2 types of lesions. Differential diagnosis of leukoplakia and LP in oral mucosa based on digital texture analysis in intraoral macrophotography is possible. Moreover, it can be used to develop computer/smartphone applications and can also be a helpful tool for general dentists to define the clinical problem before consultation with oral and maxillofacial surgeons.

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The effectiveness of whole-body cryotherapy and physical exercises on the psychological well-being of patients with multiple sclerosis: A comparative analysis

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Abstract

Background. Due to the chronic character of multiple sclerosis (MS), non-pharmacological treatment can be applied. These therapies can be a good complementation to standard pharmacological treatment.

Objectives. The aim of the study was to evaluate the effectiveness of whole-body cryotherapy (WBC) and physical exercise training on the psychological and general well-being of patients with MS.

Material and methods. The study was carried out on 60 patients, who were divided into 3 groups: cryotherapy (Cryo), physical exercise training (Gym) and cryotherapy with physical exercise training (CryoGym). The Psychological General Well-Being Index, the Hospital Anxiety and Depression Scale and the Rivermead Mobility Index were used at 2 points in time: T1 – before the first therapy session and T2 – after 14 days of therapy.

Results. Statistically significant differences in the psychosocial well-being were found in the Gym and CryoGym group. Reduction of depressive symptoms and improved functional status was noted in Cryo group. The most significant improvement was observed in the group using WBC with exercise training (CryoGym).

Conclusions. Whole-body cryotherapy with physical exercise training was an effective therapy for patients with MS. The introduction of WBC into the standard physiotherapy protocol for patients with MS is fully justified.

Key words: multiple sclerosis, physical exercise, functional status, psychological well-being, whole-body cryotherapy

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Introduction

Multiple sclerosis (MS) is an autoimmune demyelinating disease of the central nervous system. It causes focal white matter injuries. The underlying mechanisms are still unclear, although the destruction of the immune system or myelin-producing cells failure caused by genetic and environmental factors are mainly considered. Demyelination of nerve fibers slows conduction by up to 20 times and may lead to a complete conduction block in advanced stages.[1]

The polymorphism, unpredictability, chronic nature, specificity of first symptoms and a varying episodic course of deterioration in MS all lead to feelings of powerlessness, helplessness, confusion, and loneliness among the patients. Existing studies point out the importance of patient self-perception in MS. They often have low self-esteem and self-acceptance as well as a negative self-rating. This is caused not only by the progressing impairment of movement, but also by the patients' emotional disorders and the inability to cope with the disease.[2]

Approximately 40% of patients develop depressive symptoms that require treatment with antidepressants. Perception of the health status is the principal predictor of depressive symptoms.[3] Research confirms that depressive disorders are usually mild in their early stages. Some reports indicate that the pathogenesis of depressive symptoms in MS may be organic. It has been shown that depression is more common in patients with lesions in the brain than in those with lesions in the spinal cord and cerebellum.[4]

The nature of the disease poses a challenge to doctors. The unpredictability of MS and a poor recovery prognosis make the planning and treatment process very difficult. It is, therefore, important to not only focus on the physical dysfunctions, but to maintain a balance between the physical and the psychological health of the patient. The positive attitude of the patients as well as their motivation and willingness to cooperate can positively influence the treatment process and their quality of life.

Physical exercise training is often used in the management of MS. Studies have shown that properly prescribed exercise programs can improve modifiable impairments in MS.[5] Exercise is generally safe and well-tolerated.[6]

Whole-body cryotherapy (WBC) can support and complement primary treatment and facilitate physical activity.[7] In recent years, the use of WBC has gained recognition as a treatment option. The physical, psychological and clinical effect of cryostimulation is unique and cannot be compared with any other body-cooling therapy.[8] Patients with MS subjected to cryogenic temperatures have shown an improvement in physical activity and experienced a reduction in spasticity and pain. Cryotherapy is a factor that improves the functional capacity of MS patients.[9,10] Whole-body cryotherapy was successfully used in patients with MS to improve psychological well-being, but there are limited literature reports on the beneficial psychological effects of cryogenic temperatures.[9,11]

Therefore, the aim of the study was to evaluate the effectiveness of WBC and physical exercise training on the psychological and general well-being of patients with MS.

Material and methods

The study was carried out among patients diagnosed with MS. Participants were recruited by a neurologist. The study group included 60 patients, consecutively admitted to a neurologist, who met the following inclusion criteria: patient's written consent to participate in the study, physician's consent – no contraindications to participate in the study, especially no contraindications for the WBC (anemia, hypothyroidism, asthma, heart defects, impaired blood flow and heart rhythm, angina, untreated hypertension), MS in remission (at least 6 months since the last relapse), 0–6 points in the Expanded Disability Status Scale – EDSS (patient's independence in mobility and locomotion), and no other physiotherapy during the course of the study. The following exclusion criteria were also applied: progressive type of the disease, hemodynamic instability immediately prior to each WBC session, previous exposure to WBC, diagnosis and pharmacological treatment of mental disorders, and participation in any clinical test.

Participants were informed about the purpose of the study, the rules for participation and the possibility to withdraw at any stage of the study without consequences.

An approval from the Bioethics Committee of the University School of Physical Education in Wrocław was obtained.

Participants were randomly divided into 3 groups:

– Group 1 (CryoGym): 20 patients who had WBC and, immediately after leaving the cryo-chamber, participated in physical exercise training using the Thera-Band.

– Group 2 (Cryo): 20 patients who had WBC.

– Group 3 (Gym): 20 patients who participated in physical exercise training (resistance training using the Thera-Band).

Additionally, participants who had WBC were informed of the WBC procedure in the presence of a medical doctor. Hemodynamic parameters were analyzed in all the participants immediately prior to each WBC session. The cryotherapy chamber accommodated 5 patients at a time. They stayed in the vestibule with the physiotherapist for 30 s in order to adapt to the cryogenic temperature. Then, they entered the main chamber alone. The temperature was maintained at –110°C during the first session and reached –160°C on the last day of the study. The sessions were planned at a fixed time from Monday to Friday during a 2-week period. There were 10 sessions during one protocol.[12,13]

The physical exercise training (in groups 1 and 3), which aimed at strengthening muscles of the lower limbs, lasted

60 min per session. Each session began with a warm-up (10 min). It was followed by the main exercise (40 min of resistance exercises using the Thera-Band in closed kinetic chains) and a cool-down period to reduce the heart rate (10 min). Patients exercised in a lying position, gradually going to sit and standing position. Elastic bands with medium resistance (red) were used. The direction of the resistance was determined exactly. At 90° between the tape and the part of the body being trained, the resistance was maximal. Below the angle of 30° the resistance was minimal. Intensity in the range of a maximum of 8–15 repetitions was recommended. The number of sets was in the range 1–3. Exercises were performed to obtain a subjective feeling of fatigue (about 5–6 according to the 10-degree Borg scale). No pain reactions could occur during exercising. Rest periods between sets and exercises in the range of 2–4 min were recommended.[14]

Both forms of therapies (WBC and physical exercises) in all groups were carried out under the supervision of a medical doctor and a physiotherapist.

The study was conducted at 2 points in time: T1 – before initiating each of the 3 forms of therapy and T2 – 14 days after commencing the therapy and 2 days after finishing the last session, in order to minimize the impact of fatigue after exercise or euphoria and mood improvement immediately after leaving the cryochamber.

The structure of the 3 groups was similar and the characteristics of them are shown in Table 1.

The following research tools were used in the study: The Psychological General Well-Being Index (PGWBI) consists of 22 questions that assess the subjective psychological well-being and stress level of the patients within 6 subscales – anxiety, depression, positive well-being, self-control, general health, and vitality. The more points gained, the better the quality of life of a patient.[15]

The Hospital Anxiety and Depression Scale (HADS) consists of 2 independent subscales that assess anxiety (HADS-A) and depression (HADS-D). Each subscale consists of 7 items. Values 0–7 indicate normal levels of anxiety and depression, values 8–10 mean mild level of anxiety and depression, while values 11–21 suggest the probability of an anxiety or depressive disorder.[16]

The Rivermead Mobility Index (RMI) assesses patient's mobility and locomotion. The more points gained, the better the functional state of a patient.[17,18]

Statistical analysis

The following descriptive statistics were applied to the characteristics of the study group: mean, standard deviation, variables, numbers and percentages. The Shapiro–Wilk test was used to check for normal distribution

Table 1. The characteristics of each studied subgroup

Variables	Total	Groups			Kruskal–Wallis/ Pearson's χ^2
		Gym (n = 20)	Cryo (n = 20)	CryoGym (n = 20)	p-value
Age mean (SD)	49.4 (12.2)	53.3 (13.5)	45.8 (10.1)	48.8 (12.2)	0.1555
Body mass mean (SD)	70.0 (14.2)	71.7 (13.6)	68.6 (16.8)	69.6 (12.7)	0.6809
Height mean (SD)	166 (8.5)	164 (8.4)	167 (9.3)	167 (7.7)	0.3392
BMI mean (SD)	25.3 (4.0)	26.7 (4.1)	24.3 (4.1)	24.9 (3.6)	0.1449
Duration of disease from onset of symptoms [years] mean (SD)	20.3 (9.1)	21.1 (10.3)	17.9 (9.1)	24.3 (3.5)	0.5653
Gender, n [%] women men	43 (100) 16 (100)	15 (35) 5 (31)	11 (26) 8 (50)	17 (39) 3 (19)	0.1580
Marital status, n [%] married single/widow(er)	42 (100) 17 (100)	14 (33) 6 (35)	13 (31) 6 (35)	15 (36) 5 (30)	0.4400
Occupation, n [%] employed retired/disability pensioners unemployed	25 (100) 21 (100) 8 (100)	7 (28) 9 (42) 3 (37.5)	10 (40) 6 (29) 2 (25)	8 (32) 6 (29) 3 (37.5)	0.4540
EDSS mean (SD)	2.4 (1.9)	2.35 (1.9)	2.3 (1.7)	2.4 (2.1)	0.9992
Number of comorbidities mean (SD)	0.7 (0.8)	0.9 (0.9)	0.3 (0.5)	0.8 (0.8)	0.0940

SD – standard deviation; EDSS – Expanded Disability Status Scale.

of the data. The data was non-normally distributed; therefore, the Kruskal–Wallis and the χ^2 tests were used to compare the 3 groups. The effectiveness of the treatment was assessed by comparing variables measured at 2 time points (T1 and T2) and was determined with the Wilcoxon test. Statistical tests were verified at the 0.05 level of significance.

Results

There were no differences among the groups in terms of age, gender, marital status, occupation, body mass, height, BMI, EDSS, number of comorbidities, and the duration of the disease from the onset of symptoms (Table 1). No side effects after using WBC or physical exercises were observed.

There were no differences between groups at baseline in the general well-being. After therapy a statistically significant difference in the psychosocial well-being (between T1 and T2) was found in the Gym and CryoGym groups (Table 2).

There were also no differences between groups at baseline in the HADS, despite differences in the depressive subscale (HADS-D). A statistically significant reduction in the severity of anxiety symptoms between the T1 and T2 was found in the CryoGym group. Additionally, a significant reduction in depressive symptoms over a period of time was noted in the CryoGym and Cryo groups. Statistically significant differences were not noted in the remaining group (Table 3).

There were also no statistically significant differences between groups at baseline in the RMI. A statistically significant improvement in the functional status between the T1 and T2 was noted in the Cryo group (Table 4).

Results of the changes between T1 and T2 in different form of therapies among studied groups were summarized in Table 5.

Table 2. Comparison of the PGWBI between T1 and T2 in each group (Wilcoxon test) and between groups in T1 and T2 time point (Kruskal–Wallis test)

Groups	Baseline (T1)	Follow-up (T2)	Wilcoxon test	
	median (quartiles)	median (quartiles)	Z	p-value
Whole group	66.0 (49.0–78.0)	72.0 (61.0–85.0)	–4.604	0.0000
Gym	64.0 (50.2–77.7)	73.0 (58.0–90.0)	–2.417	0.0155
Cryo	71.0 (56.0–84.0)	77.0 (65.0–88.0)	–1.911	0.0561
CryoGim	67.0 (40.2–70.5)	70.5 (61.0–77.5)	–3.528	0.0004
Kruskal–Wallis test H p-value	 3.473 0.1761	 1.641 0.4402		

PGWBI – Psychological General Well-Being Index.

Table 3. Comparison of the HADS between T1 and T2 in each group (Wilcoxon test) and between groups in T1 and T2 time point (Kruskal–Wallis test)

HADS	Groups	Baseline (T1)	Follow-up (T2)	Wilcoxon test	
		median (quartiles)	median (quartiles)	Z	p-value
HADS-A	whole group	7.0 (4.0–10.0)	6.0 (4.0–7.0)	–2.920	0.0035
	Gym	5.5 (4.0–8.0)	5.5 (2.0–9.5)	–0.970	0.3320
	Cryo	7.0 (4.0–8.0)	5.0 (4.0–7.0)	–1.499	0.1336
	CryoGym	9.0 (5.0–13.0)	6.0 (5.0–7.0)	–2.556	0.0106
	Kruskal–Wallis test H p-value	 5.504 0.0638	 1.485 0.4759		
HADS-D	whole group	5.0 (3.0–8.0)	4.0 (2.0–6.0)	–3.031	0.0024
	Gym	4.0 (3.0–7.0)	4.0 (2.0–6.7)	–0.724	0.4691
	Cryo	4.0 (2.0–6.0)	4.0 (1.0–5.0)	–2.045	0.0409
	CryoGym	7.0 (5.0–11.0)	6.0 (4.0–7.0)	–2.485	0.0128
	Kruskal–Wallis test H p-value	 6.288 0.0431	 3.998 0.1354		

HADS-A – Hospital Anxiety and Depression Scale-Anxiety, HADS-D – Hospital Anxiety and Depression Scale-Depression.

Discussion

Multiple sclerosis is a specific chronic disease which causes gradual physical status decline, emotional burden, poor overall well-being, and depression.

The analysis of the results showed that cryogenic temperatures have a large impact on mental and physical health, especially in improving patients' mood. The same results were noted in studies conducted by Rymaszewska et al. Cryostimulation led to an immediate improvement in the somatic and mental well-being and caused psychomotor relaxation in patients.[19,20] Authors emphasized that this can be partially explained by a significant increase in the concentration of beta-endorphins and testosterone in the hypothalamus-pituitary-adrenal axis.[21] It is established that MS has to be dealt with on both physical and psychological levels, so the planning and implementation of physiotherapy programs can be complemented by cryotherapy in order to be more effective.[22] This was also observed in the present study. Whole-body cryotherapy significantly reduced the perceived symptoms of depression and improved the patients' functional status. Short-term exposure to extremely low temperatures induces numerous neurotransmitter changes in the central nervous system.[21] The alleviation of depressive symptoms under

Table 4. Comparison of the (RMI) between T1 and T2 in each group (Wilcoxon test) and between groups in T1 and T2 time point (Kruskal–Wallis test)

Groups		Baseline (T1)	Follow-up (T2)	Wilcoxon test	
		median (quartiles)	median (quartiles)	Z	p-value
RMI	whole group	14.0 (11.0–15.0)	14.0 (13.0–15.0)	–3.254	0.0011
	Gym	12.0 (10.0–14.0)	13.5 (11.0–14.0)	–1.890	0.0587
	Cryo	14.0 (12.0–15.0)	15.0 (13.0–15.0)	–2.366	0.0180
	CryoGym	14.0 (12.2–15.0)	14.0 (13.0–15.0)	–1.750	0.0800
	Kruskal–Wallis test H p-value	 2.097 0.3504	 1.905 0.3858		

RMI – Rivermead Mobility Index.

Table 5. Summary of the results of the applied different forms of therapies in the studied groups

Variables	Groups		
	Gym	Cryo	CryoGym
PGWBI	0.0155	0.0561	0.0004
HADS-A	0.3320	0.1336	0.0285
HADS-D	0.4691	0.0409	0.0128
RMI	0.0587	0.0180	0.0800

PGWBI – Psychological General Well-Being Index; HADS-A – Hospital Anxiety and Depression Scale-Anxiety; HADS-D – Hospital Anxiety and Depression Scale-Depression; RMI – Rivermead Mobility Index.

extremely low temperatures may be associated with an increase in catecholamine levels in areas of noradrenergic neuron clusters. The results of studies conducted by Zagrobelny also support the noradrenergic "antidepressant" mechanism of action of WBC.[23] Studies indicate that in this process, hypothalamic structures are activated, and endogenous catecholamines, ACTH, cortisol, and beta-endorphins are released.[21] This can also explain the improved functional status of patients in our study. Patients felt an improvement in their physical performance after 10 days of a 3-minute WBC sessions. Low depression symptoms, good sleep and lower fatigue have an influence on physical well-being, motivation for life and contribute to a more active lifestyle. Such data can be used by physiotherapists working with patients with MS. The patient's approach and confidence in the used method may impact the patients' motivation and further rehabilitation process. Another result of cryogenic temperatures exposition is the sudden decrease of temperature of the skin and subcutaneous tissues. The temperature of muscles also decreases (although slower). Reducing pain is connected with reducing nerve conduction velocity, inhibiting nociceptors (responsible for the pain sensation), blocking C fibers (neurons responsible for pain sensations conduction) and reducing the release of pain mediators.[24–26] This effect is most likely achieved by slowing down nerve conduction and reducing the responsiveness of peripheral sensory-motor endings, including muscular tone receptors (the Golgi apparatus in the tendons and neuromuscular spindles in the muscles) as well as partial blockade of the motor plate and γ-motoneurons.[26,27] Therefore, it seems important to look for links between reducing pain (analgesic effect of WBC) and a more effective kinesiotherapy and increased motivation for exercise, which translates into improved psychophysical efficiency of MS patients.[28]

Physical activity, including exercise training, is safe and also very important for people with MS. Now, there is also evidence that at least some of the disability that occurs after MS is due to secondary deconditioning resulting from a sedentary lifestyle adopted because of the MS symptoms and not CNS damage alone.[29] Therefore, physical activity is necessary for maintaining well functional status and health in people with MS.[30] Maintaining physical function, increased social participation and feelings of self-management and control are the most commonly identified perceived beneficial consequences of physical activity and exercising.[5] People with MS participating in regular physical activity have favorable scores in fatigue, depression and quality of life, when compared to MS patients who do not participate in regular physical activity.[31]

The mechanism of the influence of physical activities on depression is especially essential, as it is based on psychological ("faith in yourself" and the conception of distracters) and biological (for example β-endorphin and thermogenic theory) theories.[32,33]

A lot of researchers found exercising to be beneficial and presented the advantages of regular physical activity on the patient psychological well-being.[5,29,34–37] The same results were noted in our study. We observed significant improvement in psychological well-being in the group with exercise training.

But the most significant improvement was noted in the group using WBC with exercise training (CryoGym). Whole-body cryotherapy with exercise training significantly reduced anxiety levels and depressive symptoms in patients with MS. Connection physical exercises and WBC was the most effective therapy in the presented study.

Disability, fatigue, depression, and anxiety are strongly and negatively associated with the perceived physical and mental health. A study by Szilasiova et al. demonstrates that anxiety and depression are the strongest predictors of mental health and are crucial in clinical practice.[38] Therefore, it is important to choose a rehabilitation program that will affect both the physical and psychological well-being of the patients.

Cross-sectional study analyses suggest that people who exercise regularly are less likely to develop depression.[39] This is also reflected in other studies which show that regular physical activity is positively associated with

a reduction in depressive symptoms and an increase in the quality of life of those patients.[40,41] Hence, combining physical activity and cryogenic temperatures seems to be the most effective method of improving the condition of patients with MS. The results of our study support this concept.

The present study has certain limitations, which include the size of the groups. The instrument used to measure symptoms of depression is a screening instrument and does not provide a medical diagnosis. The study certainly needs to be continued and confirmed on a larger group, and study tools should be supplemented with biochemical measurements.

Conclusions

In the presented study, WBC improved the psychophysical well-being of patients with MS. The use of WBC reduced anxiety and depressive symptoms in studied patients with MS, particularly when combined with physical exercise training. The introduction of WBC into the standard physiotherapy protocol for patients with MS is fully justified.

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Silicon intake and plasma level and their relationships with systemic redox and inflammatory markers in rheumatoid arthritis patients

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Abstract

Background. The nutritional significance of silicon for the human body is highlighted by a continually growing body of evidence. In conditions of excessive reactive oxygen species and upregulated immune response, silicon has been observed to provide benefits, but its role in redox and inflammatory status has not yet been examined in rheumatoid arthritis (RA).

Objectives. The aim of this study was to assess the relationship of silicon intake and plasma level to systemic indices of redox status and inflammation in patients with RA.

Material and methods. Silicon intake and plasma levels were measured in 115 RA subjects and 129 control subjects. Serum antioxidant and oxidant levels, antioxidant enzyme activity, and albumin, uric acid, TBARS, hs-CRP, and IL-6 levels were measured and compared to the intake and plasma levels of silicon.

Results. Silicon intake and plasma silicon levels were higher in RA subjects than in the controls. In the RA group, a generally favorable correlation to redox and inflammatory markers was found for silicon in diet and in plasma; however, albumin level, smoking status, and gender interfered with these results. In the control subjects, a significant relationship was observed only between plasma silicon and non-enzymatic markers of redox status.

Conclusions. There are suggestions of silicon's involvement in managing redox and inflammatory status in RA, though further studies are warranted.

Key words: silicon, redox status, inflammatory biomarkers, rheumatoid arthritis

Introduction

A growing body of evidence suggests that silicon is essential to the human body, supported recently by the identification of the cellular silicon influx and efflux system.[1,2] Orthosilicic acid diluted in water and soluble dietary silicates are both easily absorbed by the intestines. Solid plant foods, such as cereals and certain vegetables, provide silicon in the form of the less absorbable phytolytic silica, but the high silicon content nevertheless makes them important dietary sources of this element.[3,4] The food industry utilizes silicon compounds as food additives, in the form of food-grade amorphous silica, silicates, and polymethylosiloxane, which can substantially increase the amounts of silicon in one's diet.[5] The body retains silicon mainly in connective tissues, including bone, cartilage, skin, and the aortal wall.[1] Orthosilicic acid circulating in the blood is thought to be predominantly unbound to protein.[6]

Silicon has been shown to stimulate collagen synthesis, to promote osteoblast differentiation, and to improve bone density and turnover. Silicon deficiencies may lead to abnormalities in the connective tissue and articular cartilage in rats. Serum silicon concentration positively correlates with collagen concentration in the skin and cartilage.[6] The protective role of silicon in the development of cognitive impairment and atherosclerosis has also been shown, although the latter remains controversial.[1] Studies on silicon-deprived animals suggest a contribution of silicon to immune and inflammatory responses.[7,8] In murine macrophages, silicon in the form of sodium metasilicate demonstrated the ability to reduce nitric oxide generation and to inhibit the gene expression of tumor necrosis factor-α and cyclooxygenase-2.[9] Redox status has also been shown to improve in silicon-supplemented rats with non-alcoholic steatohepatitis, including enhanced gene expression of liver antioxidant enzymes and lowered glutathione persulfide levels.[10] It should also be mentioned that the role of silicon in enhancing antioxidant capacity, including antioxidant enzyme activity, has been confirmed for plants exposed to abiotic stress.[11] On the other hand, hypersilicemia induced in rats by an excessive intake of metasilicate nonahydrate led to the accumulation of oxygen free radicals in the liver and kidneys and to the inhibition of superoxide dismutase and glutathione peroxidase.[12]

So far, no association of dietary silicon with inflammatory autoimmune diseases has been reported. Only exposure to non-dietary silicon, e.g., crystalline silica inhalation or silicone implants in the body has been found to increase autoimmune disease risk in humans, including RA.[13,14] Dietary silicon at a physiological level has in turn shown an immunomodulatory effect in rats with arthritis induced by collagen type 2 injection.[8] Rheumatoid arthritis is a chronic syndrome characterized by non-specific inflammation of mainly the peripheral joints, leading to the progressive destruction of cartilage and bone. The generation of reactive oxygen and nitrogen species at the site of inflammation has been shown to contribute to disease development.[15] It has previously been reported that an impaired ability to remove oxidative stress in RA results in a disrupted systemic redox status.[16] The beneficial activity of soluble silicon forms under excessive reactive oxygen species (ROS) and an upregulated immune response, as observed in biological fluids, cell lines, and animal models, raising the question of whether dietary intake of silicon and plasma silicon level influence systemic redox and inflammatory status in patients with RA.

Therefore, the aim of this study was to evaluate the silicon intake and plasma level in RA subjects and controls; moreover, the goal was to analyze the relationship of dietary and circulating silicon levels to selected serum indices of antioxidant, oxidant, and inflammatory status as measured in RA patients and compared to control subjects.

Subjects and methods

Patients with rheumatoid arthritis and the controls

This study included 115 RA patients who were enrolled in the study via the outpatient clinic of the Department of Rheumatology and Internal Medicine of Wroclaw Medical University; all of them fulfilled the American College of Rheumatology criteria.[17] The control subjects comprised 129 healthy people recruited from public offices and Wrocław's 3rd Age Universities. The exclusion criteria for the control group were chronic pro-inflammatory diseases and mental health issues. The study protocol was approved by the Wroclaw Medical University Ethics Board (consent No. KB-390/2012). All subjects gave written informed consent.

The RA study group underwent clinical examination by a rheumatologist in order to collect the following data: age, duration of disease, number of swollen joints, number of painful joints, the Disease Activity Score of 28 Joints (DAS 28), the presence of rheumatoid factor (RF) and anti-citrullinated protein antibodies (ACPA), and pharmacological treatment. In both groups, body mass index (BMI) was measured and declared smoking status was recorded. In all recruited subjects, an assessment of dietary intake and habits was performed by a trained dietician, and fasting blood samples were drawn on the day of the dietary interview. Serum samples were collected in order to assess systemic redox and inflammatory status. To measure silicon levels, K_2EDTA plasma samples were collected with the exclusive use of plasticware in order to prevent pre-analysis silicon contamination of the samples.

The 2 groups were matched in terms of age and cigarette smoking. The baseline characteristics of both groups are presented in Table 1.

Table 1. Group characteristics

Parameter		RA (n = 115)	Control (n = 129)	p-value
Sex of subjects, n (%)	female	91 (79.1)	82 (63.6%)	0.008
	male	24 (20.9)	47 (36.4%)	
Age [years], median (range) Q1–Q3		52 (18–82) 43–63	54 (25–79) 46–65	NS
BMI [kg/m^2], n (%)	<18.5	2 (1.7)	3 (2.3%)	NS
	18.5–24.9	48 (41.7)	44 (34.1%)	
	≥25	65 (56.5)	82 (63.6%)	
Cigarette smoking, n (%)	current smoker	33 (28.7)	41 (31.8%)	NS
	former smoker (cessation ≥ 1year) & never smoker	82 (71.3)	88 (68.2%)	
Time from RA onset [years], median (range)		8.9 (0.2–50)	–	–
Number of swollen joints, median (range)		4 (0–24)	–	–
DAS 28, median (range)		5.05 (1.49–8.52)	–	–
RF+ (cutoff 20 IU/mL), n (%)		56 (65)	–	–
ACPA+ (cutoff 25 U/mL), n (%)		44 (51)	–	–
Treatment, n (%)	methotrexate	46 (56.1)	–	–
	other non-biological DMARDs	33 (40.2)	–	–
	anti-TNF therapy	23 (28.0)	–	–
	steroids	62 (75.6)	–	–
Dietary intakes, median (range)	calories [kcal]	1590.4 (884.4–3622.5)	1708.2 (896.5–3816.2)	0.037
	carbohydrates [g]	225.5 (112.3–373.9)	233.3 (92.5–588.4)	0.019
	protein [g]	65.8 (28.4–145.7)	69.0 (31.8–174.5)	NS
	fat [g]	50.1 (20.6–136.7)	56.5 (15.5–148.2)	0.022
	water [g]	2225.7 (1057.9–3950.2)	2035.9 (717.5–5395.7)	NS
	fiber [g]	18.0 (6.8–34.6)	20.9 (6.0–48.3)	0.007
	vitamin C [mg]	83.6 (18.8–459.5)	93.0 (11.9–505.3)	NS
	vitamin E [mg α-tocopherol equiv.]	6.10 (1.89–83.21)	7.13 (2.75–19.21)	NS
	vitamin A [µg retinol equiv.]	756.3 (292.4–10484.1)	883.6 (210.8–9611.4)	NS
	Fe [mg]	8.92 (3.90–24.2)	11.45 (4.21–26.8)	0.005
	Cu [mg]	0.90 (0.49–6.81)	1.24 (0.55–2.45)	<0.000

RA – rheumatoid arthritis patients; Control – control subjects; n – number of subjects; Q1–Q3 – range between the 25th and 75th percentile; BMI – body mass index; DAS 28 – Disease Activity Score of 28 joints; RF – rheumatoid factor; ACPA – anti-citrullinated protein antibody; DMARDs – disease-modifying anti-rheumatic drugs; anti-TNF – anti-tumor necrosis factor; NS – not statistically significant.

Dietary intake assessment

Dietary intake in the 2 groups was assessed by dietary recall from the last 3 days before the interview.[18] Long-term silicon intake was assessed using a questionnaire on dietary habits developed by the Department of Food Science and Dietetics at the Wroclaw Medical University.[19] The questionnaire consists of 106 questions concerning the consumption of food groups and individual foods within those groups, calculated in portions per unit of time. The amounts of dietary silicon were calculated using data from unpublished analyses of Polish food products which were performed at the Department of Food Science and Dietetics, as well as the United Kingdom silicon food database[4] and data on silicon contents in Belgian food products.[3] As the dietary habit questionnaire has been validated in the Polish population only for the dietary intake of nutrients with established daily recommendation, but not for silicon, silicon intake from the 3-day dietary recall was also calculated to cross-check the data gathered from the dietary habit questionnaire. Silicon intake calculated using the 3-day food recall amounted to 90 ±9% of the intake reported on in the dietary habit questionnaire. Moreover, the data from the dietary recall allowed us to assess short-term dietary exposure to silicon. As silicon intake and plasma levels may differ depending on gender, the 2 variables were measured in each group as a whole and in the female and male subgroups.[6,20]

Silicon determination in plasma

The levels of silicon in plasma were determined by graphite furnace-atomic absorption spectrometry (GF-AAS), using the standard addition calibration method. A PinAAcle 900

(Perkin Elmer, Waltham, USA) atomic absorption spectrometer was used for analyses. All reagents were prepared using deionized water with a specific resistivity of 18.2 MΩ-cm. Plasma samples were diluted (1:4) with 0.01% Triton X-100 in water. From a silicon standard solution of 1000 ±3 mg/L in 0.2% HNO_3 (CPI International, Santa Rosa, USA), working standard solutions were prepared in 60 and 120 µg/L volumes. The graphite furnace was injected with 10 µL of sample, 10 µL of water or consecutive standard solution, and 5 µL of matrix modifier (0.1% Pd and 0.01% Mg). The operating conditions and parameters of the instruments for silicon measurement are summarized in Table 2. The silicon signal was linear up to 700 µg/L with the limit of quantification 5 µg/L. Due to the lack of appropriate certified reference material, the Seronorm™ Trace Element Serum L-2 (Sero AS, Billingstad, Norway) was used with an approximate concentration of silicon given (ICP-SFMS), and the recovery rate was 106%.

Table 2. Operating conditions and instrumental parameters for silicon measurement in plasma by graphite furnace – atomic absorption spectrometry (GF-AAS)

Operating conditions	Temperature [°C]	Ramp time [s]	Hold time [s]	Gas
Drying	110 130 300	1 30 20	5 40 5	argon argon argon
Mineralization	550 550	20 1	15 15	air argon
Pyrolysis	1100 1250	5 1	10 30	argon argon
Atomization	2450	0	5	–
Cleaning	2550	1	3	argon
Instrumental parameters				
Wavelength	251.61 nm			
Slit width	0.2 nm			
Lamp energy	63 mA			
Measurement mode	area under the peak			
Characteristic mass	42 pg			

Biochemical analyses in serum

The total antioxidant status (TAS) in the blood serum was measured by the spectrophotometric method with 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS), using a Randox TAS kit (Randox Laboratories, Crumlin, UK) and a Konelab 20i analyzer (ThermoSientific, USA).[21] The 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging capacity was measured spectrophotometrically in deproteinized serum (DSAS) using a Spectronic GENESYS 6 UV-visible spectrophotometer (Thermo Electron Corporation, Waltham, USA).[22] In both methods, antioxidant status was expressed in Trolox equivalents.

The total oxidant status (TOS) of the serum was measured as described by Erel.[23] In this method, the ability of serum oxidants to oxidize ferrous-ion-o-dianisidine complex is ascertained by the spectrophotometric determination of colored oxidized product using xylenol orange. The assay was calibrated with hydrogen peroxide. The results are expressed in µM of H_2O_2 equivalent. Oxidative stress index (OSI) was also calculated as a TOS:TAS ratio, using the values of both parameters expressed in the given units.[24]

Thiobarbituric acid reactive substances (TBARS) in the serum were detected spectrophotometrically after extracting pink-colored reaction products in butanol, and the TBARS concentration was expressed in nM of malondialdehyde (MDA).[25]

Serum albumin concentration was measured using an assay kit (Thermo Electron Corporation, Vantaa, Finland) with bromocresol green based on the colorimetric method. Uric acid was measured in the serum using a method based on oxidation by uricase. Serum high sensitivity C-reactive protein (hs-CRP) was determined using an hs-CRP assay kit (DiaSys, Holzheim, Germany) based on the measurement of immunoprecipitation at 540 nm. The addition of microparticles coated with anti-human CRP to the buffered samples results in immunoprecipitation recorded turbidimetrically at its end-point. Albumin, uric acid, and hs-CRP were measured with the Konelab 20i analyser.

The activity of superoxide dismutase (SOD) in serum was measured with an SOD Activity Assay kit (Cayman Chemical, Ann Arbor, USA), which uses tetrazolium salt to quantify superoxide radicals generated by xanthine oxidase and hypoxanthine. The oxidation rate of tetrazolium salt to formazan dye is inversely proportional to the endogenous activity of SOD. One unit (U) of SOD activity was defined as the amount of enzyme causing 50% superoxide radical dismutation. The activity of catalase (CAT) was measured in the serum using a CAT Assay kit (Cayman Chemical) which uses the peroxidative function of the enzyme at optimal hydrogen peroxide concentration with methanol as a substrate. One unit (U) of CAT activity was defined as the amount of enzyme causing the formation of 1 nmol of formaldehyde per minute. Measurements of SOD and CAT were performed on a Multiscan GO microplate reader (Thermo Fisher Scientific, Waltham, USA).

Serum interleukin-6 (IL-6) concentration was also measured, using a solid phase sandwich ELISA assay kit (Diaclone SAS, Besançon, France).

Statistical analyses

Statistical calculations were performed using STATISTICA v. 13.0 (StatSoft Inc., Tulsa, USA). Pearson's χ^2 test was used to assess differences in the qualitative variables between groups. The correlations between measured variables were assessed in both groups, further divided into

subgroups by gender, smoking status, and current treatment. Depending on the distribution of variables, either Student's t-test or the Mann–Whitney U test were used for group comparisons, and either Pearson or Spearman correlation analysis was used to determine associations between variables.

Results

Data on the intake of macronutrients, antioxidant vitamins, and essential microelements involved in peroxyl radical formation is included in Table 1. The diet of the experimental group provided the subjects with less energy, fat, carbohydrates, dietary fiber, iron, and copper than that of the control group, though their intakes of vitamin C and equivalents of vitamins E and A were similar. Short-term silicon intake estimated from the 3-day dietary recall amounted to 21.4 mg/day in the experimental group and was approx. 10% higher than in the control group; this difference was not observed in men, however (Table 3). The main dietary sources of silicon for the 2 study groups were cereal products (approx. 35% in total) and hot and cold non-alcoholic beverages (34 and 24% in total, respectively). Among cereal products, breads played an important role in the acquisition of dietary silicon. Non-alcoholic beverages contributed significantly more to silicon intake in the RA group than in the controls. Fruits and vegetables also provided considerable amounts of silicon in the diets of both groups, whereas fruits comprised a more substantial source for the controls.

Plasma concentrations of Si were shown to vary over a wide range in both groups, but the interquartile range between the 25th and 75th percentile was smaller in the controls than in the experimental group, including the distribution of values in the subgroups of females and males (Table 3). Significantly higher concentrations of silicon were found in the RA group than in the control group, and especially large differences were observed between the females of these groups; the males' plasma silicon concentration did not differ significantly in either group. In the controls, a negative correlation between silicon concentration and age was also noticed. A negative correlation between silicon level and the number of swollen joints was found in the RA female subgroup.

Total silicon intake estimated according to the short-term dietary recall did not correlate with silicon concentration in plasma, while dietary silicon provided by non-alcoholic beverages was shown to correlate with plasma silicon in the RA group. In the control group, the total silicon intake from cereal products correlated with plasma silicon level.

Table 3. Silicon intake and plasma level in the RA and control groups, and significant correlations found between plasma or dietary silicon and selected characteristics in the study groups

Parameter		RA	Control	p-value
Silicon intake [mg/day], median (range)	all subjects	21.4 (11.2–48.6)	19.4 (12.6–37.0)	0.046
	female	24.4 (12.9–48.6)	19.9 (12.6–37.0)	0.041
	male	20.1 (11.2–31.1)	19.8 (15.0–30.6)	NS
Silicon food sources [% of total silicon intake]	white bread	13.0	13.1	NS
	wholemeal bread	9.6	9.3	NS
	other cereal products	12.5	13.5	NS
	vegetables	11.3	13.2	NS
	fruits	9.1	14.1	0.003
	non-alcoholic beverages	19.9	11.3	<0.000
	tea & coffee	13.8	12.6	0.037
	alcoholic beverages	0.5	3.3	0.007
	other foods	10.3	9.6	NS
Plasma silicon [µg/L], median (range) Q1–Q3	all subjects	192.3 (42.7–372.9) 120.2–269.7	140.9 (59.4–312.0) 111.0–175.9	0.028
	female	196.6 (42.7–372.9) 118.7–269.1	139.1 (66.1–312.0) 104.2–177.2	0.007
	male	207.7 (44.6–366.1) 131.9–279.7	149.2 (59.4–269.8) 115.5–170.4	NS
Significant plasma silicon correlations with food consumption (r)	cereal products	–	0.52	0.001
	tea & coffee	0.33	–	<0.000
	non-alcoholic cold beverages	0.29	–	0.002
Significant plasma silicon correlations with group characteristics (r)	age	–	–0.27	0.001
	number of swollen joints	–0.32	–	0.041

RA – rheumatoid arthritis patients; Control – control subjects; Q1–Q3 – range between the 25th and 75th percentiles; NS – not statistically significant; r – correlation coefficient.

Table 4. Redox status components and markers of inflammation in RA and control groups, median (range)

Parameter	RA	Control	p-value
TAS [mM Trolox]	1.47 (1.03–1.86)	1.71 (1.20–2.14)	<0.000
DSAS [µM Trolox]	171.3 (41.1–450.3)	202.6 (67.4–523.1)	<0.000
TOS [µM H_2O_2 equiv.]	7.81 (2.25–14.94)	2.84 (0.09–49.31)	<0.000
OSI (TOS/TAS ratio)	5.19 (1.76–10.03)	1.63 (0.05–32.96)	<0.000
TBARS [µM MDA]	1.88 (1.16–4.04)	1.75 (0.68–7.98)	0.001
SOD [U/mL]	2.49 (0.32–4.57)	1.95 (0.19–3.22)	0.031
CAT [U/mL]	35.63 (4.70–76.24)	40.27 (5.48–77.91)	0.023
Albumin [g/dL]	3.92 (2.52–5.7)	4.25 (2.85–5.42)	0.006
Uric acid [g/dL]	4.4 (1.5–9.1)	5.3 (3.0–8.8)	NS
IL-6 [pg/mL]	6.39 (0.84–98.95)	2.26 (0.81–16.41)	<0.000
hs-CRP [mg/L]	4.94 (0.12–83.57)	1.51 (0.03–10.8)	<0.000

RA – rheumatoid arthritis patients; Control – control subjects; TAS – total antioxidant status; DSAS – deproteinized serum antioxidant status; TOS – total oxidant status; OSI – oxidative status index; TBARS – thiobarbituric acid reactive substances; SOD – superoxide dismutase; CAT – catalase; IL-6 – interleukin 6; hs-CRP – high sensitivity C-reactive protein; NS – not statistically significant.

Table 5. Correlations between dietary or plasma silicon with markers of redox and inflammatory status in RA and control groups

Parameter	Correlations with dietary Si		Correlations with plasma Si	
	RA	Control	RA	Control
TAS	NS	NS	0.34 (0.007)[a]	0.28 (0.001)
DSAS	NS	NS	–0.23 (0.018)	0.25 (0.014)
TOS	–0.28 (0.009)[c]	NS	–0.24 (0.032)[b]	–0.23 (0.007)
OSI	–0.26 (0.014)[c]	NS	–0.30 (0.019)[a]	–0.26 (0.003)
TBARS	NS	NS	NS	0.34 (0.017)[d]
Albumin	0.28 (0.030)[a]	NS	NS	NS
Uric acid	NS	–0.42 (0.005)[b]	NS	NS
SOD	NS	NS	NS	NS
CAT	NS	NS	0.21 (0.027) 0.39 (0.002)[a]	NS
hs-CRP	–0.35 (0.012)[a]	NS	NS	NS
IL-6	–0.19 (0.041)	NS	–0.32 (0.005)[b] –0.29 (0.006)[c]	NS

RA – rheumatoid arthritis patients; Control – control subjects; TAS – total antioxidant status; DSAS – deproteinized serum antioxidant status; TOS – total oxidant status; OSI – oxidative status index; TBARS – thiobarbituric acid reactive substances; SOD – superoxide dismutase; CAT – catalase; IL-6 – interleukin 6; hs-CRP – high sensitivity C-reactive protein; NS – not statistically significant. Statistical significance in subgroups: [a] – patients with an albumin level above the median value (3.92 g/dL); [b] – non-smokers; [c] – female subjects; [d] – male subjects.

For the assessment of systemic redox balance in the study groups, the antioxidant capacity and total oxidant load in serum were measured (Table 4). The scavenging activity of non-enzymatic serum components against ABTS radicals was determined by TAS assay. The antiradical activity of low-molecular-weight serum components was also assessed in deproteinized serum using a DPPH-stable radical (DSAS assay). Significantly lower values of both TAS and DSAS measurements were found in the RA group than in the controls. In both groups, DSAS values comprised approx. 12% of the TAS. Measuring the concentrations of the main endogenous antioxidants in blood serum revealed that in the experimental group circulated albumins were lower than in the control group; in the case of uric acid, no differences were found. The total concentration of oxidant molecules in blood serum was measured by TOS assay, which revealed higher values among the RA group than the control group. These differences in oxidative status between groups were accompanied by higher OSI values for RA patients than for controls. The TBARS assay used in this study as an indicator of lipid peroxidation also showed higher serum concentrations of MDA equivalents in the experimental group. The assessment of the enzymatic components of antioxidant status revealed more activity of serum SOD and less activity of CAT in the RA group than in the control group. Disrupted systemic inflammatory status, expressed as a higher serum concentration of hs-CRP and IL-6, was also found in the RA patients than in the controls (Table 4).

The relationships of dietary intake and plasma silicon concentration to redox and inflammatory statuses were statistically analyzed for the 2 groups (Table 5). No correlation between plasma silicon and dietary silicon level was observed in either group, though a negative correlation between Si intake and IL-6 level was found among the RA group, and negative correlations of Si intake with TOS and OSI was noted among the RA females, as well as with hs-CRP in patients with above-average serum albumin levels. Moreover, silicon intake in the RA group was associated with higher concentrations of albumin; however, in patients with albumin levels above the median, this relationship was not noticed. In the control group, dietary silicon did not correlate with redox or inflammatory status, except for a negative correlation with uric acid. Plasma silicon concentration positively correlated with TAS and DSAS values in the controls. In the experimental group, a positive correlation of plasma silicon with TAS was found in patients with serum albumin over the median value, and this relationship was accompanied by an opposite relationship with DSAS and was observed in all RA patients. Among the parameters of oxidative status, TOS and OSI negatively correlated with plasma silicon in the experimental group. In the case of TOS, this correlation was limited to non-smoking patients; for OSI it was limited to patients with above-average albumin levels. In the control group, these 2 parameters decreased with increasing plasma silicon concentrations. A positive correlation between TBARS level and plasma

silicon level was noticed, but only among the male control subjects. The activity of SOD and CAT did not correlate to plasma silicon in the controls, while a positive correlation between plasma silicon and CAT was found among the RA group, especially in those with above-average albumin levels. Plasma silicon concentration did not correlate with inflammatory markers in the control group. In the experimental group, a negative correlation with IL-6 concentration was found, but only in non-smoking patients and in the female subgroup.

Discussion

This study is the first attempt to assess the association of silicon provided by diet and silicon concentration in blood plasma with the antioxidant and oxidant status of patients with RA and with systemic markers of inflammatory status in RA. Our previous study showed that the intake of some essential nutrients, such as polyunsaturated fatty acids, vitamin E, B6, and calcium correlated with higher serum antiradical activity in RA patients; moreover, this antioxidant potential was beneficially linked with disease duration and severity.[18] Silicon also seems to be involved in redox balance and inflammation, though these observations originate from animal models and the cell cultures underwent toxic, metabolic, or immune challenges.[7–10] The effects of dietary silicon on the development, progress, and severity of autoimmune diseases have not yet been studied.

Dietary Si intake has not yet been evaluated in the Polish population and no database is available with silicon levels in Polish foods. Therefore, we assessed the amount of silicon in foods consumed by the subjects in this study by using the dietary recall method and a questionnaire on dietary habits, and by calculating silicon intake according to data on the silicon content of foods measured in our laboratory and databases on food component analysis available in European countries and published elsewhere.[3,4] So far, adequate daily intakes of Si have not been established, but the results obtained in this study are in agreement with the estimated beneficial intake range based on animal and human exposure studies (10–30 mg/day); according to the Framingham Offspring Cohort study, however, the most favorable amount of silicon in the diet associated with bone health exceeded 30 mg/day.[1,26]

The contributions of foods supplying silicon in the 2 study groups were in alignment with previous studies, though the higher intake in male subjects than female subjects was not confirmed.[3] As with Finnish, British, and American diets, cereal products were the primary food source of silicon, followed by fruits and vegetables and hot and cold beverages. In the typical Western diet, beer was among the foods found to contribute the most to the intake of silicon in men (up to 18%).[26] In our study, alcoholic beverages proved to be a much less important source of Si in the diet. The RA group, especially women, consumed a diet providing slightly more silicon than the control group, and non-alcoholic beverages containing soluble orthosilicic acid comprised a larger percentage of silicon food sources for the RA patients than the controls. Silicon sources in the diets of both groups greatly reflected the dietary habits of the adult Polish population, as recently published in the National MultiCenter Health Survey (WOBASZ II, 2013–14),[27,28] and in our previous study concerning the diet quality of RA patients vs controls.[18] These habits include consuming a high proportion of white bread to total cereal products, tea and coffee comprising much of the non-alcoholic beverages, and fruit and vegetable consumption which is lower than recommended.

A higher silicon content in the diet was accompanied by a higher plasma silicon level in the RA group than in the control group. A positive correlation of plasma silicon with beverage consumption has also been found in RA patients, which provides an easily absorbable form of silicon.[3] Reference values for fasting plasma silicon concentration in healthy adults have not yet been established. It is difficult to refer measured plasma silicon levels to the data published for different populations because of the different methods used in measuring silicon concentration and the lack of certified reference materials. Nevertheless, it may be stated that the concentrations of silicon in the plasma of the subjects in this study were similar to those obtained for other populations (100–310 μg/L).[1] The negative correlation with age found among the controls was in alignment with previous findings,[3] while in the RA patients no correlation between plasma silicon and age was noted, and in the female RA subgroup a favorable association with the disease course was found. Plasma silicon concentration did not appear to be dependent on gender, as observed by other authors.[24] Since it has been shown that plasma silicon level is physiologically regulated by kidney excretion and likely by active intestine transport, the elevated plasma silicon levels among the RA patients may suggest an alteration in this regulation linked to the disease rather than a slightly higher oral intake of silicon in these patients.[1,2] An increased serum silicon level has been reported in rats with collagen-induced arthritis, and higher serum Si levels have also been observed in sclerosis multiplex – a disease with inflammatory immunopathogenesis.[8,29]

The role of oxidative stress has been implicated in the etiology of RA, and several studies have observed a relationship between oxidative damage and the maintenance of the inflammatory process in RA.[15] In this study, we assessed extracellular antioxidant/oxidant status, since it constitutes an important modulator of intracellular redox balance in different tissues and under inflammatory conditions.[30] Systemic antioxidant status was analyzed using TAS and DSAS methods which evaluate serum radical scavenging capacity, being the first line of defense in conditions of increased ROS production.

In order to specify the potential of the circulating low-molecular-weight antioxidant pool, measurements of antiradical activity in deproteinized serum (DSAS) were taken in addition to TAS.[31] As anticipated, the RA experimental group was characterized by an imbalance in redox status, manifested in the reduced antiradical capacity of serum and its deproteinized fraction. Elevated serum TOS, OSI, and TBARS levels in comparison with the control group also indicated an overproduction of hydrogen peroxides and lipid peroxides.[23] The disrupted balance between SOD and CAT activity that was found in the RA group when compared to the control group may contribute considerably to the impaired management of hydrogen peroxide production. Activity of Cu/Zn-SOD has previously been shown to be elevated or reduced in the serum or plasma of RA patients.[16,32] The inhibition of serum CAT activity observed in the RA group was in accordance with Shah et al.,[33] though other authors have found this enzyme to be more active.[32] Insufficiently metabolized hydrogen peroxide, under reduced CAT activity, underlies further reactions to yield the highly reactive hydroxyl radical involved in lipid oxidation, and might therefore contribute to the increased formation of MDA found in the TBARS assay of the RA group. The lower serum concentrations of albumins in the RA group when compared to the control group indicate diminished cationic ligand binding by albumins, thereby facilitating the formation of hydroxyl radicals via the Fenton reaction.[31,34]

The inflammatory response in RA involves the increased expression of markers of inflammation, such as CRP and proinflammatory cytokines, including tumor necrosis factor-α (TNF-α) and IL-6, in the synovial fluid and serum.[35] In our study, both hs-CRP and IL-6 concentrations were significantly higher in the serum of RA patients than in the controls. Concentrations of IL-6 higher than those in the control group were also found for patients treated with anti-TNF therapy (data not shown). Interleukin 6 plays a crucial role in RA pathogenesis and may serve as an indicator of disease severity.[36] The immense value of CRP as an inflammatory marker in RA has also been recognized. C reactive protein, as part of an acute phase response to inflammation, stimulates proinflammatory cytokine production and may contribute directly to the inflammatory state.[15,35]

No link between dietary silicon and indices of oxidant/antioxidant status was found in the control subjects, except for a negative correlation with serum uric acid concentration. The effect of silicon on uric acid level has not yet been reported; however, the relationship noted in this study may be indirect, since dietary silicon sources (such as cereals, fruits, and vegetables) are simultaneously purine-poor foods, leading to a decrease of uric acid level in the serum.[37] The total Si intake from these foods was higher in the control group than in the experimental group (63.2% vs 55.5%, respectively). In the conditions of enhanced oxidative stress detected in the RA patients, the correlation between dietary silicon and serum markers of redox status was more pronounced and pertained to the reduction of hydrogen peroxide and lipid peroxide levels measured in the TOS assay for female patients with increased silicon content in their diet. Moreover, the ratio between oxidant and antioxidant status decreased along with increasing silicon intake in these subjects, indicating the role of Si in managing oxidant overproduction related to RA. Again, the observed relationship could be an effect of the composition of foods supplying silicon, which are mainly of plant origin and contain a wealth of antioxidants proven to be effective in ROS neutralization. However, it has been shown that silicon possesses the ability to remove hydrogen peroxide or other ROS, and its protective effects against hydrogen peroxide toxicity was observed in animal models and cell lines.[2,9,38] Moreover, it has been reported that silicon exerts an in vitro inhibitory effect on DPPH radical production in a concentration-dependent manner.[9] This specific implication of silicon in managing oxidative stress was confirmed in our study by the positive correlation between silicon concentration in plasma and antiradical capacity measures, and by the negative correlation to oxidative stress indices prominently expressed among the controls. In the RA patients, these correlations were found only in patients who were not burdened by factors influencing redox status, i.e., lower albumin levels and cigarette smoking. Uric acid contributes the most to the antioxidant pool of deproteinized serum, and the opposite relationship of silicon concentration to antiradical activity measured in deproteinized serum might result from the silicon–uric acid interactions revealed when silicon levels are elevated in the blood. Uric acid antioxidant activity has been shown in fact to be affected by certain components of the chemical milieu in the human body, including bicarbonate.[39] Moreover, in the DPPH assay, which was performed in an organic solvent, the mode of interaction between the assay components and the impact of their proportions may differ from how they are in the natural environment.[40] The compelling positive link between plasma silicon level and the marker of lipid peroxidation in male controls might be at least partially explained by the findings published by Garcimartin et al.[38] The authors of that study demonstrated that silicon in concentrations up to 100 μg/L did not induce TBARS production in neuroblastoma cells, but when concentrations reached 250 μg/L or more peroxidation was significantly higher. This suggests that Si is ineffective as a hydroxyl radical neutralizer or may even induce the production of hydroxyl radicals at the concentrations found in serum. However, in this study an organic compound of silicon was tested, and the observed effect might depend on the chemical form of silicon. On the other hand, in an aluminum challenge study on rats, the concentration of TBARS decreased after oral administration of orthosilic acid, but this effect might result from the ability of silicon to produce aluminosilicate

complexes rather than from direct antioxidant action.[41] It should also be mentioned that a high intake of iron and copper was noted among the control group, especially in the men, whose diet supplied 135% and 153% of the recommended daily allowance for these elements, respectively.[42] Moreover, a tendency toward higher plasma silicon levels was observed in the male controls with higher Fe intake (data not shown). An excessive intake of these redox-active transition metals, especially iron, has been shown to promote lipid peroxidation, so this effect might contribute to the relationship between plasma silicon level and TBARS level observed in the male subgroup.[43] Further light on the role of silicon in extracellular redox status may be shed by the relationship of Si to antioxidant enzyme activity in the serum. Although dietary and plasma silicon were not linked to SOD and CAT activity in serum among the control group, a significant positive correlation was found for CAT in the RA patients, especially among those with above-average albumin values. In RA, silicon may play a modulatory role in ROS disabling, where SOD activity is elevated and CAT is reduced. These associations suggest that silicon in the serum acts as a hydrogen peroxide neutralizer, at least partly by inducing CAT activity. The modulatory effects of silicon on SOD and CAT were also studied in a non-alcoholic steatohepatitis rat model, where strong activation of initially low SOD levels in the liver tissue of rats was reported after the rats were fed supplemental silicon, while the activities of CAT and glutathione peroxidase did not change.[10] As the authors of that study suggested, in this model the ability of silicon to neutralize hydrogen peroxide rendered the overexpression of CAT unnecessary. A favorable link between dietary and plasma Si and inflammatory response in RA was also found in our study, but some interferences were noted — given that this relationship was influenced by serum albumin level, smoking status, or gender. Rheumatoid arthritis therapy did not further modify the observed associations (data not shown).

The role of silicon in alleviating inflammatory and immune response was revealed in a study on animals with collagen-induced arthritis.[8] The involvement of silicon in the response to inflammation was also demonstrated in silicon-deprived rats, which accumulate more silicon in the liver and bones than silicon-supplemented rats in acute-phase inflammation induced by lipopolysaccharide endotoxin.[44] In murine macrophage cells, the inhibitory effect of silicon on IL-6 secretion was observed only at a concentration of 50 μM, far exceeding those found in human blood.[9] Silicon was also shown to normalize the gene expression of TNF-α upregulated in mice exposed to aluminum.[45] Since both hs-CRP and IL-6 concentrations in the blood are sensitive markers for assessing the intensity of inflammatory processes and disease severity, the results obtained in this study may suggest a beneficial role of silicon in the course of RA. Bone resorption and osteoclast induction in RA is stimulated by IL-6, so the negative correlation between Si and IL-6 in the RA group is an indication of its role in preventing bone, cartilage, and joint destruction in RA.[35]

In our previous study, systemic antioxidant capacity was shown to decrease in RA patients, as disease activity and lenght of time from disease onset increased.[18] With regard to this observation and the suggested contribution of silicon to antioxidant status in RA, we evaluated the association of dietary and plasma silicon with the clinical indices used to monitor RA activity and duration. A negative correlation between plasma silicon and the number of swollen joints was revealed among female RA patients; moreover, a non-significant positive trend was also noted for disease duration among non-smoking subjects ($r = 0.19$, $p = 0.068$). These findings may support the case for silicon's involvement in disease response in RA patients, though the effect of supplementary silicon on the number of affected joints was not found in an arthritic rat model.[8]

In conclusion, silicon may contribute to systemic redox balance. In light of the generally favorable correlation of plasma and dietary silicon to redox and inflammatory markers, the elevated plasma silicon levels in the RA patients (as compared to the controls) might suggest the involvement of silicon in managing the disturbances related to RA. Non-smoking status and non-affected serum albumin seem to be important in exposing the beneficial effects of silicon in RA. As a number of questions remain, further studies on the role of silicon in the antioxidant system and inflammatory processes are warranted.

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Characteristics of aeromedical transport, both interhospital and directly from the scene of the incident, in patients with acute myocardial infarction or acute trauma between 2011–2016 in Poland: A case-control study

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Abstract

Background. Patients with acute myocardial infarction (AMI) or acute trauma (AT) are transported by air to save time. Helicopter Emergency Medical Service (HEMS) provides both flights to and from the emergency scene, as well as interhospital transport (interHtransport).

Objectives. The objective of this study was to compare aeromedical transport and HEMS missions of AMI and AT patients regarding safety, medical procedures and the length of flights.

Material and methods. This is a case-control study analyzing the medical history records of AMI and AT patients transported between hospitals and from the scene identified using ICD-10 codes. Research of customary data (age, sex and general health status measured with Glasgow Coma Scale (GCS) and Revised Trauma Score (RTS)) was performed.

Results. There were 48,555 flights in the years 2011–2016, of which 7,645 (15.7%) were interhospital (19% AMI and 12% AT). Out of these, 40,910 (84.3%) HEMS missions were to patients on the scene (10% AMI and 13% AT). No fatalities were noted. The AMI GCS score was higher than in AT patients: 15.0 vs 14.0, respectively. The medical procedures during transport of AMI patients between hospitals and from the scene were the following: cardiopulmonary resuscitation (CPR): 6 vs 73 cases ($p < 0.001$); oxygen therapy: 41.1% vs 50.2%, respectively. The median distance was 59.4 km vs 52.1 km ($p < 0.001$), while median flight time was 45.0 min vs 38.0 min ($p < 0.001$), respectively. Regarding AT patients, the procedures performed (during interhospital and from the scene transport) were the following: CPR: 5 vs 244 cases ($p < 0.001$); intubation: 10.7% vs 17.3% ($p < 0.001$); sedation: 50.1% vs 24.3% ($p < 0.001$); oxygen therapy: 17.6% vs 36.6% ($p < 0.001$); spinal board: 17.1% vs 66% ($p < 0.001$); cervical collar: 15.9% vs 63.4% ($p < 0.001$), respectively. Interhospital transport and HEMS mission median flight distance was 135.9 km vs 56.3 km ($p < 0.001$), while median flight time was 66.0 min vs 45.0 min ($p < 0.001$), respectively.

Conclusions. Aeromedical transport is safe and very rarely requires resuscitation during the flight. The long distances of flights and time required can reflect the scarcity of trauma centers (TCs) compared to cardiovascular wards. The location of hemodynamic centers in Poland is optimal.

Key words: acute myocardial infarction, Helicopter Emergency Medical Service, aeromedical transport, patient with trauma

Introduction

Similarly to the rest of the world, in Poland aeromedical interhospital transport (interHtransport) in the rescue mode is the most frequent form of transporting both patients with acute trauma (AT) and with acute myocardial infarction (AMI).[1,2] The Medical Air Rescue Service (MARS) has at its disposal 2 kinds of aircraft: 22 EC 135 2+ and H135 P3 helicopters, forming the Helicopter Emergency Medical Service (HEMS). Of all its air bases, 4 work all year while 1 is seasonal and operates only in the summer. The other kinds of aircraft are 2 Plane Transport Teams (PTS), which mostly provide interHtransport of patients in the planned mode. In addition to carrying out flights to the immediate scene of the emergency incident, HEMS is also used for rescue transport between treatment institutions which have helipads (functioning either during the day or round-the-clock). It is essential to transport the AMI patient in a sudden critical health condition who requires intensive supervision during the flight to a hospital which has a hemodynamics department, so that percutaneous coronary intervention (PCI) can be carried out.[1,3] The responsibility for the organization and choice of transport mode between an ambulance and a helicopter falls on the dispatching doctor who is in charge of the patient. The decision can be consulted with the doctor from the interventional cardiology department. The procedures described above function both in the Polish and in the American healthcare system.[4,5] In the case of patients in a critical condition, when making the decision about transport to another center, the doctor in charge of the patient must first make sure that all the diagnostic and treatment possibilities have been exhausted and then must be guided by the principle that potential benefits should outweigh risks, including that of death in the course of transport.[6]

Transport takes place between hospitals that have adjacent helipads. In each and every case enrollment of the patients for transport is implemented by the medical dispatcher of the Operational Center of the Medical Air Rescue Service (OP MARS), who then actively participates in coordinating transport operations. Research reports from all the world all agree that when it comes to saving time, aeromedical transport of AMI patients from the place of the incident to the center implementing PCI is superior to transport from hospital to hospital.[4] In fact, under Polish conditions, where many hospitals have no land transport units, HEMS is the only possibility of transporting a patient in a critical, life-threatening condition.

Under the law, every medical legal entity in Poland is obliged to provide sanitary transport to a patient in a critical condition to the nearest appropriate medical facility. Such a policy is based on the premise that immediate treatment or continuation of treatment must be provided. In practice, transport contracts made between medical units and an enterprise carrying out sanitary transport (often located at a distance of a few dozen kilometers from the dispatching hospital) are also accepted. In such cases, air transport is the desirable alternative to land transport. The key factor is to make sure that aircraft are dispatched in an optimal way, so that more patients can be helped.

Flights to AT patients who have suffered injuries in road or construction accident and other events resulting in life-threatening situations are as frequent as cases of AMI. Patients fulfilling the criteria for enrollment in a trauma center (TC), a center for the treatment of burns or a hospital performing the replantation of limbs can be transported by air, which is beneficial from the point of view of saving time, minimizes the shaking present during ambulance transport[7,8] and also reduces the fatality rate.[9,10] When comparing land and aeromedical transport in the course of implementing vital procedures, an important role is played by the exceptional professional experience of HEMS teams.[11]

In Poland there are 14 TCs for 16 voivodeships (provinces). In this context, ambulance transport over a distance of many kilometers can lengthen the time of reaching the patients and transporting them to a place where specialist treatment can be provided. In most cases, reports regarding the air transport of AMI patients do not distinguish cases of cardiac arrest or fatality. What is featured in reports are cases of hypotension in the course of the flight.[2,12]

The aim of the present study was to compare the transport of AMI and AT patients carried out by HEMS regarding undertaken medical procedures as well as the time and distance of flights.

Material and methods

A case-control study was performed using the medical and air histories of MARS regarding patients transported in the course of interhospital operations and flights to the immediate scene of the emergency incident (HEMS missions) in the years 2011–2016 in Poland. Both the medical and flight data were recorded using Microsoft Excel (Microsoft Inc., Redmond, USA) databases.

The group that was researched were patients transported due to acute coronary syndrome, identified by the following ICD-10 codes: I20, I21 and I24. The control group comprised AT patients identified by the following ICD-10 codes: S06, T06, T29, and S68. They were the second most numerous homogeneous group of patients transported during HEMS rescue service flights.

The data that was identified and compared concerned: 1) age, sex, patient status (on the basis of Glasgow Coma Scale (GCS) and Revised Trauma Score (RTS)) and death in the course of flight; 2) the medical procedures undertaken (external heart massage, defibrillation, sedation, neuromuscular block, oxygen therapy, respiratory

therapy, intubation, or using a spinal board, a cervical collar or a painkiller; 3) the time and distance of the flight.

Statistical analysis

Descriptive statistics are presented as the numerical data and percentages of categorical variables and the median weighted with the 1st and 3rd quartile for numeric variables. Comparisons of groups of patients with AMI and those with AT were carried out using χ^2 tests and the Mann–Whitney test (for the relevant category and numerical data). Analyses were conducted using R 3.4.1 software (R Core Team, 2017; Foundation for Statistical Computing, Vienna, Austria; https://www.R-project.org/).

Results

A total of 48,555 HEMS flights were carried out between 2011 and 2016, out of which 7,645 concerned transport between hospitals in the rescue mode, including 1,429 AMI patients and 908 AT patients (19% and 12% transport flights, respectively). Out of the 40,910 missions directly to the site of the incident, 4,002 were flights to AMI patients and 5,231 to AT patients (10% and 13%, respectively).

The basic characteristics of the patients with AMI from each group are presented in Table 1. In the group of patients with AMI, females transported between hospitals accounted for 32.3%, whereas in the AT group women comprised 31.0% (not significant (NS)). Male patients comprised 67.7% and 68.9% (NS), respectively. Unidentified patients (NN) accounted for 0.1% of the flights in both groups (NS). The median age of patients transported between hospitals was 65.9 years, and from the scene of the event – 63.6 years ($p < 0.001$).

The median state of consciousness assessed using GCS was 15 points in both groups (NS). The number of GCS points was divided into 4 ranges in patients transported between hospitals was the following: <9: 27 (2.1%), 9–12: 13 (1.0%), 13–14: 24 (1.9%), and 15: 1,230 (95.1%), while in the HEMS mission it was <9: 105 (2.8%), 9–12: 51 (1.4%), 13–14: 130 (3.5%), and 15: 3,479 (92.4%). The assessment of patients on the RTS was 12 (NS).

In the group of AT patients, women transported between hospitals accounted for 24.1%, while in the group taken from the place of the event for 26.0% (NS). The corresponding percentages for men were the following: 75.4% and 72.7% (NS), respectively. Unidentified patients accounted for 0.5% of interhospital flights and 1.3% of flights from the site of the event (NS). The median age of the patients transported between hospitals was 42.1 years, and from the scene of the event – 33.1 years ($p < 0.001$) (Table 2).

The median state of consciousness on GCS in interHtransport and in flights to the event amounted to 14.0 points (NS). The number of points on GCS divided into 4 ranges for patients transported between hospitals was the following: <9: 205 (30.9%), 9–12: 58 (8.7%), 13–14: 61 (9.2%), and 15: 340 (51.2%), while the corresponding numbers for the HEMS missions were the following: <9: 1,582 (31.4%), 9–12: 459 (9.1%), 13–14: 712 (14.1%), and 15: 2,286 (45.4%). The evaluation of patients on the RTS was 12.0 points (NS).

One fatality was registered in each of the groups analyzed (AMI and AT patients). Moreover, cardiac arrest occurred in 9 (0.6%) patients with AMI in the course of interHtransport, while among patients flown from the scene

Table 1. Characteristics of the group of patients with AMI in the study

Variables	N	Inter-Htransport	N	HEMS missions	p-value
Sex, n (%) F M NN	1,438	464 (32.3) 973 (67.7) 1 (0.1)	4,002	1,239 (31.0) 2,758 (68.9) 5 (0.1)	0.573
Age, median (Q1–Q3)	1,435	65.9 (58.0–75.4)	3,975	63.6 (56.5–73.1)	<0.001
GCS, median (Q1–Q3)	953	15.0 (15.0–15.0)	2,642	15.0 (15.0–15.0)	0.037
GCS <9 9–12 13–14 15	 27 13 24 1,230	 2.1% 1.0% 1.9% 95.1%	 105 51 130 3,479	 2.8% 1.4% 3.5% 92.4%	0.009
RTS, median (Q1–Q3)	953	12.0 (12.0–12.0)	2,639	12.0 (12.0–12.0)	0.073

AMI – acute myocardial infarction; interHtransport – interhospital transport; HEMS – Helicopter Emergency Medical Service; GCS – Glasgow Coma Scale; RTS – Revised Trauma Score; F – female; M – male; NN – non notus.

Table 2. Characteristics of the group of trauma patients analyzed in the study

Variables	N	Inter-Htransport	N	HEMS missions	p-value
Sex, n (%) F M NN	916	221 (24.1) 691 (75.4) 4 (0.5)	5,231	1,361 (26.0) 3,804 (72.7) 66 (1.3)	0.038
Age, median (Q1–Q3)	895	42.1 (23.5–57.8)	4,683	33.1 (19.2–53.5)	<0.001
GCS, median (Q1–Q3)	472	14.0 (3.0–15.0)	3,313	14.0 (6.0–15.0)	0.007
GCS <9 9–12 13–14 15	 205 58 61 340	 30.9% 8.7% 9.2% 51.2%	 1,582 459 712 2,286	 31.4% 9.1% 14.1% 45.4%	0.002
RTS, median (Q1–Q3)	471	12.0 (8.0–12.0)	3,309	12.0 (9.0–12.0)	0.011

InterHtransport – interhospital transport; HEMS – Helicopter Emergency Medical Service; GCS – Glasgow Coma Scale; RTS – Revised Trauma Score; F – female; M – male; NN – non notus.

Table 3. Medical rescue procedures carried out by the HEMS (patients with AMI)

Variables	N	InterHtransport	N	HEMS missions	p-value
Defibrillation, n (%) no yes	1,439	1,433 (99.6) 6 (0.4)	4,002	3,950 (98.7) 52 (1.3)	0.008
CPR, n (%) no yes	1,302	1,296 (99.5) 6 (0.5)	3,745	3,672 (98.1) 73 (1.9)	<0.001
Intubation, n (%) no yes	1,439	1,426 (99.1) 13 (0.9)	4,002	3,947 (98.6) 55 (1.4)	0.215
Sedation, n (%) no yes	1,439	1,343 (93.3) 96 (6.7)	4,002	3,819 (95.4) 183 (4.6)	0.002
Neuromuscular block, n (%) no yes	1,439	1,423 (98.9) 16 (1.1)	4,002	3,977 (99.4) 25 (0.6)	0.098
Oxygen therapy, n (%) no yes	1,439	848 (58.9) 591 (41.1)	4,002	1,991 (49.8) 2,011 (50.2)	<0.001
Respirator, n (%) no yes	1,439	1,380 (95.9) 59 (4.1)	4,002	3,900 (97.5) 102 (2.5)	0.004

AMI – acute myocardial infarction; HEMS – Helicopter Emergency Medical Service; interHtransport – interhospital transport; CPR – cardiopulmonary resuscitation; GCS – Glasgow Coma Scale; RTS – Revised Trauma Score.

Table 4. Medical rescue procedures carried out by the HEMS (patients with trauma)

Variables	N	InterHtransport	N	HEMS missions	p-value
Defibrillation, n (%) no yes	916	914 (99.8) 2 (0.2)	5,231	5,208 (99.6) 23 (0.4)	0.490
CPR, n (%) no yes	869	864 (99.4) 5 (0.6)	5,102	4,858 (95.2) 244 (4.8)	<0.001
Intubation, n (%) no yes	916	818 (89.3) 98 (10.7)	5,231	4,324 (82.7) 907 (17.3)	<0.001
Sedation, n (%) no yes	916	457 (49.9) 459 (50.1)	5,231	3,958 (75.7) 1,273 (24.3)	<0.001
Neuromuscular block, n (%) no yes	916	788 (86.0) 128 (14.0)	5,231	4,753 (90.9) 478 (9.1)	<0.001
Oxygen therapy, n (%) no yes	916	755 (82.4) 161 (17.6)	5,231	3,316 (63.4) 1,915 (36.6)	<0.001
Respirator, n (%) no yes	916	529 (57.8) 387 (42.2)	5,231	3,631 (69.4) 1,600 (30.6)	<0.001
Spinal board, n (%) no yes	916	759 (82.9) 157 (17.1)	5,231	1,780 (34.0) 3,451 (66.0)	<0.001
Cervical collar, n (%) no yes	916	770 (84.1) 146 (15.9)	5,231	1,916 (36.6) 3,315 (63.4)	<0.001

HEMS – Helicopter Emergency Medical Service; interHtransport – interhospital transport; CPR – cardiopulmonary resuscitation; GCS – Glasgow Coma Scale; RTS – Revised Trauma Score.

there were 53 (1.3%) such cases. In patients with multi-organ trauma, cardiac arrest was observed in 19 (0.3%) patients transported from the site of the event.

A comparison of the medical procedures undertaken during the transport of patients with AMI is presented in Table 3. Clinical events that occurred before and during transport in the interhospital group and in flights from the scene of the event included chest compressions (n = 6 (0.5%) vs n = 73 (1.9%), respectively; $p < 0.001$). As far as defibrillation is concerned, it was carried out in 0.4% of patients transferred between hospitals and in 1.3% carried by HEMS missions ($p = 0.008$).

During the transport of patients in the state of a sudden health risk, medical procedures relevant to each group were implemented. There were 13 instances (0.9%) when intubation was carried out in the group of patients transported between hospitals, while in the group transported from the scene there were 55 (1.4%) such cases (NS). Ninety-six patients (6.7%) were given sedation during interHtransport, while in the group of patients transported from the scene there were 183 (4.6%) such cases (NS). The number of instances when neuromuscular block was used in patients transported between hospital was 16 (1.1%), while it was administered to 25 (0.6%) patients transported from the scene (NS). Oxygen therapy was carried out in 591 (41.1%) of patients transported between hospitals, while it was performed in 2,011 (50.2%) patients taken from the scene ($p < 0.001$). In the same group, respiratory therapy was implemented 102 times (2.5%), and in the group transported between hospitals – 59 times (4.1%) (NS).

A comparison of the medical procedures undertaken during the transport of patients with AT is presented in Table 4. For AT patients, clinical events that occurred before and during transport in the interhospital group and during flights from the scene of the event included chest compressions (n = 5 (0.6%) vs n = 244 (4.8%), respectively; $p < 0.001$) and defibrillation (0.2% patients transported between hospitals and 0.4% transferred by HEMS missions; $p = 0.490$).

Table 5. Analysis of HEMS time and distance (patients with AMI)

Variables	N	InterHtransport	N	HEMS missions	p-value
Time until reaching the patient, median (Q1–Q3)	1,412	36.0 (30.0–44.0)	3,942	23.0 (19.0–27.0)	<0.001
Time from reaching the patient until arrival at the target medical institution, median (Q1–Q3)	1,423	45.0 (37.0–54.0)	3,951	38.0 (32.0–44.0)	<0.001
Total time of operation, median (Q1–Q3)	1,401	81.0 (69.0–95.0)	3,896	61.0 (54.0–70.0)	<0.001
Distance of transport, median (Q1–Q3)	1,433	59.4 (49.8–42,107.5)	3,910	52.1 (37.1–42,116.5)	<0.001

AMI – acute myocardial infarction; HEMS – Helicopter Emergency Medical Service; interHtransport – interhospital transport.

Table 6. Analysis of HEMS time and distance (patients with trauma)

Variables	N	InterHtransport	N	HEMS missions	p-value
Time until reaching the patient, median (Q1–Q3)	889	46.0 (35.0–58.0)	5,136	23.0 (19.0–29.0)	<0.001
Time from reaching the patient until arrival at the target medical institution, median (Q1–Q3)	895	66.0 (50.0–85.0)	5,043	45.0 (37.0–55.0)	<0.001
Total time of operation, median (Q1–Q3)	875	115.0 (90.0–141.0)	4,962	70.0 (58.0–83.0)	<0.001
Distance of transport, median (Q1–Q3)	911	135.9 (66.6–42,141.1)	4,920	56.3 (34.0–42,181.3)	<0.001

HEMS – Helicopter Emergency Medical Service; interHtransport – interhospital transport.

Table 7. Number and percentage of missions carried out to the TC as part of transport between hospitals and directly from the scene of the event

Variable	InterHtransport				HEMS missions			
	TCs in Poland – 14							
	no		yes		no		yes	
	N	[%]	N	[%]	N	[%]	N	[%]
T06	19	21.1	71	78.9	234	13.9	1,454	86.1

TC – trauma center; HEMS – Helicopter Emergency Medical Service; interHtransport – interhospital transport.

Intubation in the group of patients transported between hospitals was performed 98 times (10.7%) and in the group transported from the place of the incident – 907 times (17.3%) ($p < 0.001$). The number of times sedation was applied in the case of interHtransport was 459 (50.1%), while in the group of patients transported from the scene it was 1,273 (24.3%) ($p < 0.001$). The number of times neuromuscular block was used in patients transported between hospitals was 128 (14.0%), and 478 (9.1%) in patients taken from the scene ($p < 0.001$). Oxygen therapy and respiratory therapy were applied 161 times (17.6%) and 387 times (42.2%) in patients transported between hospitals, and 1,915 times (36.6%) and 1,600 times (30.6%) in patients taken from the scene of the incident, respectively ($p < 0.001$). Spinal board was used in 3,451 (66.0%) patients transported from the incident site, and in 157 (17.1%) patients transported between hospitals ($p < 0.001$). The cervical collar was placed in 3,315 (63.4%) and 146 (15.9%) cases, respectively ($p < 0.001$).

Information on the technical parameters of the flight – time till reaching and transporting the patient as well as the total time and distance of the mission – are presented in Tables 5 and 6. Median time from take-off until reaching the patient with AMI in the case of interHtransport was 36 min. In the case of HEMS missions, it was 23 min ($p < 0.001$). The time from taking the patient from hospital until arrival at the target medical institution and then transferring the patient to the reference center was 45 min. In the case of HEMS missions, this was 38 min ($p < 0.001$). The total time of the operation was 81 min vs 61 min for interHtransport and the HEMS missions, respectively ($p < 0.001$). The median distance for interHtransport was 59.4 km, and 52.1 km ($p < 0.001$) for flights from the scene of the incident.

Median time from take-off until reaching the patient with AT in the case of interHtransport was 46 min and for the HEMS missions it was 23 min ($p < 0.001$). The time from reaching the patient until arrival at the target medical institution and then taking the patient to the reference center was 66 min for transport between hospitals, while in the case of HEMS missions it was 45 min ($p < 0.001$). The total time of interHtransport operations was 115 min and in the case of a HEMS mission it was 70 min ($p < 0.001$). The median distance of transport for interHtransport was 135.9 km, and for flights from the scene it was 56.3 km ($p < 0.001$) (Table 7).

Patients diagnosed with T06 were most frequently transported to TCs both from the scene of the incident (86.1%) as well as by interHtransport (78.9%). The median distance of transport of an AMI patient (I 20, I21 and I24) across Poland (all bases) is between 41.2 km and 49.8 km. It is noteworthy that 95% of HEMS missions are shorter than 68.8–86.0 km for relevant bases (Fig. 1).

Discussion

The present publication is the first one in Poland to assess the course and results of HEMS interHtransport and flights to the immediate scene of the emergency incident regarding AMI or AT patients.

In the years 2011–2016 a total of 7,645 transport operations were carried out between medical entities and 4,002 missions took place directly from the scene of the incident. Out of these, patients with AMI and AT constituted a vast majority. Similarly to the data published in other countries, men comprised a decisive majority of the AMI patients in both groups (67.7% were transported between hospitals and 68.9% from the scene of the event). Trauma patients were younger than AMI ones – aged 42.1 years for those transported between hospitals and 33.1 years from the scene, vs 65.9 and 63.6 years of age for AMI patients.[13]

The most frequent level of consciousness on GCS was on average 15 points (based on data on 953 patients transported between hospitals and 2,642 taken from the scene – there was a lack of data on the others). There were 27 patients with GCS below 9 points, which constituted 2.1%. In the range between 9 and 12 points, the number of patients was 13 (1.0%); 24 patients scored 13–14 points (1.9%), while 1,230 were given 15 points (95.1%). In the HEMS missions, the corresponding numbers were the following: <9: 105 (2.8%), 9–12: 51 (1.4%), 13–14: 130 (3.5%), and 15: 3,479 (92.4%). This means that the patients were in logical and verbal contact and there were no disorders of consciousness in both groups of AMI patients. In the case of AT patients, the GCS consciousness level was on average 14 points (on the basis of data regarding 472 and 3,313 patients, respectively – there was a lack of data on the others). The number of points on the GCS varied widely. There were 205 patients with GCS below 9 points, which constituted 30.9%. In the range between 9–12 points, the number of patients was 58 (8.7%); 61 patients scored 13–14 points (9.2%), while 340 were given 15 points (51.2%). In the HEMS missions, the corresponding numbers were the following: <9: 1,582 (31.4%), 9–12: 459 (9.1%), 13–14: 712 (14.1%), and 15: 2,286 (45.4%). Such results lead to the conclusion that a significant percentage of patients were unconscious or had moderately disturbed consciousness. While the assessment of patients on the RTS scale on average amounted to 12, this was also true in both groups of AT patients. Similarly to reports in the literature, the scales show that the status of the AT patients was significantly more severe in comparison to AMI patients.[10] In another publication, the level of consciousness in patients with AMI was assessed at 13 points, while of those with AT at 11.9.[13] In some cases, the implementation of additional medical procedures for the time of transport was necessary (sedation, intubation, respiratory therapy).

The clinical procedures undertaken due to the state of the AMI patients included the following: chest compression (n = 6; n = 73) and defibrillation 0.4% and 1.3%,

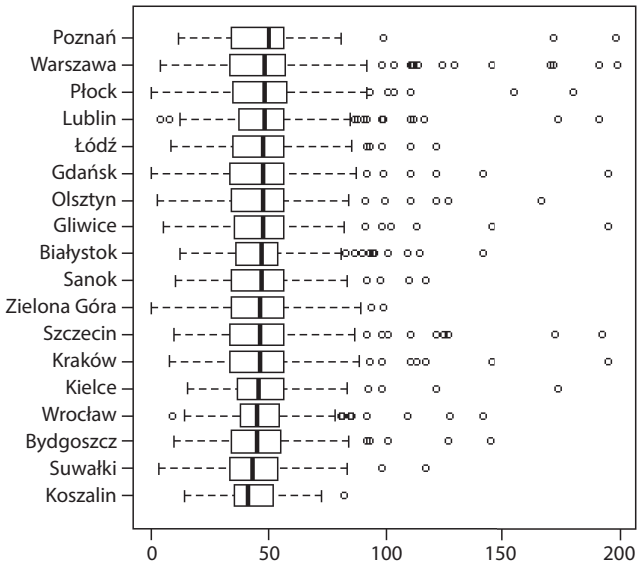

Fig. 1. Analysis of the distance of transport of patients with AMI to the departments of hemodynamics in Poland

while for patients with AT clinical procedures involved defibrillation 0.2% and 0.4%; and chest compressions (n = 5; n = 244). The disorders described occurred both before and in the course of the helicopter flight. The European Society of Cardiology guidelines recommends that patients transported between hospitals should be accompanied by personnel adequately equipped and trained to deal with life-threatening arrhythmias and cardiac arrest.[14–16] There were no fatalities in the course of the flights between hospitals. Nevertheless, there was a fatality incident which one team experienced when flying an AMI patient from the scene of the incident. Analysis showed that the occurrence of cardiac arrest (I46) was more frequent in patients with AMI (I21) than in those with multi-organ trauma (T06). On the basis of the data they received, the authors are unable to determine the moment of cardiac arrest.

In McMullan's study, it was reported that in the course of interHtransport, cardiopulmonary resuscitation was necessary only in 2 patients.[4] This may result from the proper preparation of patients before transport and accurate enrollment of patients who can benefit from aeromedical transport. Analysis showed that oxygen therapy was implemented in 591 and in 2,011 patients with AMI in interHtransport and transport from the scene of the incident, respectively, while sedation in was carried out in 96 and 183 patients, respectively, and respiratory therapy was necessary in 59 and 102 patients, respectively. In AT patients, on the other hand, the most frequently used therapy was sedation (n = 459; n = 1,273) and respiratory therapy (n = 387; n = 1,600) due to severe body injuries and the risk of secondary cardiopulmonary disorders. In this group, oxygen therapy (n = 161; n = 1,915), neuromuscular block (n = 128; n = 478) and intubation (n = 98; n = 907) were also frequent. The percentage of intubation in the American study was different than in ours: patients with AMI

and AT who received intubation comprised 24% and 22% of those transported between hospitals and from the scene of the incident, respectively, which constitutes a significant difference with respect to the data reported in the present study (AMI 0.9% and 1.4%, AT 10.7% and 17.3%, respectively).[12] In the first case (AMI patients), the difference can result from the fact that Polish patients received intensive care protecting them prior to transport by the dispatching entities and were subsequently looked after by MARS teams. The group of patients with AT demanded more careful preparation for the flight, what was analyzed on the basis of the number of undertaken medical rescue procedures, It reflects their more severe condition.

The median of the distance of flight from the dispatching center to the target in the case of AMI or AT was 59.4 km (AMI – interHtransport) and 52.1 km (AMI – HEMS mission) vs 135.9 km (AT – interHtransport) and 56.3 km (AT – HEMS mission), respectively, while the median of the transport time was 45 min (AMI – interHtransport) and 38 min (AMI – HEMS mission), and 66 min (AT – interHtransport) and 45 min (AT – HEMS mission), respectively. The data from the literature differs: the average flight distance with AMI is 70 km and flight time – 31 min, while in the case of AT patients the flight time is 121 min, while there is a lack of data for flight distance.[10,17] The reason for the differences observed is most likely due to the following factors: the density of HEMS bases and target centers, the kind of helicopters, and the organization of the land medical care that takes the patient to HEMS. Moreover, procedural differences between HEMS teams in different countries should not be excluded.

Trauma to multiple areas of the body or multi-organ injuries should ultimately be treated with therapy in TC. Therefore, at the stage of receiving the call for help, it is advisable to immediately dispatch HEMS to patients with multi-organ trauma to avoid unnecessary delay of proper treatment in a TC.

The paper presents a comparison of transport of AT patients to the hospital where there is a TC. Among AT patients, the criteria for treatment in a TC were met by patients diagnosed with T06 – injuries involving numerous body regions according to the ICD-10 codes. Patients with multiple-organ trauma most often came directly from the accident site.

Another analysis of HEMS missions between the years 2011–2013 in Poland also showed that the AT patients most often transported to a TC were those classified into the T06 group.[18]

The study also included an analysis of the distance over which patients with AMI were transported to hemodynamic departments. It was shown that in Poland there are no significant differences between the distances of transporting patients with AMI, which makes it possible to conclude that the location of hemodynamic centers in Poland is optimal.

According to the author's analysis, patients with AMI before the arrival of HEMS received pain relief from the personnel of ambulances – it was probably administered after the examination of the patient. Analgesic drugs were also provided by the hospitals to which the patients were sent. The most commonly administered drug in both groups of transport was morphini sulfas. Air teams more often than ground teams administered fentanylum, which may result from their greater experience with pain relief therapy.

In AT patients, both ambulances and hospitals administered morphini sulfas, fentanylum and ketoprofenum. The HEMS units used fentanylum and morphini sulfas in fractionated doses. Drugs from other groups were administered occasionally (detailed tables are shown in the supplementary data).

Krzyżanowski et al. in a Polish study carried out in the Pomeranian voivodeship indicated that in ground emergency medical teams, only 16% of AT patients are treated with analgesics. The most frequent drug is ketoprofenum. It was shown that 84% of all patients were transported to the hospital without painkillers.[19] The study became the reason for the Ministry of Health to implement guidelines for ground and air emergency medical teams in the area of proper pain management.

Limitations of the study

The main limitation of the study is its retrospective character and the lack of possibility to follow-up the further fate of the patients. Nevertheless, an analysis was done of all the patients transported by HEMS in the timeframe reported, thus minimizing the risk that a systematic error of the selection should occur.

Conclusions

In Poland, HEMS is more readily available and more frequently administered for transport from the place of the event than for interhospital transfer. The interhospital air transport of AMI patients compared to transport from the emergency scene requires less advanced life-saving procedures with the exception of neuromuscular block, sedation and respiratory therapy. Similarities in these areas are also observed in the group of patients with AT. Longer distances or longer transport times of AT patients reflect the existence of fewer TCs compared to hemodynamic TCs. The location of hemodynamic centers in Poland, according to the results of the study, is optimal.

Patients diagnosed with T06 carried both by interHtransport and from the scene of the incident are most often transferred to TCs.

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Appendix – supplementary data

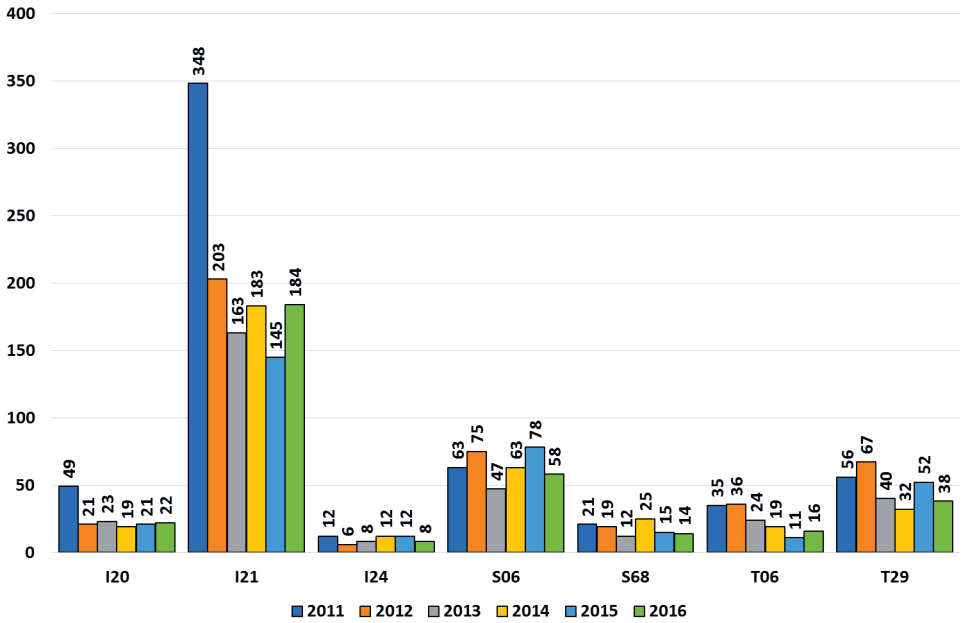

Fig. 2. The number of times interhospital aeromedical transport was used for patients with AMI and selected AT patients during the period analyzed

In the present analysis, the largest number of cases transported to interventional cardiology departments in order to implement the PCI procedure were those with the I 21 diagnosis (AMI). It is noticeable that over this timespan there is a decreasing tendency in the number of patients with AMI transported by air. The authors assume that in subsequent years interventional cardiology units were established in or near the hospitals ordering the air dispatch of such patients, which reduced the need for air transport. On the other hand, the number of times air transport that was used for AT patients remained on a similar level, probably due to the constant number of TCs. Since there are only over a dozen such centers operating in Poland now and a few replantation and burn centers, the transport of AT patients takes longer than that of AMI patients.

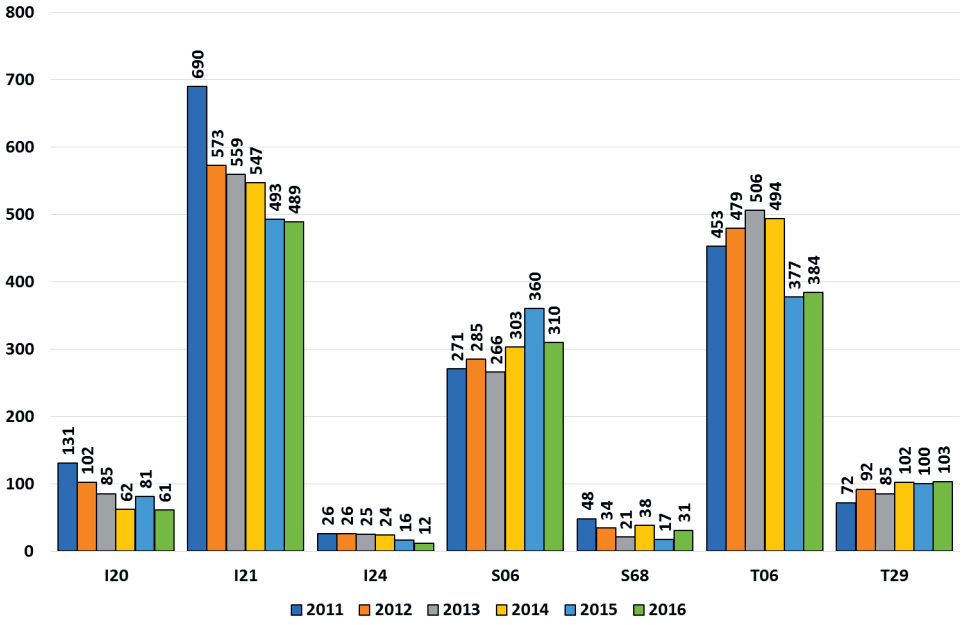

Fig. 3. The number of HEMS missions of patients with AMI and selected acute trauma patients by year

Among the patients transported directly from the scene of the incident, those diagnosed with I21, S06 and T06 were transferred mainly to interventional cardiology departments, replantation centers and TCs, respectively.

Table 8. The frequency of using selected medication by MARS teams in the course of interHtransport and HEMS missions in patients with AMI and AT

	AT				AMI			
	HEMS missions		interHtransport		HEMS missions		interHtransport	
Total number of patients	5,231	100%	916	100%	4,002	100%	1,439	100%
Sodium chlorine	3,310	63.3%	368	40.2%	970	24.2%	180	12.5%
Acidum acetylsalicylicum	7	0.1%	2	0.2%	2,967	74.1%	747	51.9%
Clexane	0	0.0%	7	0.8%	101	2.5%	194	13.5%
Clopidogrelum	4	0.1%	1	0.1%	696	17.4%	80	5.6%
Heparinum sulfas	6	0.1%	4	0.4%	1,571	39.3%	474	32.9%
Morphini sulfas	1,437	27.5%	237	25.9%	2,668	66.7%	435	30.2%
Nitroglicerin	2	0.0%	1	0.1%	653	16.3%	154	10.7%
Ondasteronum	394	7.5%	36	3.9%	482	12.0%	119	8.3%
Midazolamum	1,447	27.7%	309	33.7%	207	5.2%	135	9.4%
Metoclopramidum	245	4.7%	22	2.4%	607	15.2%	48	3.3%
Rocuronium	573	11.0%	62	6.8%	26	0.6%	4	0.3%
Propofol	783	15.0%	91	9.9%	21	0.5%	5	0.3%
Ticagrelor	0	0.0%	0	0.0%	1	0.0%	0	0.0%
Ketoprofenum	603	11.5%	51	5.6%	49	1.2%	18	1.3%
Metamizolum	97	1.9%	28	3.1%	31	0.8%	8	0.6%
Tramadol	34	0.6%	16	1.7%	2	0.0%	0	0.0%
Ketamine	164	3.1%	18	2.0%	1	0.0%	1	0.1%
Paracetamolum	44	0.8%	8	0.9%	2	0.0%	0	0.0%
Fentanylum	2,727	52.1%	297	32.4%	159	4.0%	25	1.7%
Plavix	4	0.1%	2	0.2%	1,819	45.5%	666	46.3%
Compound electrolyte so	1,134	21.7%	194	21.2%	273	6.8%	65	4.5%
Suksametonium	445	8.5%	3	0.3%	8	0.2%	0	0.0%
Wekuronuim	314	6.0%	41	4.5%	7	0.2%	5	0.3%
Atropinum	318	6.1%	19	2.1%	179	4.5%	7	0.5%
Epinephryne	306	5.8%	8	0.9%	114	2.8%	13	0.9%
Thiopental	309	5.9%	55	6.0%	8	0.2%	2	0.1%

MARS – Medical Air Rescue Service; HEMS – Helicopter Emergency Medical Service; AMI – acute myocardial infarction; AT – acute trauma; interHtransport – interhospital transport.

Table 9. The frequency of using analgesic medication by MARS teams in the course of interHtransport and HEMS missions to help patients with AMI

	HEMS missions				InterHtransport			
	ambulance – place of event		MARS		hospital		MARS	
Morphini sulfas	2,020	75.7%	648	24.3%	361	83.0%	74	17.0%
Ketoprofenum	42	85.7%	7	14.3%	17	94.4%	1	5.6%
Metamizolum natricum	21	67.7%	10	32.3%	8	100.0%	0	0.0%
Tramadol	2	100.0%	0	0.0%	0	–	0	–
Ketamine hydrochloride	1	100.0%	0	0.0%	1	100.0%	0	0.0%
Paracetamolum	2	100.0%	0	0.0%	0	–	0	–
Fentanylum	62	39.0%	97	61.0%	15	60.0%	10	40.0%

MARS – Medical Air Rescue Service; HEMS – Helicopter Emergency Medical Service; AMI – acute myocardial infarction; interHtransport – interhospital transport.

Table 10. The frequency of using analgesic medication by MARS teams in the course of interHtransport and HEMS missions to help patients with AT

Analgesic administered	InterHtransport				HEMS mission			
	ambulance – place of event		MARS		hospital		MARS	
Morphini sulfas	1,058	73.6%	379	26.4%	167	70.5%	70	29.5%
Ketoprofenum	469	77.8%	134	22.2%	45	88.2%	6	11.8%
Metamizolum natricum	73	75.3%	24	24.7%	24	85.7%	4	14.3%
Tramadol	34	100.0%	0	0.0%	16	100.0%	0	0.0%
Ketamine hydrochloride	20	12.2%	144	87.8%	12	66.7%	6	33.3%
Paracetamolum	44	100.0%	0	0.0%	8	100.0%	0	0.0%
Fentanylum	729	26.7%	1,998	73.3%	145	48.8%	152	51.2%

MARS – Medical Air Rescue Service; HEMS – Helicopter Emergency Medical Service; AT – acute trauma; interHtransport – interhospital transport.

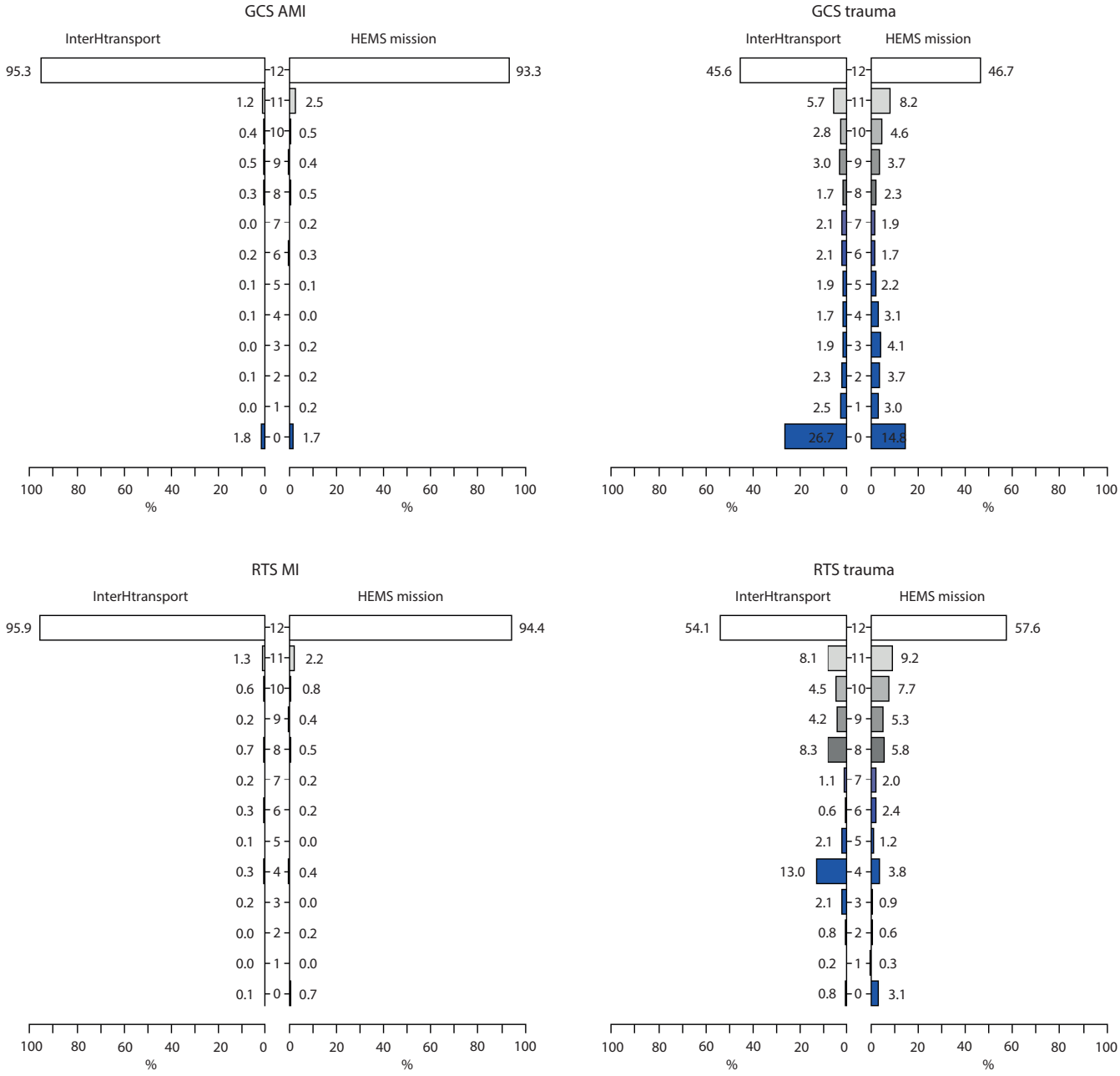

Fig. 4. RTS and GCS scale distribution in the group of patients with AMI and AT

Detection rate of crossing vessels in pediatric hydronephrosis: Transperitoneal laparoscopy versus open lumbotomy

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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. A bundle of crossing vessels (CV) supplying the lower pole of the kidney and causing mechanical obstruction of the ureteropelvic junction (UPJ) has been the subject of many discussions. During pyeloplasty, it is possible to overlook the CV. This may result in recurrent dilatation of the kidney and the need for re-surgery.

Objectives. To compare the detection rate of CV in UPJ obstruction (UPJO) depending on the operational access applied (transperitoneal laparoscopy (LAP) vs open lumbotomy (OPEN)). Assessment of features that could indicate the presence of CV.

Material and methods. Two hundred and forty-six pediatric pyeloplasties were performed between January 2006 and July 2017 in the Department of Pediatric Surgery and Urology at the Wroclaw Medical University, Poland – 111 out of them by LAP and 135 by OPEN, on 98 girls and 148 boys. A retrospective analysis of the patient records for the detection of CV and characteristics of the CV before surgery was performed.

Results. Intraoperative CV causing obstruction of the UPJ in the LAP group were recognized in 34.2% ($n = 38$) of the patients, and within the OPEN group in 12.5% ($n = 17$) ($p < 0.0001$); 90% ($n = 27$) of patients with the diagnosed CV did not show congenital hydronephrosis. In 68% ($n = 21$) of the patients there were cases of recurrent renal colic. The presence of CV was suspected in 7.2% of kidney ultrasounds and in 12.5% in computed tomography (CT) urograms.

Conclusions. The detection rate of CV in UPJO is statistically higher in LAP access than in open retroperitoneal lumbotomy. The distinguishing features of patients with CV are the lack of prenatal diagnosis for hydronephrosis and the presence of pain in the lumbar region.

Key words: hydronephrosis, ureteropelvic junction obstruction, pyeloplasty, vascular hitch, crossing vessels

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Introduction

In the pathogenesis of pediatric hydronephrosis (ureteropelvic junction obstruction – UPJO), we can distinguish intrinsic and extrinsic factors.[1] The most frequently mentioned intrinsic factors are the stenosis of the ureteropelvic junction (UPJ), fibroepithelial polyps and ureteral valves, and impaired number and expression of Cayal-like interstitial cells.[1–3] Among the extrinsic factors, crossing vessels (CV) are the first mentioned, causing obstruction of the UPJ. Less-frequently given reasons for UPJO include high abduction, ureteral twist or adhesions in the retroperitoneal space.[4–6]

A bundle of CV supplying the lower pole of the kidney and causing mechanical obstruction of the UPJ has been the subject of many discussions. Particularly debatable aspects include the detection rate and a method for dealing with recognized CV and the accompanying hydronephrosis. During pyeloplasty, in the cases of both the open and laparoscopic type, it is possible to overlook CV. This may result in recurrent dilatation of the kidney and (in some cases) also pain symptoms despite surgical treatment. It is important for a surgeon to be aware of the presence of CV and to remain vigilant when preparing the UPJ.

Crossing vessels are much more common in older children and adults, with no history of congenital hydronephrosis (prenatally diagnosed).[7] Moreover, characteristic features are colic and paroxysmal pains in the lumbar region. Subsequent ultrasound examinations may significantly differ in the aspect of the sizes of the pelvicalyceal system of sick kidney. These patients should be suspected of having an additional vascular bundle. In such cases, it is important not to overlook the CV during the surgery and to apply an appropriate surgical technique.

Objectives

The study was aimed at comparing the detection rate of CV in UPJO depending on the operational access applied (transperitoneal laparoscopy vs open lumbotomy). Additionally, we assessed the features that could indicate the presence of CV.

Material and methods

A retrospective analysis of the documentation of patients operated on from January 2006 to July 2017 in the Department of Pediatric Surgery and Urology at the Wroclaw Medical University, Poland, was carried out, where 246 pyeloplasties were performed on 98 girls and 148 boys. In the qualification for hydronephrosis surgery, renal ultrasound and renoscintigraphy were performed in all the patients. In dubious cases, computed urotomography was additionally performed. The patients were divided into 2 groups: LAP – patients operated on using a laparoscopic technique with transperitoneal access, n = 111, and OPEN – patients operated on using an open technique from a retroperitoneal approach, n = 135. Fisher's exact test with Wessa software (wessa.net).

Results

A summary of patients operated on with the different surgical techniques depending on access – laparoscopy or open technique – is presented in Table 1.

Additional CV before surgery were suspected in 7.2% of the patients in the ultrasound of the kidney and in 12.5% in computed urotomography. Intraoperative CV causing obstruction of the UPJ in the LAP group were diagnosed in 34.2% (n = 38), and in the OPEN group in 12.5% (n = 17) (Table 2) ($p < 0.0001$). Two patients from the LAP group had previously undergone an unsuccessful surgery with the open retroperitoneal approach using the Anderson–Hynes method. In these cases, the presence of CV was not recognized intraoperatively, however, they were found in the second, laparoscopic surgery.

The number (rate) of laparoscopic hydronephrosis operations grew over time, reached 23.6% in first 3 years of this study and amounted 61.2% in the last 3 years. The rate of open surgeries simultaneously decreased.

A decision on the selection of the surgical technique after the diagnosis of the CV was made intraoperatively

Table 1. Summary of all patients operated on with open techniques (OPEN group) and laparoscopic techniques (LAP group) with division into various surgical techniques

Surgical technique	LAP	OPEN
All surgical techniques	111	135
Anderson–Hynes	78	127
Fenger	4	3
Foley (Y-V Plasty)	2	2
Hellström, mod. Chapman	26	2
Other	1	1

Table 2. Summary of patients with diagnosed crossing vessels (CV) depending on the surgical access (LAP group – laparoscopy and OPEN group – classical surgery)

Variable	LAP	OPEN
Number of patients with CV	38/111 (34.2%)	17/135 (12.5%)
Median age [years]	6	5.5
Sex	M-25 (65.8%) F-13 (34.2%)	M-9 (52.9%) F-8 (47.1%)
Anderson–Hynes + posterior translocation of CV	14 (36.8%)	15 (88.2%)
Hellström, mod. Chapman (cephalad translocation of CV)	24 (63.2%)	2 (11.8%)

Table 3. Characteristic features of hydronephrosis patients with diagnosed crossing vessels (CV)

Hydronephrosis diagnosed antenatally	yes – 10% (3)	no – 90% (27)	n.d. – 25
Recurrent pain/renal colic	yes – 68% (21)	no – 32% (10)	n.d. – 24
Suspected CV in ultrasound	yes – 4 (7.2%)	no – 51	–
Suspected CV in CT examination (Uro-CT)	yes – 1 (12.5%)	no – 7	–
Median age [years]	5.75	–	–

n.d. – no data; Uro-CT – urotomography.

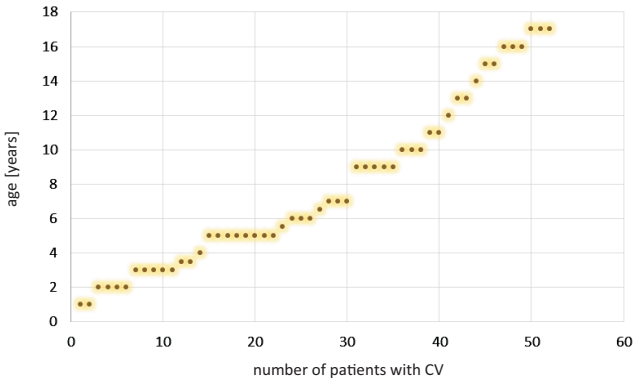

Fig. 1. Diagram showing the age of patients with crossing vessels (CV) at the time of surgery

on the basis of the UPJ anatomy and the entire disease picture. Data of the patients with CV depending on the surgical access is presented in Table 2. Data regarding the interview and imaging tests is given in Table 3. The age of patients in individual groups was also analyzed, which is given in Table 2. Diagram showing the age of patients with CV at the time of surgery is presented in Fig. 1. Crossing vessels were not revealed in any patient under 1 year of age. The median age of patients with CV did not differ from the median age of patients without CV and it was 5.75 years. Dilatation of the renal pelvis in the anterior–posterior (AP) diameter before surgery in the group without CV was 30 mm (median) (from 15 to 55), and also 30 mm in the group with CV (from 13 to 70) ($p > 0.05$).

A certain limitation to our study is the fact that some surgeons operated on the patients with the use of a laparoscopic technique and others with an open technique. Moreover, the study was performed retrospectively.

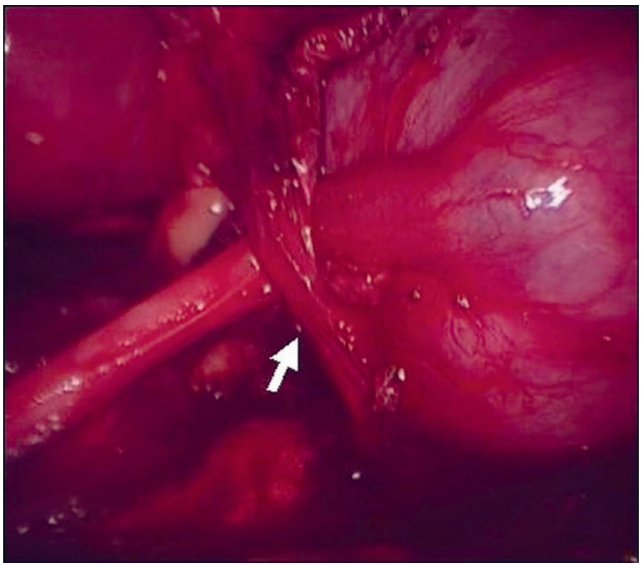

Fig. 2. Intraoperative image during laparoscopic pyeloplasty. Crossing vessel causing obstruction of the ureteropelvic junction (UPJ), marked by arrow

Discussion

The most common extrinsic factor of UPJO is the presence of CV.[1,4,5,7] In the literature, they are described as an artery or an artery and a vein supplying the lower pole of the kidney. Localized at the height of the UPJ, they can cause mechanical obstruction and the occurrence of a symptomatic hydronephrosis (Fig. 2). In a typical course of UPJO with the presence of CV in the history, recurring pain in the lumbar and abdominal region frequently occurs.[7] In the analyzed material, the symptoms of renal colic, nausea and vomiting were the dominant symptoms in patients with CV. As many as 68% of our patients presented with CV. According to the literature, in most cases, children with CV in a prenatal and neonatal interview do not have the dilatation of the kidney.[1,7] This variant of the disease is even referred to by some researchers as "adult hydronephrosis". We obtained very similar results in our material. The vast majority of the patients – as much as 90% in ultrasound, both prenatal and in the neonatal-infancy period – did not show kidney dilatation. Furthermore, in patients with CV, in subsequent ultrasound scans, the sizes of the kidney pelvis may differ significantly from one another. This is due to the fact that the UPJ obstruction is the so-called "permeable obstruction".

The renal pelvis most often in the case of CV is not very dilatated (less than 30 mm in the AP dimension), and it can be located deeply in the renal cavity.[7] A larger dilation may affect calyces. Weiss et al. showed a smaller dilatation of the kidney in the pediatric group with CV than in a group without CV.[8] Similar results were shown in the studies on adult patients with hydronephrosis.[3] In the group of patients with CV, the dilatation grade 1–2 is statistically more often enlarged while in the group without CV, the dilatation is more often grade 3–4 in the SFU (Society of Fetal Urology) scale. In our material, the dilatation of the kidney in the group of patients with CV was not significantly different from the group of patients without CV. In both groups, the median renal pelvic dilatation in the AP dimension was 30 mm. The discrepancy in comparison with the literature may be due to the fact that the cited studies also evaluated the width of the calyx

cup and in some studies the SFU scale was used. In our material, in each case we assessed the AP dimension of the renal pelvis.

The literature states that Doppler examination is effective in preoperative CV detection. Veyrac et al. showed CV in ultrasound with Doppler in 25 out of 28 cases of hydronephrosis in children, in which the presence of CV was later confirmed intraoperatively.[9] In our material, all the patients before the surgery had a kidney ultrasound examination, but mostly without a Doppler option. Only 10.8% of them were suspected of having CV. The group of patients in whom we performed urotomography was too small to draw conclusions. In the study by Weiss et al., in 32.5% of the patients who underwent functional magnetic resonance urography, the radiologists described the presence of CV in the opposite, healthy kidney.[8] These studies show that the presence of CV in children is very common, but not every vessel must cause ailments and dilatation of the kidney. Similarly, in adults it is reported that CV occur in about 20% of healthy people and do not cause any problems.[10–13] Thus, intraoperative detection of the CV and the assessment of whether this bundle is important for UPJ function is quite subjective; it depends on the surgeon's judgment and largely on his/her experience. Improper assessment and a hasty decision to abandon formal pyeloplasty in favor of vessel dislocation may result in the recurrence of hydronephrosis and the need for reoperation.

The incidence of the CV causing obstruction of the UPJ in children increases with age.[14–16] Crossing vessels are very rarely noticed in newborns and infants. Similarly, the analyzed material did not show this pathology in any child under 1 year of age. Calisti et al. showed CV in 6 out of 54 (11%) prenatally diagnosed patients with an average age of 3.5 months compared to 12 out of 30 (40%) symptomatic patients with an average age of 6.4 years.[14] In the study by Schneider et al., all CV patients were over 2 years of age, with an average age of 10 years (from 2 to 17.3 years).[15] Maheshwari et al. in a group of 82 patients showed CV in 7 (8.5%) with an average age of 7.12 years (from 4 months to 15 years).[16] In adults, the proportion of patients with CV is around 39–71% of all patients with UPJO.[3,10–13] In the analyzed material, the median age in a child with the presence of CV was 5.5 years in the OPEN group and 6 years in the LAP group, respectively. However, when comparing the age of children with and without the presence of CV, there was no difference – in both groups the median age was 5.75 years.

The aim of this study was to compare the CV detection frequency depending on the surgical technique – laparoscopy with transperitoneal access and the open retroperitoneal approach. Menon et al. described the CV detection rate in the classical retroperitoneal technique at the level of 5.1% in a group of 643 patients.[7] The low number of detected vessels can be explained by the fact that the study qualified patients under 12 years of age, and as we know from the literature, the incidence of additional vessels increases with age. In addition, these authors used the open retroperitoneal technique. In another study, Hacker et al., also in open retroperitoneal pyeloplasty, in patients aged from 6 weeks to 16 years, reported the incidence of vessels in 25% of the children (28 out of 112 patients).[1] Assem et al., using the retroperitoneal laparoscopic technique in a group of 23 pediatric patients, demonstrated the presence of CV in 4 of them (17.3%) intraoperatively.[17] Simforoosh et al. reported the presence of CV in 9 out of 63 (14.2%) children operated on using the laparoscopic transperitoneal technique.[18] The average age of the patients was 5 years. The authors reported 1 case of a patient with CV that were previously overlooked in open pyeloplasty. Data on the detection of additional vessels, reported in the literature, is divergent. This fact could be the result of the significantly different age groups of the patients. In addition, in some reports, the number of patients analyzed is too small to draw important conclusions. There is also a lack of studies comparing the detection rate of vessels depending on 2 different types of operational access in 1 medical center. In available publications, the authors focus on 1 operational access.

In this material, the CV were statistically more frequent in the transperitoneal laparoscopic approach. In the LAP group, the incidence of CV was 34.2%, and in the OPEN group 12.5%, which was statistically significant. Considering the anatomy of the kidney, the additional vascular bundle is located forward in relation to the UPJ. In the transperitoneal approach when preparing UPJ with the presence of CV, it is difficult to omit it. With this anatomy in the open retroperitoneal approach, the vessels are located at the back of the UPJ. In addition, during surgery, often in an older child, where the UPJ is located deep in the surgical field, in order to show it well and prepare, it is necessary to put on the stitches and use traction. With such a maneuver, the UPJ is pulled towards the operator, and the CV remains even deeper in the operating field and it can thus be omitted. In the analyzed material there were 2 cases (1.4%) originally operated classically from retroperitoneal approach, where the presence of CV was not recognized. In reoperation with transperitoneal access using laparoscopy these vessels were detected and cephalad translocation was performed.

Conclusions

The detection rate of crossing vessels in UPJO is statistically greater in transperitoneal laparoscopic access than in retroperitoneal open pyeloplasty. Distinctive features of patients with the presence of an additional CV are the lack of prenatal diagnosis for hydronephrosis and the presence of pain in the lumbar area.

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Could the super-pulsed CO_2 laser be used for oral excisional biopsies?

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Abstract

Background. The main purpose of a biopsy is microscopic examination and diagnosis. Keeping the margins of specimens safe and readable is always fundamental to detecting marginal infiltrations or malignant transformation. Numerous options and tools have been introduced for biopsy procedures. Lasers are one of these options that provide many enhancements to clinical and surgical biopsy procedures in comparison to scalpels.

Objectives. The aim of the present study is to quantify the thermal artefacts in histological specimens obtained using a CO_2 laser from different oral mucosal lesions and to evaluate if the resulting thermal effect hinders the histological examination. This aim is accomplished through quantitatively and qualitatively assessing the thermal effect in both the epithelium and connective tissue.

Material and methods. A super-pulsed CO_2 laser (10,600 nm) was used to obtain 10 excision biopsy samples. The parameters were a power of 4.2 W in focused mode and a frequency of 80 Hz in super-pulse mode. The histological analysis was performed with an optical microscope. Computerized imaging software was utilized to quantitatively evaluate the thermal effect in both the epithelium and connective tissue expressed in microns.

Results. The thermal effect of the CO_2 laser was limited to the surgical resection margins in all the specimens and did not hinder the histological analysis. Thermal artefacts were observed in 3 specimens. The range of thermal effects in the epithelial tissue was between 184 µm and 2,292 µm, while in the connective tissue it was between 133 µm and 2,958 µm.

Conclusions. The resulting thermal effects of using a CO_2 laser did not hamper the histological evaluation. Utilizing a laser in biopsy procedures should be tailored. Not only should laser parameters and safety margins be taken into consideration but also the working time, clinical accessibility, and the nature and water content of the tissue.

Key words: biopsy, artefacts, carbon dioxide laser (CO_2)

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Introduction

The main purpose of a biopsy is microscopic examination and diagnosis. Keeping the margins of specimens safe and readable, especially in suspected lesions or neoplastic lesions, is always fundamental to detecting marginal infiltrations or malignant transformation.[1–6] Numerous options and tools have been introduced for biopsy procedures.[7] Lasers are one of these options that provide many enhancements to clinical and surgical biopsy procedures in comparison to scalpels. A high degree of decontamination of the surgical area, minimal postoperative bleeding, and reduction of inflammation and postoperative pain have been described in studies about lasers used for biopsies.[8–14]

There are more than 10 different laser devices for dental use.[9,15] The carbon dioxide (CO_2) laser is characterized by high affinity to water and has become one of the favorite instruments for the treatment of benign lesions, such as fibromas, papillomas, labial and lingual mucosal frenula and gingival hyperplasia, as well as for premalignant lesions such as oral leukoplakias.[3,16–19] In general, cutting with a laser is accomplished through the photothermal effect, which is the conversion of light into thermal energy that heats the target tissue and eventually leads to the cutting action. Consequently, thermal effects occur at the periphery in the collected specimens.[3,11] These thermal effects may result in creating tissue artefacts that lead to alterations in the histopathological evaluation and confusion for pathologists.[1,9]

Thus, it is important to evaluate the thermal effects of CO_2 lasers on the peripheral margins of specimens in order to assess if the CO_2 laser is a reliable tool for biopsy procedures. The aim of the present study was to quantify the thermal artefacts in histological specimens obtained by CO_2 lasers from different oral mucosal lesions and to evaluate if the resulting thermal effect will hinder the histological examination. This aim was accomplished through quantitatively and qualitatively assessing the thermal effect in both the epithelium and connective tissue.

Material and methods

Ten oral lesions from 10 different patients, 5 males and 5 females, ranging in age from 23 to 72 years (mean: 48.5 years) were examined. The cases included 1 carcinoma in situ, 2 mucocele, 4 focal fibrous hyperplasia, 1 kaposiform hemangioendothelioma, 1 peripheral giant cell granuloma, and 1 granular cell tumor. The lesions were distributed as follows: 3 cases from buccal mucosa, 3 cases from the attached gingiva and 4 cases from the labial mucosa. The biopsy procedures were conducted at our outpatient clinic.

Before the biopsy procedures, all patients were informed about the advantages and disadvantages of laser surgery. They signed an informed consent form. The study was conducted following the Declaration of Helsinki according to the local Ethical Committee guidelines. Exclusion criteria included systemic disease, degenerative bone disease, chemotherapy or radiotherapy to the head and neck region, pregnancy, smoking habit, and alcohol consumption.

All the cases were photographed pre- and postoperatively. Two follow-up visits were performed. All biopsies were performed under local anesthesia using 1.8 mL of mepivacaine solution containing 1:100,000 epinephrine by the same surgeon under similar conditions.

A super-pulsed CO_2 laser (Smart US20D; DEKA Laser, Florence, Italy) with the following characteristics was used to perform the biopsy: wavelength of 10,600 nm, frequency range between 5 Hz and 100 Hz, and pulse length range between 200 µs and 80 ms. The efficiency of power transfer was measured to be greater than 85%. The 15% power loss was balanced by a suitable calibration of the internal pump to avoid dust and particle deposition over the lenses during operation.[3] All the samples were excised using dental handpiece focal 2″ with non-contact tip (tip with a mirror to deflect the laser of 120°) with a power of 4.2 W in focused mode with spot diameter between 0.2 mm and 0.4 mm at a distance of 2 mm to 4 mm from the tip and a frequency of 80 Hz in super-pulse mode.

Both 0.2% chlorhexidine spray and 0.5 mL of amino acids and sodium hyaluronate gel were prescribed 3 times daily for 1 week. All excised specimens were immediately fixed in a 10% neutral buffered formalin solution. Then, they were embedded in paraffin and stained with hematoxylin and eosin (H&E) for the histological evaluation.

The histological analysis was performed with an optical microscope (Leica Leitz Camera; Leica Camera AG, Wetzlar, Germany). A computerized digital camera (Olympus Camedia 5050; Olympus Inc., Tokyo, Japan) was used to capture 5 Mp (24-bit color depth) images (×100 magnification) of surgical resection margins (stored as JPG files). Computerized imaging software (ImageJ; National Institutes of Health, Bethesda, USA) was utilized to quantitatively evaluate the thermal effect in both the epithelium and connective tissue, expressed in microns.

Results

The thermal effects of the CO_2 laser were limited to the surgical resection margins in all the specimens and did not hinder the histological analysis. Thermal artefacts were found in 3 specimens: vacuolar degeneration at the basal keratinocytes in one of the labial mucosa specimens (Fig. 1) and diathermocautery artefacts in 2 specimens: 1 from the labial mucosa and the other from attached gingiva.

The thermal effect in connective tissue was greater than that in the epithelium in all the specimens except 1 (Fig. 2). The range of the measured thermal effect in the epithelium was between 184 µm and 2,292 µm. The range

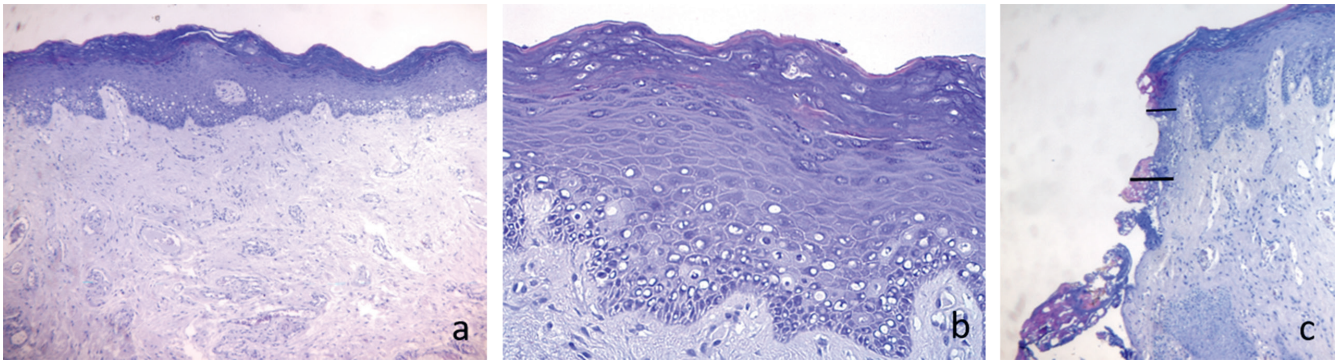

Fig. 1. A – representative photomicrograph of labial mucosa with focal fibrous hyperplasia. Original magnification ×5. B – high magnification of the lesion showing a hyperkeratotic epithelium with vacuolar degeneration of basal keratinocytes and a dense collagen matrix in the lamina propria (×20 magnification). C – surgical resection margin of the oral mucosa shows the thermal effects both in the epithelium and in the connective tissue (×10 magnification). The bars show the extension of tissue damage in the epithelium (thin bar) and in the connective tissue (thick bar). Hematoxylin and eosin (H&E) staining

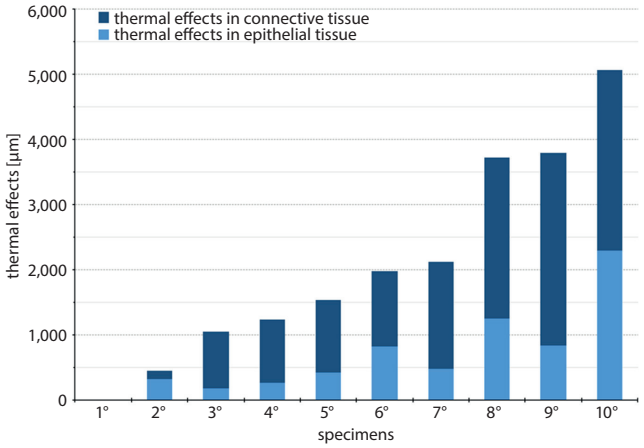

Fig. 2. Bar chart of average values of the thermal effect obtained in epithelial and connective tissue

of the thermal effect in the connective tissue was between 133 µm and 2,958 µm (Table 1). The mean of the thermal effect in the epithelium was 687 µm, while in connective tissue it was 1,407 µm. The mean total thermal effect was 2,094 µm (Fig. 3,4).

The most prominent thermal effect was observed in the specimens excised from attached gingiva. Only 1 specimen did not show any thermal effect.

Discussion

Specimens collected with a laser are usually compromised by thermal effects. It is often considered a common disadvantage that may cause tissue artefacts and marginal dysplastic changes.[20,21] For this reason, many studies have

Table 1. The evaluated marginal thermal effects and thermal artefacts in all specimens in the study

Specimen No.	Diagnosis	Site of lesion	Histologic artefact	Thermal effect in the epithelium [µm]	Thermal effect in connective tissue [µm]	Total thermal effect [µm]
1	kaposiform hemangioendothelioma	buccal mucosa	no	≃0 µm	≃0 µm	≃0 µm
2	granular cells tumor	labial mucosa	no	322.75 µm	133.4 µm	456.15 µm
3	peripheral giant cell granuloma	attached gingiva	no	184.24 µm	867.75 µm	1,052 µm
4	mucocele	labial mucosa	no	262 µm	968.26 µm	1,230.26 µm
5	focal fibrous hyperplasia	labial mucosa	vacuolar degeneration at the basal keratinocytes	429.62 µm	1,101.22 µm	1,530.83 µm
6	squamous cell carcinoma in situ	buccal mucosa	no	828.36 µm	1,151.1 µm	1,979.47 µm
7	focal epithelial hyperplasia	buccal mucosa	no	476.69 µm	1,646.86 µm	2,123.56 µm
8	focal fibrous hyperplasia	attached gingiva	no	1,245.19 µm	2,478.2 µm	3,723.39 µm
9	mucocele	labial mucosa	diathermocautery artefacts	831.74 µm	2,958.06 µm	3,789.8 µm
10	focal epithelial hyperplasia	attached gingiva	diathermocautery artefacts	2,292.94 µm	2,767.69 µm	5,060.63 µm

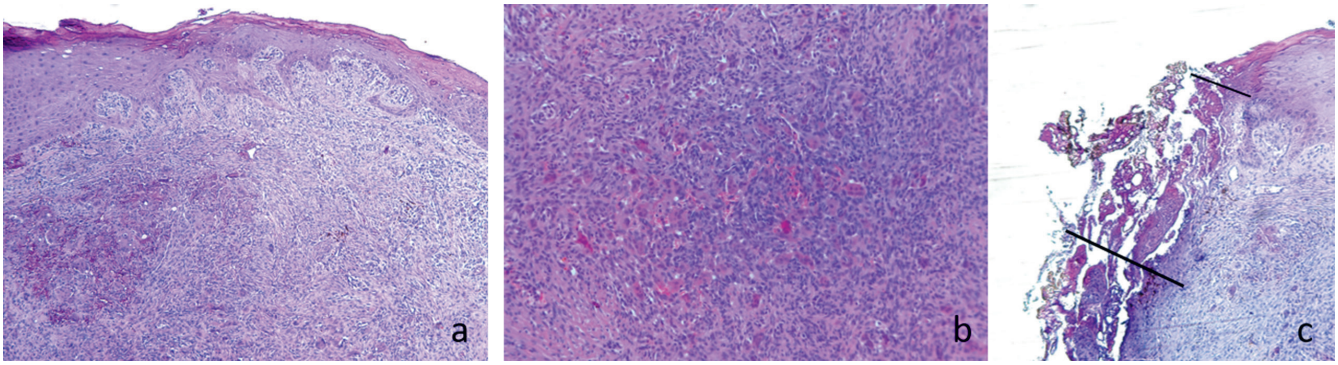

Fig. 3. A – representative photomicrograph of the oral mucosa with peripheral giant cell granuloma. Original magnification ×5. B – high magnification of the lesion showing abundant multinucleated osteoclast-like giant cells in a fibroblastic stroma (×20 magnification). C – surgical resection margin of the oral mucosa shows the thermal effects both in the epithelium and in the connective tissue (×10 magnification). The bars show the extension of tissue damage in the epithelium (thin bar) and in the connective tissue (thick bar). Hematoxylin and eosin (H&E) staining

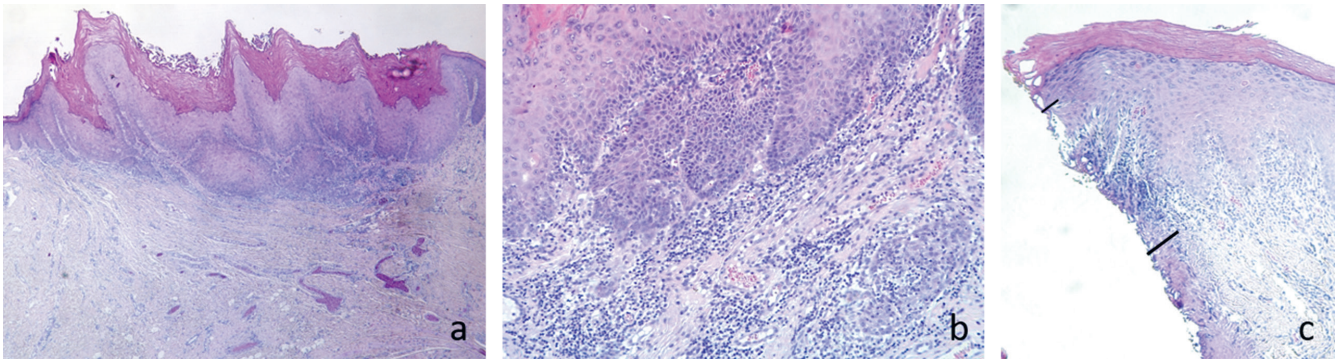

Fig. 4. A – representative photomicrograph of the oral mucosa with in situ squamous cell carcinoma arising on lichenoid keratosis. The lesion shows a pronounced hyperkeratosis and a papillary surface. Original magnification ×5. B – high magnification of the lesion showing a moderate inflammatory infiltrate in the lamina propria (×20 magnification). C – surgical resection margin of the oral mucosa shows the thermal effects both in the epithelium and in the connective tissue (×10 magnification). The bars show the extension of tissue damage in the epithelium (thin bar) and in the connective tissue (thick bar). Hematoxylin and eosin (H&E) staining

been carried out to assess this disadvantage and its impact on histological evaluation.[7,17,19,22] In an experimental study performed on 25 Sprague Dawley rats, the influence of the thermal effect caused by different CO_2 laser powers (between 3 W and 12 W) was examined, and it was concluded that the CO_2 laser, unrelated to the wattage, generates epithelial thermal damage similar to dysplastic changes. Thus, it was suggested that clinicians should take these changes into consideration.[21]

The control of power settings, spot diameter and pulse duration minimizes the thermal damage and enables achieving histologically acceptable specimens for diagnosis. Many authors consider the thermal effect of lasers that impairs the histological evaluation to be caused by the operator rather than the laser itself.[23]

Therefore, many ex vivo and in vivo studies were carried out to find the ideal parameters for the laser that minimizes this thermal effect and consequently decreases the chance of thermal artefacts.[5,9,21,24] In an ex vivo study, the histological analysis of specimens collected by different CO_2 laser parameters were compared, and it was found that efficient cutting with minimal thermal effect can be achieved by a power of 3 W in continuous wave (CW) or in pulsed wave (PW) settings at a frequency of 50 Hz.[3]

The laser beam in PW has shown reduced thermal damage compared to CW in many animal studies.[25–28] In a clinical study, the thermal damage outcomes following excision biopsy of 100 fibrous hyperplasia lesions using CO_2 laser in PW and CW mode were compared. It was concluded that both laser modes produced similar thermal damage, and researchers recommended adding a 1 mm safety margin, especially in suspicious soft tissue lesions.[25]

Other studies were carried out to compare the thermal effect of lasers compared with other tools.[7,11,16,22] Matsumoto[22] compared CO_2 lasers with an electrotome. In his study, the optical microscopic examination of specimens excised by a CO_2 laser, particularly in PW mode, produced less thermal damage than the electrotome. The thermal damage was estimated to be less than 500 μm and did not affect the pathological diagnosis.

In the present study, one of the collected specimens was carcinoma in situ, and histological evaluation was achieved without confusion. Utilizing a laser for excision biopsy of oral malignancy in an early stage has been reported.[25,29–31] The nature of the lesion and water content appear to have an impact on the thermal effect during excision, as the most prominent thermal effect in our study was observed in a focal fibrous hyperplasia lesion.

In the sample with the lowest value, the thermal effect in both epithelium and connective tissue was so minimal that it was considered by the pathologist to be proximal to 0 (≃). Additionally, the working time (different depending on the site of intervention) was reported to be a possible factor that affects the thermal effect.[9]

In fact, there is a difference in the laser parameters used in clinical and ex vivo studies. The parameters for this study were similar to the parameters recommended in the literature.[22,25] In this study, the thermal effect was prominent in all the specimens, as the average of the total thermal effect was approx. 2 mm (2,049 μm). It was generally higher in attached gingiva compared to other anatomical sites.

It is obvious that the thermal effect of the CO_2 laser will occur and cannot be prevented but can be minimized. For that reason, the control of laser parameters and working time and adding laser safety margins are suggested.[3,24] The resulting thermal effects of using a CO_2 laser did not hamper the histological evaluation. Utilizing a laser in biopsy procedures should be tailored. Not only should laser parameters and safety margins be taken in consideration but also the working time, clinical accessibility, and the nature and water content of the tissue.

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The effect of esomeprazole vs famotidine on aspirin/clopidogrel dual therapy after percutaneous coronary intervention

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Abstract

Background. Regarding drug interactions between proton pump inhibitors (PPIs) and dual antiplatelet therapy (DAPT), controversies have arisen over the possibility that PPIs may interfere with the antiplatelet effect of DAPT. However, whether this interaction is drug-specific or a class effect needs to be determined. It is not clear whether famotidine, an H2-receptor antagonist (H2RA), interacts with DAPT.

Objectives. The aim of this study was to assess the impact of esomeprazole and famotidine on the efficacy of DAPT.

Material and methods. The study involved 160 patients undergoing elective percutaneous coronary interventions and treated with DAPT and concomitant use of esomeprazole (40 mg/d) or famotidine (40 mg/d). Platelet reactivity was measured with adenosine diphosphate (ADP)-induced light transmittance aggregometry (LTA) and vasodilator-stimulated phosphoprotein phosphorylation-platelet reactivity index (VASP-PRI) at baseline, 14 and 30 days after applying randomized acid-suppressing agents.

Results. No significance differences were observed in treatment-by-period interactions with LTA values ($p = 0.298$) and VASP-PRI values ($p = 0.867$), which suggested no carryover effect in either regimen over the 30-day treatment period. Intergroup comparisons were done between the 2 groups at 3 times, and similar findings were observed at each time (all $p > 0.05$). As for intragroup measurements among the separate times, significantly lower LTA and VASP-PRI values existed on day 14 for both agents (both $p < 0.05$).

Conclusions. The antiplatelet effect of DAPT was not affected by concomitant use of esomeprazole or famotidine. These 2 agents were much less likely than CYP2C19 polymorphisms to influence aspirin/clopidogrel therapy, supporting the assertion that the pharmacodynamic interaction between aspirin/clopidogrel and acid-suppressing agents is a drug-specific rather than a class effect.

Key words: platelet reactivity, drug–drug interaction, dual antiplatelet therapy, esomeprazole, famotidine

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Coronary heart disease (CHD) is the leading cause of death worldwide.[1] Percutaneous coronary intervention (PCI) with stent implantation is increasingly used for the treatment of ischemic heart disease. In China, more than 600,000 CHD patients underwent PCI in 2016, as published in the surveillance report from the Chinese Heart Association.[2] Accumulating evidence supports the utility of antiplatelet therapy as lifelong standard care following revascularization. Dual antiplatelet therapy (DAPT) consisting of aspirin and an adenosine diphosphate (ADP) receptor blocker such as clopidogrel is used to protect against thrombotic complications[3]; however, patients with high on-treatment platelet reactivity remain at high risk of recurrent ischemic events.[4] Aspirin/clopidogrel dual antiplatelet therapy usually increases the risks of major gastrointestinal bleeding, with events increased from 0.7% in patients on aspirin alone to 1.3% in those with aspirin and clopidogrel co-therapy during 12 months of treatment.[5,6] Several societies have therefore recommended acid-suppressing agents for the prevention of bleeding complications.[7] Clopidogrel requires metabolism by liver cytochrome p450 enzymes (CYPs) to become an active metabolite. Among the CYPs, CYP2C19 is now regarded to play the most important role.[8] Although proton pump inhibitors (PPIs) used in clinical settings are inactivated by CYP2C19, the extent of inactivation by CYP2C19 is dependent on the type of PPI; therefore, the association between PPIs and increased risk of serious cardiovascular events in patients receiving clopidogrel has led to a warning label by the FDA.[9]

As a frequently prescribed PPI during the maintenance of dual antiplatelet therapy, omeprazole had been reported to competitively inhibit clopidogrel transformation, resulting in decreased clopidogrel antiplatelet activity.[9–11] Nevertheless, whether the clopidogrel-PPI interaction is a class effect or a drug-specific effect is still a matter of debate. H_2-receptor antagonist (H2RA) has been used as an alternative to PPIs. In a previous study, H2RA was reported to be effective in the prevention of ulcers with aspirin.[12] However, another study comparing PPI and H2RA regimens indicated that famotidine was inferior to pantoprazole in preventing digestive bleeding.[13] Whether famotidine causes any interference with the platelet inhibitory effect of aspirin/clopidogrel is unknown. The aim of this study was to investigate the antiplatelet efficacy of DAPT with concomitant use of esomeprazole and famotidine.

Material and methods

Patients and selection criteria

We conducted this prospective, randomized trial to assess the effects of esomeprazole and famotidine on platelet inhibition by DAPT. We recruited clopidogrel-naive hospitalized patients who had been admitted to the Department of Cardiology of the Second Affiliated Hospital of Zhejiang Chinese Medical University, Hangzhou, China. The inclusion criteria included: (A) age ≥18 years, (B) diagnosis of acute coronary syndromes, (C) performed PCI, and (D) received 600 mg clopidogrel and 300 mg aspirin loading dose between 12 h and 24 h prior to the PCI. We excluded all subjects that could be classified as having (A) class IV heart failure according to New York Heart Association (NHA) criteria, (B) thrombocytopenia (platelet count $< 100 \times 10^9$/L) or anemia (hemoglobin <10 g/dL), (C) any chronic illness, such as cancer, liver cirrhosis or end-stage renal failure, (D) a history of hemorrhagic disorder, stroke or gastrointestinal ulcer, (E) known allergies to esomeprazole or famotidine, or who (F) refused to participate in the study.

In patients without prior antiplatelet therapy, the loading was 300 mg of aspirin (Bayer HealthCare AG, Leverkusen, Germany) and 600 mg of clopidogrel (Sanofi Aventis, Bridgewater, USA). Subsequently, the patients were maintained with 100 mg of aspirin and 75 mg daily of clopidogrel. The patients in the esomeprazole group received 40 mg/day of esomeprazole (AstraZeneca LP, Wilmington, USA), while the famotidine group received 40 mg/day of famotidine (Eisai Pharmaceuticals Co., Tokyo, Japan). The administration of these drugs was at the same time as the clopidogrel/aspirin. The patients were followed up daily during their hospital stay. After discharge, drug therapy compliance was assessed through a telephone call every 3 days and outpatient clinic visits on the 14th and 30th day. All the patients were instructed to bring their drug bags for examination.

The study complied with the Declaration of Helsinki and was approved by the Ethics Committee and Institutional Review Board of the Second Affiliated Hospital of Zhejiang Chinese Medical University. All the participants provided their written informed consent. An independent data safety monitoring committee was instituted for the adjudication of adverse clinical events.

Study endpoint

The study endpoint was residual platelet reactivity, which was assessed with the value of ADP-induced light transmittance aggregometry (LTA) and vasodilator-stimulated phosphoprotein phosphorylation-platelet reactivity index (VASP-PRI). Blood sampling for evaluating platelet function was conducted at 3 timepoints: (1) baseline (prior to randomization), (2) on the 14th day of treatment with randomized acid-suppressing agents and (3) on the 30th day of treatment with randomized acid-suppressing agents.

The baseline characteristics recorded included demographic data, cardiovascular risk factors and concomitant medications. All the investigators who evaluated the clinical endpoints were blinded to the results of platelet function activity.

Platelet function tests

All platelet function tests were performed on the same day and within 2 h of sampling. Platelet aggregation was performed using LTA, as described in a previous study.[14] In short, whole blood was centrifuged at 800 rpm for 10 min to obtain platelet-rich plasma (PRP); platelet-poor plasma (PPP) was obtained by a 2nd centrifugation of the blood fraction at 2,500 rpm for 10 min. Light transmission was adjusted to the 100% line with PPP and a 0% baseline with PRP before the addition of the agonist. The agonist used was 20 µmol/L of ADP. Then, 0.45 mL PRP was incubated at 37°C for 3 min, after which the agonist was added to the PRP. Maximal platelet aggregation (MPA) and late platelet aggregation (LPA) values (5 min after the addition of ADP) of on-treatment platelet aggregation were measured. The results were given as MPA and LPA values according to the formula:

$$[\text{Disaggregation } (\%) = 100 \times (1 - \text{LPA}/\text{MPA})].$$

VASP-PRI was also performed as described in a previous study.[15] Briefly, citrated blood samples were incubated with prostaglandin E1 (PGE1) alone or with PGE1+ADP, and both were fixed with paraformaldehyde. After a cellular permeabilization, VASP in its phosphorylated state was labeled with a primary monoclonal antibody against serine 239-phosphorylated VASP (clone 16C2), followed by a secondary fluorescein isothiocyanate-conjugated polyclonal goat anti-mouse antibody. Final analyses were performed using quantitative flow cytometry (Biocytex Inc., Marseille, France). A platelet reactivity index (PRI) was calculated using mean fluorescence intensity (MFI) in the presence of PGE1 alone or PGE1+ADP according to the formula:

$$\text{PRI } (\%) = [\text{MFI}_{(\text{PGE1})} - \text{MFI}_{(\text{PGE1+ADP})}]/\text{MFI}_{\text{PGE1}} \times 100.$$

Statistical analysis

Calculation of the sample size for the present study was based on previous experience.[10,14] We estimated that a study sample size of 120 would enable detection of a one-half standard deviation (SD) difference (i.e., a 10% difference in platelet reactivity between groups) with an 80% statistical power and a 5% alpha risk. To ensure that this sample size would be available for analysis, 60 extra patients were randomized and included. All the laboratory data was normally distributed and was described as mean ±SD or n (%). The baseline variables of the 2 groups were compared using Pearson's χ^2 test when appropriate for categorical baseline variables and the two-sample t-test for continuous baseline variables. Repeated measures analysis of variance (ANOVA) was used to evaluate the values of ADP-induced LTA and VASP-PRI at 3 points in time.[16] Intergroup comparison of the respective regimens at each time point and intragroup comparison of the same regimen among the different timepoints were compared with the two-sample t-test. A p-value <0.05 was considered statistically significant throughout the analyses. The statistical analysis was performed using SPSS v. 22.0 software (IBM Corp., Armonk, USA).

Results

Between March and October 2017, 188 clopidogrel-naive hospitalized patients were qualified for the study according to the inclusion and exclusion criteria. Among these, 18 patients refused to participate in the study and 10 developed gastrointestinal complications prior to the initial test; therefore, a total of 160 participants were enrolled. The baseline characteristics are presented in Table 1. All the participants were randomly divided into

Table 1. Baseline characteristics of the subjects

Variables	Esomeprazole (n = 80)	Famotidine (n = 80)	p-value
Men, n (%)	50 (62.5)	48 (60.0)	0.746
Age [years]	62.3 ±8.3	61.8 ±9.1	0.717
BMI	29.0 ±5.3	28.2 ±5.7	0.359
Medical history, n (%)			
Hypertension	32 (40.0)	27 (33.8)	0.413
Hyperlipidemia	39 (48.8)	43 (53.8)	0.527
Current smoker	31 (38.8)	28 (35.0)	0.623
Diabetes	21 (26.3)	18 (22.5)	0.581
PCI data, n (%)			
Chronic CAD	23 (28.8)	19 (23.8)	0.472
STEMI	17 (21.2)	21 (26.2)	0.457
NSTEMI	8 (10.0)	11 (13.8)	0.463
UA	32 (40.0)	29 (36.2)	0.625
Biochemistry detection			
FBG [mmol/L]	6.17 ±1.97	5.86 ±2.12	0.340
TG [mmol/L]	1.56 ±0.76	1.71 ±0.83	0.235
TC [mmol/L]	3.70 ±0.91	3.84 ±1.06	0.371
HDL-C [mmol/L]	1.16 ±0.39	1.05 ±0.32	0.385
LDL-C [mmol/L]	2.11 ±0.93	2.31 ±0.84	0.155
HbA1C [%]	8.27 ±1.88	8.02 ±1.95	0.410
Creatinine [mmol/L]	90 ±18	93 ±21	0.334
Current treatment, n (%)			
β-blocker	52 (65.0)	46 (57.5)	0.330
Heparin	60 (100)	60 (100)	N/A
CCB	9 (11.2)	12 (15.0)	0.482
ACEI/ARB	51 (63.8)	48 (60.0)	0.625
Insulin	4 (5.0)	7 (8.8)	0.349
Statin	60 (100)	60 (100)	N/A

TG – triglyceride; TC – total cholesterol; FBG – fasting blood glucose; CR – creatinine; UA – uric acid; STEMI – ST-elevation myocardial infarction; NSTEMI – non-ST-elevation myocardial infarction; UA – unstable angina; BMI – body mass index; ACEI – angiotensin-converting enzyme inhibitor; ARB – angiotensin receptor blocker; CCB – calcium channel blocker; N/A – not applicable.

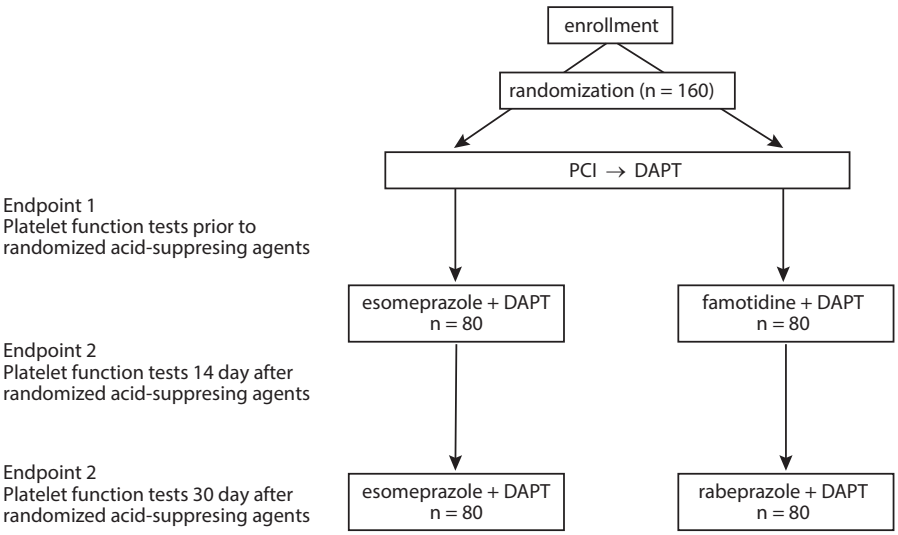

Fig. 1. Progress of the patients during the study

DAPT – dual antiplatelet therapy;
PCI – percutaneous coronary intervention.

an esomeprazole group (n = 80) and a famotidine group (n = 80) with the concomitant use of DAPT. The study design of the present investigation is illustrated in Fig. 1. The rate of compliance with the study drug was 100%, and no patients were lost to follow-up. The baseline characteristics of both groups were comparable. None of the patients experienced bleeding or cardiac death.

Measurements were performed for ADP-induced LTA and VASP-PRI at baseline, day 14 and day 30 (Fig. 2,3). At days 14 and 30, a reduction was documented in both LTA and PRI values when compared to the baseline (both $p < 0.001$). No statistically significant differences were observed in treatment-by-period interactions with LTA values ($p = 0.298$) and VASP-PRI values ($p = 0.867$) between the 2 regimens, which suggested no carryover effect in either regimen over the 30-day treatment period.

Intergroup comparisons between the esomeprazole and famotidine groups were done at 3 separate times (Table 2). The baseline LTA value was 41.4 ±7.9 in the esomeprazole group and 40.3 ±6.4 in the famotidine group ($p = 0.316$), while the baseline VASP-PRI value was 68.4 ±11.4

Table 2. Platelet function tests in users of esomeprazole vs famotidine at baseline and after 14 and 30 days of randomized acid-suppressing agents

Platelet function test	Esomeprazole (n = 80)	Famotidine (n = 80)	p-value
ADP-induced LTA [%]			
Baseline	41.4 ±7.9	40.3 ±6.4	0.316
Day 14	35.2 ±6.1	36.2 ±6.5	0.330
Day 30	35.6 ±5.5	36.8 ±6.4	0.235
VASP-PRI [%]			
Baseline	68.4 ±11.4	68.3 ±11.9	0.912
Day 14	62.2 ±10.5	63.3 ±9.1	0.497
Day 30	62.6 ±10.9	63.0 ±9.3	0.820

The values are expressed as the mean ±SD, unless otherwise indicated.
ADP – adenosine diphosphate; LTA – light transmittance aggregometry; VASP – vasodilator-stimulated phosphoprotein; SD – standard deviation.

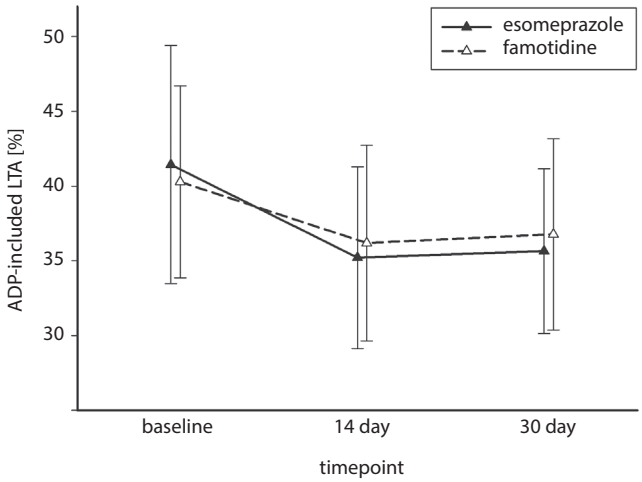

Fig. 2. 20 µmol/L ADP-induced LTA values across the baseline, day 14 and day 30 of randomized acid-suppressing agents

ADP – adenosine diphosphate; LTA – light transmittance aggregometry.

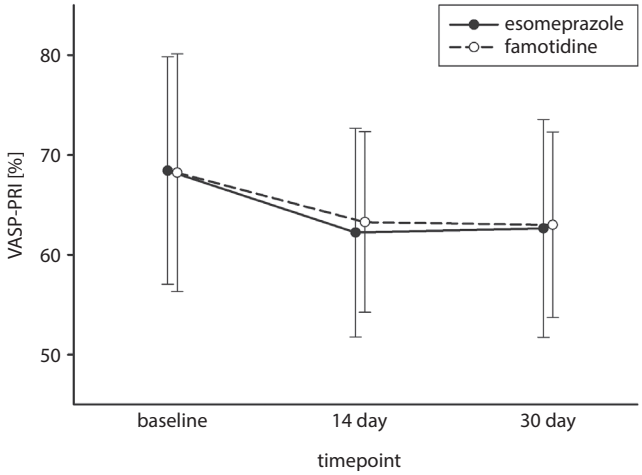

Fig. 3. VASP-PRI values across baseline, day 14 and day 30 of randomized acid-suppressing agents.

VASP – vasodilator-stimulated phosphoprotein; PRI – platelet reactivity index.

in the esomeprazole group and 68.3 ±11.9 in the famotidine group (p = 0.912). Similar findings were also observed on day 14 (p = 0.330 for LTA and p = 0.497 for VASP-PRI) and day 30 (p = 0.235 for LTA and p = 0.820 for VASP-PRI).

As for intragroup measurements among the different points in each group, statistically significant differences existed between the baseline and day 14 in both LTA (both p < 0.001 for esomeprazole and famotidine) and VASP-PRI values (p < 0.001 for esomeprazole and p = 0.004 for famotidine). Moreover, comparing day 14 and day 30 of randomized acid-suppressing agents, similar LTA values (p = 0.634 for esomeprazole and p = 0.567 for famotidine) and VASP-PRI values (p = 0.805 for esomeprazole and p = 0.852 for famotidine) were shown in the 2 groups. No cardiovascular events such as cardiovascular death, nonfatal myocardial infarction, gastrointestinal bleeding, or ischemic stroke were recorded in either group in the 30-day treatment period.

Discussion

In the present prospective, randomized, case-control study, we administered esomeprazole (40 mg/day) and famotidine (40 mg/day), commonly used doses for preventing upper gastrointestinal disorders in patients receiving a combination of aspirin and clopidogrel. This study demonstrated that over the 30-day treatment period, there was no significant difference in antiplatelet effects between the 2 treatment groups. There are linear unconditionally stable results in the duration of treatment between the regimens, and no carryover effect was found in the esomeprazole or famotidine group in the setting of CHD patients receiving DAPT. Neither esomeprazole nor famotidine reduced the platelet inhibitory effect of DAPT in CHD patients who had undergone PCI. This study complements previous research, and also leads to further interest in PPIs or H2RA for upper gastrointestinal protection in DAPT users.

Concomitant use of acid-suppressing agents is usually prescribed to reduce upper gastrointestinal bleeding, but a major concern is that certain PPI drugs might abrogate antiplatelet efficiency. Because the implications correlated with the reduced pharmacodynamic effects in patients undergoing DAPT as a result of PPI drug interaction remain controversial, several studies have provided warnings against the concomitant administration of certain PPIs.[17,18] Most of the available results on PPI-clopidogrel interaction is with omeprazole, a moderate CYP2C19 inhibitor,[19,20] but limited data is available on the pharmacodynamic effects of other PPIs, such as esomeprazole. Because famotidine is excreted by the kidneys, hepatic enzymes (such as CYP2C19) do not metabolize such agents, and no interactions with clopidogrel and H2RAs have been reported.[21] Therefore, to avoid drug-drug interaction, famotidine may be an alternative for patients treated with DAPT. Uotani et al. reported that anti-platelet drug-induced gastric injury was alleviated by famotidine without attenuation of antiplatelet functions.[22] However, the sample size in their study was only 20, and the subjects were all young, healthy volunteers, not CHD patients after undergoing PCI and taking DAPT.

There have been several reports on the relationship between DAPT and the concomitant use of esomeprazole and famotidine therapy, but a definite conclusion has not yet been determined.[23–25] Chan et al. showed that both treatments were comparable in preventing upper gastrointestinal bleeding in patients undergoing DAPT,[23] while another study by Ng et al. reported that PPIs were superior to H2RAs in the prevention of upper gastrointestinal bleeding.[24] Our findings were similar to the only existing study comparing the influence of the 2 agents on the platelet inhibitory effect of DAPT in which famotidine was found to have similar effects to esomeprazole during DAPT.[25] However, in that report, only 88 patients completed the study protocol and they were mostly male (84%); moreover, geographical locations and racial differences could be considered another important factor. In view of this, limited data is available on the pharmacodynamic interaction between esomeprazole and famotidine and DAPT.

This study has certain limitations. Firstly, we did not distinguish the genetic polymorphism of CYP2C19. It may be argued that CYP2C19 polymorphisms could have affected the pharmacodynamic response to clopidogrel. However, the influence of CYP2C19 loss-of-function allelic variations on clopidogrel-mediated effects is considered relatively small (5–12%).[8,26] Notably, prior studies have failed to identify any influence of CYP2C19 polymorphisms on adverse outcomes in PPI-treated patients.[27,28] Secondly, this study was limited by a relatively short follow-up period, and by the cohort of average-risk DAPT users without a prior history of gastrointestinal bleeding. It has been shown that a history of upper gastrointestinal bleeding is the most important risk factor with DAPT use. Whether there are any unintended effects of concomitant use of acid-suppressing agents in longer follow-up and high-risk DAPT users should be studied further.

Conclusions

The objective of this study was to demonstrate that the concomitant use of esomeprazole or famotidine did not antagonize DAPT in Chinese patients who had undergone PCI. Our research indicates a lack of any statistically significant differences between esomeprazole and famotidine users in LTA and VASP values in treatment-by-period interaction, which suggests that neither agent had a carryover effect on DAPT during the 30-day treatment. All these results showed that esomeprazole and famotidine were much less likely than CYP2C19 to influence the patients'

response to clopidogrel, indicating that the pharmacodynamic interaction between clopidogrel and acid-suppressing agents is a drug-specific effect rather than a class effect.

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Resistant hypertension: Renal denervation or pharmacovigilance? Insights from a renal denervation screening program

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Abstract

Background. With emerging new therapeutic concepts including renal denervation (RDN), there is a renewed interest in resistant hypertension (ResH). Among patients suspected of having ResH, a definitive diagnosis needs to be established.

Objectives. This study presents observations from a standardized single-center screening program for RDN candidates, including medical therapy modification and reassessment.

Material and methods. All patients referred to our center for RDN underwent a standardized screening protocol. Candidates were recruited from among patients receiving no less than 3 antihypertensive drugs, including diuretics with office blood pressure (BP) >140/90 mm Hg. The assessment included 2 measurements of BP and ambulatory BP monitoring (ABPM). If needed, pharmacotherapy was intensified and the diagnosis of ResH was reconfirmed after 6 weeks. If ResH was persistent, patients were hospitalized with repeated ABPM on day 4. Further, renal CT-angio was performed and a multidisciplinary team discussed the patients' suitability for RDN.

Results. A total of 87 patients with a ResH diagnosis were referred for RDN. Mean office BP was 159/92 (±7.0/6.5) mm Hg and mean ABPM was 154/90 (±9.0/4.8) mm Hg. The initial medication included angiotensin convertase inhibitors (ACE-I, 78%), angiotensin receptor blockers (12%), β-blockers (85%), calcium channel blockers (36%), and diuretics (93%). During the 18 months of the RDN program, 5 patients underwent RDN and 2 further had ineligible renal anatomy. A new diagnosis of secondary hypertension was made in 21 patients. However, in 59 patients, BP control was achieved after optimization of medical therapy, with a mean ABPM of 124/74 mm Hg. The final treatment included ACE-I (100%), β-blockers (92%), indapamide (94%), amlodipine (72%), and spironolactone (61%). Medication in most of these patients (88%) included single-pill triple combination (52.5%) or double combination (35.6%).

Conclusions. Patients with elevated BP screened for RDN require a rigorous diagnostic workup. Up to 2/3 of patients can be managed with strict pharmacotherapy compliance and pharmaceutical intensification, including single-pill combinations and improved drug compliance. Hasty use of RDN may be a result of poor drug optimization and/or compliance. It does remain a viable treatment option in thoroughly vetted ResH patients.

Key words: compliance, arterial hypertension, resistant hypertension, renal artery denervation

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Arterial hypertension (HA) remains a major public health concern with substantial morbidity and mortality, affecting nearly 25% of all adults in the industrialized world. More specifically, the national health registry data in Poland suggest that approx. 10.5 million people suffer from HA, accounting for approx. 32% of the adult population.[1] Over the past decades, a great deal of research and literature has focused on HA leading to a general consensus about the pathomechanisms, pharmacotherapy and other treatment modalities; however, there remains a subset of patients that do not benefit from the standard treatment algorithm. These patients are thought to suffer from resistant hypertension (ResH), which is defined as blood pressure (BP) that remains above the goal in spite of optimal doses of 3 antihypertensive agents of different classes, ideally including a diuretic.[2] However, in situations where elevated office BP is due to white-coat hypertension, improper BP measurement or medication non-compliance, patients are considered not to have true ResH, but rather so-called pseudo-ResH.[3]

In the case of true ResH, a failure of pharmacotherapy leads to more invasive methods of treatment, which are based conceptually on the role of the autonomic nervous system in the pathogenesis of HA. Initially, invasive techniques involved surgical sympathectomy of abdominal organs,[4] which is usually successful in anti-hypertensive effects but often results in unbearable gastrointestinal distress. Thus, a consensus was reached that renal artery denervation (RDN), a more selective procedure, may be an option for patients with ResH. The authors of the largest clinical trials examining RDN – HTN 1,[5] HTN 2[6] and HTN 3[7] – established strict inclusion criteria and procedural guidelines for the consideration of RDN. According to the practice-based guidelines, patients suspected of ResH undergo serial consultations along with confirmatory testing. Due to the complicated screening and verification of clinical suspicions of RDN, there needs to be a consensus on the diagnosis and final qualification for RDN therapy. In this paper, the authors seek to analyze the causes of disqualifications from RDN in patients with a suspected diagnosis of ResH.

Material and methods

We conducted a prospective study, enrolling 87 consecutive patients with a preliminary diagnosis of ResH who were hospitalized in either the cardiology or nephrology wards at the Medical University of Lodz, Poland. For the purposes of the study, ResH was defined as BP that remained above the goal in spite of optimal doses of 3 antihypertensive agents of different classes, 1 ideally being a diuretic, in accordance with the most updated guidelines set forth by the European Society of Hypertension and the European Society of Cardiology.[2] Participation in the study required informed consent, which outlined all the study procedures and potential side effects. The study protocol was broken down into multiple phases: a preliminary phase to confirm the diagnosis of true ResH and a confirmation/hospital phase. The preliminary phase included screening for true ResH, composed of a detailed medical history and HA analysis, outlined in Fig. 1.

Preliminary phase observations helped to exclude patients with a diagnosis of secondary HA or those with an increased vascular risk (i.e., abdominal aortic aneurysm or atherosclerosis obliterans) in invasive RDN. The patient interview was focused on a detailed history of the patient's HA and past medical and pharmacotherapy. A small subset of patients was found to be mismanaged according to treatment guidelines and a definitive diagnosis of ResH could not be made. In these cases, the pharmacotherapy was optimized and the preliminary stage of the study was repeated after 6 weeks to assess for true ResH. When a diagnosis of ResH was confirmed during the initial or reassessment visit (after pharmacotherapy optimization), a patient was enrolled in the study and admitted to the hospital.

The second phase of the study, considered the confirmation/hospital stage, is outlined in Fig. 2. A continuation of pharmacotherapy along with routine BP monitoring was followed by blood tests to further exclude any other causes of HA or risks in undergoing RDN.

After all inclusion criteria were met, the patients were screened and qualified to undergo the RDN procedure. The Symplicity^{TM} renal denervation system (Medtronic, Dublin, Ireland) was used to carry out the RDN procedure. Each RDN procedure was conducted by a properly trained operator – a cardiologist, experienced in percutaneous coronary angioplasty procedures and supported by a highly qualified licensed technician sent by the manufacturer. Post-procedure hospitalization lasted an average of 3 days. Ambulatory BP monitoring (ABPM) and a blood test were conducted on the last day of hospitalization just before discharge, and again at 6 months (±2 weeks) and 12 months (±4 weeks). The approval of the Medical University of Lodz Bioethics Committee/Institutional Review Board was obtained for this study.

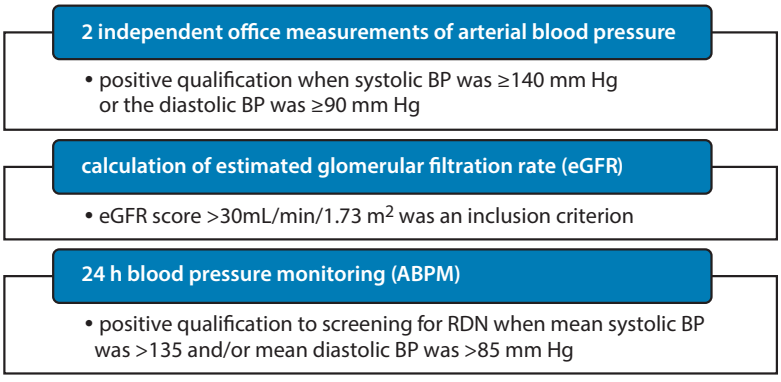

Fig. 1. Preliminary phase testing

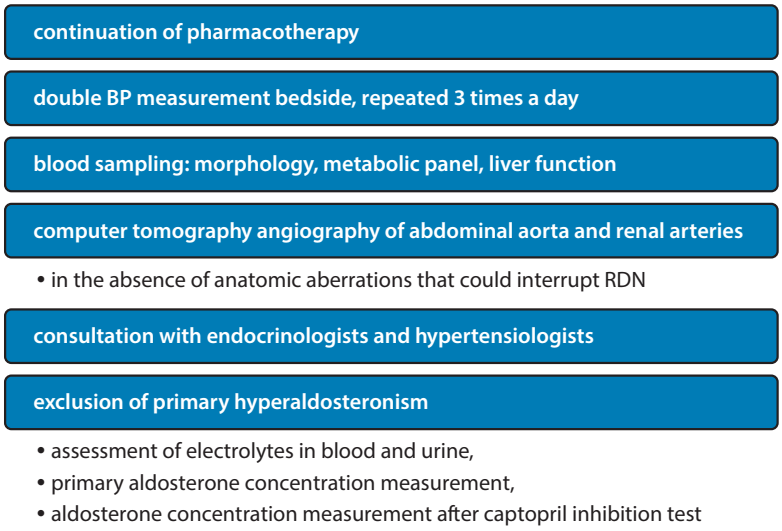

Fig. 2. Hospital procedures of study protocol

Statistical analysis

The quantitative data was compared to a standardized bell curve with the Kolmogorov–Smirnov test (Lilliefors modification). When the data was compatible to normal distribution, a mean and standard deviation were used (mean ±SD). A variance analysis for dependent samples was performed. The calculations were carried out on MedCalc Statistical Software v. 17.11.5 (MedCalc Software bvba, Ostend, Belgium).

Results

A total of 87 patients (33 female, 54 male) were enrolled in the study. The demographics are shown in Table 1. The mean office BP measurement in the study group was 159/92 (±8.7/6.5) mm Hg and the mean ABPM measurement was 154/90 (±9/4.8) mm Hg. During the 18 months of enrollment, only 5 patients fully satisfied the inclusion criteria and were qualified to undergo RDN. The primary choice for vascular access was the right femoral artery, and no local complications were noted after the procedure.

All the patients who underwent the RDN procedure were followed up at 6 and 12 months, in accordance with the protocol. A modification of pharmacotherapy was required in 1 patient – a reduction of β-adrenolytic due to asymptomatic bradycardia. The treatment of other patients was not altered during the observation time. Office BP and ABPM measurements revealed a reduction of overall BP as compared to the initial measurements at the time of enrollment. Blood pressure measurements from consecutive visits are shown in Table 2.

The patients that underwent RDN all had satisfactory primary renal function with estimated glomerular filtration rates (eGFR) >60 mL/min/1.73 m^2. There was no significant deterioration of renal function

Table 1. Demographic and clinical characteristics of the study population

Parameter	Mean ±SD
Age [years]	63.58 ±10.01
Gender (female/male)	33 (37.9%)/54 (62.1%)
Diabetes mellitus type 2	22 (25.3%)
Coronary artery disease	24 (27.6%)
Hypercholesterolemia	23 (26.4%)
Atrial fibrillation	11 (12.6%)
History of stroke	8 (9.2%)
eGFR (MDRD) [mL/min/1.73 m^2]	73.67 ±23.11
Creatinine [mg/dL]	1.06 ±0.29
Time of HA therapy [years]	15.01 ±5.99

SD – standard deviation; eGFR – estimated glomerular filtration rate; MDRD – eGFR estimation formula; HA – arterial hypertension.

Table 2. Office BP measurements and ABPM measurements during the follow-up period in patients that underwent RDN

Parameter	M0 BP (mean ±SD)	M6 BP (mean ±SD)	M12 BP (mean ±SD)	p-value
Mean office BP measurements				
Systolic BP	158.80 ±6.40	134.70 ±4.40	130.50 ±1.32	<0.001
Diastolic BP	88.30 ±4.56	85.10 ±4.55	81.60 ±6.19	0.14
ABPM results				
24-hour systolic BP	141.40 ±4.83	127.60 ±8.38	130.60 ±6.39	0.015
24-hour diastolic BP	82.60 ±4.34	70.80 ±7.82	71.20 ±6.30	0.026
Systolic BP – day	145.00 ±5.24	132.20 ±11.67	135.40 ±6.19	0.017
Diastolic BP – day	86.20 ±5.26	75.80 ±10.71	75.60 ±7.50	0.053
Systolic BP – night	132.00 ±4.36	120.20 ±5.50	123.00 ±4.18	0.049
Diastolic BP – night	75.80 ±6.91	61.60 ±7.86	65.40 ±5.90	0.088

BP – blood pressure; M0, M6, M12 – consecutive follow-up visits at the end of hospitalization, after 6 and 12 months post hospitalization; SD – standard deviation; ABPM – ambulatory blood pressure monitoring.

in the follow-up period. The eGFR values based on the Modification of Diet in Renal Disease (MDRD) Study Equation are shown in Table 3.

There were 82 patients that did not qualify for RDN procedure, all with a diagnosis of elevated BP despite optimal pharmacotherapy. The mean office BP measurements and mean ABPM scores for this group are shown in Table 4. For the patients who were disqualified from RDN, the reasons have been outlined in Table 5.

Secondary hypertension due to various etiologies was diagnosed in 21 (25.6%) of disqualified patients. The most frequent cause of secondary hypertension was a significant stenosis of at least 1 of the renal arteries (12 cases (14.6%)). These patients were advised to continue with a diagnostic workup and treatment with vascular surgery. Seven patients (8.5%) were found to have primary hyperaldosteronism and 2 (2.4%) were discovered to have an active adrenal aldosterone-secreting adenoma and were referred to endocrinologists. Another 2 patients (2.4%) had an abnormal renal artery diameters and kidneys with multiple vascular supplies.

Another 59 patients (72%) were diagnosed with pseudo-ResH, defined as an initial diagnosis of ResH but with suboptimal pharmacotherapy or patient noncompliance. At the end of the screening phase, optimization of therapy and counseling on compliance lead to this group achieving a satisfactory BP and disqualification from RDN. All the patients diagnosed with pseudo-ResH had initial BP measurements that could qualify them for RDN. The mean office BP and ABPM scores in the screening phase and after final effective pharmacotherapy administration are shown in Table 6.

In this pseudo-resistant group, a satisfactory BP was achieved at various points in the screening process. In all cases, pharmacotherapy was optimized during the initial screening visit and BP measurements were repeated at 6 weeks. The majority of patients (36) achieved satisfactory BP measurements at this point. In 23 of these cases, ResH was the working diagnosis until the first hospitalization. Despite some patients having BP measurements that qualified for RDN during the initial screening, not all of them were treated with diuretics. The pharmacotherapy regimen of the screening failure group on presentation and final pharmacotherapy regimen are shown in Fig. 3,4.

In most cases, the addition of hypertensive therapy was based on single-pill combinations with 2 or 3 substances. In 31 patients (52.5%), we chose a triple combination pill of perindopril, amlodipine and indapamide. In 21 patients (35.6%) a combination pill of perindopril and amlodipine was utilized.

In summary, an initial diagnosis of ResH was ruled out due to the discovery of reversible causes of high BP in 82 cases (94%). Improper treatment or noncompliance issues were the cause of this in 59 of the patients (68%).

Table 3. The eGFR values of the patients who underwent renal denervation calculated with the MDRD formula in mL/min/1.73 m^2

Patient No.	eGFR (baseline)	eGFR (at 6 months)	eGFR (at 12 months)
1	105.37	100.88	117.45
2	85.01	89.29	87.81
3	77.50	72.69	83.93
4	85.64	91.10	87.75
5	80.81	76.17	75.08

eGFR – estimated glomerular filtration rate; eGFR 0, 6, 12 – values of estimated glomerular filtration rate during the first visit and during follow-up visits in the 6^{th} and 12^{th} month.

Table 4. Office BP measurements and ABPM measurements during the follow-up period in patients disqualified from RDN

Parameter	Mean +SD
Mean office BP measurements	
Systolic BP	159.20 ±8.88
Diastolic BP	92.20 ±6.54
ABPM results	
24-hour systolic BP	153.95 ±8.89
24-hour diastolic BP	89.86 ±4.56
Systolic BP – day	158.45 ±8.89
Diastolic BP – day	91.44 ±6.15
Systolic BP – night	149.38 ±8.96
Diastolic BP – night	88.27 ±7.06

RDN – renal denervation; BP – blood pressure; SD – standard deviation; ABPM – ambulatory blood pressure monitoring.

Table 5. Main causes of disqualification from renal denervation during screening (number of patients)

- Non-optimal pharmacotherapy (59)
- Secondary hypertension (21)
  - significant stenosis in renal artery (12)
  - primary hyperaldosteronism (7)
  - active adrenal adenoma (2)
- Renal artery anatomy improper to RDN procedure (2)

Table 6. Office BP measurements and ABPM measurements including BP scores during the screening and final BP final scores after new pharmacotherapy optimization

Parameter	BP screening scores (mean ±SD)	BP final scores (mean ±SD)	p-value
Mean office BP measurements			
Systolic BP	158.6 ±8.0	125.30 ±5.81	<0.001
Diastolic BP	93.4 ±5.4	81.70 ±4.45	
ABPM results			
24-hour systolic BP	153.3 ±7.9	123.9 ±1.9	<0.001
24-hour diastolic BP	89.9 ±4.7	74.4 ±1.9	
Systolic BP – day	157.8 ±7.9	129.6 ±2.5	
Diastolic BP – day	91.6 ±6.5	79.6 ±2.5	
Systolic BP – night	148.7 ±8.0	118.3 ±3.3	
Systolic BP – night	88.2 ±6.9	69.3 ±3.0	

BP – blood pressure; SD – standard deviation; ABPM – ambulatory blood pressure monitoring.

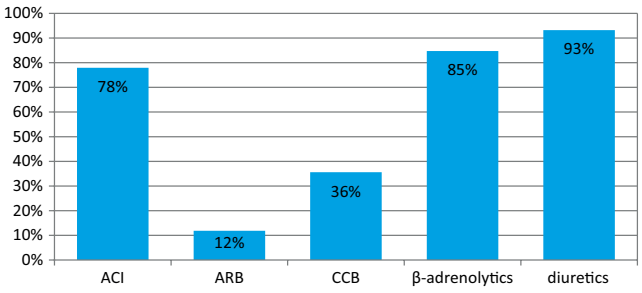

Fig. 3. Initial pharmacotherapy in patients with pseudoresistant hypertension

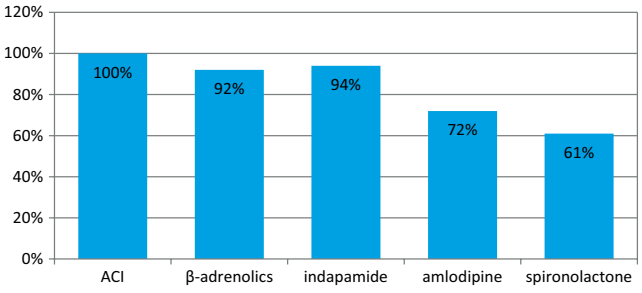

Fig. 4. Final pharmacotherapy in patients with pseudoresistant hypertension

Discussion

Resistant hypertension presents a clinical challenge often requiring an arduous diagnostic workup, pharmacovigilance, optimization of anti-hypertensive medications, and a detailed medical history to rule out non-compliance. Our study, to the best of our knowledge, is the first to analyze a population of patients initially presenting with a presumed diagnosis of ResH that in actuality suffered from uncontrolled hypertension caused by suboptimal pharmacotherapy or poor compliance issues.

Symplicity HTN-1 was the first clinical trial examining percutaneous transcatheter RDN in the treatment of ResH.[4] The study consisted of a cohort of 50 patients who underwent RDN; a sustainable BP lowering effect was observed during consecutive follow-up visits at 1, 3, 6, 9, and 12 months. The cohort was then increased to 153 patients and the follow-up period was extended to 3 years.[8] The hypotensive effect of RDN was still present 36 months from the date of the procedure. Blood pressure measurements were on average 32 mm Hg lower for systolic BP and 14.4 mm Hg lower for diastolic BP as compared to the measurements at the initial screening. It was observed that the percentage of local femoral site complications was not any higher than those in similar procedures such as coronary angiography. Renal denervation did not have a negative impact on renal function. Other studies have also reported similar observations.[9] Nevertheless, RDN is an invasive procedure and should only be performed in definitive cases of ResH.

There should be no ambiguity in the qualification of patients with ResH for RDN procedures. The results of our study show that ineffective medical therapy with 3 or more hypotensive drugs (including diuretics) is not enough to establish a definitive diagnosis. High BP in an outpatient setting may be a symptom of various disorders or of psychosomatic causes such as "white coat hypertension", which may be defined as persistently elevated BP (≥140/90 mm Hg) "in the presence of a healthcare worker, particularly a physician" in patients not taking medication, with an average awake ABPM < 135/85 mm Hg.[10] To obtain unadulterated objective measurements of BP, 24-hour ABPM is required.[11] When suboptimal BP control is confirmed, it still requires exclusion of potentially reversible causes of HA. In our study 25.6% of the RDN disqualifications were caused by potentially reversible or secondary causes of hypertension, which generally have a prevalence of 5–10% in hypertensive patients.[12] Renal diseases such as renal artery disease, glomerular and tubular diseases are responsible for almost 50% of cases of secondary hypertension.[12] It is also important to note that diseases with significant deterioration of glomerular filtration excluded most of these patients from the RDN procedure in our study. Patients with serious renal artery stenosis need a more significant diagnostic workup due to stimulation of the renin-angiotensin-aldosterone system.

As demonstrated by our study and the literature, exclusion of secondary causes of HA is necessary. The most common and available methods to exclude this diagnosis are imaging techniques, most commonly renal Doppler ultrasonography.[13] An RDN screening requires more specific methods that can in addition visualize the aorta and femoral arteries. Computed tomography (CT) with contrast is more appropriate, providing precise vascular imaging and providing visualization of the kidneys and adrenal glands to rule out any possible pathologies. Appreciation of the quantity and diameter of renal arteries is also necessary in preparing for RDN. In addition, renal and adrenal gland anomalies may be responsible for high BP which may be confirmed through imaging techniques.

Another significant cause of HA are pathologies resulting in the disruption of normal hormonal activity.[14] In our study, we excluded 2 patients from RDN due to the presence of adrenal tumors discovered using CT. Detailed examination and diagnostic workup confirmed the hormonal, aldosterone-exerting activity of these tumors. Other patients with endocrine-disrupting properties had either primary hypertrophy of the renal cortex or primary hyperaldosteronism, diagnosed in 7 subjects in this group. Computed tomography was not useful in these cases, but, as the literature suggests, renal scintigraphy is more specific.[15] Renal artery anatomy is another important issue that can influence the course, effectiveness and safety of the renal denervation procedure. Short, bendy and narrow vessels should be noted and may exclude patients from RDN.[16] Furthermore, there is a group of patients with more than 1 artery supplying blood to the kidney. The general prevalence of this anomaly is seen in up to 28%[17] or 34%[13] of patients with HA. In our study, we disqualified 2 patients from undergoing RDN due to the presence of multiple renal arteries in 1 kidney.

The most common reason for disqualification from RDN in our study was inappropriate or suboptimal medical therapy which was identified in the initial screening phase of the study. In 72% of the cases, modifications of pharmacotherapy led to optimized BP control. A detailed medical history interview often revealed multiple therapeutic modifications leading to non-compliance. Numerous studies have been performed over the past decades discussing patient attitudes and compliance. It has been reported that satisfactory compliance is seen in only about 50% of cases. Partial compliance is seen in approx. 30–40% of patients, and 5–10% admit that they take their drugs selectively or do not take them at all.[18] Achievement of optimal BP control by adding another drug as opposed to increasing the dose of the current regimen is a strategy confirmed by numerous studies.[19] On the other hand, the effectiveness of therapy increases when the number of pills is reduced.[20,21] The only way to reconcile these issues is single-pill combination therapy.[19] In our study, patients disqualified from RDN during the initial screening phase due to potential drug compliance issues were ultimately transitioned to a single-pill triple combination (52.5%) or single-pill double combination (35.6%). In 28% of our cases, optimal BP control was achieved with the initiation of new pharmacotherapy, but only during the hospitalization period. These findings affirmed that drug compliance was the most important cause of false positive reports of ResH.

Conclusions

Renal artery denervation is an innovative method of treatment for ResH but remains debated, and its indication is decreasing. Improvements in technical skill and greater availability of devices for performing RDN are necessary. Additional studies must also be performed to further assess the benefits of the procedure.

The presence of uncontrolled high BP does not automatically warrant a diagnosis of ResH, regardless of duration. Such cases always require thorough diagnostic testing to exclude secondary causes of hypertension. It is the up to the due diligence of healthcare workers to provide a conscientious choice of pharmacotherapy, taking into account the patient's capabilities and needs, to allow for good compliance.

Analyzing the methodology of our study, we hypothesize that the best way to achieve compliance is single-pill combinations of anti-hypertensives, but larger studies focused on the issues of compliance are required to verify this assumption.

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Retrospective analysis of transvaginal ultrasound-guided aspiration of simple ovarian cysts

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

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Abstract

Background. The widespread availability of ultrasonography means that transvaginal ultrasonography has become a routine procedure during gynecological examinations, even in asymptomatic patients. Nowadays the imaging technology offered by ultrasonography and tumor biomarkers give us an opportunity to implement transvaginal ultrasound-guided aspiration as a less radical treatment of simple ovarian cysts (SOC).

Objectives. The aim of the study was a retrospective evaluation of the diagnostic and therapeutic efficacy of transvaginal ultrasound-guided aspiration of SOC in postmenopausal and premenopausal patients.

Material and methods. A total of 84 women, divided into a premenopausal group (38/84) and a postmenopausal group (46/84), underwent transvaginal ultrasound-guided aspiration of small SOC (40–80 mm in diameter). Simple cysts were defined ultrasonographically according to the International Ovarian Tumor Analysis (IOTA) guidelines as cysts with negative risk of ovarian malignancy algorithm (ROMA) scores and CA125 levels. Simple ovarian cyst-related data was obtained from medical documentation (diagnostic tests, medical reproductive and surgical history, and clinical status during SOC aspiration). Follow-up data was collected by means of a telephone interview and medical database. The survey included questions focused on cyst recurrence during the 24-month period following the aspiration of SOC.

Results. We had 100% compatibility with ultrasound diagnosis and cytological examination of aspirated fluid. The cumulative rate of cyst recurrence among 84 patients was 20.2% (17/84). There was a higher percentage of cyst recurrence in the premenopausal group: 27% (10/38) vs 15.2% (7/46) in the postmenopausal group, but the difference was not statistically significant (hazard ratio (HR) = 1.89, 95% confidence interval (95% CI) = 0.72–4.97; p = 0.19). Recurrent cysts were treated with laparoscopic cystectomy, adnexectomy or a second aspiration in accordance with individual indications.

Conclusions. Ultrasound-guided aspiration of small (<80 mm) adnexal SOC is a diagnostic and alternative therapeutic procedure, which allows cytological examination and may reduce the need for surgery, which is especially beneficial for women of reproductive age.

Key words: transvaginal ultrasound-guided aspiration, simple ovarian cysts, simple cyst recurrence

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The widespread use of medical ultrasonography, including transvaginal sonographic imaging, has become a routine procedure during gynecological examinations even in asymptomatic patients. One of its beneficial effects is frequent identification of adnexal masses, including simple ovarian cysts (SOC). Fortunately, the majority of these unilocular, anechoic, thin-walled cysts are benign in character.[1] The management of SOC is dependent on the patient's age. Traditionally, due to the risk of malignancy, adnexal cysts or even palpable ovaries in postmenopausal women have been treated surgically, using either laparoscopy or laparotomy. Regarding premenopausal women, more than 66% of diagnosed ovarian cysts are functional.[1,2] That is why the initial treatment of SOC in younger women starts with oral contraceptives, while laparoscopic cystectomy is only performed as second-line therapy.[2]

Nowadays, the imaging diagnostics offered by ultrasonographic technology and tumor biomarkers give us an opportunity to use transvaginal ultrasound-guided aspiration as a less radical treatment of SOC. This procedure is associated with a number of advantages, including fertility preservation, less risk of pelvic adhesions and lower surgery-related morbidity rates, while the lack of any histopathological examination may be perceived as the main disadvantage of this procedure.

The aim of our study was to retrospectively evaluate the diagnostic and therapeutic efficacy of transvaginal ultrasound-guided aspiration of SOC in post- and premenopausal patients.

Material and methods

The study involved 105 female patients who had been qualified for fine-needle aspiration of SOC at the Department of Gynecology and Gynecological Oncology of the Mother's Memorial Hospital Research Institute, Łódź, Poland, in the years 2011–2015. During follow-up, contact was lost with 21 patients, who were therefore excluded from our study. Ultimately, the study involved 84 patients, who underwent follow-up ultrasonographic examination 24 months after the cyst aspiration procedure. The patients were divided into 2 groups: premenopausal (n = 38) and postmenopausal (n = 46).

Medical data on the SOC were collected from patient records stored in the hospital database. Each patient was told on discharge not to neglect control ultrasonographic examinations at the outpatient unit 3 and 6 months after the cyst aspiration. If the hospital database showed no cyst recurrence data during the 24-month time interval after the procedure, telephone enquiries with the patients were conducted for any complementary information.

A SOC was defined as qualified for aspiration when it was sonographically identified as a unilocular, anechoic, thin-walled tumor, without blood flow in a power Doppler (PD) examination, and with a 40–80 mm diameter. Additionally, all the patients had CA125 levels and risk of ovarian malignancy algorithm (ROMA) scores measured before aspiration and had no other gynecological disorders or lesions. They also had negative results on inflammation tests (white blood cell count (WBC)) and C-reactive protein (CRP)).

In order to avoid laparoscopic cystectomy, the patients in the premenopausal group were qualified for ultrasound-guided aspiration of a SOC if they had complained of periodic pelvic pains, which are especially characteristic of cysts over 50 mm. Progressive cysts that occurred during a 3–6-month observation period despite oral contraceptive treatment were also included. The essential criteria for cyst aspiration included negative ROMA and serum CA125 levels. Additionally, 2 premenopausal patients with SOC between 40 mm and 50 mm in diameter were qualified for fine-needle aspiration because of cancerophobia (2/38) (Table 1).

Table 1. Characteristics of the study groups

Variable	Premenopausal n = 38	Postmenopausal n = 46	p-value
Age	31.7 ±9.1	61.4 ±7.5	
Indications for FANC			
periodic pelvic pain	30	10	
enlargement or persistent simple cyst despite contraception	36	–	
persistent over a year	–	46	
cancerophobia	2	–	
Mean diameter of the cyst [mm]	62.3 ±21.2	57.2 ±19.5	>0.05
Aspirated volume [mL]	89.2 ±87.7	89.5 ±113.9	>0.05
CA 125 [IU/mL]	11.5 ±7.35	13.99 ±8.26	>0.05
Inflammatory cells in fluid (lymphocytes, neutrophils)	3/38 (7.9%)	2/46 (4.36%)	>0.05
Recurrence rate	10/38 (27%)	7/46 (15.2%)	>0.05
Second aspiration	1/10 (10%)	2/7 (28.5%)	>0.05
Laparoscopy or laparotomy in recurrence	7/10 (70%)	4/7 (57.1%)	>0.05
Positive microbiological examination	13/38 (34.2%)	16/46 (34.8%)	>0.05

In the postmenopausal group, the presence of an asymptomatic SOC with a diameter over 40 mm and normal ROMA and CA125 levels, persistent for over a year, qualified the patient for ovarian aspiration instead of laparoscopic cystectomy. Ten of the postmenopausal patients were qualified for ultrasound-guided aspiration because of mild pelvic pains.

Women with pelvic pain were examined in order to exclude any other causes of the pain before ultrasound-guided transvaginal aspiration of the SOC was finally decided. All the patients provided their informed consent before the procedure and had transvaginal ultrasonographic follow-ups 3, 6, 12, and 24 months after the procedure, performed either at an outpatient clinic or on a subsequent admission to hospital. Recurrence was defined as the presence of an adnexal cyst on the same ovary with the largest diameter of 30 mm or greater and still persistent at 3–6 subsequent months in premenopausal women (to distinguish between the formation of a new functional cyst and a true recurrence) and a cyst larger than 10 mm in postmenopausal women. Similar criteria for ovarian cyst recurrence are reported in the literature.[3]

The mean age in the premenopausal and postmenopausal groups was 31.7 years and 61.4 years, respectively. The right ovary was affected in 54.76% of the patients (46/84). In 2 cases, cysts were located bilaterally, and in 42.8% of the cases (36/84), they were left-sided (Table 1).

Procedure

The ultrasound-guided aspiration procedures were performed by a gynecologist trained in gynecological surgery, using a 5–7 MHz intravaginal probe. No anesthesia was administered in 25/84 of the patients (29.7%), while short intravenous anesthesia was requested by 59 of the patients (70.2%). After disinfecting the vagina, a 22-gauge needle attached to a vaginal probe was used for the aspiration. It was ejected when the probe was fixed and the SOC was clearly visualized. The needle was sonographically guided directly into the SOC and all the fluid was aspirated into a syringe. The fluid aspirated from the cyst was then sent for cytological and microbiological examination. The whole procedure took approx. 3–4 min, without any complications for any of the patients either during or after the intervention.

Statistical analysis

The χ^2 test was used for non-parametric values, while parametric values were compared with Student's t-test. The cyst recurrence rate was compared between groups, using the Kaplan–Meier (K–M) survival estimator. Diagrams were plotted showing the survival rate without ovarian cyst recurrence during 24 months. The diagrams were compared using the log-rank test and Cox's proportional hazard models to calculate the relative chance for cyst recurrence during the period evaluated. The level of statistical significance was set at $p < 0.05$. The statistical analysis was carried out using STATISTICA v. 12 software (StatSoft Inc., Tulsa, USA).

Results

The cytological examinations did not reveal any atypical cells in aspirated fluid. Inflammation cells were found in 5 cases; however, this did not correlate either with the patients' symptoms or the results of the microbiological examinations. In addition, in 29/84 patients (34.5%), the results of the microbiological examinations were positive. Positive microbiological test results revealed Gram-positive *Staphylococci* and *Streptococci*: *Staphylococcus epidermidis* (13/29; 44.8%), *S. kloosii* (5/29; 17.2 %) and *Streptococcus agalactiae* (11/29; 37.9%). It is worth pointing out that all of the cultured bacteria belonged to typical bacterial flora of the human vagina. All the patients had normal WBC counts and negative CRP levels, so no antibiotic therapy was needed.

The cumulative rate of cyst recurrence among the 84 patients was 20.2% (17/84). There was higher percentage of cyst recurrence in the premenopausal group than in the postmenopausal group (27% 10/38, K–M estimate = 0.73) vs 15.2% (7/46, K–M estimate = 0.85)), but the difference was statistically insignificant: hazard ratio (HR) = 1.89 (95% confidence interval (95% CI) = 0.72–4.97; p = 0.19) (see Fig. 1).

In the 10 cases of cyst recurrence among the premenopausal patients, 6 laparoscopic cystectomies, 1 adnexectomy and 1 2nd aspiration were performed; 2 patients declined further interventions during the follow-up period. In the 7 cases of recurrence in the postmenopausal group, 2 patients underwent 2nd aspirations and 4 had adnexectomies; 1 patient declined further interventions during the follow-up period. Second aspirations were performed when the patient did not agree to laparoscopy. Histopathological studies, performed after laparoscopic cystectomy and adnexectomy, revealed the presence of SOC.

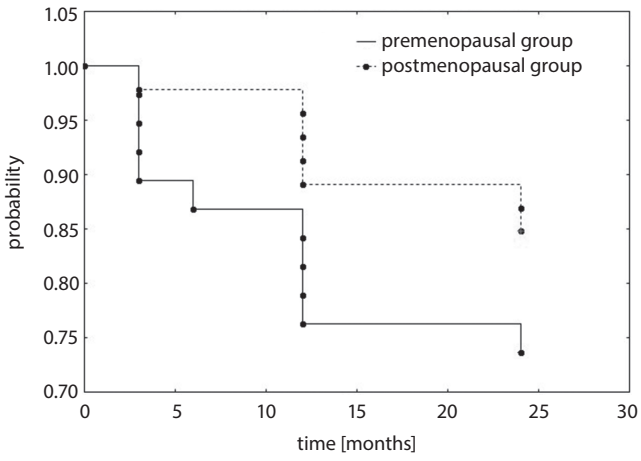

Fig. 1. Probability of survival without recurrence of the ovarian cyst

Discussion

When applied hastily, transvaginal ultrasound-guided aspiration of an ovarian lesion may be perceived as a rather controversial approach, not only from the diagnostic but also from the therapeutic point of view. First, it may pose a risk of spreading atypical cells from an unsuspected malignant tumor. Up to 24% of ovarian tumors in premenopausal women and up to 60% in postmenopausal women are malignant.[4] Second, aspiration is thought to be ineffective due to continued fluid production by the cyst wall. In the literature, the incidence rates of cyst recurrence after aspiration range 11–75%, but in some studies endometrial and dermoid cysts were included.[3,5–8]

Fortunately, a SOC is quite easy to diagnose with ultrasonography, ROMA and CA125 biomarkers. Moreover, malignant transformation is a rather rare process in SOC. In our study, the initial sonographic diagnosis of SOC was 100% compatible with the results of the cytological examinations. Gupta et al. showed high specificity (98.0%) and sensitivity (85.7%) levels for the diagnostics of ovarian/adnexal lesion malignancy with fine-needle aspiration, with a positive predictive value of 97.7%, a negative predictive value of 87.7% and accuracy of 92.0%. They compared cytology results from fine-needle aspiration cytology (FNAC) with the results of histopathological studies.[9] Similarly, Ray et al. revealed high concordance between the results of FNAC and histopathological examinations: The sensitivity of cytological diagnosis was 83%, the specificity was 97% and its accuracy was 93%.[10]

Preoperative cytodiagnostics of ovarian masses were conducted by Pal et al., who carried out ultrasound-guided FNAC in 70 cases. Their cytological examinations reached over 93% accuracy in relation to histopathological diagnoses; the sensitivity and specificity rates of their cytological examinations were 95.23% and 95.83%, respectively. Based on their results, the authors stated that ultrasound-guided FNAC was a quick, economic and safe procedure in diagnosing ovarian masses with brilliant accuracy.[11] Fine-needle aspiration of ovarian masses was also found to be highly specific and moderately sensitive for the detection of ovarian malignancies by Zhou et al.[12] Nagamine et al. confirmed the high sensitivity (75%) and specificity (100%) of FNAC in the identification of malignant lesions in a study involving intraoperative FNAC before ovarian tumor excision.[13]

Lokich et al. evaluated the use of ROMA as a diagnostic tool in women with adnexal masses, identifying low malignancy risk cases which could safely be treated with conservative, non-surgical methods. The use of ROMA seems to be valuable particularly in premenopausal women, where the incidence of epithelial ovarian cancer is low, while cases of benign disease are numerous. In conclusion, they asserted that ROMA in conjunction with detailed ultrasound imaging could safely identify women with low-malignancy adnexal masses, appropriate for conservative therapy. Their results confirmed that nonsurgical management should be considered for SOC identified with ultrasonography and negative ROMA and CA125 levels.[14]

The patients qualified for fine-needle aspiration at our department presented SOC under 80 mm in diameter. Cysts with diameters above 80 mm were qualified for surgery. According to García-Tejedor et al., a higher risk of recurrence was found for cysts larger than 70 mm (odds ratio (OR) = 4.2; 95% CI = 1.2–14.1).[3] Yamamoto et al. estimated a higher risk of malignancy in tumors ≥70 mm in size. They found the Risk of Malignancy Index (RMI) including parameters like menopausal or premenopausal status, ultrasonographic findings, CA125 level, and tumor size to be 91.0% specific with a sensitivity of 86.6% in preoperative evaluation of adnexal masses regarding malignancy.[15]

According to a statement of the Society of Radiologists in Ultrasound from 2010, in premenopausal women, cysts >3 cm and ≤5 cm should be described in imaging impressions as almost certainly benign and not in need of follow-up.[16] The major ultrasonographic features of ovarian malignancy, described by the IOTA Group and including solid or complex ovarian tumors, pathological velocity etc., also account for the size of the tumor. The mean diameter of benign adnexal masses in the IOTA study was 63 mm, while the mean diameter of malignant tumors was 100 mm. However, ultrasonographic findings of a benign character of a tumor are not always confirmed in histopathological evaluations. In the IOTA study, 2 patients out of 1,066 had borderline tumors, although no signs of malignancy were revealed in ultrasonography. This is, in our opinion, why patients with SOC, regardless of their age, should have, as a minimum, ultrasonographic examination in their follow-up.[17]

García-Tejedor et al. showed a 39% (34/87) recurrence rate of SOC in their follow-up examinations.[4] Similar results, although in a rather small study group, were obtained by Nikolaou et al., who observed a 35.2% (n = 12) recurrence rate after transvaginal ultrasound-guided aspiration of serous ovarian cysts.[8] In our study, the general recurrence rate was considerably lower: 20.2% (17/84); it was 27% in the premenopausal group and 15.2% in the postmenopausal group. Probably this difference depends on size of the tumors. In our study, only small ovarian cysts, i.e., ≤80 mm, were qualified for aspiration.

In general, the recurrence rate of benign ovarian cysts in our previous study was 19% (excluding endometriomas) in premenopausal women who had undergone laparoscopic cystectomies, monitored during a 24-month follow-up. Comparing our recurrence rates after fine-needle aspiration in the premenopausal group with those following surgical treatment (27% vs 19%), it becomes fairly obvious that transvaginal ultrasound-guided aspiration of SOC is a procedure worth considering in young women to avoid surgery-related ovarian reserve reduction, thus preserving fertility, especially in cases of subsequent recurrence.[18]

Our study demonstrated insignificantly lower recurrence rates of SOC in the postmenopausal women vs the premenopausal group (15% vs 27%, respectively). This shows that fine-needle aspiration may, in reasonable cases, be considered a less radical management method for SOC in postmenopausal patients. In some studies, even conservative management of SOC in postmenopausal women is taken into account, particularly for cysts ≤50 mm. Spontaneous disappearance of ovarian cysts ≤50 mm in postmenopausal patients has been reported by many authors, with prevalence rates up to 50% (8.3–50%).[19–21] The previously reported recurrence of SOC and sactosalpinx in postmenopausal women after FNAC was 25%.[22]

Better results after the procedure with lower recurrence rates (15%) of SOC were achieved in premenopausal women on oral contraceptives by Koutlaki et al. However, the researchers noted much higher recurrence rates in postmenopausal groups (10/17 (58.9%)).[23] Our study results confirm that transvaginal fine-needle aspiration of SOC is a reliable alternative to surgery, demonstrating many advantages, such as excellent tolerance, low risk and low recurrence rates, especially in premenopausal women.

Gupta et al. also published promising results performing percutaneous aspiration and subsequent methotrexate injection into SOC and endometriomas. In follow-up ultrasonography in their study, cysts had disappeared in 120 patients (90.9%) and persisted in 12 patients (9.1%).[24]

Transvaginal ultrasound-guided aspiration of SOC, performed in properly qualified patients, may reduce to a minimum the risk of procedure-related malignant transformation of the lesions. Furthermore, the low recurrence ratio, comparable to that following laparoscopic cystectomies, shows that it may be considered second-stage procedure after conservative management of SOC or regarded as a less radical treatment in difficult recurrent cases of SOC with negative CA125 levels and ROMA scores. Transvaginal ultrasound-guided aspiration can eliminate risk of pelvic adhesions or morbidity due to surgery, and can preserve fertility, which can be especially important in young women.

Summing up, transvaginal ultrasound-guided aspiration of SOC should be considered an alternative method for the treatment of selected cases of adnexal cysts in women of all ages.

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Peripheral neurotoxic effects of cisplatin on rats and treatment with rutin

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Abstract

Background. Cisplatin, used in cancer treatment, has toxic and apoptotic effects on the peripheral nervous system. Rutin, also known as vitamin P, has antioxidant and antiapoptotic activity.

Objectives. The purpose of this study was to investigate the biochemical and histopathologic efficacy of rutin on neurotoxic and apoptotic effects caused by cisplatin in the peripheral nervous system.

Material and methods. Twenty-four albino Wistar male rats were divided into the following 4 groups: control group (CG), only cisplatin-injected group (CIS), cisplatin and rutin 50 mg/kg (RG-50)-injected group, and cisplatin and rutin 100 mg/kg (RG-100)-injected group. Analyses were performed on sciatic nerve tissue of experimental animals. Analyses of malondialdehyde (MDA), total glutathione (tGSH), glutathione reductase (GSHRd), glutathione-s-transferase (GST), and superoxide dismutase (SOD) were performed. Caspase-3 expression in nerve tissue was also investigated. The analyzed groups were compared with CG.

Results. Biochemical investigation shows that there is a statistically significant difference between CG and only CIS and RG-50. Control group and RG-100 were found to be similar. Cisplatin-induced changes were observed in histopathological analysis of the nerve tissue. The RG-100 and CG were found to be similar. The caspase-3 expression in the neural tissue was compared between groups. Control group and CIS were found to be different. Control group and RG-100 were found to be similar.

Conclusions. Antioxidant and antiapoptotic effectiveness of rutin was detected against the toxic effects caused by cisplatin in the peripheral nerve tissue.

Key words: apoptosis, antioxidants, cisplatin, rutin, caspase-3

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Introduction

Cisplatin is an old and important drug used in the treatment of cancer. It is used in many types of cancer. The main side effects are ototoxicity, nephrotoxicity and neurotoxicity, which are dose-limiting. Cisplatin causes peripheral neuropathy due to its toxic effect on peripheral nerve dorsal root ganglia.[1] The occurrence of peripheral neuropathy causes a reduction in dose and early termination of treatment. Pathophysiological mechanisms leading to the formation of peripheral neuropathies are oxidative damage, inflammation, mitochondrial dysfunction, DNA damage, and apoptosis.[2] Due to the absence of protection, like a blood-brain barrier in central nervous system, peripheral nerves can easily be exposed to toxic effects. Patients complaining of numbness in the form of glove-socks, clinical absence or reduction of deep tendon reflexes, decrease of distal vibration, and proprioceptive sensations are observed. Morphologic changes and deterioration of mitochondrial DNA transcription and replication in neuronal mitochondria were demonstrated during cisplatin treatment. Energy deficit, which is one of the causes of peripheral neuropathy, can take place due to the deterioration of mitochondrial functions. Cisplatin leads to an increased level of reactive oxygen species (ROS), which, in turn, causes an increase in lipid peroxidation and a decrease in catalase and glutathione peroxidase activity, consequently leading to apoptosis due to an excessive increase in caspase-3 expression.[3]

Oxidative stress stems from an insufficient detoxification of the biological system with increased ROS. Lipid peroxidation causes many compounds that are harmful to the cells. Arachidonic acid is an omega-6 fatty acid that can be a source of hydrogen atoms for free radicals found in cell membranes. Malondialdehyde (MDA) is the major metabolite of arachidonic acid and a reliable biomarker of oxidative stress. Following the MDA level makes it is possible to follow also lipid peroxidation. The MDA level in blood plasma or tissue homogenates is one of the most useful factors in determining oxidative stress.[4] Neurotoxicity has been associated with increased oxidative stress and decreased glutathione levels. Glutathione-s-transferase (GST) is a key enzyme in the defense of the cells against oxidative stress. The main function of GST is detoxification of ROS and other oxidative stress products through reduced glutathione.[5] Glutathione reductase (GSHRd) is responsible for providing reduced glutathione, which is one of the most important factors in controlling intracellular ROS. It acts as an electron donor for antioxidant enzymes, such as GST.[6] Superoxide radical anion forms from free radicals in the cell after aerobic respiration. Superoxide dismutase (SOD) transforms and consumes this formed radical. As a result, it is important to prevent oxidative stress.[7] Oxidative stress can lead to apoptosis by activating the pathways. Apoptosis occurs after the activation of caspase enzymes. Caspase-3 is at the end of this path and can be activated by both the internal and external pathway. For this reason, caspase-3 may be considered to reflect the general characteristics of apoptosis.[8]

Rutin is an important flavonoid that is consumed in a daily diet and is found in many vegetables and fruits. It is also known as vitamin P. The protective and anti-inflammatory activity of rutin has been shown in many studies. It inhibits the peroxidation of low-density lipoprotein (LDL). It also reduces oxidative stress and inflammation and normalizes caspase-3 expression.[9] Rutin, due to its free radical consumer efficacy, prevents the toxic effects of oxidative stress by inhibiting the effects of ROS and shows neuroprotective activity.[10]

The toxic effect of cisplatin on the peripheral nerves was investigated in many studies. However, there was no study showing the protective effect of rutin in preventing these side effects of cisplatin. Therefore, the aim of this study was to investigate the neuroprotective and antiapoptotic effectiveness of different doses of rutin on the side effects of cisplatin in biochemical and histopathologic rat experiments. For this purpose, MDA, GST, GSHRd, and SOD levels, and caspase-3 expression were measured in experimental animals and compared with the control group.

Material and methods

Animal experiments were performed in accordance with the National Guidelines for the Use and Care of Laboratory Animals and were approved by the local animal ethics committee of Atatürk University, Erzurum, Turkey (Ethics Committee approval No. 1700238627 dated August 25, 2017).

Study animals

A total of 24 albino Wistar male rats weighing 250–265 g were used in the study. The animals were obtained from Atatürk University Medical Experimental Application and Research Center. Before the experiments, the animals were housed and fed in groups at room temperature (22°C) for 7 days under appropriate conditions.

Chemical substances

The following chemical substances were used for the experiments: cisplatin vials (50 mg/100 mL; Cisplatin-Ebewe) were provided by Liba Laboratuarları A.Ş. (Istanbul, Turkey), rutin (in tablet form) was provided by Solgar America (Leonia, USA) and thiopental sodium was obtained from IE Ulagay Ilac Sanayii Turk A.S. (Istanbul, Turkey).

Experiment groups and procedure

The animals were divided into only cisplatin-treated group (CIS), cisplatin + 50 mg/kg rutin (RG-50)-treated group, cisplatin + 100 mg/kg rutin (RG-100)-treated group, and control group without any treatment (CG).

In the course of the experiment, RG-50 (n = 6) received 50 mg/kg of rutin and RG-100 group (n = 6) received 100 mg/kg of rutin by oral gavage. In previous experimental studies, these doses of rutin were found to be effective.[11] For CIS (n = 6) and CG (n = 6), only distilled water was intraperitoneally (ip.) injected as solvent in the same volume (0.5 mL). One hour after rutin and distilled water, cisplatin 5 mg/kg was administered ip. to all groups, except CG. It is a common practice that drugs protecting tissues against the toxic effect of cisplatin are given to experimental animals 1 h before cisplatin.[12] Rutin and distilled water were administered once a day for 8 days. Cisplatin was administered every 2 days for 8 days.

At the end of this period, the sciatic nerve was removed from the animals, which were killed with a high dose of thiopental sodium. The attained samples were investigated biochemically and histopathologically. All results obtained from the experiments were compared with CG.

Malondialdehyde analysis

For the MDA measurement, we adhered to the method used by Ohkawa et al.[13] This method is based on the spectrophotometric measurement of the absorbance of the pink complex formed by MDA with thiobarbituric acid (TBA) at high temperature (95°C) and 532 nm wavelength. The homogenates were centrifuged at 5,000 g for 20 min and these supernatants were used to determine the amount of MDA. Briefly, 250 µL of homogenate, 100 µL of 8% sodium dodecyl sulfate (SDS), 750 µL of 20% acetic acid, 750 µL of 0.08% TBA, and 150 µL of purified water were vortexed into the capped test tubes. The mixture was allowed to incubate at 100°C for 60 min, after which 2.5 mL of n-butanol was added and spectrophotometric measurements were taken. The resulting red color quantities were read at 532 nm using 3 mL cuvettes, and the MDA concentration of the samples was determined by taking the dilution coefficients into account using the standard graphic generated with the previously prepared MDA stock solution.

Total glutathione analysis

The amount of GSH in the total homogenate was measured according to the method used by Sedlak and Lindsay, with some modifications.[14] The sample was weighed and homogenized in 2 mL of 50 mmol/L of Tris–HCl buffer containing 20 mmol/L of ethylenediaminetetraacetic acid (EDTA) and 0.2 mmol/L of sucrose at pH 7.5. The homogenate was immediately precipitated with 0.1 mL of 25% trichloroacetic acid, and the precipitate was removed after centrifugation at 4,200 rpm for 40 min at 4°C, and the supernatant was used to determine GSH level. A total of 1,500 µL of measurement buffer (200 mmol/L Tris–HCl buffer containing 0.2 mmol/L of EDTA at pH 7.5), 500 µL of supernatant, 100 µL of 5,5-dithiobis (2-nitrobenzoic acid), also known as Ellman's reagent (DTNB) (10 mmol/L), and 7,900 µL of methanol were added to a tube, vortexed and incubated for 30 min in 37°C. The DTNB was used as an chromogen and it formed a yellow-colored complex with sulfhydryl groups. The absorbance was measured at 412 nm, using a spectrophotometer (Beckman DU 500; Beckman Coulter, Brea, USA). The standard curve was obtained using a reduced glutathione.

Glutathione reductase analysis

Glutathione reductase activity was determined spectrophotometrically by measuring the rate of NADPH oxidation at 340 nm according to Carlberg and Mannervik method.[15] After tissue homogenization, supernatant was used for GSHRd measurement. After the NADPH and glutathione disulfide (GSSG) addition, chronometer was on and absorbance was measured with spectrophotometric methods for 5 min by 30-minute intervals at 340 nm.

Glutathione-s-transferase activity

Glutathione-s-transferase activity was performed according to the method used by Habig and Jakoby.[16] Briefly, the activity of the enzyme was assayed spectrophotometrically at 340 nm in a 4 mL cuvette containing 0.1 M of PBS (pH 6.5), 30 mM GSH, 30 mM of 1-chloro-2,6-dinitrobenzene and tissue homogenate.

Superoxide dismutase analysis

Superoxide dismutase analysis performed according to the method used by Sun et al.[17] When xanthine is converted into uric acid by xanthine oxidase, SOD forms. If nitro blue tetrazolium (NBT) is added to this reaction, SOD reacts with NBT and a purple-colored formazan dye occurs. The sample was weighed and homogenized in 2 mL of 20 mmol/L phosphate buffer containing 10 mmol/L of EDTA at pH 7.8. The sample was centrifuged at 6,000 rpm for 10 min and then the brilliant supernatant was used as an assay sample. The measurement mixture containing 2,450 µL of measurement mixture (0.3 mmol/L of xanthine, 0.6 mmol/L of EDTA, 150 µmol/L of NBT, 0.4 mol/L of Na_2CO_3, 1 g/L bovine serum albumin), 500 µL of supernatant, and 50 µL of xanthine oxidase (167 U/L (unit /liter)) was vortexed. Then, it was incubated for 10 min. At the end of the reaction, formazan appeared. The absorbance of the purple-colored formazan was measured at 560 nm. As more of the enzyme exists, the least $O2^-$ radical that reacts with NBT occurs.

Histopathologic analyses

The sciatic nerve tissue attained from the rats was fixed in 10% formalin solution for 24 h. Four micron thick sections were obtained from the paraffin blocks after routine

tissue procedure and stained with hematoxylin and eosin (H&E). All sections were evaluated using a light microscope (Olympus BX 52; Olympus, Tokyo, Japan) by a pathologist who did not know which treatment protocol was applied to samples.

Immunohistochemical procedures

For immunohistochemical staining, primary antibodies of caspase-3 antibody Cat: RB-1197-P0, Lot: CPP32, Ab-4 (Santa Cruz Biotechnology, Dallas, USA, TX:1/100, and Cell Signaling Technology Inc, Danvers, USA) were used. Sections were stained using a fully automated immunohistochemistry (IHC) device (Leica Bond-Max; Leica Biosystems, Melbourne, Australia). After being processed in the IHC device, sections were dehydrated through a graded series of ethanol to xylene and enclosed with a mounting medium (Entellan; Merck Millipore, Darmstadt, Germany). From the rat sciatic nerve samples incubated in 10% formalin solution for IHC processing, 4 µm thick sections were cut on a positively charged microscope slide. The results of the analysis under Olympus BX51 microscope were evaluated based on the caspase-3 staining of the sciatic nerve using the grading system described below. In this evaluation, diffuseness and intensity were considered separately. Diffuseness represents the areas the dye can be found and the intensity represents the intensity of coloration. For diffuseness, grade I represent coloration in less than 10%, grade II represents coloration 10–50%, and grade III represents coloration in more than 50% of the cells. For intensity, grade I represent mild, grade II represents intermediate and grade III represents intense coloration of the cells.

Statistical analysis

The results were presented for continuous variables as mean ± standard deviation (SD), median and minimum–maximum. The normality of distribution for continuous variables was confirmed with the Kolmogorov–Smirnov test. For comparison of independent continuous variables between 3 groups, analysis of variance (ANOVA) was used. Homogeneity of variances was confirmed with Levene's test. While comparing 2 groups, least significant difference (LSD) was used as post-hoc test. The statistical level of significance for all tests was considered to be 0.05. A statistical analysis was performed using the IBM SPSS v. 19 package program (IBM Corp., Armonk, USA).

Results

The results of biochemical analysis in the 4 groups are shown in Table 1. When MDA (µmol/g protein) mean levels were compared in the study groups, a statistically significant difference was found between the groups ($p < 0.001$). In order to determine the group that made the difference, binary comparisons were made, and CG and RG-100 were found to be similar (p = NS). Cisplatin-injected group and RG-50 were found to be different from each other, RG-100 and CG (for all other comparisons $p < 0.001$).

When tGSH (nmol/g protein) mean levels were compared in the study groups, a statistically significant difference was found between the groups ($p < 0.001$). In order to determine the group that made the difference, binary comparisons were made, and CG and RG-100 were found to be similar (p = NS). Cisplatin-injected group and RG-50 were found to be different from each other, RG-100 and CG (for all other comparisons $p < 0.001$).

When GSHRd (U/g protein) mean levels were compared in the study groups, a statistically significant difference was found between the groups ($p < 0.001$). In order to determine the group that made the difference, binary comparisons were made and CG and RG-100 were found to be similar (p = NS). When CG was compared with CIS and RG-50, it was concluded that the averages were statistically different (p = 0.002, p = 0.012, respectively). When

Table 1. Antioxidant levels according to study groups

Variables	Groups				p-value
	CG	CIS	RG-50	RG-100	
MDA	1.3 ±0.4 1.2 (1.0–1.8)	4.5 ±0.4 4.5 (4.1–5.1)	2.6 ±0.4 2.6 (2.0–3.1)	1.5 ±0.3 1.5 (1.2–2.0)	<0.001
tGSH	3.6 ±0.4 3.7 (3.0–4.1)	0.8 ±0.2 0.9 (0.5–1.1)	1.8 ±0.2 1.9 (1.5–2.1)	3.1 ±0.4 3.1 (2.7–3.6)	<0.001
GSHRd	5.2 ±1.3 5.4 (3.5–6.6)	1.3 ±0.2 1.4 (1.0–1.6)	2.6 ±0.5 2.8 (1.9–3.2)	4.6 ±1.1 4.7 (3.0–5.9)	<0.001
GST	6.4 ±0.3 6.4 (6.0–6.9)	2.1 ±0.3 2.1 (1.7–2.3)	4.1 ±0.3 4.1 (3.7–4.6)	6.1 ±0.4 6.0 (5.6–6.6)	<0.001
SOD	5.6 ±0.5 5.6 (4.8–6.2)	1.3 ±0.4 1.2 (1.0–2.1)	2.4 ±0.4 2.4 (1.8–2.9)	5.1 ±0.5 5.2 (4.2–5.7)	<0.001

Variables are shown as mean ± standard deviation (SD), median (minimum–maximum); CG – control group; CIS – cisplatin-injected group; RG-50 – cisplatin and rutin 50 mg/kg-injected group; RG-100 – cisplatin and rutin 100 mg/kg-injected group; MDA – malondialdehyde; tGSH – total glutathione; GSHRd – glutathione reductase; GST – glutathione-s-reductase; SOD – superoxide dismutase.

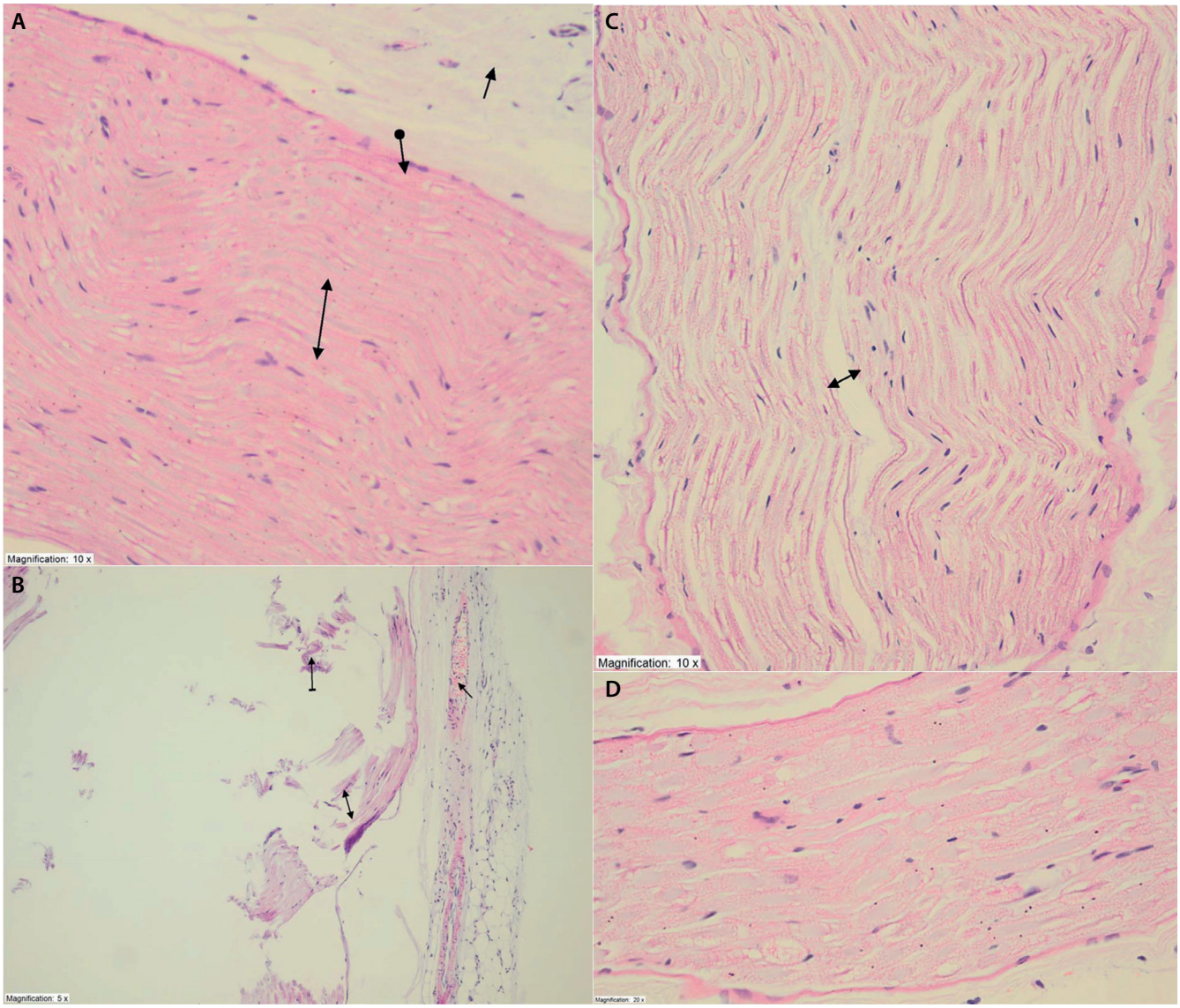

Fig. 1. A – healthy nerve tissue; the straight arrow points to the epineurium, the arrow with a circle at the endpoints to the perineurium, the two-way arrow shows the nerve fibers; B – CIS, destruction (arrow with a line at the end), edema (two-way arrow), dilate blood vessels (simple arrow) are seen in the nerve tissue; C – RG-50, there is only edema on the tissue (two-way arrow); D – RG-100, appearance similar to normal nerve tissue

the CIS was compared with RG-50 and RG-100, a statistically significant difference was found ($p = 0.006$, $p = 0.002$, respectively). When RG-50 was compared with RG-100, a statistically significant difference was found between GSHRd averages ($p = 0.018$).

When the mean levels of GST (U/g protein) were compared in the study groups, statistically significant difference was found between the groups ($p < 0.001$). In order to determine the group that made the difference, binary comparisons were made and CG and RG-100 were found to be similar (p = NS). Cisplatin-injected group and RG-50 were different from each other, RG-100 and CG (for all other comparisons $p < 0.001$).

When the mean levels of SOD (U/g protein) were compared in the study groups, statistically significant differences were found between the groups ($p < 0.001$). In order to determine the group that made the difference, binary comparisons were made and CG and RG-100 were found to be similar (p = NS). When CG was compared with CIS and RG-50, the averages were statistically found to be different ($p < 0.001$ and $p < 0.001$, respectively). When the CIS was compared with RG-50 and RG-100, a statistically significant difference ($p = 0.009$ and $p < 0.001$, respectively) was found. When RG-50 and RG-100 were compared, there was a statistically significant difference between SOD averages ($p < 0.001$).

Biochemical examinations of CG, CIS, RG-50, and RG-100 are shown in Table 1. Histopathological examinations of CG, CIS, RG-50, and RG-100 are shown in Fig. 1A–D. Figure 1A shows a healthy tissue section in CG. Figure 1B demonstrates destruction, edema and dilate blood vessels in the sciatic nerve tissue formed by cisplatin in CIS. Figure 1C illustrates only edema in the sciatic nerve tissue in RG-50. Figure 1D shows similar appearance to normal nerve tissue in RG-100.

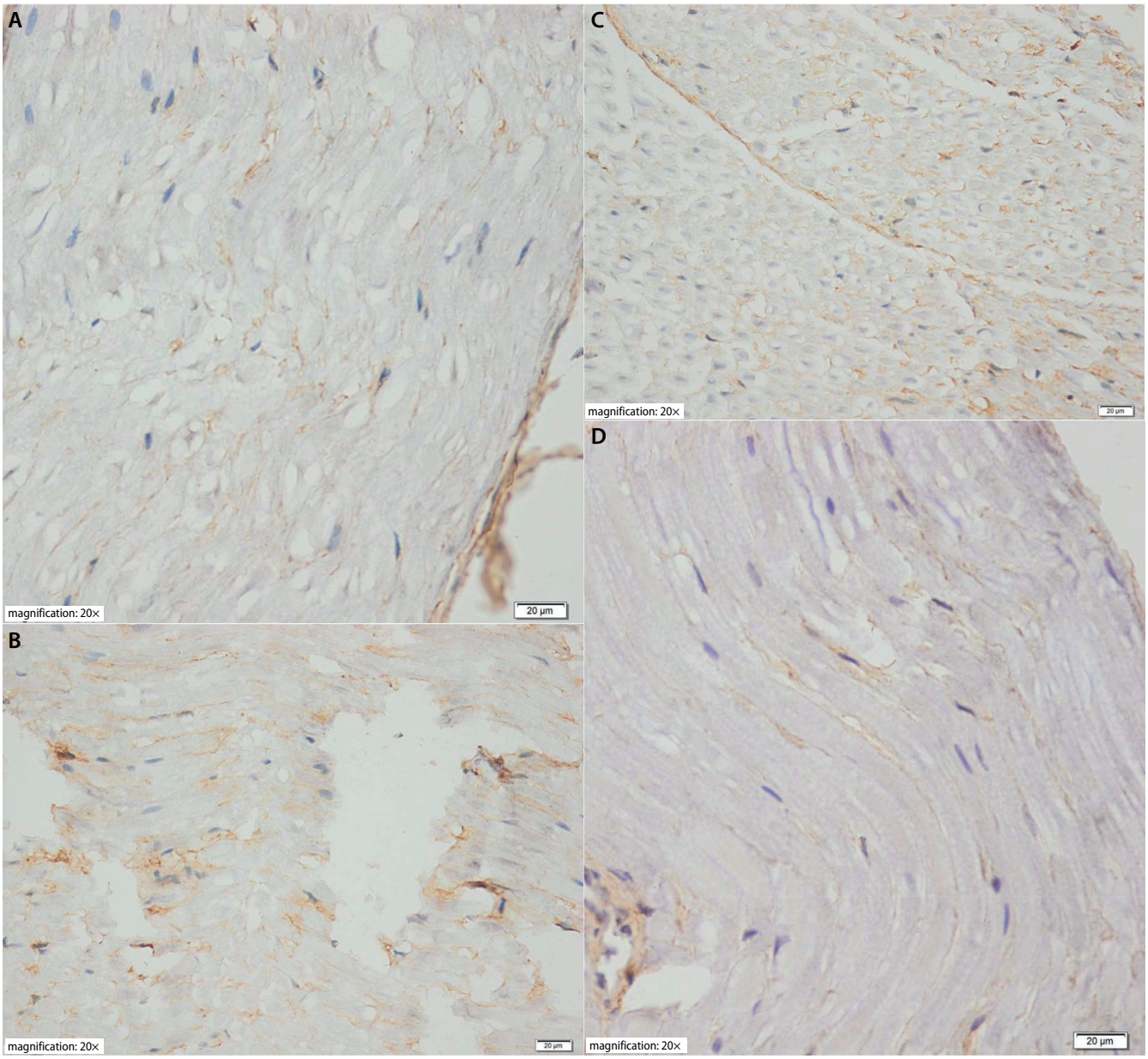

Fig. 2. A – the findings of caspase-3 expression, grade I density (mild) in the CG; B – grade II density (intermediate) in CIS; C – grade II density (intermediate) in the RG-50; D – grade I density (mild) in the RG-100

Analysis of caspase-3 expressions of CG, CIS, RG-50, and RG-100 are shown in Fig. 2A–D. Caspase-3 expression is more extensive in CIS and RG-50 than in CG and RG-100.

Discussion

The peripheral neurotoxic effects of cisplatin have been demonstrated in many studies. Neurotoxicity is the major dose-limiting side effect of cisplatin. Cisplatin increases the production of free oxygen radicals and reduces antioxidants, thereby destroying the oxidant and antioxidant balance. It also increases MDA levels.[18]

Rutin reduces MDA levels by decreasing lipid peroxidation.[19] In the present study, a significant increase in MDA levels was observed in the samples treated with cisplatin. By contrast, significant reductions in MDA levels appeared after the use of rutin. This result is in line with the previously mentioned studies.

Total GSH levels are reduced by the use of cisplatin. Total GSH levels increase and become similar to CG with rutin (it increases tGSH activity depending on the dose). The present study is consistent with the study by Abarikwu et al., who showed that rutin had a protective effect on tGSH levels.[20]

Glutathione reductase is responsible for providing reduced glutathione in the cell.[6] In the present study, GSHRd levels were decreased significantly in CIS compared to CG. GSHRd levels were significantly increased depending on the dose in both RG-50 and RG-100 after use of rutin. Thus, GSHRd levels in RG-100 reached similar values with

the CG. These results are consistent with the work of Umarani et al., who observed a significant increase in GSHRd activities after application of rutin in 50 mg/kg and rutin 70 mg/kg doses to rats.[19]

In the present study, GST levels were decreased significantly in CIS compared to CG. Glutathione-s-transferase levels were increased in RG-50 and reached the same values as in CG with the use of rutin in 100 mg/kg dose. In one study, cisplatin caused a significant reduction of GST levels.[21] In another study, GST levels significantly increased with the use of rutin.[22] Both results were replicated in our experiment.

In the present study, SOD levels were the lowest in CIS and were different from CG. The use of rutin in doses of 50 mg/kg and 100 mg/kg resulted in increased SOD levels similar to those in CG. In some experiments, the use of cisplatin decreased the SOD levels in cells.[23] Superoxide dismutase levels were observed to increase with the use of rutin.[24] Our findings are in line with the previous data.

A histopathological examination has demonstrated that cisplatin causes toxic effects in peripheral nerves. It has been shown that these toxic effects were improved with increasing rutin doses and became similar to CG. In earlier studies, the peripheral neurotoxic effect of cisplatin in sciatic nerves had histopathological evidence.[25] It has been shown that rutin reduces neuronal damage and gliosis.[26]

An increase in caspase-3 expression was observed in CIS as well as in RG-50. Decrease in caspase-3 in RG-100 expression was similar to that in CG. It has been shown that caspase-3 expression increases with the use of cisplatin. A decrease in caspase-3 expression was detected in experiments with animals who received cisplatin together with rutin. The present study is compatible with the study by Arjumand et al., who showed that rutin attenuated cisplatin-induced apoptosis by reducing caspase-3 expression in Wistar rats.[27]

Conclusions

Our biochemical, histopathological and immunochemical investigations have confirmed that cisplatin causes oxidative damage in the sciatic nerve. It has been found that 100 mg/kg of rutin reduces oxidative nerve tissue damage induced by cisplatin more significantly than 50 mg/kg of rutin. Our experimental results and the existing literature suggest that rutin is an important agent in preventing dose-limiting side effects of cisplatin.

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Quality of life and clinical outcomes in Polish patients with high activity rheumatoid arthritis treated with leflunomide (Arava®) in Therapeutic Program: A retrospective analysis of data from the PLUS study

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Abstract

Background. Rheumatoid arthritis (RA) is a chronic autoimmune disease. Therapy is based on disease-modifying agents. Methotrexate (MTX) is used in first-line therapy and, in the case of failure, its alternatives include leflunomide, which was recommended in Poland within the National Health Fund Therapeutic Program.

Objectives. The purpose of the study was to evaluate the parameters of quality of life of Polish patients with high RA activity during treatment with leflunomide. Additional aims were to evaluate the effectiveness and safety of treatment.

Material and methods. We performed a retrospective analysis of the data from the PLUS study. The PLUS study comprised 887 adult patients from 30 centers. During the study patients received leflunomide in a maintenance dose of 20 mg or 10 mg once daily. Before the study, 100 mg of leflunomide had been administered daily for 3 days, followed by a maintenance dose of 20 mg/day or 10 mg/day for at least a month before enrollment. The PLUS study observation time was up to 12 months with 1 control visit every 3 months. The patients' quality of life was assessed with Health Assessment Questionnaire Disability Index (HAQ-DI). Erythrocyte sedimentation rate (ESR), Disease Activity Score (DAS28) and CRP (C-reactive protein) concentration were used to assess the disease activity.

Results. Six hundred seventy-nine patients completed the study. The HAQ-DI decreased after 3 months of observation (mean value 1.46 vs baseline 1.63; $p = 0.001$) and remained stable. The percentage of patients with HAQ-DI less than 1 and greater than 2 increased from 12.2% to 17.8% and decreased from 33.2% to 20.3%, respectively ($p < 0.0001$); DAS28 progressively decreased on subsequent visits. C-reactive protein and ESR decreased after 3 months and remained stable. Adverse events were observed in 4.4% of patients.

Conclusions. Treatment with standard leflunomide doses is safe and allows for significant clinical improvement.

Key words: leflunomide, disease-modifying antirheumatic drugs, rheumatoid arthritis

Introduction

Rheumatoid arthritis (RA) is a chronic, autoimmune and systemic connective tissue disease characterized by symmetrical arthritis and the presence of extra-articular manifestations. Its prevalence in Poland is about 0.45% of the adult population, affecting approx. 131,000–157,000 patients.[1,2] Treatment is based on synthetic or biological disease-modifying anti-rheumatic drugs (DMARDs). According to the European League Against Rheumatism (EULAR) recommendations,[3] methotrexate (MTX) 25–30 mg/week is the first-line drug. In the case of contraindications, intolerance or ineffectiveness, other drugs are used, including leflunomide, which is considered an alternative to MTX according to American College of Rheumatology (ACR) recommendations.[4] Leflunomide at low doses is a reversible inhibitor of the dihydroorotate dehydrogenase enzyme resulting in decreased synthesis of pyrimidines. At higher concentrations, it also inhibits tyrosine kinases interfering with cell signal transduction. Finally, it exerts immunomodulatory, anti-inflammatory and possibly immunosuppressive and antiproliferative effects. Its effectiveness is similar to that resulting from low doses of MTX and its therapeutic effect is visible after 4–6 weeks. In the next 4–6 months, the patient's condition can be further improved.[5] In Poland, leflunomide was initially available in the Therapeutic Program, created and funded by the National Health Fund (NHF), which strictly defined patient's inclusion and exclusion criteria, and specified ways of monitoring treatment and disease activity, schedule of control visits, the type and timing of additional tests, and method of data recording. Analysis of these data makes it possible to obtain reliable and reproducible information collected on a large group of Polish patients.

We performed a retrospective analysis of the data from the PLUS study (the study was conducted between 2007 and 2009). The primary objective of the analysis was to evaluate the parameters of quality of life of Polish patients with high-activity RA during treatment with leflunomide (Arava®, Sanofi-Aventis Deutschland GmbH; Frankfurt am Main, Germany). Additional aims were to evaluate the effectiveness and safety of treatment.

Material and methods

The PLUS study was a multicenter, non-interventional, observational, and prospective study of RA patients enrolled in the Therapeutic Program of National Health Fund in Poland and treated with leflunomide (Arava®, Sanofi-Aventis). The PLUS study was conducted between 2007 and 2009. The study was composed exclusively of patients who were already enrolled in the Therapeutic Program (already treated with leflunomide). Each patient provided informed consent to participate in the Therapeutic Program. All procedures performed by the physicians were carried out according to the rules and requirements of the Therapeutic Program (no additional procedures were performed). The patients' data and outcomes were obtained from 30 of the 50 wards and outpatient rheumatology units in Poland which used leflunomide (Arava®) in 2007 under the Therapeutic Program in accordance with its rules, with the said patients agreeing to the data transfer. According to the Polish law at the time, the Ethics Committee approval was not necessary for the PLUS study (leflunomide was used in accordance with the Therapeutic Program guidelines and summary of product characteristics, patients already treated with leflunomide were enrolled in the study, no additional diagnostic and monitoring procedures were performed). The study protocol was sent to the Pharmacovigilance Department of the Office for Registration of Medicinal Products, Medical Devices and Biocidal Products. We performed a retrospective analysis of the data from the PLUS study. This retrospective analysis was approved by the Military Institute of Medicine Ethics Committee.

Patient selection

Patients suffering from RA and treated with leflunomide as part of the Therapeutic Program at least a month prior to the inclusion were included into the PLUS study. The inclusion criteria of the Therapeutic Program were as follows: 1) RA diagnosed according to 1987 ACR criteria[6]; 2) age of 18 years or more; 3) presence of poor prognostic factors of the disease; 4) MTX (or other DMARD) treatment failure or contraindications to MTX; 5) high RA activity according to the Ritchie index score, morning stiffness >30 min, erythrocyte sedimentation rate (ESR) >28 mm/h, C-reactive protein (CRP) >2 mg/dL; 6) both complete blood count and alanine transaminase level within normal limits; 7) consent for appropriate contraception during the participation in the Therapeutic Program and 2 years after treatment cessation. The exclusion criteria from the Therapeutic Program were: 1) inadequate response after 6 months of therapy; 2) bone marrow failure (anemia, neutropenia, leukopenia, and thrombocytopenia); 3) presence of severe medical conditions, such as congestive heart failure, unstable coronary artery disease, chronic respiratory insufficiency, chronic renal insufficiency, or chronic liver failure; 4) presence of malignancy or premalignant state; 5) current or planned pregnancy and/or breastfeeding during the 2 years after the end of the treatment; 6) drug and/or alcohol abuse. Contraindications to leflunomide listed in Polish summary of product characteristics (SmPC) and participation in any other clinical trial were also considered as exclusion criteria.

Study medication protocol

During the study, leflunomide was used according to Therapeutic Program guidelines and SmPC. Patients who had already received leflunomide for at least 1 month

were qualified for the observational study. Such patients received leflunomide in maintenance doses of 20 mg or 10 mg once daily (depending on the activity of the disease, tolerance of the treatment and physician's decision). Some patients concurrently used other conventional synthetic DMARDs, corticosteroids and non-steroidal anti-inflammatory drugs.

Quality of life, efficacy and safety analyses

Observation time was 12 months with visits in months: 0 (visit V1), 3 (V2), 6 (V3), 9 (V4), and 12 (V5), or until the end of treatment because of ineffectiveness or adverse drug effects. During each visit, quality of life was assessed using Health Assessment Questionnaire Disability Index (HAQ-DI).[7,8] Disease activity was assessed based on the Disease Activity Score (DAS28), CRP concentration and ESR.[9] During each visit, a complete blood count (routine procedure required in the Therapeutic Program) was performed and an interview concerning a history of adverse reactions was conducted.

Statistical analysis

All statistical analyses were performed with Stata v. 10 software (StataCorp, College Station, USA). The analysis was performed for patients who completed the full study participation – this was ascribed to individuals for whom the V1 and V5 forms were submitted with the visit dates entered, and for whom the time between visit 1 (V1) and visit 5 (V5) exceeded 10 months. The distributions of frequencies of categorical variables, measured during subsequent visits, were compared with the use of marginal distribution testing. The existence of a linear relationship between the variables with ordered categories was assessed with linear trend testing. The generalized estimating equation for continuous variables was used to analyze the changes in the mean HAQ-DI, DAS28, ESR, and CRP. The result was expressed as a mean change (beta) in the studied parameter at each subsequent visit in relation to visit 1. On following visits, the observed changes were tested to determine whether the results had changed, and in the case of a statistically significant results, the changes between individual visits were compared. The generalized estimating equation for binary variables was used to evaluate a decrease in HAQ by at least 0.22 at subsequent visits. All performed tests were two-tailed.

Results

Eight hundred and eighty-seven patients (84% women) were enrolled in the PLUS study, of whom 679 (76.6%) completed the study (data from visits V1–V5 was available and the duration between visits was greater than 10 months). One hundred and eighty-six patients dropped out the study earlier. In the case of 22 patients, it was not possible to determine whether the study was completed due to the lack of dates of V1 or V5 in case report forms (CRFs).

Patients over 50 years old accounted for 66.5%, and 22.5% of patients were 40–49 years of age. Patients 30–39 years old comprised 7.8% and patients 18–29 years old 3.2% of the study population. The mean duration of RA was 9.4 ±7.2 years (range: 0.25–43.8 years). Rheumatoid arthritis lasted longer than 2 years in 92.7% of patients. High disease activity was reported in 57.5% of patients according to DAS28, moderate activity in 32.1% of patients, low activity in 4.7% of patients, and 5.6% of patients were in remission.

The majority of patients (74.4%) used at least 2 classical synthetic DMARDs before leflunomide therapy, including therapy with at least 3 DMARDs in 25% of patients. Only 1 drug was used in 25.6% of patients. The most commonly used drugs were MTX in 95.5% and sulfasalazine (SSZ) in 58.8% of patients. In 56% of patients, both MTX and SSZ were used in the past, while 1.4% of patients had never been treated with any of these drugs. The reason for changing the previous therapy to leflunomide was lack of efficacy of the previous treatment in 76% of patients, intolerance in 15%, and both inefficiency and intolerance to DMARDs in 9% of patients.

The average duration of treatment with leflunomide prior to enrollment was 1.43 ±0.92 years. At study entry leflunomide was used in a standard maintenance dose of 20 mg daily in 98.37% of patients, and 10 mg in 1.63% of patients. The reason for early termination of patient participation in the study was lack of efficacy in 32 patients (3.6%), intolerance to treatment in 11 patients (1.2%) and other reported causes in 8 patients (0.9%) (e.g., the decision of the patient). In the remaining 135 patients (15.2%), no reason was given for early discontinuation.

At the time of inclusion in the study, the mean value of the HAQ-DI was 1.63 ±0.62, and 54.6% of patients were characterized by moderate disability (HAQ-DI value 1–2), 33.2% by severe disability (HAQ-DI > 2) and 12.2% by mild disability (HAQ-DI < 1). The mean HAQ-DI decreased significantly during the first 3 months of observation (1.63 ±0.62 on V1 vs 1.46 ±0.64 on V2, $p < 0.001$). Afterwards, it remained stable (mean HAQ-DI on V3, V4 and V5 was 1.42 ±0.64, 1.40 ±0.63 and 1.38 ±0.61, respectively). The percentage of patients for whom HAQ-DI was less than 1 increased between V1 and V5 from 12.2% to 17.8%, while the percentage of patients with HAQ-DI > 2 decreased from 33.2% to 20.3%. The difference in the distribution of HAQ-DI between V5 and V1 was significant ($p < 0.0001$) (Table 1).

The proportion of patients with a reduction in HAQ-DI of at least 0.22 (difference considered significant in terms of treatment) was 39.5% on V2, 47% on V3, 50.7% on V4, and 50.3% on V5 (Table 2).

The likelihood of obtaining a reduction of at least 0.22 was significantly higher on V3, V4 and V5 as compared to V2 ($p \leq 0.001$). The decrease in HAQ-DI of at least

Table 1. Distribution of HAQ-DI on baseline and follow-up visits

HAQ-DI	V1 (baseline)[a]	V2 (3 months)[b]	V3 (6 months)[c]	V4 (9 months)[d]	V5 (12 months)[e]
<1	69 (12.2%)	109 (18.3%)	107 (18.0%)	111 (18.4%)	107 (17.8%)
1–2	309 (54.6%)	335 (56.4%)	351 (59.0%)	368 (61.1%)	371 (61.8%)
>2	188 (33.2%)	150 (25.5%)	137 (23.0%)	123 (20.4%)	122 (20.3%)

[a] Data available for 566 patients; [b] data available for 594 patients; [c] data available for 595 patients; [d] data available for 602 patients; [e] data available for 600 patients.

Table 2. Significant change in HAQ-DI on follow-up visits (as compared to baseline visit)

Variable	V2 (3 months)[a]	V3 (6 months)[b]	V4 (9 months)[c]	V5 (12 months)[d]
Decrease in HAQ-DI of at least 0.22	219 (39.5%)	258 (47%)	276 (50.7%)	272 (50.3%)
p-value for comparison vs V2	–	p = 0.001	p < 0.001	p < 0.001

[a] Data available for 554 patients; [b] data available for 549 patients; [c] data available for 544 patients; [d] data available for 541 patients.

Table 3. Clinically significant change in HAQ-DI (≥0.22) dependent on duration of leflunomide therapy prior to enrollment

Visit	Duration of leflunomide therapy prior to enrollment ≤6 months	Duration of leflunomide therapy prior to enrollment >6 months	p-value
V2[a] (3 months)	48/114 (42.1%)	114/322 (35.4%)	p = 0.2
V3[b] (6 months)	66/115 (57.4%)	124/317 (39.1%)	p < 0.001
V4[c] (9 months)	61/111 (55.0%)	135/316 (42.7%)	p = 0.026
V5[d] (12 months)	61/111 (55.0%)	138/316 (43.7%)	p = 0.04

[a] Data available for 436 patients; [b] data available for 432 patients; [c] data available for 427 patients; [d] data available for 427 patients.

Table 4. Distribution of DAS28 on subsequent visits

DAS28	V1 (baseline)[a]	V2 (3 months)[b]	V3 (6 months)[c]	V4 (9 months)[d]	V5 (12 months)[e]
<2.6	38 (5.6%)	42 (6.3%)	48 (7.2%)	47 (7.1%)	56 (8.4%)
2.6 ≤ DAS28 < 3.2	32 (4.7%)	64 (9.6%)	56 (8.5%)	72 (10.9%)	102 (15.4%)
3.2 ≤ DAS28 < 5.1	217 (32.1%)	316 (47.4%)	374 (56.5%)	373 (56.3%)	367 (55.2%)
≥5.1	389 (57.5%)	245 (36.7%)	184 (27.8%)	170 (25.7%)	140 (21.0%)

[a] Data available for 676 patients; [b] data available for 667 patients; [c] data available for 662 patients; [d] data available for 662 patients; [e] data available for 665 patients.

0.22 on V5 was not significantly associated with age, sex, duration of illness, or number of previously taken disease-modifying drugs. However, a significant difference in the frequency of a decrease of HAQ-DI of at least 0.22 was observed between patients treated with leflunomide ≤6 months and >6 months prior to enrollment beginning from V3 (Table 3).

At the time of inclusion in the study, the mean value of DAS28 was 5.27 ±1.51. High disease activity was observed in 57.5%, moderate in 32.1%, low in 4.7%, and remission in 5.6% of patients. The mean and median DAS28 decreased with increasing duration of observation and all the differences in comparison to V1 were statistically significant (p < 0.001). The mean value for V2, V3, V4, and V5 was 4.60 ±1.29, 4.44 ±1.22, 4.37 ±1.23, and 4.21 ±1.22, respectively. Changes in DAS28 after 6 and 12 months as compared to V1 were significant and were −0.83 (95% CI = −0.92–−0.74, p < 0.001) and −1.06 (95% CI = −1.15–−0.97, p < 0.001), respectively. Mean changes (beta) of DAS28 for subsequent visits compared to the preceding visit beginning with V2 were small and did not reach statistical significance (−0.16 for V3, −0.08 for V4 and −0.15 for V5).

The percentage of patients for whom DAS28 was <2.6 increased from 5.6% on V1 to 8.4% on V5, and of patients with low disease activity from 4.7% to 15.4%, respectively. At the same time, the proportion of patients with high disease activity (DAS28 > 5.1) decreased from 57.5% on V1 to 21% on V5 (Table 4) (p < 0.0001).

It was found that the shorter duration of therapy with leflunomide at the time of enrollment, the greater the percentage of patients with a decrease in DAS28 and the smaller percentage of patients with an increase in DAS28 (at each visit, the linear trend was statistically significant, p ≤ 0.001)

Table 5. Change in DAS28 (compared to baseline) depending on the duration of leflunomide therapy prior to enrollment

Visit	Test for linear trend	Change in DAS28	Duration of leflunomide therapy prior to enrollment			
			1–3 months	3–6 months	6–12 months	≥12 months
V2, n = 536	p = 0.001	decrease	65 (89.0%)	46 (72.0%)	46 (67.7%)	218 (65.9%)
		increase	7 (9.6%)	15 (23.4%)	19 (27.9%)	99 (30.0%)
V3, n = 532	p < 0.001	decrease	67 (92.0%)	45 (70.3%)	45 (68.2%)	212 (64.4%)
		increase	6 (8.2%)	16 (25.0%)	19 (28.8%)	112 (34.0%)
V4, n = 532	p < 0.001	decrease	66 (89.2%)	48 (73.9%)	45 (69.2%)	212 (64.6%)
		increase	8 (10.8%)	17 (26.2%)	20 (30.8%)	113 (34.4%)
V5, n = 537	p < 0.001	decrease	64 (86.5%)	50 (76.9%)	49 (72.1%)	218 (66.1%)
		increase	10 (13.5%)	14 (21.5%)	19 (27.9%)	110 (33.3%)

Table 6. Mean ESR and CRP on baseline and follow-up visits

Variable	V1 (baseline)	V2 (3 months)	V3 (6 months)	V4 (9 months)	V5 (12 months)
ESR [mm/h]	38.77 ±22.10	30.98 ±18.54	30.87 ±18.98	30.35 ±19.18	30.45 ±20.10
CRP [mg/L]	19.23 ±22.35	12.61 ±15.78	12.37 ±15.77	11.83 ±13.39	11.52 ±14.10

ESR – erythrocyte sedimentation rate; CRP – C-reactive protein.

(Table 5). Men more often than women experienced remission on V5 (OR = 3.7, 95% CI = 1.6–8.6, p = 0.002).

Patients with a history of ineffective combined therapy with MTX and SSZ (prior to treatment with leflunomide) obtained clinical improvement on V5 less often than other patients (OR = 0.56, 95% CI = 0.36–0.87, p = 0.01).

The mean ESR value on V1 visit was 38.77 ±22.1 mm/h and mean CRP concentration was 19.23 ±22.35 mg/L. Both ESR and CRP decreased on V2 and remained stable until the end of observation (Table 6). Changes in ESR after 6 and 12 months as compared to baseline were significant: −8.08, 95% CI = −9.52–−6.63, p < 0.001; and −7.34, 95% CI = −9.8, −6.9, p < 0.001, respectively. Similarly, changes in CRP levels on V3 and V5 compared to V1 were significant: −7.05, 95% CI = −8.5–−5.6, p < 0.001; and −8.0, 95% CI = −9.4–−6.6, p < 0.001, respectively.

A total of 45 adverse events occurred in 39 patients (4.4%). The most common complaints were gastrointestinal complications, including diarrhea, nausea, vomiting, and abdominal pain that occurred in 21 patients (46% of all reported adverse events), and skin lesions in 6 patients. Increased activity of serum transaminases occurred in 7 patients – it exceeded 3 times upper limit of normal (ULN) and was the reason for discontinuation of treatment in 4 patients. Four serious adverse events were reported, including an increase in transaminases activity over 3 times ULN in a patient with concomitant cholelithiasis, exacerbation of purulent skin lesions observed several years before treatment with leflunomide, an episode of severe hypertension after 9 months of treatment with leflunomide in a patient with previously well-controlled hypertension, and fatal myocardial infarction.

The most common therapeutic procedure in case of adverse events was the decision to stop treatment in 26 patients (67%). In 96% of these cases, it resulted in a resolution of symptoms, but no attempts were made to return to treatment. In 1 patient who discontinued treatment due to an increase in transaminases activity, the decision was made to re-introduce the drug and hypertransaminasemia did not recur. In 11 (28%) patients who experienced adverse events, a decision to change the dosage was elected, while full-dose treatment was continued in 2 patients (5%).

Discussion

Rheumatoid arthritis in its natural course inevitably leads to joint damage, organ involvement and premature death. It is well-known that the most important factor determining the outcome of the disease is its activity. Several indices may be used to assess the activity of RA, with DAS28 being widely used in Europe. Objective markers of joint damage are erosions and joint space narrowing on X-ray, with deformations and ankylosis in advanced cases. For the patient, however, the most important factors are physical impairment, life activity limitation and reduction of health-related quality of life. The method that allows for the measurement of physical disability is HAQ. According to ACR criteria, it is recommended as part of the assessment of improvement for use in all clinical trials. It includes 20 questions grouped into 8 categories, and the patient provides responses on a scale from 0 (performed without any difficulty) to 3 (cannot be performed at all). The need to use additional help is also taken into consideration. The answers are then summed, and their mean is called HAQ Disability Index (HAQ-DI). There are also other ways to express HAQ index, but this method,

as the most widely used in RA, was adopted for the present study.[7,8]

The assessment of the physical functioning of the patient is influenced by joint pain and range of motion. A reduction in range of motion, hand grip strength, a greater number of swollen joints, and an increase in pain intensity correlates with limited physical functioning. Therefore, HAQ comprises components that are both constant (irreversible) and variable (reversible). Joint damage manifested by erosions, joint space narrowing and radiographic ankylosis is irreversible and is a constant component. In contrast, active inflammation and accompanying pain are variable components of disability. Therefore, the baseline physical functioning of the patient assessed in clinical trials depends on the disease activity, severity and duration. Smaller HAQ improvement is observed in patients with long-lasting RA compared with patients with a shorter duration of RA and is a result of irreversible joint damage. According to Aletaha et al., each additional year of average duration of RA decreases the effect size of the HAQ by 0.02, which corresponds to a decrease in average HAQ improvement of 0.01.[10]

As shown in many randomized controlled trials, evaluating the efficacy of treatment in RA, a change in HAQ correlates well with other disease activity measures and the severity of the disease over time, distinguishes accurately active treatment from placebo, and aptly predicts long-term morbidity and mortality.[11] Improvement of HAQ correlates significantly with other indicators such as Short-Form Health Survey (SF-36) not only in the physical aspects, but also in the functioning in society, emotional life and general sense of health. In early RA, a deterioration in HAQ allows for the prediction of early job loss and death, and correlates with the progression of disability and increased costs of the disease. A decrease of 0.22 in HAQ-DI is considered a minimal clinically significant change and the effectiveness of treatment may be expressed with the percentage of patients who achieved it. Singh et al.[12] showed that an increase of 1 point in HAQ-DI in the first 2 years of the disease causes a 90% increase in disability and an 87% increase in the cost of treatment in the next 3 years along with a 75% increase in disability and 74% increase in costs in the next 8 years. Yelin and Wanke[13] demonstrated that RA patients in the top quartile of the disability generate an annual cost that is 2.55 times greater and a cost of hospitalization that is 6.97 times greater than that of patients in the lowest quartile. Therefore, it must be assumed that the stabilization of HAQ for 12 to 24 months can significantly reduce the medical and overall costs of treatment of these patients.[11,14]

In our large study, initially involving 887 patients, 66% were patients over 50 years old and the mean age was similar to that reported in randomized trials of leflunomide (53.3–58.8 years)[15–19] and in observational studies (46–65 years).[20–27] The mean duration of RA was 9.4 years, which was markedly longer than 3.5–7.6 years cited in randomized trials[15–19] but similar to most of the abovementioned observational studies (8.0–12.1 years)[20–22,24,25]; in 2 of the cited studies, the mean duration of RA was similar to that of the randomized trials (4.1–5.1 years),[23,27] while 1 study involved patients with early RA.[26] It is particularly important to note that only 7.3% of the patients had a disease duration <2 years compared to 37.6–45.2% of patients in randomized trials.[15–19] In this study, 74.4% of the patients previously experienced treatment failure with 2 DMARDs and 25% with 3 DMARDs, while in the randomized trials, patients received previously 0.7–1.1 DMARDs.[15–19] Almost all patients (95.5%) in this study received MTX in the past, but the dosage among patients is not known. The dose was probably 10–15 mg/week because this is apparent from other studies on the prescribing behavior of Polish physicians.[28–29] In 85% of patients, treatment with leflunomide was indicated in the case of of prior therapy failure, and only 15% experienced side effects. Therefore, this was a negatively selected group of patients in whom the efficacy of treatment with another DMARD was poor, but, in contrast to clinical trials, this accurately reflects the usual practice based on Polish recommendations modeled on EULAR recommendations. At baseline, 62% of patients were taking leflunomide ≥12 months (with an average of 1.43 years), which, in the context of a maximal therapeutic effect in the first 3 months,[15–18,27,30–32] also affects treatment outcome.

Final analysis included 679 patients who completed the survey with established endpoints. This large group of Polish patients were treated with leflunomide and the dosing regimen was similar to previously published studies of 63–501 patients observed in randomized trials and observational studies.[15,16,18,27,33] Baseline HAQ-DI was 1.63, which indicated moderate disability (55% had HAQ value 1–2, and 33% had >2), and was similar to that in randomized trials (1.3–1.7). However, it should be emphasized that the majority of patients in this study had already been treated with leflunomide for more than a year at that time, and considering that the NHF program required as an inclusion criterion disease activity expressed by DAS28 > 5.1, the actual value at the beginning of treatment was probably greater. Despite this, from the 3rd month (V2) significant improvement in HAQ-DI was observed (–0.17, $p < 0.001$). From the 6th month (V3) it reached the level of minimal clinical importance (–0.22, $p < 0.001$), and it was maintained throughout the 12th month (V5) at the same level (–0.26, $p < 0.001$). The percentage of patients with HAQ-DI < 1 increased from 12.0% to 17.4% while the percentage of patients with HAQ-DI > 2 decreased from 32.9% to 18.3%. These differences were highly significant ($p < 0.0001$). The percentage of patients in whom a decrease of at least 0.22 was reported increased from 39.5% on V2 to 50.3% on V5 ($p < 0.001$), but the differences between subsequent visits beyond V2 did not reach statistical significance. Thus, the biggest chance of improvement was observed during the first 3 months of observation; after

that time, there was further improvement, but to a much lesser degree. A significant difference in the frequency of a decrease of HAQ-DI of at least 0.22 was observed between patients treated with leflunomide ≤6 months and >6 months prior to enrollment (beginning from V3), it can be suggested that the observed improvement in HAQ-DI is mostly attributable to patients with short leflunomide therapy prior to enrollment. The frequency of change in HAQ-DI of at least 0.22 on V5 was not associated with age, sex, duration of RA, or number of previously taken DMARDs. The magnitude of HAQ-DI improvement in the present study is different from randomized trials, in which it ranged from −0.45 to −0.89 and was similar to values observed for low doses of MTX used as a control (from −0.26 to −0.37).[15–17] Similarly, clinically significant improvement in HAQ-DI was higher in randomized trials than in the current study (71–78% vs 50.3%). The observed differences can be explained by the previously discussed selection of patients treated in the present study (patients with a longer duration of disease and failure of treatment with 2 or more first-line drugs), and by the fact that they were already treated with leflunomide for an average of 1.4 years, i.e., after the time of its greatest effectiveness.

Evaluation of the effectiveness of treatment on subsequent visits with the use of the DAS28 index was a secondary objective of the study. Its baseline value of 5.27 was high and it significantly decreased to the final value of 4.21 after 1 year. The biggest change was observed during the first 3 months of observation. The proportion of patients in remission or with low disease activity (DAS28 < 3.2, target therapeutic effect recommended by EULAR) increased between V1 and V5 from 10.4% to 23.8%. At the same time, the proportion of patients with high disease activity (DAS28 > 5.1) decreased from 57.5% to 21% ($p < 0.0001$). It was also found that the shorter the duration of leflunomide therapy prior to enrollment, the greater the percentage of patients with improvement of DAS28 and the smaller ratio of patients with a worsening of DAS28. In previous randomized trials, 70% improvement was achieved in 20% of patients treated with leflunomide and 50% improvement in 33–34% of patients after 12 months of treatment.[16,18] The efficacy of treatment was better than placebo and small doses (7.5–15 mg/week) of MTX,[16,18] and significantly worse than the dose of 10–15 mg/week of MTX,[19] and comparable to SSZ.[18]

An important finding of our study is a persistent beneficial effect of the leflunomide treatment during long-term observation. The mean duration of leflunomide treatment before enrollment was 1.43 years. All parameters assessed in this study (i.e., HAQ-DI, DAS28, ESR, and CRP) decreased mainly in the first 3 months of observation and then remained stable or continued to decrease, but much more slowly. This strong effect of leflunomide on HAQ-DI and disease activity in the first period of observation seems to be attributed to patients with a shorter duration of leflunomide treatment. This finding is not surprising as the beneficial effect of leflunomide is visible mostly in the first few months of treatment. Our results are in agreement with the results of previous studies. In randomized trials, drug efficacy was maintained at month 24, with 26% sustaining 70% improvement and 56% sustaining 50% improvement. These results were better than those for low doses of MTX (mean: 12.5 mg/week) and SSZ.[15,17] Similarly, the effectiveness of the treatment at 4 and 5 years was maintained,[27] wherein the improvement of 70% was observed in 19.6% of patients and 50% improvement in 43% of patients. The efficacy of treatment decreased after the reduction of the maintenance dose from 20 mg/day to 10 mg/day.

The ESR and CRP levels, an objective indicator of improvement, were also analyzed in the current study. Mean and median ESR and CRP levels decreased after 3 months of observation and remained stable until the end of the follow-up. Erythrocyte sedimentation rate and CRP changes after 6 and 12 months as compared to the first visit were statistically significant and were −8.08 ($p < 0.001$) and −7.34 mm/h ($p < 0.001$) for ESR, and −7.05 ($p < 0.001$) and −8.0 mg/L ($p < 0.001$) for CRP. These changes were consistent with the data reported in the literature, where ESR decreased by 6.3 mm/h to 17.7 mm/h and CRP levels were reduced by 2.2 mg/L to 27 mg/L.[15–17,19,23,25,26]

Treatment ineffectiveness was the reason for the premature exclusion of 32 patients (3.6%) from the study. In randomized studies, treatment ineffectiveness was the cause for excluding 5–17% of patients treated with leflunomide, 3–22% of those treated with low doses of MTX, 3% of patients treated with SSZ, and 32–53% of those treated with placebo (after 6 months).[16,17,19] However, our results cannot be compared with those from clinical trials, as no reason of discontinuation is known in 135 of our patients.

Adverse events occurred in 39 patients (4.4%), mostly gastrointestinal complications. A significant increase of activity of transaminases was observed only in 7 patients, and in 4 patients it was the cause for treatment discontinuation. Discontinuation of therapy in 26 patients resulted in resolution of adverse events in 96% of patients.

The safety profile of leflunomide in this observational study is better than reported in many previous studies assessing the safety of the treatment for 2 years, and was similar to this observed in patients treated longer than 2 years.[15,17,19,27] This may suggest that many patients were excluded from treatment with leflunomide before enrollment in the current study. However, it should be emphasized that the number of adverse events may be underestimated in our study, as 135 patients discontinued the study without given reason. Types of observed adverse events were consistent with the known side effects of leflunomide.[15–27] According to the data in published studies, adverse events were the cause of discontinuation of treatment in 6.3–29% of patients.[16,18,20–22,24,26,27] In a study by Strand et al., the most common side effects were the following: gastrointestinal complications (in 60.4% of patients, and

in 5.5% they were the cause of therapy discontinuation), rash (in 22.4% of patients, and in 2.2% as the cause of withdrawal of treatment), exacerbated and new hypertension (in 13.1% of patients, and in 1.1% as the cause of withdrawal), and reversible alopecia (in 9.9% of patients).[16] Asymptomatic increase of activity of transaminases was observed in 11% of patients, and in 7.1% it was the cause of discontinuation of treatment.[16] During the current study, there were no toxic effects on bone marrow and maintenance of normal morphological values was observed during the entire follow-up period (data not shown). Similarly, no significant changes of these parameters were reported in other cited studies.[15–18]

According to data from previous studies, adverse events that led to treatment discontinuation were more frequent than after small doses of MTX (22% vs 10.4%)[16] and less frequent than after SSZ (14% vs 19%).[18] The number of adverse events did not increase in the 2nd year of treatment and was 18.9%; they also did not change in nature.[15] Similarly, their character did not change within 5 years of treatment.[27] However, the number of adverse events decreased over time because when they were observed, patients were excluded from treatment, and such a mechanism may explain the results of the presented work.

Conclusions

The results of our study, including a large group of patients, indicate that treatment with standard doses of leflunomide allows for significant clinical improvement as measured by HAQ-DI and DAS28 in most patients. The long-term treatment seems to be relatively safe.

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Prospective multicenter Polish Stress Echocardiography Registry (PolStress-Echopro) – the role in clinical practice

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Abstract

Background. Stress echocardiography (SE) is becoming an increasingly frequently performed diagnostic examination in Poland. After the published retrospective PolSTRESS Registry, this prospective study is the first one available so far.

Objectives. The aim of the study was to analyze SE tests, taking into account the clinical characteristics of the patients, indications, applied protocols, and diagnostic and therapeutic decisions.

Material and methods. Reference cardiological centers in Poland were asked for a 1-month prospective analysis of the data obtained. The study included 189 SE examinations. To evaluate coronary artery disease (CAD) (178 tests), all 17 centers performed dobutamine SE (DSE) (100%), 3 centers (17%) performed pacing, while cycle ergometer and treadmill SE were performed by 1 (5%) and 2 (11%) centers, respectively. In patients with valvular heart disease (VHD) (11 tests), 3 centers (16%) performed SE to evaluate low-flow/low-gradient aortic stenosis (AS), 4 (22%) in asymptomatic AS and 1 (5%) to evaluate mitral regurgitation.

Results. For CAD assessment, a positive result was found in 37 (20%) patients, negative in 109 (61%) and nondiagnostic in 32 (19%). In the CAD group, coronarography was performed in 41 (23%) people. The analysis of the significance of the SE results for decision-making on interventional measures revealed that 30 patients (from the total study population of 189) were referred for the intervention.

Conclusions. The most commonly used SE is the DSE. Negative test results allowed in almost half of the patients to resign from invasive coronarography. Stress echocardiography should be more frequently used in patients with VHD in the qualification for invasive treatment.

Key words: coronary artery disease, stress echocardiography, valvular heart disease

Introduction

Stress echocardiography (SE) is a widely known method for the diagnosis of coronary artery disease (CAD) and valvular heart disease (VHD), among others, which involves the use of various types of functional tests stimulating contractility and/or inducing ischemia together with echocardiographic evaluation of contractility and/or vascular evaluation of valve flows.[1] In CAD diagnosis, SE, depending on the type of load, is characterized by a high sensitivity and specificity of the test: exercise SE – 80–85% and 80–88% respectively, dobutamine SE (DSE) – 79–83% and 82–86%, SE with vasodilatatory agent – 72–79% and 92–95%.[2] Stress echocardiography is recommended as an initial test to diagnose stable CAD in patients with a pre-test probability of 66–85% (intermediate) or a left ventricular ejection fraction (LVEF) below 50% in patients without typical symptomatology. In addition, it is performed in patients with abnormalities in resting electrocardiograpgy (ECG), which prevent accurate interpretation of ECG changes during loading, in symptomatic patients after prior revascularization and in order to assess the functional severity of indirect lesions found in coronarography.[2] In the context of valvular heart defects, SE is used in the functional evaluation of a defect, among others, in patients with low flow-low gradient aortic stenosis (LF-LG AS) (classical and paradoxical). The main features of the study were asymptomatic severe aortic stenosis (AS), secondary mitral regurgitation (MR), severe asymptomatic organic mitral valve regurgitation, and significant asymptomatic mitral stenosis (MVA) <1.5 cm^2.[3–5]

Aim

The aim of the study covered a detailed analysis of SE tests performed in Poland, including the clinical characteristics of patients, indications for stress diagnostics and applied protocols. Eventually it describes the obtained results in terms of further therapeutic decisions based on a prospective registry covering a complete 2-month study.

Material and methods

Based on the analysis of the annual retrospective Polish Stress Echocardiography Registry (Pol-STRESS Registry),[6] large medical institutions including cardiology centers and the majority of universities in Poland were asked to prepare a prospective analysis of data obtained at further diagnosis stages. The tests were performed for 1 month in 2017 by cardiologists or internal medicine practitioners trained in the field of SE. Requirements related to equipment standards and operator's experience have been specified by the Echocardiography Working Group of the Polish Cardiac Society and the appropriate national certification documents.

For a positive SE result in the assessment of myocardial ischemia, the authors agreed on the worsening of contractility in at least 2 segments (in 3 segments for high risk stratification) of the echocardiographic left ventricular model and for the determination of myocardial viability – a typical 2-phase response (improvement of contractility at dobutamine dose up to 20 µg/kg/min and its deterioration at 40 µg/kg/min). In asymptomatic severe AS, patients qualified for the study were previously diagnosed with aortic valve area (AVA) <1 cm^2 (aortic valve area index (AVAi) < 0.6 cm^2/m^2) and mean transvalvular gradient (P_{mean}) >40 mm Hg at rest. Poor prognosis featured: AS symptoms, decrease in blood pressure, increase in P_{mean} by at least 20 mm Hg, risk of pulmonary hypertension with systolic pulmonary artery pressure (SPAP) >60 mm Hg, and decrease of LVEF. In LF-LG AS (patients with LVEF < 40%, AVA < 1 cm^2 and P_{mean} < 40 mm Hg at rest) the test indicated severe AS when LVEF or stroke volume (SV) improved by more than 20%, P_{mean} elevated >40 mm Hg and AVA remained <1.0 cm^2. The lack of LVEF reserve (stress ejection fraction–rest ejection fraction) was an indication of a poor prognosis. In MR (ischemic etiology), the aim was to assess the EF reserve and the dynamic effective regurgitant orifice (ERO) component with an increase of over 13 mm^2. Statistical descriptive data included in the study is presented in numerical and percentage form.

Results

As many as 17 teaching hospitals and large regional centers in Poland were involved in the study. A total of 189 SE tests were performed, including 178 diagnosing CAD and 11 for VHD assessment (asymptomatic AS, low-flow/low-gradient AS, MR). Diagnostic tests for CAD were performed in all centers (100%) and examinations to evaluate VHD varied from 1 center (5%) for mitral valve disease, to 3 centers (17%) for both asymptomatic AS and LF-LG AS. To evaluate CAD, the protocols used included: DSE (all centers, 100%), cycling ergometry (1 center, 5%), treadmill (2 centers, 11%), and pacing using atrial mode (3 centers, 17%). In order to evaluate VHD, stress methods were applied in sequence: 1) in MR – DSE (1 center, 5%); 2) in LF-LG AS – DSE (2 centers, 11%) and a cycle ergometer (1 center, 5%); 3) in patients with asymptomatic AS – DSE (3 centers, 17%) or cycle ergometer (1 center, 5%).

The study population contained 189 people, including 87 men (46%) and 102 women (54%). The average age of the patients was 66.4 years. According to the medical history of the group studied, 63 (33%) people were patients with suspected CAD and 115 (60%) were those with previously diagnosed CAD based on a history of myocardial infarction (44 people), coronary angiography, and in some patients based on previously performed non-invasive tests.

A significant VHD had been found in the remaining 14 (7%) people. Eighty-five (45%) individuals were subjected to coronary angiography, 58 (30%) of which were treated with percutaneous coronary angioplasty (PCI) and 16 (8%) with subsequent coronary artery bypass surgery (CABG). In 2 patients, coronary angiography was used for evaluation for valvular intervention, and in 11 cases it preceded the implantation of an electrotherapy device. Clinically, 31 (17%) individuals presented symptoms of typical chest angina, 89 (47%) of atypical angina and 25 (15%) patients reported non-coronary chest pain (Fig. 1).

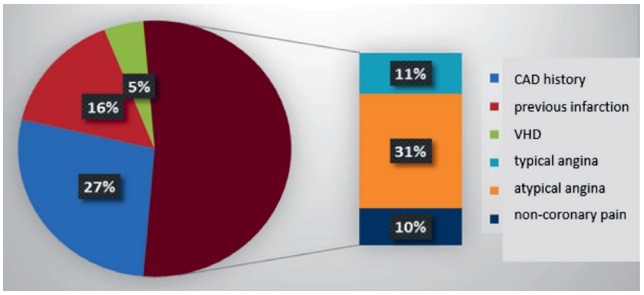

Fig. 1. Initial clinical characteristics of patients

CAD – coronary artery disease; VHD – valvular heart disease.

The analysis of SE studies performed in patients tested for CAD (without previous diagnosis of CAD) revealed that the majority of patients, 45 (71%), presented intermediate pre-test probability (PTP) of the disease before the test, estimated in accordance with the 2013 European Society of Cardiology (ESC) guidelines.[2] Low PTP was found in 17 (27%) patients – SE was performed for the verification of ailments and possible functional CAD – and high PTP was described in 1 individual (2%) – SE was performed for risk stratification and possible revascularization. In all patients analyzed for CAD, 28 (16%) were assessed for myocardial viability, the remaining 150 (84%) were subjected to provoking test of possible ischemia. Within the 11 VHD assessment studies, 6 (55%) involved the analysis of asymptomatic severe AS, 4 studies (36%) were performed in patients with LF-LG AS and in 1 case (9%) the study was related to a diagnosis of MR (Fig. 2). The ECG analysis revealed that 166 (88%) patients had regular sinus rhythm at baseline, 13 (6.8%) had atrial fibrillation and 10 (5.2%) presented pacemaker rhythm. Intraventricular conduction

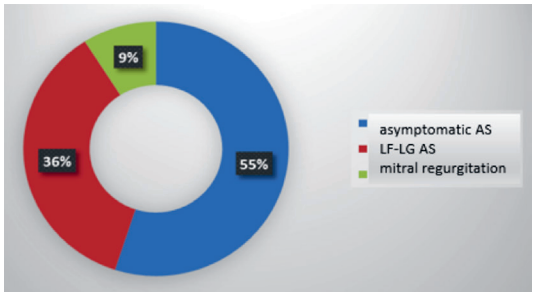

Fig. 2. Valvular heart disease in SE examinations

AS – aortic stenosis; LF-LG AS – low-flow low-gradient aortic stenosis.

disorders, being the main element that hindered the interpretation of regular ergometry on the treadmill or bicycle with ECG recording only, included 16 (8.5%) patients with LBBB, 2 (1%) with RBBB and 16 (8.5%) cases with non-specific intraventricular disorders. The LVEF was 55%, which included LVEF >50% in 158 subjects (83%), 40–50% in 15 (8%) and less than 40% in as many as 16 (9%).

Most of the tests (150, 79%) were carried out in accordance with the recommendations of the Echocardiography Section of the Polish Cardiac Society,[1] and the remaining 39 (21%) in accordance with the guidelines of the European Society of Cardiology.[4] The differences in the 2 protocols are not significant and include, in the first case, early onset of atropine administration (from a dose of 30 μg/kg/min dobutamine vs 40 μg/kg/min), and for the end test criteria, ST-segment elevation >1 mm (vs >2 mm) on an ECG. The vast majority of tests, 163 (86%), were performed by a cardiologist, 20 tests (10.0%) by a doctor specializing in cardiology (fellow) and 6 tests (4%) by an internal medicine doctor. The stress protocol was predominantly with the use of dobutamine (162, 86%). The remaining tests were performed using a cycle ergometer (12 tests), treadmill (5 tests) or using the stimulation of 10 tests. Dobutamine stress echocardiogram with a full dose of dobutamine (40 g/kg) was used in 121 people (75% of DSE) and 20 g/kg in 41 (25% of DSE) (Fig. 3). Dipyridamole or adenosine were not used in the studies. A positive SE score for the assessment of myocardial ischemia or VHD was obtained in 46 (24%) people, negative in 110 people (58%) and non-diagnostic in 33 (18%). For the assessment of CAD, a positive result was reported in 37 (20%) patients, negative in 109 (61%) and non-diagnostic in 32 (19%). For the evaluation of VHD, a positive result was reported in 9 (82%) patients, while in negative and non-diagnostic in 1 individual each (9%) (Fig. 4,5). The average stress time was 14.4 min (21.6 min for DSE and 7.3 min for exercise SE).

The most common reason for terminating the test according to the protocol was obtaining a heart rate limit or applying maximum doses of drugs. This concerned 141 tests. The reasons for the premature termination of the test included: achievement of the test objective, that is, echocardiographically positive result – 39 (20%); or observed

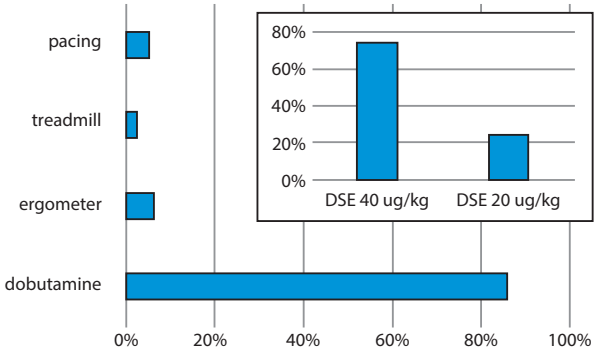

Fig. 3. SE protocols

DSE – dobutamine stress echocardiography.

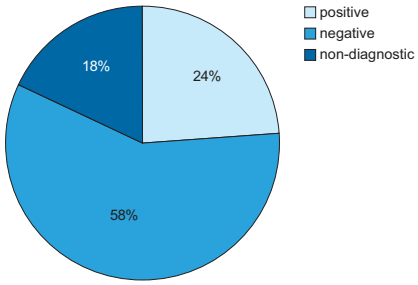

Fig. 4. Results of all SE examinations obtained

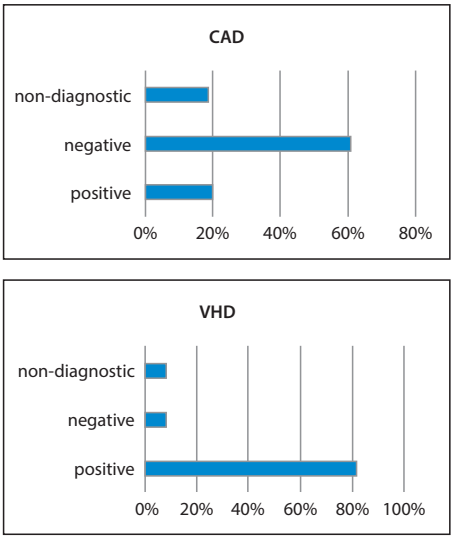

Fig. 5. Results obtained taking into account the diagnostic purpose

CAD – coronary artery disease; VHD – valvular heart disease.

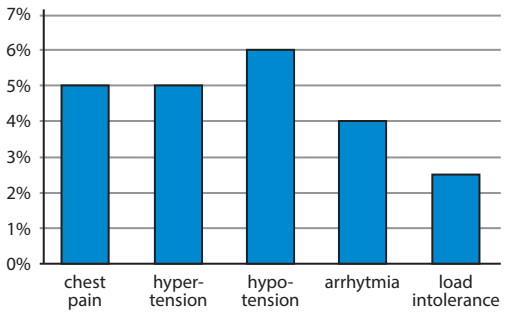

Fig. 6. Adverse effects during SE

adverse reactions – chest pain 9 (5%), excessive increase (5,2%) or decrease (6, 3%) of blood pressure or arrhythmia 8 (4%). Five (2.5%) tests were discontinued due to patient's intolerance of the stress agent or 3 (1.5%) at his request (Fig. 6).

The most serious complications of SE (myocardial infarction and death) were not observed. After evaluating the group with CAD and SE performed for the assessment of potential ischaemia, 41 (23%) patients were referred to coronary angiography; 20 of those had hemodynamically insignificant lesions, 19 people had critical and in 2 patients no coronary artery lesions were detected (Fig. 7).

Among 37 patients with positive SE results, 16 patients were diagnosed with critical lesions when subjected

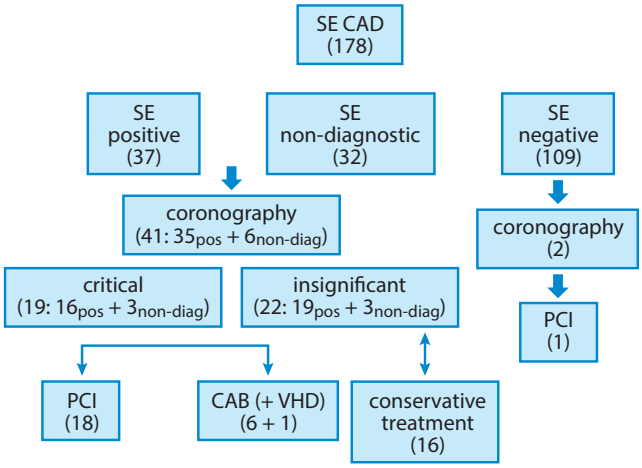

Fig. 7. Diagram of CAD treatment based on SE results obtained (number of patients)

SE – stress echocardiography; CAD – coronary artery disease; VHD – valvular heart disease; PCI – percutaneous coronary intervention; CABG – coronary artery bypass graft.

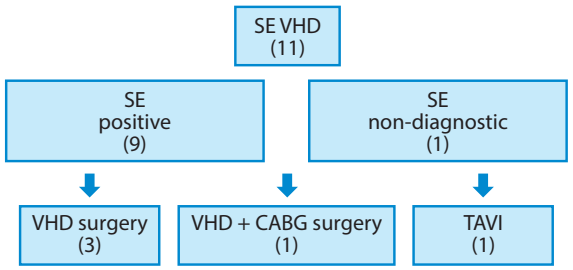

Fig. 8. Diagram of VHD management on the basis of SE results obtained (number of patients)

SE – stress echocardiography; VHD – valvular heart disease; CABG – coronary artery bypass graft; TAVI – transcutaneous aortic valve implantation.

to coronary angiography. In 2 patients, despite the positive SE result, no angiography was performed. However, among 109 people with negative SE results, 2 patients were subjected to coronary angiography, which revealed in the 1st case insignificant lesions, and in the 2nd critical lesions qualified for PCI were described. The analysis of the significance of the SE results for the decision-making on interventional measurements revealed that 30 patients (from the total study population of 189) were referred for the intervention. That included 19 individuals for percutaneous revascularization (44% of all who underwent angiography and 95% with significant lesions), 6 (15%) for surgical revascularization, 3 for valvular surgery, 1 patient qualified for transcutaneous aortic valve implantation (TAVI), and 1 for hybrid CABG and valve surgery (Fig. 7,8).

Discussion

The study presented shows the results of the first Polish prospective multicenter registry of echocardiographic

stress tests (PolStress-Echopro). In conjunction with the recently published[6] retrospective registry, Pol-STRESS is a large database reflecting the current situation and significance of SE in everyday clinical practice in Poland. The analysis involved 17 large cardiology centers, in which a total of 189 SEs were performed. In order to make the registry more objective and reliable, and not limiting its interpretation at the same time, many data points (14.5%) were rejected in the course of further analysis due to the lack of full access to necessary documentation. In Poland, the most frequently performed stress test is the electrocardiographic exercise test on a treadmill or cycle ergometer, which is mainly due to its wide availability, not the diagnostic power. The sensitivity and specificity of this study are 45% and 85%, respectively, which, in comparison to SE (83% and even 95% with vasodilator), significantly influences the interpretation of the outcome and subsequently the fate of the patient.[2] This data in terms of ischemia makes SE the dominant and preferred tool in CAD diagnostics.[7]

An important factor which elucidates accessibility concerns depends on the reimbursements considerations under the healthcare system. Hence, this published registry is considered to raise the awareness among the healthcare community of the increasing role of echocardiographic functional tests, which may encourage them to increase the availability of non-invasive diagnostics measures. All centers performed SE with the use of dobutamine, and only 3 were based on the physiological response of the patient during exercise, although it is known for its higher sensitivity in detecting cardiac ischemia in comparison with dobutamine and dipyridamole.[8] Moreover, exercise SE, in addition to providing additional prognostic data on physical performance, can also be used to diagnose patients with valve dysfunction or diastolic insufficiency, which can be frequently co-responsible for a patient's symptoms.[9,10] Unfortunately, the limited availability of supine stress ergometers in Poland and the application of relatively cheap provocation agents affects the type of protocol used. Among all institutions included in the study, only 3 used a previously implanted pacemaker. The usage of fast pacing allows the physician to perform the test as accurately and safely as using the previously described modalities, but can influence the result interpretation in the case of ventricular pacing or the occurrence of the Wenckebach point below the target pulse rate.[1,10] This small number was related to the patient profile and the limited group of patients with a pacemaker. Furthermore, none of the centers used adenosine or dipyridamole. The knowledge of key features and contraindications to a particular type of stress has significant importance in terms of appropriate method selection, making the result even more specific.[10]

The main reason of implementing SE remained CAD and this indication dominated in all centers. Stress echocardiography is recommended in symptomatic patients and in moderate PTP to detect myocardial ischemia, as well as in those without typical angina and LVEF < 50% or after incomplete revascularization.[2] This is convergent with our results, where most patients presented intermediate PTP. Only 1 individual was diagnosed with high PTP, which also seems to be reasonable in terms of incident risk and direct qualification for invasive measures. However, Papachristidis et al.[11] proved that SE can also influence this group of patients. In "only" 41% of those with high PTP was the SE score positive; 57% of them were subjected to coronary angiography and 24.6% were treated with PCI. Compared to the routine approach in accordance with ESC recommendations, a significant reduction in procedure costs was observed, with only a small percentage of serious adverse cardiac and cerebrovascular events (Major Adverse Cardiac and Cerebrovascular Events – MACCE). In current recommendations, SE also plays a key role in more challenging cases of VHD such as: asymptomatic AS, LF-LG AS (both classic low-gradient with P_{mean} < 40 mm Hg and EOA < 1 cm^2, and – paradoxically – with preserved LVEF and low SV < 35 mL/m^2), or in MR. Unfortunately, the use of advantages of SE in VHD is limited to only a few centers, which is significantly different from the United Kingdom National Health Service Survey data (almost 99% of centers), where, except for the VHDs listed in PolStress-Echopro, severe MVA (25% of centers) and asymptomatic severe aortic regurgitation (18% of centers) were evaluated.[12] New guidelines for the treatment of VHDs which appeared recently should influence the wider management of SE in the assessment of VHD, thus improving further therapeutic decisions and patients' prognosis.[13,14] The guidelines contain some minor modifications related to the outcome of SE performed in asymptomatic AS and in primary asymptomatic MR.[13] The class IIb C indication for valvular surgery in asymptomatic AS when the mean gradient increased by >20 mm Hg in the SE exercise test, was excluded. Similarly, in patients with primary asymptomatic MR, exertional increase in systolic pressure in the pulmonary artery >60 mm Hg is no longer considered an indication for surgery.

The areas of SE applications are not only limited to CAD and VHD (including valve prostheses) – SE may and should also be performed for other indications such as diastolic dysfunction, cardiomyopathy (dilated, hypertrophic), resynchronization therapy, pulmonary hypertension, congenital heart diseases (coarctation of the aorta, atrial septal defect, tetralogy of Fallot, single-chamber heart), and the athlete's heart.[15]

In the study presented, tests used for CAD assessment revealed the negative SE result in 61% of patients, which resulted in resignation from invasive testing that could have involved the possibility of serious complications. However, one of the patients with negative DSE who was referred to coronarography had critical coronary lesions.

It should be noted that among the 37 individuals with positive DSE, only 16 had hemodynamically significant lesions. This might be due to inadequate interpretation of the SE outcomes and false-positive results in cases such as: poor test conditions, changes in the basal segment

of the inferior wall, the presence of asynchrony of the ventricular septum in a patient with LBBB, in a patient after cardiac surgery and in the lesion in the left coronary artery circumflex branch.[10] As many as 19% of tests did not have diagnostic power, which corresponds with data from the available literature (5–20%).[16] The use of contrast agents would increase the accuracy of diagnosis, but also raise the cost of the study, thus it was not used in any center.[17]

The PolStress-Echopro registry has once again demonstrated the great safety of SE, which, considering its high sensitivity and specificity, makes it a unique diagnostic tool in patients with CAD and VHD. The most frequent stress effects resulted in stenocardial pain (subjective, not related to CAD severity) and fluctuations in blood pressure, which required premature discontinuation of the study. There were no serious adverse events such as death or myocardial infarction observed. For comparison, those complications are described in the UK-NHSS register (1 and 8 centers respectively).[12] Nevertheless, SE is considered a safe diagnostic method, with mortality rate and myocardial infarction estimated at <0.01% and 0.01–0.1%, respectively.[18,19]

An undoubted limitation of this study that needs to be mentioned is the absolute number of the exams analyzed and the time of observation. Both in the retrospective PolSTRESS Registry and the currently referenced prospective PolStress-Echopro, the number of SEs performed for the purpose of CAD assessment was predominant. This data is consistent and should be interpreted in a coherent and comprehensive manner, as well to confirm the conclusions of the work presented below on the necessity of wider dissemination of this method in the assessment of VHD. However, when analyzing the number of tests and the time of observation between both registries, the results obtained are proportional to the time of observation.

Conclusions

The most commonly used echocardiographic stress test in the diagnosis of coronary heart disease is DSE. Negative test results in the CAD diagnoses made it possible to avoid invasive coronary angiography in almost half of the patients. The proven high value and SE safety in assessing the significance of selected heart valve defects should lead to more frequent use of these methods in the qualification of patients for surgical treatment.

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Fibronectin and its soluble EDA-FN isoform as biomarkers for inflammation and sepsis

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Abstract

Fibronectin (FN) is a widely distributed glycoprotein which is present in different bodily fluids, on the surface of cells and in the extracellular matrix (ECM). It plays roles in various processes, including cell adhesion, migration, growth, proliferation, and tissue repair. Fibronectin exists in 2 forms: a soluble, inactive molecule, called plasma FN (pFN), which is synthesized by hepatocytes in the liver, and an insoluble cellular form (cFN), which is produced locally by different types of cells and is a key component of the ECM. Fibrinogen fibrils ensure structural support for cell adhesion and promote cell migration, proliferation and apoptosis. Additionally, FN controls the availability of growth factors. The plasma form of FN is a crucial component of the fibrin clot in the early wound-healing response, while the cellular form of FN supports efficient platelet adhesion, activation, aggregation, and procoagulant activity. Alternative splicing of the FN gene results in the generation of protein variants which contain the additional isoforms – extra domain A of FN (EDA) and extra domain A of FN (EDB); these are associated with, e.g., tissue remodeling, fibroblast differentiation, inflammation, and tumor progression. Fibronectin also serves as a target for a large number of bacterial proteins, and as part of a 3-component bridge (FN, integrin and FN-binding proteins – FnBPs) it contributes to bacterial colonization of endothelial and epithelial cells. Fibronectin has been identified in sepsis in humans as a negative acute-phase protein, and a low level of FN seems to be a marker of a poor prognosis for a patient. Here, the role of FN in inflammatory processes and sepsis is presented.

Key words: extra domain A of fibronectin (EDA), fibronectin-binding proteins (FnBPs), inflammation, sepsis, fibronectin

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Fibronectin structure

Fibronectin (FN) is a glycoprotein widely distributed in human bodily fluids, on cell surfaces and in tissues. It is a key component of the extracellular matrix (ECM).[1] Fibronectin occurs in both soluble and insoluble forms. The concentration of FN in the blood plasma of healthy humans is approx. 300 mg/L,[2] and this type of soluble FN – called plasma FN (pFN) – is synthesized by hepatocytes.[1] Insoluble, cellular FN (cFN) is a component of the ECM; it is produced by different types of cells (e.g., fibroblasts, smooth muscle cells, endothelial cells, platelets, and monocytes) and is deposited locally.[1,3] Fibronectin comes from a single gene, through alternative mRNA splicing. This gene encodes a large number of variants.[3]

The FN molecule is a dimer which is comprised of 2 nearly identical polypeptides linked by 2 disulfide bonds at their C-termini (Fig. 1). Each monomer is composed of 3 types of homologous repeating units or modules (12 of type I, 2 of type II and 15–17 of type III) which create structurally and functionally independent domains with binding sites for ECM proteins (e.g., collagen), cell surface receptors (e.g., integrins, bacterial FN receptors, cytokines), blood proteins (e.g., fibrin), and glycosaminoglycans (e.g., heparin).[1,3]

Fibronectin is encoded by a single gene which, through alternative splicing, generates 20 possible variants in humans. Protein diversity is obtained through the inclusion or exclusion of a type III homology segment (extra domain A of FN (EDA) and extra domain B of FN (EDB)) and by the presence of a V region (IIICS), which can be assembled in 4 ways or completely excluded.[1] Plasma FN lacks extra EDA and EDB segments, and its V region is included in only 1 subunit.[1] Cellular FN bears variable proportions of EDA and EDB segments.[3] Both forms, pFN and cFN, are reported to be incorporated into the fibrillar network of the ECM.[1,3]

Human FN is 5–9% sugars attached as N- and O-linked oligosaccharides. In the case of pFN, there are N-linked glycans located exclusively in the gelatin and cell-binding domain. O-glycans are present in the connecting segment between the fibrin–heparin-binding and collagen-binding domains, and in the V region.[4] The oligosaccharide part of FN protects the molecule against proteolytic degradation, enhances FN affinity to gelatin and promotes adhesion and the spread of fibroblasts.[4]

Fibronectin function

Fibronectin is involved in cellular processes such as cell adhesion, migration, growth and survival, and it is required for embryonic development.[1,3] Plasma FN supports hemostasis and regulates thrombosis, and it significantly accelerates healing by limiting the extent of inflammation.[5] As a key component of a fibrin clot, pFN supports hemostasis by being rapidly deposited at the injured vessel wall and by supporting platelet aggregation via pFN–fibrin complexes.[5] The role of cFN is to form and maintain tissue architecture and to regulate cellular processes.[1]

The EDA and EDB FN domains play an important role during embryogenesis, vascular development, cell migration, and cell differentiation.[1,3,6] Only very low levels of FN with an EDA and/or EDB domain circulate in the blood plasma of healthy people.[1] The inclusion of EDA domain in FN in adults is associated with pathological processes such as atherosclerosis,[7] lung fibrosis,[8] liver fibrosis,[9] diabetes,[10] and cancer.[11] In contrast to EDA, no receptor for EDB has yet been identified in vivo,[12] and the biological role of this domain has not yet been fully clarified. The research of Kraft et al. showed that EDB FN enhances bacterial removal by activating αvβ3 integrin.[12]

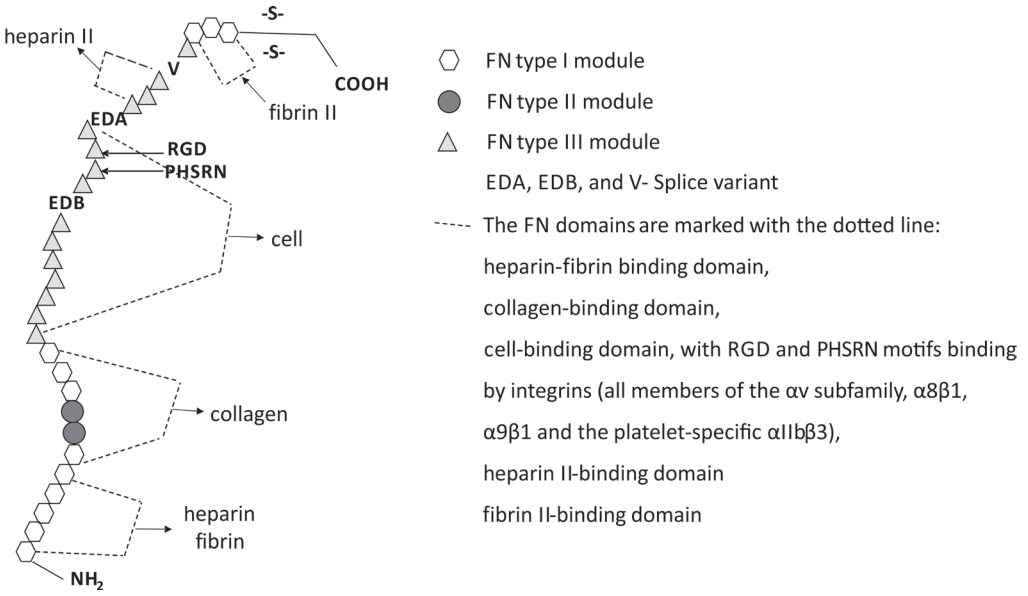

Fig. 1. Fibronectin structure. Fibronectin is composed of a series of FNI repeats (white hexagons), FNII repeats (dark grey circles), conserved FNIII repeats (light grey triangles), and alternatively spliced FNIII repeats (EDA, EDB and V). Types I, II and III repeats are composed of 40, 60 and 90 amino acids, respectively. Binding domains for collagen, fibrin, heparin, and cells are indicated

Fibronectin matrix assembly

Fibronectin matrix assembly is a cell-dependent process, and fibril formation requires interactions of the N-terminal type I modules (FNI_{1-5}) of FN with cell surface receptors. Fibronectin has a number of cryptic sites which remain hidden when the protein exists in its globular, soluble form and which become exposed when FN undergoes conformational changes associated with fibril formation.[13] The β_1-integrin $\alpha_5\beta_1$ is the major cell surface protein which controls FN assembly (Table 1), but other cell surface receptors apart from integrins also participate in FN matrix assembly.[13] The integrins link the cell surface FN with the intracellular actin cytoskeleton, forming a connection which is crucial for FN matrix assembly. The binding of FN to integrins and other receptors induces a reorganization of the actin cytoskeleton and activates the intracellular signaling complex. Contractile forces aid in converting inactive FN into the active extended molecule, and a concentration of active FN dimers at integrin clusters promotes FN–FN interactions and fibril assembly.[15]

Interaction of fibronectin with matrix components

Fibronectin assembly is initiated by binding of soluble FN to cell surface receptors (integrins) that induce conformational changes and expose cryptic binding sites in bound FN.[13] Fibronectin fibrils formed in a process of assembly are a major component of the provisional extracellular matrix of tissues, and the FN matrix is involved in the deposition of other ECM molecules as collagens, fibrillin, fibulin, and tenascin-C.[1] Shi et al. showed that the deposition of FN into the ECM regulates the deposition and remodeling of ECM collagen I, partially by regulating collagen I endocytosis.[21] The binding of the integrin α5β1 and syndecan-4 to FN generates the adhesive signaling required for the replacement of damaged tissue. The tenascin-C which is expressed at the edges of the wound and contacts the fibrin–FN provisional matrix serves to inhibit FN-initiated signaling events by blocking syndecan-4 binding.[20,21] Additionally, the FN matrix can sequester growth factors, including several vascular endothelial growth factors (VEGFs), transforming growth factor β1 (TGF-β1) and the latent complex of TGF-β1.[1,22] Vascular endothelial growth factor binds to FN type III domains to promote cell proliferation.[23] Myofibroblast differentiation is dependent on the presence of both TGF-β and EDA-FN.[24]

Fibronectin is a ligand for the TLR4 receptor (a member of the receptor family that regulates the NFκB-dependent synthesis of cytokines).[25] Kelsh et al. showed that 2 type III domains of FN can activate TLR signaling to induce an inflammatory response in fibroblasts.[19] In response to mechanical forces, the EDA and III-1 domains of FN can unfold to either reveal or hide biological active sites in a matrix which are involved in FN polymerization, cell adhesion or bacterial colonization. FN-EDA and $FNIII_1$ individually activate the same signaling pathways in dermal fibroblasts to induce a similar signature of inflammatory genes.[19] In the extracellular environment, FN interacts with the enzyme transglutaminase 2 (TG2).[26] On the cell surface, TG2 acts as an integrin-binding co-receptor for FN, which promotes cell adhesion and migration. It has also been shown that TG2 interacts with the heparin sulfate chains of syndecan-4.[26] This type of impact seems to be required in response to extensive tissue damage and ECM degradation. Increased TG2 expression during

Table 1. Integrin and non-integrin receptors for fibronectin (FN)[14–19]

Receptor	Binding sites in FN	Main function/effect
αvβ5 αIIbβ3 αvβ1 αvβ5 αvβ8 αvβ3 αvβ6 α8β1	$FNIII_{9-10}$	primary receptor for FN matrix assembly; triggers FN fibril formation in vitro hemostasis, FN matrix assembly adhesion regulation of interleukin 8 secretion angiogenesis development and wound healing; triggers FN fibril formation in vitro cell adhesion and neurite outgrowth attachment, cell spreading and neurite outgrowth
α4β1 α9β1 TLR4 (non-integrin)	EDA	fibro-inflammatory response in dermal fibroblasts cellular migration and FN matrix assembly induction of an inflammatory response in fibroblasts
αvβ3	EDB	bacterial phagocytosis
α4β1 α4β7	V	cell-matrix contact (by leucocyte extravasation and a number of immunological and inflammatory events) homing of specific lymphocyte populations to mucosal sites
TLR4 (non-integrin)	$FNIII_1$	induction of an inflammatory response in fibroblasts
Syndecan-4 (non-integrin)	$FNIII_{13}$	essential for signaling from the fibrin-FN provisional matrix and controls events such as matrix contraction
α5β1	FNI_5	regulation of endothelial cell functions and tumor growth

EDA and EDB – isoforms of *FN* gene.

wound healing probably enhances the adhesive/signaling functions of cell surface TG2 and may compensate for deficiency in the integrin-dependent adhesion and assembly of FN matrices. Telci and Griffin suggest that TG2 protected from matrix metalloproteinases (MMPs) in a complex with FN is likely to ensure an adhesion-mediated cell survival mechanism in situations of matrix breakdown during wound healing.[27] Fibronectin has the ability to form a cross-linked complex with FN fragments (FN type III domains) and can indeed participate in the exchange and/or addition of β-strands during the formation and maturation of FN fibrils.[28]

The involvement of fibronectin in inflammatory diseases and sepsis

Inflammation is a dynamic process characterized by the recruitment of leukocytes and plasma proteins from the blood into tissues, where the inflammatory response is activated to neutralize and eliminate potentially injurious stimuli, immune surveillance, optimal repair, and regeneration after injury.[29] Dysregulation of these processes forms the background of many complex diseases (e.g., sepsis, infectious disease, trauma, allergy, autoimmune disorders, cancer, neurodegenerative diseases, and atherosclerosis).[29] Sepsis is defined as a life-threatening organ dysfunction caused by a dysregulated host response to infection.[30] It is a systemic inflammatory response to an infectious pathogen that may be significantly amplified by endogenous factors. Sepsis is now known to involve early activation of both pro- and anti-inflammatory responses, along with major modifications to non-immunological pathways such as cardiovascular, neuronal, autonomic, hormonal, bioenergetic, metabolic, and coagulation.[30]

Fibronectin has been shown to be involved in inflammation. In animals, it is a positive acute-phase protein, with higher blood levels during inflammation,[31] but in humans, plasma FN is regarded as a negative acute-phase protein.[32] Fibronectin promotes chemotaxis and influences leukocyte function.[33] Additionally, Rossen et al. demonstrated that FN acts as a modulator of leukocyte function.[34] In the case of sepsis, the role of FN in promoting the adhesion and infiltration of bacteria to human cells seems to be important.[35]

EDA-FN isoforms in inflammatory diseases

The main role of EDA-FN in adults is associated with wound healing,[3] tissue fibrosis,[3,8,25] thrombosis, and the maintenance of vascular wall integrity.[5] The amount of the EDA isoform is very low or undetectable in the blood plasma of adults, but it significantly increases under pathological conditions such as fibrosis (in the lungs, liver and kidneys), diabetes, atherosclerosis, hypertension, ischemic heart disease, stroke, rheumatoid arthritis, and cancer. EDA-FN has also been recognized as a factor which promotes chronic inflammation.[1,36] The main source of EDA is the smooth muscle cells of blood vessels.[36]

In atherosclerosis, the role of EDA-FN is twofold. On the one hand, the EDA-FN isoform is involved in the initiation and progression of atherosclerosis, but on the other hand, the presence of EDA-FN is associated with a better, more stable plaque phenotype.[37] Similarly, in diabetes the EDA domain plays a dual role. It expands the extracellular matrix, resulting in diabetic structural vascular abnormalities, but prevents endothelial dysfunction by providing feedback defense against excessive generation of reactive oxygen species (ROS).[10] Bhattacharyya et al. showed that the EDA domain of FN promotes chronic cutaneous fibrosis through the TLR-4 receptor.[25] Additionally, several studies have demonstrated that the EDA domain (as well as the EDB domain) is highly expressed in many tumors, e.g., breast cancer,[38] liver cancer,[11] lung cancer,[39] colorectal cancer,[40] and bladder cancer.[41] Both isoforms of FN are expressed by cancer cells, tumor-associated fibroblasts and newly forming blood vessels in angiogenesis. The data of von Au et al. showed that circulating FN plays an important role in tumor growth. Fibronectin content in the tumor correlates with the number of blood vessels and tumor growth in mouse models.[41] Attieh et al. reported that cancer-associated fibroblasts induce invasion of cancer cells by FN assembly and integrin αvβ3 expression.[43] An ongoing study related to the role of EDA-FN in sepsis showed that an absence of this isoform contributes to poor sepsis outcomes in a murine model.[44]

Fibronectin in sepsis

The potential pathological consequences of incorrect host defense against infection include autoimmunity, inflammatory tissue damage, organ dysfunction, and sepsis. The lack of sensitive diagnostic tests for sepsis combined with its various non-specific signs and symptoms makes diagnosis very difficult. Many biomarkers have been evaluated for application in sepsis, but none of them has sufficient specificity or sensitivity to be used in clinical practice. C-reactive protein (CRP), procalcitonin (PCT) and interleukin 6 (IL-6) are used only for additional assessments.[45] Fibronectin is one of various sepsis biomarkers which have been proposed in the field of sepsis diagnosis. Martin et al. proposed that a plasma FN level lower than 120 mg/L could suggest a diagnosis of sepsis in its early stages. The lower level of pFN in patients who fulfill the criteria for a diagnosis of clinical sepsis may be associated with the constant reparatory process exerted by pFN.[46] According to data from Reichsoellner et al., the highest levels of FN occur in patients with a systemic inflammatory response but with no blood infection; it is lower in patients with Gram-positive and Gram-negative bacteremia, and the lowest levels appear in fungemia.[47]

A decreased level of pFN is associated with acute inflammation, surgical trauma and disseminated intravascular coagulation. According to Mamani et al., decreased levels of pFN and increased levels of CRP may be considered reliable diagnostic markers for sepsis.[48] Interestingly, during sepsis in animal plasma, pFN levels are higher.[49]

The data from recent years about the increasing levels of EDA and EDB isoforms of FN in pathological conditions may be of more interest than pFN in relation to sepsis. As described above, the absence of the EDA-FN isoform contributes to poor sepsis outcomes in a murine model.[44] However, Satoi et al. reported that the concentration of plasma EDA-FN in patients with sepsis who survived was significantly lower than that in non-survivors.[50]

Fibronectin has been identified as the target for a large number of bacterial proteins generally considered to be adhesins; therefore, FN seems to play a prominent role in the pathogenesis of sepsis. Furthermore, FN-binding surface proteins are found in many bacterial species (e.g., *Streptococcus pyogenes* or *Staphylococcus aureus).* They promote the adhesion of bacteria to human cells and enter into them.[35,51] The most common pathogens that cause sepsis include *S. aureus*, *Streptococcus*, *Enterobacteria* (*Escherichia coli*, *Klebsiella* and *Proteus*), *Neisseria meningitidis* and *Candida* spp. *Staphylococcus aureus* is a commensal bacterium in humans and it causes a variety of diseases, including sepsis.[51] Two FN-binding protein homologues – FnBPA and FnBPB – expressed by *S. aureus*, are involved in adhesion to the cell surface and internalization.[52] *Staphylococcus aureus* associates through FnBP with the type I repeats of host-derived FN. The ligand-binding regions of FnBPs (Fig. 2) interact with a 29 kDa N-terminal region of FN (the fibrin/heparin binding domain consisting of FN type I_{1-5} repeats). Fibronectin deposited on the pathogen surface is recognized by the cellular FN receptor integrin α5β1, which binds to the RGD motif (a key motif responsible for cell binding) within this matrix protein.[52] The additional bacterial binding sites on FN are located at the $FNIII_{12}$ module and $FNIII_9$/$FNIII_{10}$ modules.[53] Nyberg et al. demonstrated that plasma FN bound to the bacterial surface downregulates the virulence of *S. pyogenes* by limiting bacterial spread.[35] Kraft et al. reported that EDB FN enhances in vitro phagocytosis to a larger extent than plasma FN.[12] Additionally, αvβ3 integrin (classically described as the vitronectin receptor) plays a direct role in bacterial phagocytosis in mammals.[12]

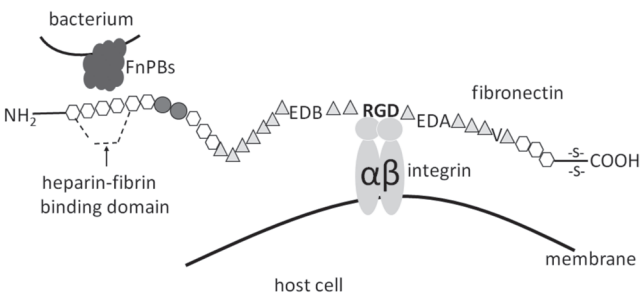

Fig. 2. Schematic diagram of *S. aureus*–FN interactions. The FN molecule acts as a bridge between the surface-bound FnBP and α5β1 integrin

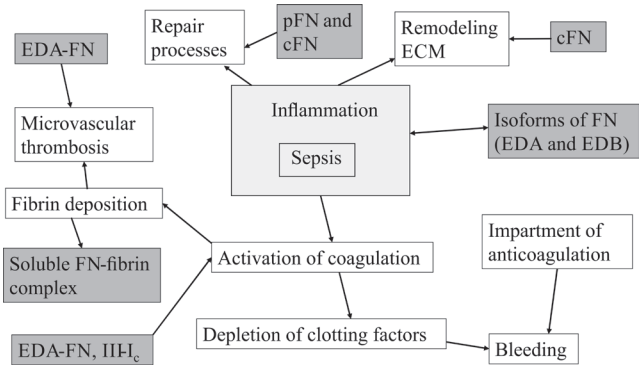

Fig. 3. Involvement of FN in inflammation processes. The diagram shows the participation of plasma and cellular FN at various stages of inflammation

The binding of FnBPs to FN mediates the adherence of *S. aureus* not only to extracellular matrices but also to the surface of a number of host cells, including endothelial cells,[54] epithelial cells[55] and fibroblasts.[56] An additional aspect to which attention should be paid when discussing the role of FN in the processes of inflammation and sepsis is the activation of the coagulation system (Fig. 3). In sepsis, systemic inflammation permanently leads to activation of the coagulation system and inhibition of the anticoagulant mechanism and fibrinolysis.[57] Increased fibrin generation and impaired breakdown as a main result of upregulating plasminogen activation inhibitor type 1 both lead to the deposition of microvascular clots. Platelet activation is an alternative stimulation of fibrin formation and it plays an important role in the development of coagulation abnormalities in sepsis.[57] Some blood plasma components, such as fibrinogen and fibrin, create high-molecular FN forms[7,58] which may reflect changes in vascular endothelium and ECM remodeling in inflammatory diseases.

Conclusions

Fibronectin is a crucial ECM protein regulating ECM-dependent cell adhesion, migration and differentiation. Both plasma and cellular forms of FN play important roles during tissue repair. Plasma FN is a significant component of fibrin clots, taking part in hemostasis stabilizing the clot structure, provisional matrix formation and the repair process. Cellular FN supports efficient platelet adhesion, activation, aggregation, and pro-coagulant activity.[1,11] The deposition of FN into the ECM regulates the inclusion and remodeling of ECM collagen I, in part by regulating collagen I endocytosis.[21] In addition to the plasma FN, isoforms of cellular FN play a key role in pathology. An elevated EDA-FN level is associated with clinical conditions, including diabetes and atherosclerosis, and may result in increased thrombosis in patients at a high risk

of cardiovascular events.[59] Many microbial pathogens invade the host by expressing surface receptors that bind specifically to FN, and – as Nyberg et al. have shown – pFN bound to the bacterial surface downregulates virulence by limiting bacterial spread.[35] The binding of FN to integrin induces a reorganization of the actin cytoskeleton and activates the intracellular signaling complex. Integrins, along with lipopolysaccharide, are a key receptor of phagocytes. The EDB-FN isoform of cFN enhances phagocytosis more than pFN.[12]

In summary, as shown previously, FN plays an important role in the host response to infection, being involved in maintaining vascular integrity, wound healing and triggering the blood clotting process.[35,51] Additionally, it mediates important interactions with phagocytes throughout the inflammatory process, and as part of a 3-component bridge, FN contributes to bacterial colonization of endothelial and epithelial cells. The understanding of the role of FN in inflammation, especially its EDA and EDB isoforms, seems to be crucial to the understanding of how FN could help in the development of therapeutic strategies to treat inflammatory diseases, including sepsis.

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Neglecting regression to the mean continues to lead to unwarranted conclusions: Letter regarding "The magnitude of weight loss induced by metformin is independently associated with BMI at baseline in newly diagnosed type 2 diabetes: Post-hoc analysis from data of a phase IV open-labeled trial"[1]

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Abstract

As the prevalence of type 2 diabetes mellitus and obesity increases worldwide, scientifically rigorous research is needed in this field to determine effective interventions for the prevention and treatment of these chronic diseases. In a recent study published in this journal, Zhou et al. conclude that metformin, a drug used for treatment of type 2 diabetes mellitus, can be used effectively for weight loss, and that this effect is even more pronounced in individuals who weigh more at baseline. Unfortunately, we believe these results to be due to the regression to the mean (RTM) phenomenon, which weakens the causal inference proposed in this study. The conclusions of Zhou et al. that metformin is an effective strategy for weight loss in individuals with type 2 diabetes mellitus are not substantiated due to the lack of a control group and failure to consider other factors that may have confounded these results.

Key words: statistics, metformin, body weight loss

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Regression to the mean

Regression to the mean (RTM), though identified by Francis Galton over a century ago and well known to statisticians, is a statistical phenomenon that is commonly ignored or misunderstood and which leads to inappropriate causal conclusions in general,[2] in biomedical research overall[3] and in obesity research in particular.[4–7] The phenomenon of RTM, if not addressed, can bias causal inference, and will occur in statistical analyses when examining 2 variables that are not perfectly correlated at a coefficient of 1.0.[3,6] In the case of Zhou et al., these variables are BMI or body weight at 2 time-points during the study.

Zhou et al. focus the results and subsequent conclusions of this paper on the 2.4-kilogram weight loss observed over a period of 16 weeks. This level of weight change may not be due to the use of metformin alone. Without the use of a control group to compare changes in mean body weight over the course of the study, the authors cannot estimate the effect of metformin on weight loss separately from RTM or other factors which may lead to weight loss.

The second finding in this paper from Zhou et al. was the observation that individuals with higher body weight at baseline had lost more weight due to metformin by the end of the 16-week period. Even ignoring the tendency to increase type 1 errors – which the stepwise regression method the authors used demonstrates[8] – this result is most likely due to RTM. By selecting a subgroup to analyze based on baseline measurements, there will be greater RTM than by observing the sample as a whole. If this subgroup of individuals has a higher baseline value for a given variable – body weight in this example – then it is expected that their second observation will also be high, but not as high as the first one, as they will have regressed towards the mean.[4] The conclusions made by the authors state: "[w]e found that after metformin treatment, the proportion of obese patients decreased ($p = 0.005$)." The proportion of overweight patients also decreased, whereas the portion of normal weight patients increased, although not significantly is influenced by RTM. Furthermore, because weight change in this case is computed as the difference between weight at week 16 and baseline weight, it will be functionally associated with body mass index (BMI), which was used in the stepwise regression model. Because of both RTM and the formula of weight change itself, higher baseline weight will almost always be associated with greater weight loss.

This examination of participants at the extreme ends of the distribution will lead to greater RTM than if the sample had been examined in its entirety.[4] This finding, which was the second major conclusion of the paper, is a result of RTM and therefore should not be considered evidence of any greater efficacy of metformin on weight loss in patients with obesity.

Conclusions

Without a control group, the causal statements and conclusions offered by Zhou et al. are not justified. Zhou et al. mention the lack of a control group as a limitation in the discussion, yet the conclusions are nevertheless causal and fail to address other potential confounding factors for the results observed in the study. The central finding that metformin treatment is associated with greater weight loss among those who were heaviest at baseline is predictable in view of RTM. Again, without a control group, this result cannot be taken as an indicator of differential efficacy as a function of baseline BMI. The article underscores the ongoing lack of understanding of RTM and its capacity to contribute to misleading conclusions in obesity research.

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Adropin and irisin in arterial hypertension, diabetes mellitus and chronic kidney disease

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Abstract

Despite great advances in medicine, the proper treatment of arterial hypertension (AH), diabetes mellitus (DM) and chronic kidney disease (CKD) remains a major challenge. Untreated, undiagnosed AH or DM may lead to the development of CKD and consequently to the occurrence of cardiovascular events. Adropin and irisin are newly discovered proteins which may play a role in the development and progression of the chronic diseases mentioned above. Endothelium dysfunction could be a bonding point. The following review paper focuses on adropin and irisin concentrations and their correlations in AH, DM and CKD. Lower adropin concentrations have been measured in patients with primary AH when compared to healthy volunteers. Irisin has reduced blood pressure on nitric oxide (NO)-dependent pathways in experimental studies; a negative correlation between irisin and blood pressure values has also been observed in preeclamptic women. Irisin also plays a role in insulin sensitivity and metabolic disorders. Lower irisin levels have been observed in patients with DM type 2 in comparison to a nondiabetic control group. It is also lower in the serum of pregnant women with gestational DM. A negative correlation between irisin and estimated glomerular filtration rate (EGFR) has also been noted. Adropin and irisin are newly described myokines measured in human plasma in healthy and disease status. Their exact function has not been specified yet and requires further studies.

Key words: diabetes mellitus, hypertension, kidney, proteins

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The relationship between arterial hypertension (AH), diabetes mellitus (DM) and cardiovascular as well as renal events has been demonstrated in many available studies and meta-analyses.[1–3] Despite huge progress in medicine, the number of patients with AH is still rising and a large percentage of people remain undiagnosed for a long time.[4,5] These facts demand further research to find the pathophysiological pathways of these disorders in order to develop new therapeutic possibilities. Adropin and irisin, newly characterized proteins, may be important elements in the pathophysiological pathways of these disorders. They are both myokines regulating energy homeostasis and metabolic processes.[6,7]

Adropin was characterized for the first time in 2008 as a secreted peptide consisting of 76 amino acids. This newly discovered peptide hormone is encoded by the *ENHO* gene and is expressed in the liver, kidney, heart, small intestine, body fluids, and central nervous system. Researchers suggest that adropin has a role in endothelial dysfunction, insulin resistance and energy homeostasis.[8] Recent data confirms that this bioactive protein has an ability to take part in cell-to-cell communication.[9]

Irisin, a membrane protein whose precursor is the fibronectin type III domain containing protein 5 (FNDC5), has been described as a hormone produced by myocytes. Experimental studies have shown that its level is maintained by PPAR-γ coactivator-1 (PGC1)-α in the circulation. The irisin sequence has been measured with mass spectrometry and a great resemblance has been found between humans and mice.[10,11]

In this review, we will focus on the possible role of irisin and adropin in patients with chronic kidney disease (CKD), DM and AH.

Adropin, irisin and hypertension

Hypertension is a risk factor for cardiovascular events. Many patients with diagnosed AH have inadequate blood pressure control. Insufficient or inadequate treatment can contribute to the end-stage CKD.[12] Available population studies suggest that a large percentage of the population suffer from high blood pressure.[13] Published experimental studies indicate an association between serum adropin levels and AH. Researchers have analyzed adropin levels in a group of 40 obese hypertensive children and 15 healthy volunteers; no correlation was found between adropin concentration and high blood pressure, but interestingly, a significantly lower concentration of adropin was measured in the obese children.[14] Another study used adropin and endothelin-1 (ET-1) enzyme-linked immunosorbent assay (ELISA) kits to show an association between adropin and endothelin-1 concentrations in patients with essential AH. A significantly lower adropin level was observed in participants with primary AH when compared to the control group. The authors concluded that adropin may be a predictor of primary AH. The study also showed that the molecular weight of adropin is very low and that this hormone is easily filtered by the kidneys. Consequently, the serum level of adropin is lower than its concentration in urine, especially in hypertensive patients.[15]

Adult patients with blood pressure values over 180/110 mm Hg had significantly lower serum levels of adropin than the normotensive controls. However, adropin levels cannot be used as a marker of target organ damage (TOD) because of its similar levels in the groups with and without TOD.[16]

There are also studies confirming an association between irisin and blood pressure. Irisin mediates energy homeostasis and it is produced by muscle cells, among others. On the basis of these facts and the correlation between metabolic disorders and AH, the association between low serum irisin levels and arterial relaxation in hypertensive and normotensive rats was analyzed. Irisin stimulates the production of nitric oxide (NO) and endothelial NO synthase (eNOS). Moreover, the vasorelaxing effect was connected with activation of the 5'-AMP-activated protein kinase (AMPK) and blocking of protein kinase B (Akt). Summarizing, the researchers observed that irisin reduced blood pressure on the AMPK-Akt-eNOS-NO dependent pathway and led to vasorelaxation in hypertensive rats. This mechanism was dose-dependent. A higher level of NO improved endothelial function.[17]

Another study focused on correlations between irisin and AH in pregnant and preeclamptic women. The researchers used ELISA kits to analyze the level of irisin in the blood. The study included 67 pregnant patients (31 healthy volunteers and 36 preeclamptic women). Interestingly, a negative correlation between irisin and systolic and diastolic blood pressure was observed in preeclamptic women. A negative correlation between age and irisin levels in preeclamptic women was also noticed.[18] Two years before, the influence of irisin on blood pressure was analyzed in an experimental study by the same researchers. They showed that central administration of this peptide hormone raised blood pressure, while its peripheral administration caused a hypotensive effect in both hypertensive and healthy rats.[19] The exact mechanisms regarding the different effects of irisin on blood pressure are unknown. Weizhen et al. suggested that central irisin administration raises blood pressure as a consequence of increased cardiac output by activation of the hypothalamus. The hypothalamus influences blood pressure through adrenergic sympathetic activity. Irisin regulates blood pressure peripherally by influencing blood vessels, specifically in endothelial and smooth muscle cells. It could be a messenger connecting the brain and the cardiovascular system.[20]

Adropin, irisin and diabetes mellitus

Diabetes mellitus type 2 is a metabolic disorder characterized by increased blood glucose, caused by a lack of insulin and/or insulin resistance. Diabetes mellitus

is a globally widespread health problem. Long-term hyperglycemia causes complications such as coronary artery disease, diabetic retinopathy and impaired renal function.[21] There are many experimental and clinical studies concerning irisin concentrations in subjects with DM type 2. It has been proven that irisin plays a role in insulin sensitivity and metabolic disorders.[11] Lower irisin levels have been observed in patients with DM type 2 in comparison to a nondiabetic control group.[22] On the other hand, obese nondiabetic patients had significantly higher levels of irisin in comparison with thin nondiabetic participants. Irisin was closely related to body mass index (BMI), homeostatic model assessment of insulin resistance (HOMA-IR) and fasting insulin.[23] Chinese researchers found that serum irisin was significantly lower in 104 patients with newly diagnosed DM type 2 as opposed to the control subjects (104 healthy volunteers).[22] A meta-analysis carried out by Zhang et al. demonstrated significantly lower irisin levels in patients with newly diagnosed DM.[24]

The same results were also observed in the maternal serum levels of irisin in women with gestational DM.[25] Irisin concentrations were significantly lower in the study group in comparison to healthy pregnant women. Interestingly, there was no statistically significant correlation between circulating irisin levels in cord blood in the same study and the control group of pregnant women.[25]

In addition, decreasing adropin expression was observed in cord serum and also in maternal blood in women with gestational DM.[26,27] Increased levels of adropin seemed to influence abnormal fetal growth in women with gestational DM, possibly through placentation dysfunctions. No significant differences in adropin levels in pregnant women with gestational DM type 1 or gestational DM type 2 were noticed. Moreover, a positive correlation between adropin and glycated hemoglobin (HbA1c) was observed.[28]

Not many studies have analyzed irisin and adropin concentrations in breast milk. Lower concentrations of these hormones have been observed in patients with gestational DM. Circulating irisin and adropin levels in breast milk reflected their concentrations in plasma.[29]

Another research group measured irisin levels in patients with DM type 2 and suggested that plasma irisin seems to be connected to metabolic factors in healthy subjects but not in diabetic patients.[30] Serum levels of irisin in the control group were associated with total cholesterol, triglycerides, age, diastolic blood pressure, and fasting blood glucose. As in earlier studies, the authors observed decreased irisin concentrations in patients with DM type 2.[30] The data presented reflected a reduced level of irisin in patients with renal insufficiency and DM type 2. Blood pressure and age have been shown to correlate with irisin in patients with DM type 2 and normal renal function.[31] A research team from China found that irisin ameliorated disturbed endothelial function in patients with DM type 2.[32] They observed improvements in vascular function after the administration of 0.5 mg/kg/day of irisin in mice suffering from DM type 2 over a period of 2 weeks. The authors also showed protective effects of irisin on the diabetic aortic endothelium. Some aorta segments were exposed to irisin (1 μg/mL) ex vivo. In their observations, irisin reduced the production of NO synthase, the glycosylated subunit component of NADPH oxidase (gp91phox) as well as peroxynitrite. Irisin may have an inhibitory effect on PKC-beta/NADPH oxidase and NF-kB/iNOS pathways.[32] Lower irisin levels were measured in patients with newly diagnosed DM type 2. Interestingly, multivariate regression showed a positive association between irisin concentrations and flow-mediated dilation levels.[33] The same authors, in an experimental study, showed that excessive expression of FNDC5/irisin ameliorated insulin sensitivity and decreased hyperglycemia as well as hyperlipidemia.[34]

A study published in 2017 presented a correlation between irisin and the AMP-activated protein kinase (AMPK) pathway. Irisin improved glucose and lipid metabolism and lowered the insulin resistance of hepatic cells.[35] A Korean research group carried out the first prospective study focused on serum irisin levels as a risk factor for the occurrence of incident DM.[11] The study included 3,500 patients. Incident DM was diagnosed in patients with a level of fasting glucose ≥126 mg/dL, or on the basis of glycated hemoglobin ≥6.5%, or in patients using medicines to lower glucose levels during the study. Interestingly, the authors suggested that circulating irisin can be used as a factor to predict DM in a healthy population.[11] In a meta-analysis carried out in 2016, irisin levels correlated positively with an insulin resistance index.[36] In this meta-analysis, 17 studies were taken into consideration, involving a total of 1,912 non-diabetic, non-pregnant adults. The authors observed a stronger positive correlation between irisin levels and insulin resistance subgroups of this meta-analysis with fasting blood glucose ≥6.1 mmol/L in comparison to patients with fasting blood glucose <6.1 mmol/L.[36] This correlation was statistically significant in Americans and Asians but not in Europeans.[36–38]

Adropin concentrations in the serum and various organs of rats with streptozotocin-induced DM were analyzed.[39] The myokine levels were measured with ELISA kit and determined on the basis of the mass of the tissues. The concentration of adropin in liver, pancreas, kidney and cerebellum tissues as well as in serum was higher in rats with DM in comparison to the healthy rats.[39] A group of Turkish researchers published results pointing to a correlation between adropin concentration and endothelium dysfunction based on flow-mediated dilatation (FDM) in volunteers with DM type 2. They observed lower serum adropin levels in individuals with endothelial dysfunction in comparison to a control group. It is important that serum adropin appeared as a marker of endothelial dysfunction.[40] It has been suggested that adropin may be a potential predictor of coronary artery disease: adropin concentrations in diabetic patients correlated with

the advancement of coronary atherosclerosis.[41] It has not been confirmed if adropin has the same results in experimental and clinical studies, nor if experimental studies may be extrapolated to humans.

Irisin, adropin and kidney function

Chronic kidney disease, an increasingly widespread health problem, is diagnosed on the basis of a reduced estimated glomerular filtration rate (eGFR) as well as albuminuria. Knowledge and understanding of CKD predictors can prevent complications and protect against the development of the terminal stage of renal failure. One risk factor for CKD is obesity, which is currently considered a worldwide epidemic problem.[42,43]

In patients with CKD, irisin was associated with fat mass, BMI and eGFR. The lowest irisin levels were observed in patients with the 5th stage of CKD.[31]

Adult obese Chinese patients with higher concentrations of irisin had considerably lower incidence of CKD in comparison to obese subjects with reduced irisin levels.[44] A correlation between irisin and eGFR was also observed: The group of patients in the 5th stage of CKD had the lowest level of this hormone. No link between circulating irisin and microalbuminuria was confirmed.[30] Another study showed that serum irisin was decreased in the CKD group. Furthermore, they found that in peritoneal dialyzed patients serum irisin levels were higher than in hemodialyzed patients. On the one hand, the research group indicated that glomerular filtration rate (eGFR) and plasma bicarbonate were identified as irisin concentration predictors, but on the other hand, no association of this myokine and body composition markers was observed.[45] Another study reported significantly lower plasma irisin concentrations in hemodialysis patients when compared to healthy volunteers; moreover, no association between irisin levels after resistance exercise training (RETP) in hemodialysis individuals was observed.[46]

Another study investigated whether irisin levels may be taken as a risk factor of sarcopenia and carotid atherosclerosis in peritoneal dialysis patients. Lower irisin levels were observed in the peritoneal dialysis group when compared to a control group, and thus the thesis was supported.[47]

Lower high-density lipoprotein (HDL) cholesterol levels are often linked to CKD. A correlation between lower irisin levels and decreased HDL cholesterol in individuals with CKD has also been observed.[48,49]

There is not much data available reflecting adropin levels in patients with CKD as a result of DM. A negative correlation of this new peptide and the progression of renal insufficiency (based on creatinine concentration, GFR and blood urea nitrogen) has been confirmed.[50] Adropin may play an important anti-inflammatory function in DM patients by reducing the mRNA expression of interleukin 6 and TNF-α.[50,51] Interestingly, a study published in 2016 focusing on adropin-associated genes in hemodialysis patients showed lower adropin concentrations in RXRA homozygotes (rs749759 and rs10776909) as opposed to an *EHNO* gene (rs2281997), where the adropin concentration was higher.[52]

Conclusions

Adropin and irisin are newly described myokines measured in human plasma in healthy and in diseased individuals. Their levels correlate with kidney function, the presence of DM, AH, and lipid status, but the exact function of adropin and irisin has not been specified yet. Further clinical and experimental studies are needed to clarify their role.

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Dietary support in insulin resistance: An overview of current scientific reports

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Abstract

Over the past 30 years, a significant increase in the prevalence of insulin resistance (IR) has been observed. It is associated with more frequent occurrence of impaired glucose tolerance, diabetes, excessive weight, cardiovascular diseases, and endocrine disorders. The results of current studies do not indicate a necessity to exclude dairy products from the diet of insulin-resistant individuals. In addition, it has been found that moderate amounts of alcohol as part of a balanced, low-energy diet do not have a negative effect on insulin sensitivity. The authors of recent studies emphasize the importance of reducing the intake of simple sugars, especially from sweet drinks, sweets and excessive fruit juice consuption. Many studies have demonstrated the beneficial effects of consuming complex, low-glycemic-index carbohydrates that are rich in dietary fiber. An insulin-resistant patient's diet should be rich in whole grains and high amounts of non-starchy vegetables and raw fruit. The beneficial effect of the Dietary Approaches to Stop Hypertension (DASH diet) and the Mediterranean diet has been confirmed. The positive correlation between low-carbohydrate and very-low-carbohydrate diets requires confirmation in long-term studies with the participation of insulin-resistant patients. Research shows the benefits of increased calorific intake during the first half of the day, especially from a high-energy and low-glycemic-index breakfast. Furthermore, many researchers indicate that slow and mindful eating is a significant component of an appropriate diet for insulin-resistant individuals.

Key words: insulin resistance, diet, glycemic index

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Insulin resistance (IR) is an important issue in modern medicine. Over the past 30 years, a significant increase in the detection of IR has been observed.[1] Insulin resistance is associated with lower insulin sensitivity of the body tissues. It may be due to abnormal functioning and signaling of insulin receptors, an excessively high level of insulin-binding antibodies or an abnormal insulin molecule structure.[2] This condition is associated with an increased incidence of hyperinsulinemia, impaired glucose tolerance and diabetes.[3]

The frequency of IR depends on age,[4] body weight,[3] sex,[5] genetic predisposition,[6] physical activity, and lifestyle.[7] In addition, it has been shown that it can be associated with stress and overstimulation of the sympathetic nervous system.[8] The occurrence and development of IR are strongly correlated with inflammation.[9]

The prevalence of IR is estimated at between 10–30%, depending on the study population. In a study involving 1,500 adolescents aged 13–18, IR (defined as a Homeostatic Model Assessment of Insulin Resistance (HOMA-IR) >4) was diagnosed in approx. 10.8% of girls and 14.3% of boys. The proportion increased to 29.1% and 44%, respectively, in the obese group, and decreased to 4.7% and 7.2%, respectively, in the normal body weight group.[10] In a study on Asian adults over 30 years of age, its incidence was estimated at 21.5% in women (HOMA-IR ≥ 1.64) and 25.1% in men (HOMA-IR ≥ 1.56).[11] In one European population (Estonian adults), the percentage of insulin-resistant individuals (HOMA-IR ≥ 1.92) amounted to approx. 12.4% in people without metabolic syndrome and 58.8% in people with it.[12]

Insulin resistance is strongly related to overweight and obesity.[3] In addition, it correlates with a higher prevalence of dyslipidemia, cardiovascular disease[13] and endocrine disorders such as polycystic ovary syndrome (PCOS), hypercortisolemia and hyperprolactinemia.[2]

Lifestyle, eating habits and physical activity are important factors that can sustain or increase the risk of IR. A well-adjusted, balanced diet and regular meal frequency may improve glucose/insulin homeostasis.

Alcohol consumption and insulin sensitivity

Even though a significant reduction or exclusion of alcohol is recommended to overweight and obese patients with metabolic complications, studies on IR have not shown any clear negative influence of alcohol consumption on the course of the disease. Some of them indicate that moderate consumption of ethanol has a positive impact on the parameters related to insulin homeostasis. In a study involving 1,542 Japanese men aged 46–58 years, it was observed that daily consumption of 30 g or more of ethanol was related to a 40% decrease in the risk of IR (defined as HOMA-IR ≥ 1.81 and fasting plasma insulin levels ≥50 pmol/L).[14] In another study with 249 patients with IR (HOMA-IR ≥ 2.5), the authors observed a negative correlation between alcohol consumption and HOMA-IR values in all alcohol-consumption groups, i.e., light (40–140 g/week), moderate (140–280 g/week) and heavy (>280 g/week).[15]

However, the authors of meta-analysis of 14 studies found no significant impact of moderate alcohol consumption (i.e., below 40 mL/day) on IR. Some results suggest a possible positive influence of moderate consumption only in women.[16]

A 20-year analysis of changes in insulin sensitivity among people aged 18–30 years, based on the quantitative insulin sensitivity check index (QUICKI) showed that alcohol consumption did not affect insulin sensitivity. However, smoking was significantly associated with its deterioration.[17]

Some authors note that abstinence from alcohol may increase insulin sensitivity in particular organs. In a study involving 8 non-obese men with elevated fasting glucose levels who consumed approx. 32.1 g (±20 g) of alcohol per day, it was shown that refraining from alcohol for a week increased the insulin sensitivity of the liver. However, this was not related to muscle insulin sensitivity. It appears that reduced insulin sensitivity of the liver is associated with a higher percentage of visceral adipose tissue, correlating with metabolic disorders, aggravated inflammation and IR in patients with type 2 diabetes. These observations need confirmation in a larger study group.[18]

Milk, dairy products and IR

In conjunction with theories regarding insulin index or insulinotropic effect of consuming milk and dairy products,[19] the merits of their consumption in a group of patients with carbohydrate metabolism disorders and IR is often considered. In a study with 78 metabolically obese but normal weight (MONW) men and 154 non-obese metabolically healthy men, daily dairy consumption was associated with a 56% lower risk of MONW, which was defined by HOMA-IR ≥ 2.48. Furthermore, more frequent consumption of dairy products, i.e., over 1.4 portions/day compared to under 0.8 portions/day, was associated with lower HOMA-IR values.[20] Similar results were obtained in a randomized study involving 23 healthy subjects. It was observed that consumption of more than 4 portions of low-fat dairy products per day for 12 months was associated with a 9% decrease in fasting insulin level and an 11% decrease in HOMA-IR values, compared to people consuming fewer than 2 portions per day.[21] In another study evaluating milk intake alone in a group of 19 people, daily consumption of approx. 1.5 L of UHT or pasteurized milk for 21 days in a row did not influence HOMA-IR values.[22]

However, not all research results confirm a neutral or positive effect of dairy consumption. In a study involving 47 patients with excessive body weight, following

a high-dairy diet (4–6 portions of dairy products daily, e.g., 250 mL of milk, 200 g of yogurt, 40 g of hard cheese, 120 g of ricotta) for 4 weeks, it was associated with higher fasting insulin levels than after 4 weeks of a high-red meat, low-dairy diet (less than 1 portion of dairy products daily). Among women following a high-dairy diet, a decrease in insulin sensitivity assessed with HOMA-IR and the Matsuda insulin index (Matsuda-IS) was observed.[23]

These studies do not indicate that it is necessary to exclude dairy products in order to increase insulin sensitivity. Assessments of the beneficial effect of dairy consumption on the occurrence and severity of IR require confirmation in studies with larger research groups.

The relationship between consumption of high-fiber products and occurrence of IR

Fruit and vegetables are at the base of the food pyramid and are a source of essential vitamins and minerals. However, not all studies confirm that consuming them has a clearly positive effect on IR. It seems that the quality of carbohydrates and total daily dietary fiber intake play a more important role in terms of this disorder.

In a study involving 89 overweight patients at risk of cardiovascular disease, no changes in insulin response (measured with the metabolic clamp technique) were observed after a 12-week period of increased fruit and vegetable consumption. No significant differences were found for 2, 4 or 7 additional servings of fruit and vegetables per day.[24] Similarly, in a study involving 6,000 Indian citizens, no impact of grain legumes consumption on the level of insulin sensitivity was found.[25] Moreover, in a group of 8-to-10-year-olds, the number of portions of fruit and vegetables was not related to the values of IR indices such as HOMA-IR and Matsuda-IS.[26]

However, in another study based on the Food Frequency Questionnaire (FFQ) and 3-day food records with 24-hour recall in 250 Koreans aged 20–81 years, it was observed that there is a negative correlation between green and yellow vegetable consumption and fasting insulin level as well as HOMA-IR values.[27]

The proportion of the total weight of consumed vegetables and fruits (excluding juices) on the one hand and other consumed food on the other should be taken into account when planning a diet for a person with impaired glucose/insulin homeostasis. Cavallo et al. found that lower fruit and vegetable intake, compared with that of other foods consumed, were associated with lower fasting insulin levels.[28]

A meta-analysis of 12 studies concerning the impact of fruit juice consumption (about 50–500 mL per day) on fasting glucose and insulin level and HOMA-IR values did not show any significant relation between this habit and fasting glucose and insulin levels. Only 3 studies confirmed a significant correlation between juice consumption and increased HOMA-IR values. However, this result could be related to high heterogeneity of the study group. A random effect model analysis did not confirm the significance of the correlation between juice consumption and increased HOMA-IR values.[29] Similar results were obtained by Simpson et al.[30] However, another study, involving children at the age of 12–17 years, found a relationship between more frequent consumption of fruit juices and higher HOMA-IR values among the female participants.[31]

Silveira et al. observed a positive effect of consuming approx. 750 mL of antioxidant-rich blood orange juice in a group of insulin-resistant individuals (HOMA-IR > 2.71) with normal body mass index (BMI) values.[32] After 8 weeks of juice consumption, fasting insulin levels were reduced by 25% and HOMA-IR values were reduced by 28% as compared to the results from the beginning of the study. This correlation was not observed among the participants with excessive body weight. It seems that the positive effect could be the result of a decrease in inflammation associated with the consumption of the antioxidants contained in the juice.

Although the influence of fruit juice consumption on IR is unclear, it has been confirmed that whole fruit consumption may be associated with lower HOMA-IR values.[28]

Dietary fiber from whole grains and vegetables is an important component of the diet of insulin-resistant individuals. Many studies showed a positive correlation between its consumption and the values of indicators used to assess this condition. In a study involving 1,494 middle-aged Finns, based on 4-day food intake records and biochemical parameters, it was estimated that the total intake of dietary fiber and wholegrain bread was associated with lower Matsuda-IS values, regardless of the physical activity level or waist circumference. High dietary fiber intake correlated with lower secretion of insulin. Among the female participants, it was observed that dietary fiber intake was related to the level of insulin secretion in the early phase of the oral glucose tolerance test and inversely correlated with HOMA-IR values. In addition, total fat intake below 30–35% of the energy from the diet (E) and a high intake of saturated fatty acids (SFA) were positively associated with HOMA-IR values and negatively with Matsuda-IS values.[33] Similarly, a study of 674 children found that the total daily dietary fiber intake and the consumption of whole grains such as bread and cereals were inversely correlated with fasting insulin levels.[34]

A meta-analysis of 3 studies concerning the effect of complex carbohydrate consumption on insulin and glucose levels showed that eating breakfasts high in complex carbohydrates (12–21 g) was associated with better glucose and insulin responses, compared to meals with a very low content of complex carbohydrates (approx. 0.1–0.9 g), the same energy content (approx. 19–22% of E) and similar levels of other macroelements.[35]

MacNeil et al. obtained similar results by assessing the effect of an increased supply of dietary fiber on glucose and insulin levels after breakfast in a group of 12 diabetic patients.[36] In this study, it was observed that replacing 20 g of simple carbohydrates with complex carbohydrates in a breakfast bagel (total carbohydrate content 50 g) significantly reduced glucose and insulin blood levels after the meal compared to the results after eating a product with the same total carbohydrate content but lower dietary fiber content (2 g).

Results from studies investigating the influence of complex carbohydrates suggest that they have a positive impact on insulin and glucose levels. However, this topic requires further studies involving larger numbers of subjects to determine the appropriate amount of carbohydrates in the meals of insulin-resistant individuals.

Sweetened drinks, sweet products and fast foods, and IR

Sweetened drinks, juices and sweets are a major source of excess simple sugars in the diet. Reducing the intake of these sugars is a particularly important element in the treatment of insulin-resistant patients. Simple sugars increase both postprandial glycemia and insulinemia.

In a study with the participation of over 2,000 people, a significant relationship was observed between HOMA-IR index values and the frequency of consumption of sweetened drinks (estimated using the FFQ). This effect was not found in the case of artificially sweetened beverages. In addition, it was assessed that people who consumed more than 3 portions of sweetened drinks per week showed a higher risk of pre-diabetes.[37]

Similar observations were made by O'Connor et al. in a study of 9,700 respondents.[38] Based on an FFQ containing 130 products, a positive correlation was observed between the intake of simple sugars from drinks (juices, carbonated and non-carbonated soft drinks, sugar-sweetened beverages) as well as added sugars (sugars from 100% juices, table sugar, honey, and syrups) and the occurrence of IR. People in the 3rd quartile of consumption of these products showed the highest HOMA-IR values.

In another study, it was estimated that higher intake of sweetened soft drinks, low-calorie soft drinks, onions, garlic, French fries, salty snacks, as well as bread and rolls made from refined flour correlated with higher HOMA-IR values. An inverse relationship was observed in relation to medium- and high-fiber cereal, jam, marmalade, honey, French dressing, vinaigrette sauce, and wholegrain bread.[39]

Sweets and sweet snacks are a significant problem in children's diets. In a study involving 1,912 Greek children aged 9–13 years, it was observed that increased consumption of candies, lollipops, jellybeans, fruit in sugar syrup, and salty snacks was associated with a higher incidence of IR (HOMA-IR > 3.16).[40] However, in another study, involving 1,153 people aged 18–69 years and based on a partial FFQ, it was found that HOMA-IR values and fasting insulin levels were significantly lower among regular consumers of chocolate. Among the respondents, 80% were considered regular consumers, eating about 25 g of chocolate per day. It was reported that daily consumption of 100 g of chocolate was associated with HOMA-IR index values lower by approx. 0.16 units.[41] However, the study did not assess what percentage of daily energy intake came from chocolate consumption and whether or not this consumption leads to weight gain, which seems highly significant in the context of IR.

Diets used in IR

In many studies about insulin sensitivity, besides the assessment of the impact of particular components of diet on the glucose-insulin metabolism, the effectiveness of comprehensive dietary plans on the condition of insulin-resistant patients has also been evaluated. Many researchers have found that diets with modified levels of particular FA, low-glycemic-index diets, the Dietary Approaches to Stop Hypertension (DASH diet), and the Mediterranean diet have positive effects.[42–52]

The relationship between IR and the Mediterranean diet, the DASH diet and a vegetarian diet

An analysis of the data obtained from the patients of the Third National Health and Nutrition Examination Survey (NHANES III) showed that scores indicating the similarity of the respondents' diet to the Mediterranean diet were associated with lower BMI and waist circumference. Lower waist circumference correlated with lower glycated hemoglobin levels, fasting insulin levels and HOMA-IR index values.[42]

Mattei et al. compared degree of similarity of patients' diets to well-known diets such as the Mediterranean diet (using Mediterranean diet score (MeDS)), the DASH diet and the American Heart Association (AHA) diet (using AHA-diet score), and assessed their quality using the Healthy Eating Index – 2005 and the Alternative Healthy Eating index 2010.[43] It was found that the Mediterranean diet had the strongest association with lower insulin levels, lower HOMA-IR values and higher levels of insulin sensitivity. A high content of homemade vegetable soups, non-processed home-cooked meat, oatmeal, grain legumes, fish, whole milk, and beer in the diet had the strongest association with higher insulin sensitivity.

A 24-week study evaluated the impact of following a vegetarian diet and a diabetic diet on the level of insulin sensitivity in type 2 diabetics with IR.[44] In the vegetarian diet,

60% of E came from carbohydrates, 15% from protein and 25% from fats. Animal products were limited to 1 portion of low-fat yogurt per day. The diabetic diet provided 50% of E from carbohydrates, 20% from proteins and less than 30% from fats, where <7% of E came from SFA. The diet contained less than 200 mg of cholesterol per day. Both diets were isocaloric, with energy values reduced by 500 kcal per day in relation to the total metabolism. In both groups, the diets were supplemented with 50 μg per day of vitamin B12. From the 13th week of the study, the nutritional strategies were combined with personalized exercises. After the end of the study, a significantly higher level of insulin sensitivity was assessed among the people following a vegetarian diet. In addition, a greater decrease in visceral and subcutaneous fat, a significant decrease in inflammation and decreased leptin levels were observed in this group.

Women with IR often have coexisting polycystic ovary syndrome (PCOS). In a study involving 60 such patients, it was estimated that 2 weeks on a reduced-calorie DASH diet (about 350–700 kcal below the total daily energy expenditure), providing 16–18% of energy from proteins, 30% from fats and 52–55% from carbohydrates, contributed to a higher reduction in fasting insulin levels, a higher reduction in HOMA-IR index values and a higher increase in insulin sensitivity levels compared to diets with a similar distribution of macroelements but obtained from other food sources. In addition, following the DASH diet was associated with an improvement in sex hormone levels.[45]

Similar results were obtained by Asemi et al.[46] In a group of 48 women with PCOS, following the DASH diet was associated with a significant reduction in insulin levels and HOMA-IR index values compared to a diet with the same distribution of macroelements, but obtained from other food sources. For 8 weeks, the subjects in both the study group and the control group followed diets which provided 18% of E from protein, 52% from carbohydrates and 30% from fats. The nutrition plans differed only in the number of portions of individual food products. The study group (the DASH diet) consumed less than 2,400 mg of sodium per day. Their diet was rich in vegetables, fruits, whole grains, and low-fat dairy products.

Low-carbohydrate diets and IR

Apart from modification of the quality and quantity of carbohydrates in the diets of insulin-resistant individuals, many researchers also assess the impact of the quantity and quality of dietary fats on glucose and insulin metabolism.

In a study involving 513 Japanese aged 35–70 years, based on data from FFQs containing 46 products and an assessment of dietary habits, it was found that diets similar to Western diets (i.e., with high content of fat and meat as well as a lot of fried meals) were significantly associated with high and abnormal HOMA-IR index values.[47]

In an 8-week study looking at PCOS women with excessive body mass, it was noted that adhering to a low-starch diet with very low dairy consumption was associated with a significant improvement in fasting insulin levels (52%), insulin levels 2 h after an oral glucose tolerance test (37%) and HOMA-IR index values (51%, i.e., 3.9 ±1.5 vs 1.9 ±0.9) compared to the results from before the dietary intervention. The diet includes low-starch vegetables, low-sugar fruits, lean meat, fish, sea foods, eggs, avocados, olives, nuts, seeds, olive oil, and coconut oil. In addition, approx. 30 g of hard cheese and 180 mL of red wine per day were permitted; the patients consumed unlimited amounts of the other permitted ingredients. Compliance with the diet was checked 3 times during the study using a 3-day food intake record. The average nutrient content in the diet was approx. 1,422 ±199 kcal, 72.1 ±16.5 g fat, including 31.9 ±9.1 g monounsaturated FA, 15.8 ±6.8 g polyunsaturated FA, 19.5 ±7.8 g SFA, 94.3 ±22.8 g carbohydrates, and 98 ±25.1 g protein.[48]

However, in another study involving healthy people aged 40–75 years with BMI values of 25–35 kg/m^2, it was observed that even 1 day on a diet that provides 80% of E from fats, 15% from carbohydrates and 5% from protein, where 63% of fats come from SFA (42% palmitic acid), 29% from monounsaturated FA (oleic acid from canola oil) and 4% from polyunsaturated FA, contributed to an increase in whole-body IR in people with normal and impaired glucose tolerance. A decrease in insulin sensitivity was also observed after a single breakfast rich in SFA.[49]

Similar results were reported by Clayton et al.,[50] who assessed the relationship between breakfast composition and changes in insulin sensitivity over a period of 12 weeks. The subjects, who were divided into 2 groups, consumed isocaloric breakfasts consisting of 2 eggs and extras (43% carbohydrates, 25% protein, 32% fats) or a bagel (68% carbohydrates, 17% protein, 15% fats). Up to week 6, significantly higher insulin sensitivity was observed in the group consuming eggs. However, these values returned to the original state in week 12, when no significant differences were found in fasting insulin and fasting glucose levels between the study groups. The results of the study do not confirm the positive effect of a protein-fat breakfast.

Fat intake may affect epigenetic changes in adipose tissue. It has been observed that adding to the diet 750 kcal from palm oil or refined sunflower oil per day over a period of 7 weeks induces genetic changes in adipose tissue (for example *Mc2R* gene). Excessive intake of PUFA from sunflower oli was associated with changes in FTO gene expression and insulin receptor gene expression that are connected with IR.[51]

A meta-analysis of 19 studies showed that replacing 5% of the energy derived from carbohydrates with SFA or polyunsaturated FA was associated with reductions in fasting insulin levels, by 1.1 pmol/L and 1.6 pmol/L, respectively. Replacing the energy with monounsaturated FA did not show such an effect. However, introducing

monounsaturated FA instead of SFA or carbohydrates was associated with lower insulin levels 2 h after an oral glucose tolerance test. In addition, replacing 5% of the energy derived from carbohydrates or SFA with energy from polyunsaturated FA resulted in a reduction of HOMA-IR index values by 3.4% and 4.1%, respectively.[52]

Diets modifying the quantity and quality of carbohydrates and IR

Many researchers calculate the appropriate amount of carbohydrates for people with IR on the basis of glycemic indices and loads.[53–55] The majority emphasize the importance of the qualitative composition of carbohydrates in products.

A 12-week study examined 20 people with the same level of physical activity (5 times a week, approx. 1 h, up to 85% of maximum heart rate) who followed high- or low-glycemic-index diets. No significant differences in insulin sensitivity levels (measured with oral glucose tolerance tests and muscle biopsies) were found between the study groups.[53]

Another study assessed that following a diet with a low glycemic index and low glycemic load (approx. 34 and <125, respectively) had a beneficial effect on fasting glucose levels and was associated with a 4% decrease in insulin-like growth factor-1 (IGF-1) levels, compared to a diet with a glycemic index of approx. 78 and a glycemic load >250. In addition, it was noted that the consumption of a low-glycemic-index breakfast significantly correlated with a better insulin response compared to a high-glycemic-index meal both among people with normal body weight and those with excessive body weight.[54]

Daily meal frequency, eating habits and IR

In addition to the quantity and quality of nutrients, many researchers indicate that daily meal frequency and eating habits are important factors in planning a diet for people with IR.

In a study involving 6 healthy people, Morgan et al. showed that the consumption of 60% of E at breakfast, 20% at lunch and 20% at dinner was significantly associated with better insulin sensitivity compared to the consumption of approx. 20% of E at breakfast, 20% at lunch and 60% at dinner. In addition, they observed higher insulin sensitivity levels in a group following a low-glycemic-index diet (average glycemic index: 34) compared to a group following high-glycemic-index diet (average glycemic index: 84). Meal frequency, their calorific value and glycemic index all influenced the values of indicators used to diagnose IR. Based on the results of the study it can be suggested that people trying to regulate both glycemia and insulinemia should avoid eating abundant dinners with a high glycemic index.[55]

In a study on the impact of meal frequency on the overall insulin sensitivity in a group of 54 patients with type 2 diabetes, it was found that consuming 2 meals a day, i.e., breakfast between 6 am and 10 am and lunch between 12 am and 4 pm, over a period of 12 weeks was associated with higher insulin sensitivity, measured with the oral glucose insulin sensitivity (OGIS) index, compared to the consumption of 6 smaller meals. Both study groups followed the same diet, with energy content reduced by 500 kcal, in which 55% of E came from carbohydrates, 20–25% from protein and <30% from fats (<7% from SFA). In addition, the diet contained less than 200 mg of cholesterol and approx. 30–40 g of dietary fiber.[56]

Similar results were obtained in a study involving 36 people with excessive body weight. It was observed that increased calorie intake in the first half of the day, i.e., 70% of E coming from the first 3 meals (breakfast, a morning snack and lunch), was associated with lower HOMA-IR index values, greater weight loss, and greater reduction of waist circumference and body fat percentage compared to a diet providing 55% of E in the first half of the day. Regardless of daily meal frequency, the subjects of both groups followed an isocaloric Mediterranean diet with energy content reduced by 600 kcal for a period of 3 months. The diet provided 55–59% of E from carbohydrates, 25–30% from fat and approx. 15–16% from proteins. The subjects who obtained better results consumed approx. 25% of E at breakfast, 10% in a morning snack, 35% at lunch, 10% in an afternoon snack, and 20% at dinner, while the other group consumed 15% of E at breakfast, 5% in a morning snack, 35% at lunch, 15% in an afternoon snack, and 30% at supper.[57]

Similar results were obtained in a study involving 60 thin PCOS women who followed an isocaloric diet (1,800 kcal, approx. 124 g carbohydrates, 191 g protein and 62 g fat) with different calorie distribution throughout the day for 3 months. It was found that the consumption of 980 kcal for breakfast, 640 kcal for lunch and 190 kcal for dinner was associated with an 8% reduction in fasting glucose levels and a 53% reduction in fasting insulin levels compared to the results of subjects who consumed 190 kcal for breakfast, 640 kcal for lunch and 980 kcal for dinner. In addition, it was noted that the diet with the high-calorie breakfast was associated with a 20% decrease in the area under the glucose curve and a 49% decrease in the area under the insulin curve after oral glucose tolerance tests. What is more, the diet with the high-calorie breakfast was associated with 56% lower HOMA-IR values and 35% lower HOMA of β-cell function (HOMA-B) values. It was also associated with the regulation of sex hormone levels and increased rates of ovulation.[58]

A study involving 956 people investigated how daily meal frequency, the manner of eating and the socio-cultural context were related to the occurrence of IR. With

the use of the Mealtime Habits Quality (MHQ) scale, the authors assessed 4 eating habits: the amount of time available for a meal, distractions while eating, the place and company during the meal, as well as independence in choosing the portion and composition of the meal. The individuals with higher scores on the MHQ scale were those who ate without rush, had enough time for a meal, ate without distractions (reading, working, watching TV), chose the quantity and quality of products themselves, and ate most meals at home, often with their families. Over a period of 8 years, it was observed that the incidence of IR (HOMA-IR > 3.2) was lower in this group compared to people with low MHQ scores (1.2% vs 12.5%). People with low MHQ scores had an approx. 12 times higher risk of IR, while those with an average score had about 5 times higher risk of IR compared to the group with high MHQ scores.[59]

Studies concerning meal frequency during the day and their calorific value have provided interesting observations in small research groups. Confirmation in larger randomized trials is needed in order to use the findings as a basis for formulating a diet for insulin-resistant people.

Summary

Based on the available literature regarding the relationship between diet and IR it seems that reduction of body mass resulting from following a diet with calorific value reduced by approx. 500–600 kcal in relation to total daily energy expenditure should be the basic dietary recommendation for people with impaired glucose/insulin homeostasis.

Observations from recent studies do not indicate a necessity to exclude dairy products from the diet of insulin-resistant people. It has been suggested that the consumption of calcium-rich foods, such as dairy and milk products, may have beneficial effects on insulin sensitivity.

The quantity and quality of carbohydrates is a key element in the diet of people with IR. Various authors indicate the merits of reducing the intake of simple sugars, especially those derived from sweet drinks, sweets and excessive fruit juices (more than 2 cups of juice as a equivalent of 2 fruit portions – according to USDA). Many of them have demonstrated the beneficial effect of eating slowly digested complex carbohydrates rich in dietary fiber. The diet of people with IR should be rich in whole grains and significant amounts of non-starchy vegetables and fruit. Insulin-resistant patients should consume carbohydrate products with a low glycemic index to improve insulin sensitivity.

Studies involving insulin-resistant people confirm the beneficial effects of the DASH diet and Mediterranean diet. In contrast, the benefits of low- and very-low-carbohydrate diets require confirmation in long-term studies with large study groups. However, it seems that less restrictive limitation of carbohydrates (up to approx. 40%) through the reduction of simple sugars and starch intake may have a beneficial effect on insulin homeostasis.

Studies evaluating the relationship between diet and IR provide interesting observations and possible dietary recommendations. However, due to study limitations, their utility for insulin-resistant patients requires confirmation in studies with larger cohorts.

Studies of the influence of the quality of consumed fats on IR showed that a high intake of SFA has a negative impact on IR. However, it has been observed that increased consumption of monounsaturated FA and polyunsaturated FA (mainly omega-3) has a positive effect on the parameters associated with IR.

In addition, it has been noted that moderate amounts of alcohol as part of a balanced low-calorie diet did not have a negative impact on insulin-resistant people. Similar observations have been made with regard to chocolate. It seems that moderate amounts of these products can be a positive element that increases patients' compliance with nutritional recommendations.

The effectiveness of dietary recommendations among people with IR may depend not only on the quality of the products, but also on daily meal frequency and meal energy content. It has been suggested that increased calorie intake in the first half of the day, with particular emphasis on an abundant, low-glycemic-index breakfast containing complex carbohydrates, contributes to reductions in IR. In addition, many researchers recommend slow and mindful eating as part of a healthy diet for insulin-resistant people.

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Down syndrome as a cause of abnormalities in the craniofacial region: A systematic literature review

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Abstract

Down syndrome (DS) is the most often diagnosed chromosomal disorder in newborns. The incidence is 1:792 live births. The article describes the irregularities and characteristics found in trisomy 21, which greatly affect the functioning of the stomatognathic system. The most significant disorders include the following: false macroglossia, muscular hypotonia and gothic palate. These abnormalities affect articulation, breathing, food intake, and swallowing. We analyzed the morphological characteristics of the craniofacial region in children with DS based on the current literature review. The following databases were used for the analysis: MEDLINE (via PubMed), Scopus, Infona, and Dentistry & Oral Sciences Source. In the course of the study, 199 pieces of literature were analyzed; the analysis also included 18 articles on children and adults with DS. It also took into account the structure of the palate, dental and skeletal defects, size of the tongue, muscular hypotonia, and temporomandibular joint dysfunction. Down syndrome is still a current subject of research. Although macroglossia, hypotonia, malocclusion, and temporomandibular joint abnormalities are not features exclusive to DS, numerous dysfunctions and parafunctions as well as retarded psychomotor development greatly complicate the treatment. Therefore, interdisciplinary treatment of patients with trisomy 21 and early treatment in the first months of life with the use of the Castillo-Morales plate are very important, as they ensure better adaptation to the subsequently used apparatus and reduce the risk of disorders of the stomatognathic system.

Key words: Down syndrome, hypotonia, dental defects, false macroglossia, Castillo-Morales plate

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Down syndrome (DS) was named after John Langdon Down, a British doctor who first described its features in 1866. In 1959, a French doctor Jerome Lejuene identified the cause of this anomaly. The estimated incidence is 1:792 live births.[1] The disorders in persons with DS include mental retardation, unfused lumbar vertebral arches and heart defects, among others.

The facial skeleton and neurocranium include hypoplastic mandible[2] with reduced nasolabial angle, reduced dimensions of the permanent teeth[3] and more frequent occurrence of periodontal diseases as compared to the neurotypical group.[4] Malocclusion resulting from vertical and transverse irregularities of the dental arches is more frequent in patients with trisomy 21.[5] This affects, among others, the demastication and texture of food, swallowing, articulation, and breathing. The abovementioned features are not pathognomonic symptoms.

Orthodontic abnormalities require early treatment of people with trisomy 21. It is mainly conditioned by macroglossia and reduced volume of the palate. These features along with retarded psychosomatic development and muscular hypotonia lead to the occurrence of parafunctions and dysfunctions, which in turn affect the frequency of malocclusions in this group of people. The most clearly marked defect is skeletal class III, which is more severe in older age groups. This affects the lengthening of the lower part of the face and irregularities in the proportions of the facial skeleton.[6]

Muscular hypotonia is characteristic of trisomy 21. Most commonly, it affects the tongue and lips, which is visible on physical examination. Muscles lifting the mandible show different degrees of muscular insufficiency, but in the case of bruxism, severe hypotonia of the masseter muscles can be observed.[7]

The objective of this paper was to analyze the morphological characteristics of persons with DS, i.e., the palate, dental defects, skeletal defects, muscular hypotonia, temporomandibular joint abnormalities, and size of the tongue.

Material and methods

Non-randomized controlled trials (NRCT), systematic reviews (SR) and case series (CS) with sample sizes of 16 or more patients published since 1986, without any restriction in language or publication status, were eligible for inclusion in this review and were considered in our study. Children and adults with DS were matched in terms of age and gender with patients without DS. There was no restriction for the presenting malocclusion, indication for treatment or type of orthodontic treatment undertaken. Studies that investigated malocclusion, temporomandibular joints, measurement macroglossia, hypotonia, and hard palate were reviewed.

Comparison of the outcomes between 2 groups was performed: 1st group – patients with DS; 2nd group – patients without DS. All of the following symptoms were taken into consideration: tongue thrusting, abnormal breathing, eating disorders, lisping, and infantile swallowing.

The following databases were searched from January 24, 2018 to February 12, 2018:
– MEDLINE (via Pubmed),
– Scopus,
– Infona,
– and Dentistry & Oral Sciences Source,
using the following Medical Subject Heading terms:
– Down syndrome,
– dental defects,
– trisomy,
– hypotonia,
– malocclusion,
– macroglossia,
– Castillo-Morales.

The search strategy for PubMed is presented in Table 1. At first, the potentially appropriate studies were identified

Table 1. Search strategy

PubMed/MEDLINE, Scopus, Infona, Dentistry & Oral Sciences Source		
Search No.	Search	
1.	((Down syndrome) AND hypotonia)	185
2.	((Down syndrome) AND macroglossia) AND hypotonia	6
3.	(((trisomy) AND dental defects) OR malocclusion) AND Castillo-Morales	1
4.	((Down syndrome) AND hypotonia) AND malocclusion	1
5.	(Castillo-Morales) AND hypotonia	6

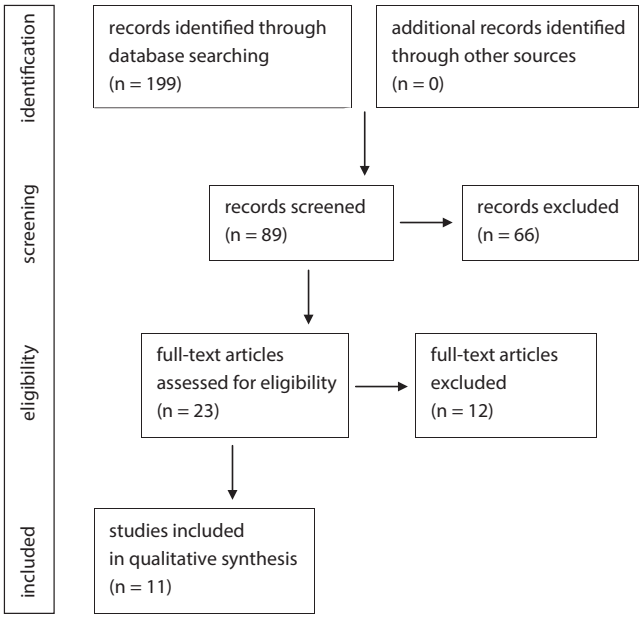

Fig. 1. Flow diagram showing the screening of studies in this systematic review

by title; subsequently, the abstracts were read and the irrelevant studies were rejected. Data was extracted with reference to the participants, age, methods, and outcomes.

Results

The abovementioned irregularities indicate the need for complex therapy in the oral cavity. Castillo-Morales proposed a treatment for people with, i.a., muscular hypotonia and macroglossia.[12] The protocol takes into account the simulating and acrylic plate, i.e., braces with a component aimed at verticalization of the tongue in the back. It is important to start the treatment early (2–3 months of age) as it reduces the negative impact on the psychosomatic development within the oral cavity. The authors refer readers who want to familiarize themselves with the above method of treatment to the article about Castillo-Morales protocol.[12]

Tables 2–5 set forth the results of the analysis of the material contained in the articles selected in accordance with the search strategy described in the Materials and Methods section.

Discussion

Macroglossia occurs when a tongue at rest goes beyond the dental arches or alveolar process and the alveolar part of the mandible, as in the case of edentulism. This abnormality occurs, i.a., in hypothyroidism, amyloidosis, Beckwith–Wiedemann syndrome, and DS.[16] There are no clear figures that would indicate macroglossia. The division comprising the true and false form of this abnormality[17] was created with regard to symptomatic treatment. Patients with DS[11] have a smaller tongue (2.432 mm^2) compared to that of the control group (2.767 mm^2). The dimensions of the facial skeleton are also reduced, but the size of the tongue compared to bone parameters remains greater (0.54) than that of the 2nd group (0.46). Therefore, in the case of trisomy 21, false macroglossia occurs.

The potential complications of this anomaly include impaired speech, deformed mandible, malocclusion, or even airway obstruction, which is why it is important to start treatment early. The therapy includes exercise and the use of appliances which expand the dimensions of the oral cavity and (in a modified version) allow the tongue to be kept behind the dental arches. The most aggressive form of treatment is surgical treatment.

Another analyzed feature is the palate.[13] Patients with DS show decreased growth and volume of the palate in the first months of life by approx. 151 days as compared to the neurotypical population, but the analyzed parameters (width and depth) remain unchanged with regard to persons with no genetic predispositions. People aged 10–40 years with DS have narrower palates, but the antero-posterior dimensions and height are similar.[9] Detailed studies[10] analyzed the antero-posterior length, height and volume of the hard palate as well as the size of the dental arches. The results (48 people in the study group and 48 people in the control group at the age of 6–16 years) revealed that the palate was significantly higher in patients with DS, and all other parameters were significantly lower than in the control group. Therefore, it should be concluded that narrowed gothic palate frequently occurring in older children is an acquired feature resulting from other irregularities. One of them is the aforementioned macroglossia. The results of these studies are inconclusive

Table 2. Analysis of the materials contained in the articles

Author (year)	Study	Participants	Methods of outcomes	Results include	Conclusions
Abeleira et al.[8] (2014)	NRCT	SG: 40 patients with DS; age: 10–40 years; sex: 25 M, 15 F CG: age- and sex-matched persons were selected	CBCT images Measurement of: – overall tooth length – crown height – root length – mesiodistal diameter – vestibular-palatine diameter – crown-to-root ratio – cervical circumference	✓ dental defects o hypotonia o malocclusion o palate o tongue ✓ skeletal defects o temporomandibular joints	Microdontia of the permanent teeth and progressive age-related reductions in tooth sizes in persons with DS.
Abeleira et al.[9] (2015)	NRCT	SG: 40 patients with DS, age: 10–40 years; sex: 25 M, 15 F CG: 40 age- and sex-matched persons were selected	CBCT images Measurements of: – anteroposterior length (aAPL) – arch length – anteroposterior length – maximum height – sagittal arch – interdental width – height – skeletal width – coronal arch	o dental defects o hypotonia o malocclusion ✓ palate o tongue ✓ skeletal defects o temporomandibular joints	Interdental and skeletal widths were greater in controls than in DS statistically significant differences between males and females with DS.

SG – study group; CG – control group; F – female; M – male; DS – Down syndrome; CBCT – cone-beam computed tomography; NRCT – non-randomized controlled trial; ✓ – included in research; o – not included in research.

Table 3. Analysis of the materials contained in the articles

Author (year)	Study	Participants	Methods of outcomes	Results include	Conclusions
Allareddy et al.[6] (2016)	CS	27 children and young adults with DS; age: 3–25 years	Analysis of lateral cephalometric radiographs	o dental defects o hypotonia ✓ malocclusion ✓ palate o tongue ✓ skeletal defects o temporomandibular joints	Patients with DS typically show skeletal class III malocclusion.
Bhagyalakshmi et al.[10] (2007)	NRCT	SG: 48 children with DS; sex: 26 M, 22 F CG: 48 children without DS; sex: 26 M, 22 F	Electronic calipers, scale, divider, dental impression material, impression trays, plaster stone, casting tray The parameters measured were: • linear width • curvilinear width • mean height • palatal arch length • anteroposterior length • volume • palatal index	o dental defects o hypotonia o malocclusion ✓ palate o tongue ✓ skeletal defects o temporomandibular joints	The hard palate in children with DS was found to be high-arched and narrow, with acutely aligned palatine plates.
Guimaraes et al.[11] (2008)	NRCT	CG: 16 patients with DS CG: 16 age- and gender-matched controls	On sagittal and axial MR images, parameters for tongue size the bony craniofacial confines of the retroglossal pharynx, the size of the tongue relative to the craniofacial bony parameters.	o dental defects o hypotonia o malocclusion o palate ✓ tongue ✓ skeletal defects o temporomandibular joints	Children with DS do not have true macroglossia but have relatively large tongues compared to the bony confines of the oral cavity.

SG – study group; CG – control group; F – female; M – male; DS – Down syndrome; NRCT – non-randomized controlled trial; CS – case series; SR – systematic review; MR – magnetic resonance; ✓ – included in research; o – not included in research.

Table 4. Analysis of the materials contained in the articles

Author (year)	Study	Participants	Methods of outcomes	Results include	Conclusions
Klimek-Jaworska et al.[12] (2014)	SR	SG(I): 57 children 2 months–3 years of age; SG(II): 50 children 3 months–5 years of age	• Reduction in the severity of the orbicularis oris muscle hypotonia • tongue position, lip closure and facial expression	o dental defects ✓ hypotonia o malocclusion o palate ✓ tongue o skeletal defects o temporomandibular joints	An early implementation of such therapy facilitates the optimal development of motor functions of the orofacial complex.
Klingel et al.[13] (2017)	NRCT	SG: 40 patients with DS; age: 221.3 ±132.4 days; sex: 20 F, 20 M CG: 40 infants; age: 53.9 ±87.2 days; sex: 20 M, 20 F	Width, depth and length of the palate were used as well as the palatal index and 3-dimensional volume.	o dental defects o hypotonia o malocclusion ✓ palate o tongue ✓ skeletal defects o temporomandibular joints	The palate of DS infants in the first 6 to 9 months of life is normally shaped but considerably smaller compared to healthy individuals.
Nęcka et al.[7] (2007)	NRCT	SG: 22 patients with DS CG: 23 persons with hypotonia	Electromyography of mimic and mastication muscles tone	o dental defects ✓ hypotonia o malocclusion o palate o tongue o skeletal defects o temporomandibular joints	No significant differences between the tonus of temporal and masticatory muscles of people suffering from DS; no statistically significant differences were observed during the orbicular muscles examination.

SG – study group; CG – control group; F – female; M – male; DS – Down syndrome; NRCT – non-randomized controlled trial; SR – systematic review; ✓ – included in research; o – not included in research.

as to the reduced parameters of the palate, but all results point to the reduction of its volume. Considering the determinants of development, which include environmental impact, quality of life, lifestyle, and national differences, it should be noted that the narrow gothic palate often found in older children is an acquired feature. This causes disorders, the most serious of which is hypoxia.

People with trisomy 21 often have disorders of the stomatognathic system. These include the following: hypoplasia, diastema, mandibular prognathism, anterior open bite,

Table 5. Analysis of the materials contained in the articles

Author (year)	Study	Participants	Methods of outcomes	Results include	Conclusions
Oliveira et al.[5] (2008)	CS	112 pairs of mothers and their children with DS; age: 3–18 years	Data was collected with a questionnaire given to the mothers and through a clinical examination of the child or adolescent. Univariate, bivariate and multiple logistic regression (backward stepwise) analyses were conducted.	o dental defects o hypotonia ✓ malocclusion o palate o tongue ✓ skeletal defects o temporomandibular joints	Age, nail or finger biting, mouth posture, and upper airway infections were associated with malocclusions in these patients.
Salazar et al.[14] (2016)	CS	40 patients with DS	temporomandibular joint examination	o dental defects o hypotonia o malocclusion o palate o tongue o skeletal defects ✓ temporomandibular joints	The relationship between habits and pain in people with DS.
Tosello et al.[15] (2002)	NRCT	18 children; age: 8–12 years; divided into 3 groups: 1. normal occlusion 2. class II division 1 3. atypical swallowing and/or incompetent lips	electromyographically in resting position and in several movements	o dental defects ✓ hypotonia ✓ malocclusion o palate o tongue ✓ skeletal defects o temporomandibular joints	The 3rd group showed very marked activity of the lower orbicularis oris and mentalis muscles.

DS – Down syndrome; NRCT – non-randomized controlled trial; CS – case series; ✓ – included in research, o – not included in research.

lip incompetence, and deepened overbite. Temporomandibular joint dysfunction in patients with DS was found in 77.5% of patients.[14] Malocclusion occurred in 66% of patients,[18] with the dominance of skeletal class III defects. Taking into account the cephalometric analysis,[6] it is dominated by the skeletal class III and increased proportion of the lower part of the face with regard to the overall height, wherein the skeletal class III is more clearly indicated in the older age groups. Analysis of incisors,[8] canines and first molars, i.e., the length of the tooth, crown height and root length, tooth neck perimeter, mesiodistal width, buccolingual dimension, and the ratio between the crown height and root length, with the use of computed tomography, draws attention to the reduction in tooth dimensions while the ratio between the crown height and root length is maintained; no sexual dimorphism was found (only the length of the roots of the incisors remains greater in men). Crown height, mesiodistal diameter and the ratio between the crown and the root gradually decrease with age. This confirms microdontia in patients with trisomy 21.

Muscular hypotonia in persons with DS affects the whole organism. An analysis[7] of temporal muscles (L/R) (L = left, R = right), masseter muscles (L/R) and orbicularis oris muscles with the use of an electromyograph during various physiological functions, i.e., swallowing, chewing, rest position of the mandible, position of the lips as for whistling, maximum intercuspation, indicates significantly higher tension of the orbicularis oris muscle in children with DS compared to the neurotypical group and no statistical differences for the temporal and masseter muscles. At the same time, during the physical examination, insufficiency of the orbicularis oris muscle is clearly expressed, which is contrary to the electromyography (EMG) indication. Careful analysis reveals an increase in the tension of the orbicularis oris muscle when the lips are positioned for whistling, while higher signals on the measuring device result from the conscious tightening of muscles by the patient.

Comparing this data with the results of studies on 3 groups[15]:

1. persons with malocclusions,
2. persons with persistent visceral swallowing,
3. persons with lip incompetence,

who did not undergo orthodontic treatment, in the case of joined lips, the EMG shows higher muscle potential in group 3 and high muscle activity while sucking a lollipop, with no differences between the subgroups while sucking a dummy, a straw or a thumb. This indicates a conscious tightening of fibers, which confirms the abovementioned data.

Conclusions

People with DS have different craniofacial morphology. The treatment plan should take into account the occurrence of false macroglossia, dental irregularities, hypoplasia, and diastema. A well-arched gothic palate is an acquired feature, thus orthodontic activities should prevent the development of this anomaly. Attention should be paid to the reduced tension of the orbicularis oris muscle, which, in conjunction with false macroglossia, affects the incidence of anterior open bite.

Although macroglossia, hypotonia, malocclusion, and temporomandibular joint abnormalities are not features exclusive to DS, numerous dysfunctions and parafunctions as well as retarded psychomotor development greatly complicate the treatment. Therefore, interdisciplinary

treatment of patients with trisomy 21 and early treatment in the first months of life with the use of the Castillo-Morales plate are very important, as they ensure better adaptation to the subsequently used apparatus and reduce the risk of disorders of the stomatognathic system in the future.

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