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Original papers

Reviews

Role of thiamine in Huntington's disease pathogenesis: In vitro studies

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Abstract

Background. Oxidative stress accompanies neurodegeneration and also causes abnormalities in thiamine-dependent processes. These processes have been reported to be diminished in the brains of patients with several neurodegenerative diseases.

Objectives. The aim of this work was to conduct a comparative analysis of the impact of supplemented thiamine on the viability of human B lymphocytes with CAG abnormal expanded huntingtin gene (mHTT) (GM13509) and control, B lymphocytes without mHTT (GM14467) through the following studies: determination of the supplemented thiamine concentrations, which are effective for cell growth stimulation after incubation in thiamine deficit conditions; determination of cell capability to intake the exogenous thiamine; evaluation of exogenous thiamine influence on the profile of the genes related to thiamine and energy metabolism; determination of ATP synthesis and activities of thiamine-dependent enzymes, KGDHC and BCKDHC in the intact cells and upon the exogenous thiamine.

Material and methods. The following methods were used: EZ4U test for cell growth analysis; HPLC for determination of thiamine intake and ATP synthesis, qRT-PCR for evaluation of the gene profiles and spectrophotometric method for KGDHC and BCKDHC activities determination.

Results. Maximal cell growth stimulation was observed at 2.5 mM in GM14467 up to 135% of the control culture and at 5.0 mM in GM13509 cells up to 165% of the control culture. Native levels of total ATP and KGDHC and BCKDHC activities in both cell types were comparable and did not changed upon thiamine deficit or supplementation. GM13509 cells showed more of an increase in growth stimulation upon thiamine supplementation than GM14467 cells and this effect was reflected in the increase of intracellular thiamine concentration.

Conclusions. The above results and reported changes in expression of GAPDH, IDH1 and SLC19A3 genes observed upon thiamine deficit conditions suggest that intracellular thiamine status and energy metabolism can have a role in HD pathogenesis.

Key words: thiamine, energy metabolism, Huntington's disease, ATP synthesis

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Huntington's disease (HD) is an autosomal dominant neurodegenerative disease with age-dependent complete penetrance. Like many neurodegenerative diseases, it is characterized by a cascade of events leading to neuronal death. In HD subjects there is strong evidence for reduced glucose consumption in the brain, even in presymptomatic mutation carriers. Reduced ATP synthesis was found in immortalized HD striatal neuronal cell lines.[1] According Mochel et al., various mechanisms that underlie the energy deficit in HD phenotype have been proposed, including impaired oxidative phosphorylation, oxidative stress, impaired mitochondrial calcium handling, abnormal mitochondria trafficking, and deregulation of key factors of mitochondrial biogenesis or decreased glycolysis.[1] Subcellular abnormalities were observed not only in tissues of the central nervous system (CNS) but also in peripheral tissue cells, such as fibroblasts, lymphocytes and erythrocytes of HD subjects.[2] This suggests that clues to the HD pathogenesis may be detectable outside the brain. In fact, decreased ATP/ADP ratio was observed in HD patient-derived lymphoblastoid cell lines, which inversely correlated with the length of the mutant polyglutamine tract.[1] Mutant huntingtin was reported to affect the activity of mitochondrial complex I in the skeletal muscles of patients with HD.[3] Schapira mentioned that complex I deficiency had been found in HD platelets.[4]

Respiratory chain defects increase reactive oxygen species (ROS) production. Evidence of enhanced oxidative stress in HD brains includes an increase in the accumulation of lipofuscin, a product of unsaturated fatty acids peroxidation or oxidative modification of proteins and lipids, also increased in HD brain and in animal models.[1] Gil-Mohapel et al. in their review presented an overview of preclinical and clinical studies that have indicated a link between oxidative stress, neurodegeneration and cell death in HD.[5] Oxidative stress accompanies neurodegeneration and also causes abnormalities in thiamine-dependent processes. These processes have been reported to be diminished in the brains of patients with several neurodegenerative diseases.[6] Aikawa et al. showed that encephalopathic rats had brain thiamine levels lower than 20% that those of the control group and ATP concentrations were 89.5% of normal controls.[7] Thiamine diphosphate (TDP) is the essential cofactor for key enzymes of energy metabolism. The TDP-dependent enzymes in brain include cytosolic enzyme transketolase, a key enzyme of the pentose phosphate shunt and 3 mitochondrial enzyme complexes, i.e. pyruvate dehydrogenase complex (PDHC), which links glycolysis and Krebs cycle (tricarboxylic acid cycle), α-ketoglutarate dehydrogenase complex (KGDHC), postulated to be a rate-limiting step of Krebs cycle and the branched chain α-keto acid dehydrogenase complex (BCKDHC), which provided a means for branched chain amino acids to enter Krebs cycle.[6] Pekovich et al. showed that mRNA levels of transketolase and E1β subunit of pyruvate dehydrogenase complex in human fibroblasts, lymphoblasts and neuroblastoma cells were lower in thiamine-deficient cultures.[8] Decreased activity of thiamine-dependent enzymes leads to a cascade of events that include focal decreases in energy status, oxidative stress, lactic acidosis, decreased glucose utilization, immediate-early gene induction and inflammation.[9] As showed by Gibson and Zhang, vitamin E and butylated hydroxyanisole provide significant neuroprotection to thiamine deficient neurons in cultures and regarded that thiamine as a selected antioxidant may be useful in terms of revealing the role of thiamine dependent processes in disease and other conditions that lead to altered neuronal function.[6]

The aim of this study was to conduct a comparative analysis of the impact of thiamine supplementation on the viability of human B lymphocytes with CAG expanded huntingtin gene and normal cells through the studies on the following items: (1) determination of the supplemented thiamine concentrations that are effective for cell growth stimulation after incubation in thiamine deficit conditions (obtained by the treatment of cells with pyrithiamine hydrobromide); (2) evaluation of cell capability to intake the supplemented thiamine from the culture medium; (3) determination of the profile of genes related to thiamine and/or energy metabolism in the intact cells and upon the exogenous thiamine treatment; (4) determination of mitochondrial ATP synthesis and activity of thiamine-dependent enzymes, KGDHC and BCKDHC in the intact cells and upon the exogenous thiamine.

Material and methods

Cells

EBV-immortalized B lymphocyte cell lines derived from B lymphocytes collected from peripheral blood of healthy female donor – GM14467 and B lymphocytes from peripheral blood of HD female donor with expanded CAG repeat located in the coding region of the huntingtin gene – GM13509 (both B lymphocytes cell lines were purchased in Coriell Institute for Medical Research, USA), were kept in RPMI 1640 with glutamine, 10% FBS and 1% antibiotics solution (penicillin, streptomycin, amphotericin) (Lonza, Walkersville, USA).

Cell viability

Cell cultures (3×10^5 cells/mL; 25 mL) were growing for 3 days with pyrithiamine hydrobromide, 600 nM (Sigma-Aldrich). Then pyrithiamine was rinsed off, the cells were seeded onto 96-well plate and exposed to thiamine

hydrochloride (Sigma-Aldrich) (0.2–6.0 mM) for 24 h. After that time, the EZ4U test (Biomedica) was carried out according to the manufacturer's instruction. All experiments were related to the reference cultures to eliminate the impact of thiamine present in the RPMI 1640. The reference cultures included: the cells kept for 4 days in unmodified RPMI 1640 (control), the cells kept with pyrithiamine – throughout all incubation time (4 days) (TDM Pyri), the cells kept for 3 days with pyrithiamine and for the next 24 h with the intact RPMI 1640 (TDM). The latter one, TDM was treated as 100%.

Two concentrations of thiamine for each cell line were selected for further studies, i.e. the minimal and maximal values, which increased cell viability. The results were expressed as % of viable cells vs. thiamine concentration with ± SD values calculated from 23 independent experiments.

Intracellular thiamine concentration

Cell cultures (3×10^5 cells/mL; 25 mL) were growing for 3 days with pyrithiamine hydrobromide, 600 nM. Then pyrithiamine was rinsed off, the cells were seeded into the next flask and exposed for 24 h to thiamine hydrochloride at the concentrations 0.5 and 2.5 mM for GM1447 cells and 1.0 and 5.0 mM for 13509 cells. Thiamine concentrations were selected on the basis of the EZ4U test. All calculations regarded the reference cultures as mentioned above. Thiamine and TDP were determined after derivatization with potassium ferricyanide. Cell lysates, which were prepared in trichloroacetic acid, were derivatized as mentioned above and then injected onto the chromatographic column.

HPLC conditions

Column: PRP-1; 150 × 4.1 mm, 5 μm with precolumn; column temperature: 25°C; mobile phase: buffer (sodium dihydrogen phosphate 50 mM + tetrabutylammonium hydrogensulfate 8.8 mM), pH = 8.0: acetonitrile (9 : 1 v/v); flow rate: 1.0 mL/min; fluorometric detection at: Ex = 365 nm; Em = 433 nm; injection volume: 100 μL. The results were presented as ng/mg protein.

Protein assay

Protein was determined with Bio-Rad Protein Assay (Bio-Rad).

Statistical analysis

All the data was presented as mean ± SD of 23 independent experiments and analyzed with Medistat System (microcomputer statistical system for medicinal purposes, v. 2.1; 1992). The results with $p < 0.05$ were considered significant.

Profile of genes related with thiamine and energy metabolism

RNA isolation

RNA was extracted from tested cell lines using the GeneMATRIX Universal RNA/miRNA Purification Kit (EURx Ltd. Biotechnology, Gdynia, Poland; www.eurx.com.pl) according to the manufacturer's protocol. The purity was verified by optical density (OD) ratio $OD_{260\ nm}/OD_{280\ nm}$ and $OD_{260\ nm}/OD_{230\ nm}$ ranging from 1.85 to 2.05 and the integrity was evaluated by electrophoresis on Gel – RNA Flash Gel System (Lonza Rockland Inc., Rockland, USA). RNA templates before reverse transcription were additionally digested by DNaseI (Thermo Fisher Scientific Inc., USA; http://www.thermoscientificbio.com/fermentas) according tothe manufacturer's protocol for preparation of DNA-free RNA.

Reversed transcription (RT)

Total RNA was reverse-transcribed using the PrimeScript RT-PCR kit II (Takara Bio Inc., Otsu, Shiga, Japan) for first-strand cDNA synthesis using 2.5 μM oligonucleotides primer and 5.0 μM random hexamer for the priming method according to the manufacturer's recommendations. Synthesis was started with the incubation of the transcription mixture at 37°C for 30 min to initiate the reverse transcriptase reaction. Finally, reverse transcription was stopped by heating the reaction mixture for 5 s at 85°C.

qRT–PCR

Gene expression was performed using qPCR analyses and was designed for genes listed in Table 1 using the Mx3005P qPCR System (Stratagene, La Jolla, USA). qRT-PCR was carried out with SYBR Premix Ex Taq (TaKaRa, Japan) using 400 ng cDNA template into a sample-specific working solution prepared for 96-well plate according to Lonza Inc. (US) recommendations for StellArrays (www.lonza.com/arrays).

The relative transcription level was calculated based on ΔΔCt (cycle threshold) type of analysis using the 2-ΔΔCt method in order to Fold Change (FC) value determination between compared groups. Normalization to 18S rRNA was used as internal control, and 9 additional gene normalizers, simultaneously distinguished by GPR software analysis, were performed for each gene tested.[10]

Statistical analysis

The data obtained from qPCR was analyzed by Global Pattern Recognition statistical software (GPR; https://array.bhbio.com/BHB/GUI/AP/GPR.aspx). The GPR was

Table 1. Subset of genes analysed with qPCR)

Gene ID	Gene symbol	Protein name	Enzyme commission number
Control and normalizer genes			
2	HSGenomic	HSGenomic	
1	Hs18s	Hs18s	
60	ACTB	PS1TP5-binding protein 1; actin,	
3251	HPRT1	hypoxanthine phosphoribosyltransferase 1	EC2.4.2.8
549	AUH	AU RNA binding protein/enoyl-CoA hydratase	EC4.2.1.18
Thiamine metabolism closely related genes			
27010	TPK1	thiamine pyrophosphokinase 1	EC2.7.6.2
79178	THTPA	thiamine triphosphatase	EC3.6.1.28
7086	TKT	transketolase	EC2.2.1.1
8050	PDHX	pyruvate dehydrogenase complex, component X	no data
5160	PDHA1	pyruvate dehydrogenase (lipoamide) alpha 1	EC1.2.4.1
1629	DBT	dihydrolipoamide branched chain transacyl+6ase E2	EC2.3.1.168
1737	DLAT	dihydrolipoamide S-acetyltransferase	EC2.3.1.12
593	BCKDHA	branched chain keto acid dehydrogenase E1, alpha polypeptide	EC1.2.4.4
594	BCKDHB	branched chain keto acid dehydrogenase E1, beta polypeptide	EC1.2.4.4
60386	SLC25A19	solute carrier family 25 (mitochondrial thiamine pyrophosphate carrier), member 19	
80704	SLC19A3 (THTR2)	solute carrier family 19, member 3	
6573	SLC19A1	solute carrier family 19 (folate transporter), member 1	
10560	SLC19A2 (THTR1)	solute carrier family 19 (thiamine transporter), member 2	
Glycolysis, citrate cycle (TCA cycle, Krebs cycle) related genes			
2597	GAPDH	glyceraldehyde-3-phosphate dehydrogenase	EC1.2.1.12
5091	PC	pyruvate carboxylase	EC6.4.1.1
48	ACO1	aconitate hydratase	EC4.2.1.3
3417	IDH1	isocitrate dehydrogenase 1	EC1.1.1.42
3419	IDH3A	isocitrate dehydrogenase 3 (NAD+) alpha	EC1.1.1.41
4967	OGDH	oxoglutarate (alpha-ketoglutarate) dehydrogenase (lipoamide)	EC1.2.4.2
1738	DLD	dihydrolipoamide dehydrogenase, E3	EC1.8.1.4
1743	DLST	dihydrolipoamide S-succinyltransferase (E2 component of 2-oxo-glutarate complex)	EC2.3.1.61
8802	SUCLG1	succinyl-CoA synthetase alpha subunit	EC6.2.1.4
6389	SDHA	succinate dehydrogenase complex A	EC1.3.5.1
2271	FH	fumarate hydratase	EC4.2.1.2
4190	MDH1	malate dehydrogenase	EC1.1.1.37
47	ACLY	ATP citrate	EC2.3.3.8
1431	CS	citrate synthase	EC2.3.3.1

used to determine the gene transcript level changes for the GM13509 with reference to the GM14467 (calibrator) using Ct values obtained for all the genes tested. The GPR FC value was calculated for each gene. The results with $p < 0.05$ were considered significant.

ATP synthesis

Each reference culture and sample was prepared in parallel, i.e. with or without oligomycin (1 mg/mL) and P^1,P^5-di(adenosine-5') pentaphosphate (Ap5A) (0.15 mM) added before the measurement. Thiamine concentrations were as given above. All calculations regarded the reference cultures as described in cell viability. ATP synthesis was measured in whole cell lysates obtained by sonification of the cells in ice for 10 min. Cell lysates were centrifuged and supernatants were directly injected onto the chromatographic column.

HPLC conditions

Column: Nova-Pak C18 150 × 3.9 mm 4 µm; column temperature: 25°C; gradient system: mobile phase A: sodium dihydrogen phosphate 25 mM + tetrabutylammonium hydrogensulfate 100 mg/L, pH = 5.0; mobile phase B: sodium dihydrogen phosphate 200 mM + tetrabutylammonium hydrogensulfate 100 mg/L + acetonitrile 100 mL/L, pH = 4.0; flow rate: 0.7 mL/min; detection wavelength: 260 nm; injection volume: 50 µL.[14] The results were presented as nmol/mg protein.

Statistical analysis

All the data was presented as mean ± SD of 2 independent experiments and analyzed with Medistat System (microcomputer statistical system for medicinal purposes, v. 2.1; 1992). The results with $p < 0.05$ were considered significant.

KGDHC and BCKDHC activity

Cell cultures (2×10^5 cells/mL, 75 mL) were growing for 3 days in a culture medium containing 600 nM pyrithiamine hydrobromide and further treated as described above. The reference cultures included: the cells kept for 4 days in unmodified RPMI 1640 (control), the cells kept with pyrithiamine – throughout all incubation time (4 days) (TDM Pyri), the cells kept for 3 days with pyrithiamine and for the next 24 h with the intact RPMI 1640 (TDM). After incubation, the culture medium was removed by centrifugation and the cells were washed twice with phosphate buffered saline solution without Ca/Mg (PBS). Cell lysates were obtained by resuspension of the cell pellets with lysis buffer (50 mM Tris-HCl; 5 mM EDTA, pH 7.4) to get final concentration 50×10^6 cells/mL.

KGDHC activity assay

The assay was performed with the use of 96-well plates. Enzymatic activity assay was conducted at 37°C in reaction mixture containing Tris-HCl pH 7.4 buffer (50 mM), 1 mM $MgCl_2$; 0.05 mM $CaCl_2$; 0.05 mM EDTA; 0.3 mM thiamine pyrophosphate (TPP); 0.1 % w/v Triton X-100; 0.3 mM DTT (dithiothreitol); 3 mM NAD (β-nicotinamide adenine dinucleotide); 0.2 mM CoA (coenzyme A). Reaction was started after 20 µL substrate solution was added (3 mM α-ketoglutaric acid disodium salt) to the well containing preheated reaction mixture (160 µL) and cell lysate (20 µL). All reagents were purchased from Sigma Aldrich. The method was based on monitoring the NADH formation by reading the absorbance change at 340 nm within the time frame of 10 min. The absorbance rate was corrected by respective blank substraction (reaction performed with water added instead of substrate solution). KGDHC specific activity was calculated with regard to molar extinction coefficient of NADH and expressed in nmol/(min × mg protein).

BCKDHC activity assay

The assay was performed with the use of 96-well plates. Enzymatic activity assay was conducted at 37°C in a reaction mixture of the composition described for KGDHC assay but with the addition of 5 mM L-carnitine. The method was based on monitoring the decrease of substrate k-Ile (keto-isoleucine sodium salt (k-Ile), 3 mM, Sigma Aldrich) content in the time monitored by specific colorimetric reaction with DNPH (2,4-dinitrophenylhydrazine, POCH) at 450 nm.[15] The absorbance rate was corrected by a respective blank substraction (reaction performed as above). BCKDHC specific activity was calculated with regard to calibration curve prepared from k-Ile solution in a range of 1.5–3.0 mM and expressed in nmol/(min × mg protein).

Statistical analysis

Data analysis was performed using Systat software v. 13 (Systat Software Inc., USA). The results of KGDHC and BCKDHC activity, expressed in nmol/(min × mg protein) and as the percentage relative to control, were obtained in 5–7 independent experiments. Resulted values were analyzed using descriptive statistics and presented as mean ± SD. Differences between groups were assessed using the analysis of variance (one-way or two-way ANOVA) at a significance level $\alpha = 0.05$ with appropriate post-hoc test selection (Gabriel test for between-groups comparison; Dunnett procedure to test differences from control group). Results with $p < 0.05$ were considered significant.

Fig. 1. Influence of thiamine supplementation on GM14467 cells. ▲– the cells kept with pyrithiamine hydrobromide (600 nm) throughout the whole incubation time (4 days) (TDM Pyri), ■ – the cells kept for 3 days with pyrithiamine and for the next 24 h in the intact RPMI 1640 (TDM); ◆ – the cells exposed to thiamine for 24 h. Bars represent the mean ± SD

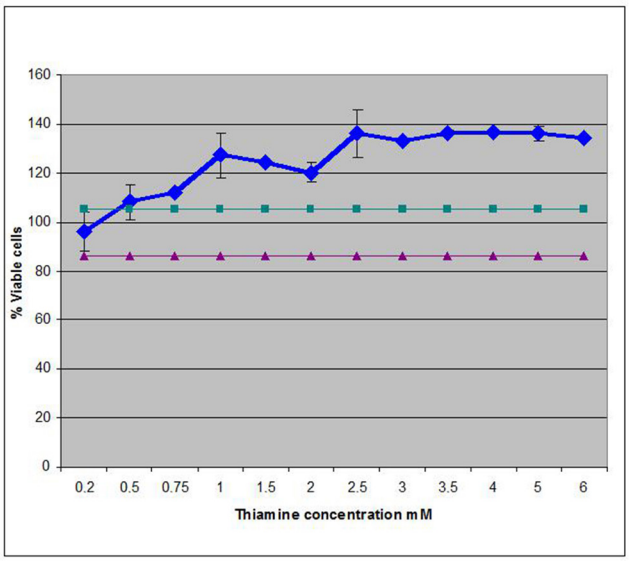
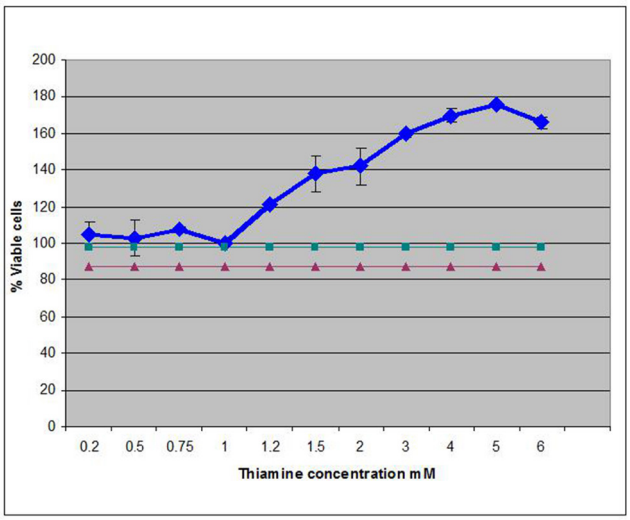


Fig. 2. Influence of thiamine supplementation on GM14467 cells. ▲– the cells kept with pyrithiamine hydrobromide (600 nm) throughout the whole incubation time (4 days) (TDM Pyri), ■ – the cells kept for 3 days with pyrithiamine and for the next 24 h in the intact RPMI 1640 (TDM); ◆ – the cells exposed to thiamine for 24 h. Bars represent the mean ± SD



Results

Cell viability

To keep thiamine deficit conditions pyrithiamine hydrobromide, a competitive inhibitor of the thiamine transporter was used at the concentration 600 nM, which ensured ca. 80% of cell viability. The effectiveness of this concentration was proved in HPLC analysis as reducing the intracellular thiamine concentration below 1 ng/mg protein. As shown in Fig. 1, the significant increase in GM14467 viability to ca. 135% as referenced to the TDM level was caused by thiamine used at the 2.5 mM. Upon the higher concentration, plateau in the cell growth was noticed. In GM13509 growth stimulation ca. 165% appeared upon thiamine supplementation at the 5.0 mM. For the higher thiamine concentrations, a decrease in GM13509 cells viability was observed (Fig. 2). At lower doses of thiamine, i.e. 0.5 mM for GM14467 and 1.0 mM for GM13509 the cell growth stimulation was comparable in both cell lines and reached the growth level as in the reference cultures TDM, i.e. ca. 100% (Fig. 1 and 2).

Intracellular thiamine concentration

Both native cell lines were not different in the intracellular levels of thiamine and TDP, but the increase of the exogenous thiamine concentration was slightly reflected in the intracellular thiamine level. The GM14467 and GM13509 cells cultivated in deficit conditions (TDM, TDM Pyri) as well as the cells in the control cultures had the level of thiamine below 1 ng/mg protein and TDP between ca. 0.3–0.5 ng/mg protein. In GM14467, cells exposed to 0.5 mM of thiamine hydrochloride and in GM13509 cells exposed to 1.0 mM of that compound the intracellular levels increased insignificantly above 3 ng/mg protein ($p > 0.05$). The only significant increase of the intracellular thiamine level after treatment with exogenous thiamine hydrochloride was noted in GM13509 cells exposed to 5.0 mM of that compound and reached 12.06 ng/mg protein as compared with the control cultures TDM and TDM Pyri ($p = 0.0273$ and $p = 0.0216$, respectively).

The TDP levels upon exogenous thiamine were comparable in both kinds of cells but any correlation with the exogenous thiamine concentrations in the medium was not observed (data not shown).

Profile of the genes related to thiamine and energy metabolism

To identify genes that could usefully classify HD patients and healthy controls, 27 genes mostly related to thiamine and energy metabolism were selected for analysis with qRT-PCR technique (Table 1).

The differences in mRNA expression level were presented as GPR FC values and reflected the gene transcripts changes found in the GM13509 cells with mHTT in relation to control GM14467 cells. As shown in Table 2, the intact cells with expanded CAG fragment differed from normal cells in the higher expression of 3 genes: MDH1, GAPDH and SLC19A3 (GPR FC 1.3 ÷ 3.4). The expression of DLD, IDH1, SDHA and SLC19A1 decreased in GM13509 cells cultured in thiamine deficit (TDM-Pyri) or upon low thiamine concentration (TDM) (GPR FC -2.11 ÷ -1.28), but the exogenous thiamine did not make any changes on mRNA levels of tested genes. Some genes,

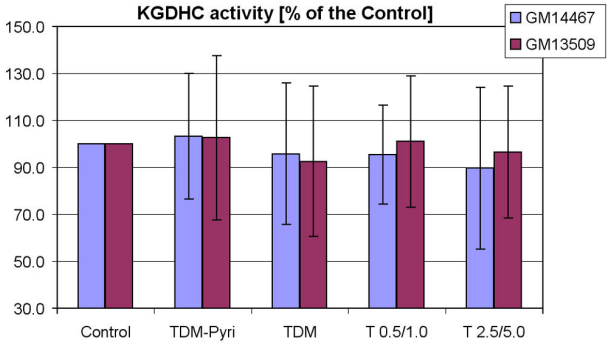
Table 2. Altered thiamine and energy metabolism related genes expression in lymphocytes B with mHTT (GM 13509)* compared with control lymphocytes B with normal HTT (GM 14467)** in deficit or presence of thiamine

Gene transcript	Control		TDM-Pyri		TDM		Thiamine 1.0*/0.5 mM**		Thiamine 5.0*/2.5 mM**	
	GPR FC	p-value	GPR FC	p-value	GPR FC	p-value	GPR FC	p-value	GPR FC	p-value
ACO1	-2.20	0.21	-1.70	0.30	-1.41	0.24	-1.39	0.20	**1.64**	**1.64**
DBT	-1.65	0.15	-1.17	0.11	-1.52	0.10	**-1.51**	**0.03**	-1.18	-1.18
DLAT	-1.66	0.20	-2.17	0.07	**-1.41**	**0.03**	**-1.22**	**0.04**	-1.00	-1.00
DLD	-1.48	0.22	-1.85	0.16	**-1.28**	**0.04**	-1.04	0.11	1.25	1.25
DLST	-1.85	0.36	-2.43	0.08	-1.54	0.17	**-2.38**	**0.00**	**-1.84**	**-1.84**
GAPDH	**2.83**	**0.01**	**5.99**	**0.00**	**2.40**	**0.01**	**3.49**	**0.01**	**1.68**	**1.68**
IDH1	-2.21	0.27	-2.38	0.17	**-1.39**	**0.03**	-2.05	0.87	-1.12	-1.12
MDH1	**1.32**	**0.03**	-1.17	0.08	1.33	0.07	1.41	0.21	1.19	1.19
OGDH	-1.42	0.36	-1.35	0.27	-1.38	0.27	**-2.08**	**0.01**	-1.02	-1.02
SDHA	-2.10	0.09	-3.63	0.11	**-1.37**	**0.03**	-2.22	0.61	-1.45	-1.45
SLC19A1	-1.40	0.51	**-2.11**	**0.01**	-1.22	0.28	-1.45	0.20	-1.18	-1.18
SLC19A2	-1.56	0.27	-1.97	0.14	-1.45	0.22	**-1.70**	**0.03**	1.05	1.05
SLC19A3	**3.44**	**0.01**	**8.37**	**0.03**	**1.61**	**0.03**	**6.86**	**0.02**	-1.13	-1.13
SLC25A1	1.11	0.05	-1.22	0.10	1.04	0.13	**1.80**	**0.02**	1.16	1.16

Bolded GPR FC values with $p < 0.05$ were considered significant.

such as: DBT, DLST, ACO1, OGDH, SLC19A2, SLC25A19 and DLAT in mutated cells showed lower mRNA expression than the normal B lymphocytes under thiamine supplementation to the medium (GPR FC -2.38 ÷ 1.51), but a deficit of the vitamin did not affect these genes. We have found only 2 genes whose expression answered both thiamine deficit and supplementation, i.e. GAPDH and SLC19A3. Increased expression of both in relation to the normal cells was observed in B lymphocytes HD which were kept in thiamine deficit conditions (TDM-Pyri) (ca. 6-fold GPR and above 8-fold GPR, respectively) and after thiamine supplementation at the concentration 1.0 mM (GAPDH – 3.5-fold GPR and SLC19A3 above 7-fold GPR). Thiamine used at a higher concentration, 5.0 mM, caused above a 1.5-fold decrease in GAPDH in GM13509 cells and a not significant difference in expression of SLC19A3 as compared to GM14467 cells.

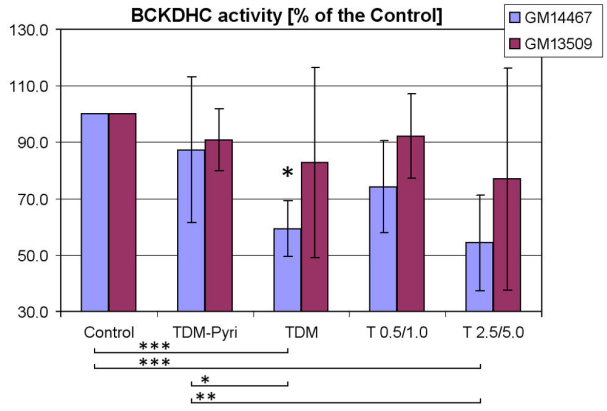
Fig. 3. KGDHC activity expressed as a percentage of the control in the cultures kept under thiamine deficit (TDM Pyri and TDM) and under 24 h – thiamine supplementation, i.e. 0.5 and 2.5 mM for GM14467 cells and 1.0 and 5.0 mM for GM13509 cells. Bars represent mean ± SD



ATP synthesis

The obtained results include a total ATP synthesis and ATP synthesized specifically in mitochondria, which was possible by the use of 1 mg/mL oligomycin, and 0.15 mM Ap5A.[11] Oligomycin is an inhibitor of mitochondrial ATP synthesis and Ap5A is an inhibitor of adenylate kinase, which inhibits direct ADP phosphorylation – one of the sources of ATP not specific for mitochondria. So, the rate of specific mitochondrial ATP synthesis was determined by the difference between the results obtained in the presence and absence of oligomycin and Ap5A, according to Marriage et al.[13]

Fig. 4. BCKDHC activity expressed as a percentage of the Control in the cultures kept under thiamine deficit (TDM Pyri and TDM) and under 24 h – thiamine supplementation, i.e. 0.5 and 2.5mM for GM14467 cells and 1.0 and 5.0 mM for GM13509 cells. Bars represent the mean ± SD. Asterix indicates significant differences with the following p values: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$



In this study, total ATP levels in the native GM13509 cells and GM14467 cells were similar (257 ± 26 ng/mg protein and 265 ± 42 ng/mg protein, respectively, $p > 0.886$) and the exogenous thiamine did not stimulate total or specifically mitochondrial ATP synthesis significantly ($p > 0.05$) (data not shown).

KGDHC and BCKDHC activity

As shown in Fig. 3, no significant differences in KGDHC activity were noticed between GM14467 and GM13509 cells in thiamine deficit conditions or under thiamine supplementation. The specific activity of this enzyme in the native cells was comparable: 13.20 ± 2.19 nmol/(min × mg protein) in GM14467 and 12.89 ± 0.80 nmol/(min × mg protein) in GM13509 cells ($p > 0.05$). The cells were similar also in regard of the native BCKDHC specific activities, i.e. 2.88 ± 0.87 nmol/(min × mg protein) in GM14467 and 2.72 ± 0.85 nmol/(min × mg protein) in GM13509 cells ($p > 0.05$). The only significant difference between both cell types was found in TDM, in which BCKDHC had higher activity for GM13509 cells than for GM14467 cells (Fig. 4) ($p < 0.05$). Thiamine deficit and supplementation did not affect BCKDHC activity in GM13509 cells. Surprisingly significant results were obtained in GM14467 cells, i.e. the 4-day treatment of these cells with pyrithiamine (TDM Pyri) did not result in the reduction of BCKDHC activity compared to the control. However, a significant decrease of BCKDHC activity was noticed in TDM (3 days with pyrithiamine and 24 h in the intact RPMI) as compared to control and to TDM Pyri ($p < 0.001$ and $p < 0.05$, respectively). A higher dose of thiamine (2.5 mM) caused a significant reduction of BCKDHC activity as compared to the control and to the cells kept under thiamine deficient condition, i.e. TDM Pyri ($p < 0.001$ and $p < 0.01$, respectively), which suggests a negative correlation between BCKDHC activity and thiamine concentration in GM14467 cells.

Discussion

In this study we analyzed the influence of the exogenous thiamine supplementation on energy metabolism in human B lymphocyte cell lines in in vitro cultures. We studied the intracellular levels of thiamine and TDP under deficit and supplemented conditions, the profiles of the genes related to thiamine and the energy processes, ATP synthesis and the activities of some thiamine dependent enzymes included in the Krebs cycle as KGDHC and BCKDHC. All these parameters were defined at the concentrations of thiamine selected on the basis of cell viability tests as the stimulating the cell growth.

The use of pyrithiamine hydrobromide as a competitive inhibitor of the thiamine transporter resulted in the lowest intracellular thiamine concentrations defined under deficit conditions (below 1 ng/mg protein).[14] The TDP values defined in our studies inside the native both cells (ca. 0.3–0.5 ng/mg protein) were similar to the levels determined in the other cells.[15] These initial results obtained in B lymphocytes may suggest that the level of the phosphorylated form of thiamine is not a feature of the neurodegeneration phenotype. Bettendorff et al. obtained similar results in brains of patient with frontal lobe degeneration of the non-Alzheimer's type.[16] The brain levels of the total thiamine, thiamine monophosphate (TMP) and TDP were also not significantly different from control values in the case of the patients with Friedreich's ataxia and spinocerebellar ataxia type 1.[17] However, more studies are required on TDP intracellular levels to evaluate the reliability of the phosphorylation processes in the examined cells. Because of that, the results obtained in this study have been interpreted only with regard to the non-phosphorylated thiamine levels. Fig. 1 and 2 present that the stimulation of proliferation of GM13509 cells up to the 135%, as compared to the reference culture (which is the maximal level of growth obtained in GM14467 cells at 2.5 mM of thiamine), was reached at ca. 1.5 mM of thiamine. These results can suggest increased growth potential of B lymphocytes with mHTT upon the exogenous thiamine.

The analysis of the genes connected with energy metabolism, which has been made in the intact B lymphocytes, showed that energy in fact may be one of the features of HD, although the thiamine related genes did not seem to be a key point in the gene profile characterizing HD. Native GM13509 cells had a higher than GM14467 cell expressions of GAPDH gene encoding glyceraldehydes-3-phosphate dehydrogenase (GADPH), which is involved in glycolysis and MDH1 gene that encodes malate de-

hydrogenase (MDH1), one of the key enzymes of Krebs cycle. GAPDH binds specifically to huntingtin and the β-amyloid precursor protein in HD and Alzheimer's disease (AD), respectively, which may affect their functional diversity, including energy production.[17] In fact, Mazzola and Sirover evidenced an impairment of GAPDH glycolytic activity of 27 and 33% in AD and HD, respectively.[18] According to other authors, the overexpression of GAPDH enhances nuclear translocation of mHTT and cytotoxicity.[19] On the other side, some studies revealed another role of GAPDH, among others in transcription or posttranscriptional regulation, apoptosis, pointing to the role of that enzyme in neurodegenerative diseases, including HD.[18]

An interesting observation obtained in this study is that SLC19A3 gene, widely expressed and capable of transporting thiamine by the encoded protein, showed the increased expression (ca. 3.5-fold GPR) in the intact cells with mHTT towards control.[20] The increase in GAPDH and SLC19A3 transcripts level was shown also in B lymphocytes with mHTT upon thiamine deficit and upon supplementation with 1 mM of the vitamin, which is difficult to explain, especially in deficit conditions.

mRNA of the genes which encode the thiamine–related enzymes and the other enzymes involved in energy metabolism in human B lymphocytes upon thiamine deficit and consequently upon thiamine supplementation was not changed. This is in contradistinction to the claims made by other authors, who described lower mRNA of transketolase and E1β subunit of PDHC in thiamine deficient cultures of human lymphoblasts and fibroblasts.[21] Strand et al. reported the decreased expression of IDH3A in skeletal muscles of R6/2 mice with mHTT, which is a gene that encodes one of the isocitrate dehydrogenase isoenzymes, which participates in Krebs cycle.[22] In our study, such an effect resulting from HD was not observed. Instead of this, the 2-fold decrease in the isocitrate dehydrogenase gene (IDH1) expression was noticed in GM13509 cells upon thiamine deficiency which suggests that low thiamine intracellular level could somehow be reflected in energy metabolism of peripheral tissues cells derived from HD subjects.

We did not observe the differences in the total ATP levels between the intact cells, which is partly consistent with Milakovic and Johnson, who reported decreased ATP synthesis only in mitochondria of clonal striatal cells established from Hdh^{Q7} (wild type) and Hdh^{Q111} (mutant huntingtin knock-in) mouse embryos but not total ATP.[23]

On the basis of our study, it can be said that an increase of cell viability observed in both types of cells after thiamine supplementation was not related to total or specifically mitochondrial ATP synthesis. Marriage et al. observed the CoQ_{10} but not of B-complex influence on ATP synthesis in peripheral lymphocytes.[13] The authors pointed out that the beneficial effects of single vitamins have often been due to a relative deficiency of the vitamin or to a specific defect in an enzyme that utilizes the vitamin as a cofactor. It is also possible that the time exposure of the cells to thiamine used in our study was too short. Ham et al. in cerebral capillary cells observed the influence of thiamine on a metabolism after 3–7 days of incubation with the vitamin.[24]

Thiamine deficit can be reflected in the activities of thiamine dependent enzymes on the cellular level. The same potential benefits of exogenous thiamine or thiamine phosphates are studied especially in the neurodegenerative diseases because of the proved role of thiamine in the pathogenesis of many neurological syndromes. Thus, a decrease of KGDHC, transketolase or PDHC activities during thiamine deficiency were found in autopsied brain tissue from neuropathologically proven AD patients.[25] 30% inhibition of KGDHC was observed in rats' whole forebrain mitochondria after pyrithiamine exposure.[26] Mastrogiacomo et al. measured the activity of KGDHC in postmortem brain samples from confirmed AD cases in both the presence and absence of TPP.[27] In each brain area examined, TPP produced a greater stimulatory effect on KGDHC activity in AD group as compared with the controls. The consequences of thiamine deficit were discussed also in non-CNS tissues.[28,29] Shi et al. indicated a region and time dependence of the KGDHC activity, mRNA and immunoreactivity of KGDHC subunits in response to thiamine deficit in brain tissues.[30]

On the other hand, KGDHC is not always susceptible to restoration. As shown by Blass et al. in cultured skin fibroblasts of DAT (dementia of the Alzheimer type) cases KGDHC and transketolase activities were reduced to 50–60% or 80–90% of normal, respectively but treatment with large doses of thiamine has not been beneficial.[28]

After analyzing the native activities of KGDHC and BCKDHC obtained in GM14467 and GM13509 cells in this study, no significant changes were found. As was shown by Krzysztoń-Russjan et al., BCKDK (branched chain ketoacid dehydrogenase kinase) on the transcription level was increased in nuclear blood cells of HD subjects.[31]

In turn, no significant abnormalities of KGDHC were also indicated in red blood cells and cultured fibroblasts of AD.[29] In this study the negative correlation noted between BCKDHC activity and thiamine concentration found in GM14467 cells is difficult to interpret, but it proves the cell specificity of energy metabolism.

Conclusions

B lymphocytes cell line obtained from HD subject seems to be more "sensitive" to the exogenous thiamine than control cells in relation to stimulation of cell growth, although they do not differ from control cells in energy metabolism expressed at the intracellular ATP level. This effect can be related to the increased intake of thiamine hydrochloride

by the cells but not controlled by SLC19A3 gene and it is rather not related to energy metabolism dependent on thiamine. However, some differences in mRNA expression of the genes related to the thiamine and energy metabolism noticed between B lymphocytes, normal and HD prompt that thiamine can have a role in HD pathogenesis and that the supplementation of this vitamin may be important to improve the impaired energy metabolism in HD cells. More studies are needed to confirm these suggestions.

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The role of certain gene polymorphisms involved in the apoptotic pathways in polycythemia vera and essential thrombocytosis

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Abstract

Background. Polycythemia vera (PV) and essential thrombocytosis (ET) are hematological disorders characterized by excessive production of mature and functional blood cells. These cellular disorders are thought to be associated with impaired apoptosis, which is one of the major cellular death mechanisms in hematopoietic cells.

Objectives. In this study, our objective was to examine the association between potential polymorphisms of the Bcl 2, Bax, Fas and Fas Ligand genes involved in apoptosis and the occurrence of PV and ET.

Material and methods. A total of 93 patients diagnosed with PV (n = 38) or ET (n = 55) at the Department of Hematology were included in this study, and 93 healthy individuals served as controls. DNA isolation was performed in blood samples obtained from both groups of subjects to determine the Bcl 2, Bax, Fas, and Fas L genotypes using the real-time PCR method.

Results. No statistically significant differences between controls and patients were found in terms of Fas -670 G > A (rs1800682), Fas -1377 G > A (rs2234767), Fas L IVS2 -124 A > G (rs5030772), Bax -248 G > A (rs4645878) and Bcl 2 -938 C > A (rs2279115) polymorphisms, genotypes, and allele frequency ($p > 0.05$).

Conclusions. The results show that polymorphisms in the Bcl 2, Bax, Fas, and Fas Ligand genes involved in the apoptotic pathways may not play a role in the pathogenesis of PV and ET.

Key words: polymorphism, polycythemia vera (PV), essential thrombocytosis (ET), apoptotic pathway genes

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Clonal myeloproliferative neoplasms (MPN) are classified into 2 major groups. While classical MPNs are represented by chronic Ph-positive myeloid leukemia (CML), carrying the Philadelphia (Ph) translocation and the BCR-ABL (Breakpoint Cluster Region-Abelson proto-oncogene) fusion gene, atypical MPNs consist of Ph-negative CML without the Philadelphia (Ph) translocation or the BCR-ABL fusion gene, polycythemia vera (PV), essential thrombocytosis (ET) and primary myelofibrosis (PMF). They generally occur in adulthood, with an annual incidence rate of 5–10 cases per one million people. Despite extensive, decades-long clinical and laboratory research, the etiology of BCR-ABL-negative myeloproliferative diseases is not exactly known. Although myeloproliferative diseases consist of a heterogeneous group of disorders, they are characterized by the excessive growth of multipotent stem cells in one or several blood cell lines.[1–3] In 2005, the JAK2 V617F mutation was discovered and was present in the majority of PV patients and 40–60% of ET and PMF patients. This type of mutation is referred to as a "Class I" mutation and is generally different from Class II mutations, which cause the MDS syndrome phenotype and occur in the active cellular growth signal mediators, including important molecules involved in the differentiation process. On the other hand, Class III mutations include multiple repetitive somatic mutations in the genes responsible for epigenetic regulation.[4,5]

Apoptotic mechanisms function through 3 pathways: the extrinsic pathway requiring cell surface and death receptors, the intrinsic pathway of mitochondrial origin, and the perforin/granzyme pathway mediated by cytotoxic T-cells. The aberrations in these mechanisms are involved in the pathogenesis of a number of conditions, including degenerative and autoimmune disorders and hematological cancers.[6,7] Deeper insight into the role of apoptosis in malignant conditions will not only shed more light on the pathogenetic mechanisms, but also may provide certain clues on how to develop more effective therapeutic strategies.

Allelic variations in the promoter region of genes may result in qualitative or quantitative changes through their effects on the transcription factor binding site or other regulatory sites of the gene. Many single-nucleotide polymorphisms (SNPs) are known to be required for the development of malignancies or in pathways such as apoptosis, which play an important role in resistance to chemotherapeutic agents.[8,9] Thus, in this study, the role of SNPs such as Fas -670 G > A (rs1800682), Fas -1377 G > A (rs2234767), FasL IVS2 -124 A > G (rs5030772), Bax -248 G > A (rs4645878) and Bcl 2 -938 C > A (rs2279115) involved in the apoptotic pathway was explored in terms of their effects on the molecular pathogenesis of 2 MPNs: PV and ET.

Material and methods

Study population

A total of 93 individuals between 28 and 85 years of age (mean age of 59.53 ± 15.52) diagnosed with PV and ET between 2010 and 2012 at the Department of Hematology, Medical Faculty of Mersin University were included in this study. The control group consisted of 93 individuals between 40 and 81 years of age (mean age of 51.96 ± 10.73) with no disease history. Thus, blood samples obtained from a total of 186 subjects were analyzed.

Extraction of genomic DNA

After written informed consent was obtained from each of the study subjects, 4–5 mL of peripheral venous blood was placed into centrifuge tubes containing 1 mL of 2% EDTA. The DNA extraction was performed using Miller DNA isolation with a salting out precipitation method.[10]

Genotyping

The genotyping of the Fas -670 G > A (rs1800682), Fas -1377 G > A (rs2234767), FasL IVS2nt -124 A > G (rs5030772), Bax -248 G > A (rs4645878) and Bcl 2 -938 C > A (rs2279115) gene polymorphisms was performed using pre-designed TaqMan SNP Genotyping Assays (Applied Biosystems, Foster City, USA). The Assays-on-Demand SNP genotyping kit was used for the Polymerase Chain Reaction (Applied Biosystems). Single nucleotide polymorphism amplification assays were performed according to the manufacturer's instructions. In brief, 25 µL of reaction solution containing 30 ng of DNA was mixed with 12.5 µL of 2X TaqMan Universal PCR Master Mix (Applied Biosystems) and 1.25 µL of pre-developed assay reagent from the SNP genotyping product (C_9578811_10 for Fas gene -670 G > A [rs1800682]; C_12123966_10 for Fas gene -1377 G > A [rs2234767]; C_32334221_10 for FasL gene IVS2nt -124 A > G [rs5030772]; C_27848291_10 for Bax gene -248 G > A [rs4645878]; and C_3044428_30 for Bcl 2 gene -938 C > A [rs2279115], Applied Biosystems) containing two 900 nM primers and two 200 nM MGB TaqMan probes. Reaction conditions consisted of preincubation at 60°C for 1 min and at 95°C for 10 min, followed by 40 cycles at 95°C for 15 s and at 60°C for 1 min. Amplifications and analysis were performed in an ABI Prism 7500 Real-Time PCR System (Applied Biosystems), using the SDS 2.0.3 software for allelic discrimination (Applied Biosystems).

Statistical analysis

The Independent Samples t-test and one-way ANOVA were used to compare continuous variables between groups. Analysis of the association between groups

and genotypes/alleles was done by χ^2 or likelihood ratio tests according to the expected value rule for crosstabs. The Hardy-Weinberg equilibriums were controlled in both control and study groups for all genotypes. Descriptive statistics were presented by mean and standard deviation for continuous variables, and with frequencies and percentages for categorical variables. Statistical analyses were done by SPSS v. 15 statistical package and p-values less than 0.05 were considered statistically significant.

Results

The group of patients with PV or ET consisted of 51 men and 42 women with a mean age of 59.53 ± 15.52 (ranging from 28 to 85 years). Thirty-eight patients (19 women, 19 men) presented with PV, while 55 (32 women, 23 men) patients had ET. There was a statistical difference in terms of the mean age between PV-ET patients and controls (p = 0.001). The Fas -670 G > A (rs1800682), Fas -1377 G > A (rs2234767), FasL IVS2 -124 A > G (rs5030772), Bax -248 G > A (rs4645878) and Bcl 2 -938 C > A (rs2279115) polymorphism distribution in patients with PV or ET was similar to those observed in healthy controls. As a result, there was no association between the Fas -670 G > A (rs1800682), Fas -1377 G > A (rs2234767), Fas L IVS2 -124 A > G (rs5030772), Bax -248 G > A (rs4645878) and Bcl 2 -938 C > A (rs2279115) polymorphisms and PV-ET (Table 1).

Discussion

MPNs are multipotent hematopoietic stem cell disorders that are characterized by the uncontrolled growth of mature blood cells.[11] There are no distinct boundaries between these disorders, which makes them capable of presenting in a number of different disease categories with the ability to evolve into each other.[12]

The allelic variations in the promoter region of the genes may cause qualitative or quantitative alterations through their effects on the transcription factor binding site or other regulatory sites. Many SNPs are known to be required for the development of certain malignant conditions and for pathways, such as apoptosis, which are important in terms of resistance to chemotherapy.[8,9] Thus, our objective was to examine the role of the Fas -670 G > A (rs1800682), Fas -1377 G > A (rs2234767), FasL IVS2 -124 A > G (rs5030772), Bax -248 G > A (rs4645878) and Bcl 2 -938 C > A (rs2279115) SNPs in the molecular pathogenesis of the 2 MPNs: PV and ET.

Single nucleotide changes of Fas -670 G > A (rs1800682) and Fas -1377 G > A (rs2234767) occur in the promoter region, at the repeating sequences of the binding sites for STAT1 (signal transducers and activators of transcription 1) and SP1 (Stimulatory Protein 1). These two polymorphisms lead to a reduced expression of Fas and Fas L genes. On the other hand, the Fas L IVS -124 (rs5030772) polymorphism is located in the second intron of the Fas L gene. Our results showed no significant associations between the genotype ratios and allele frequencies of the Fas -670, Fas L -1377 and Fas L IVS -124 polymorphisms between controls and patients with PV or ET. The deregulation of the Fas signal pathway is involved in the mechanisms of immune escape and tumorigenesis, and it is also associated with the differentiation, invasion and metastasis of cancer cells. In adult T-cell leukemia (ATL), where activated T lymphocytes were observed due to human T lymphotrophic virus type 1 (HTLV-1) infection, Farre et al. showed that the presence of the Fas -670 polymorphism was associated with clinical manifestations and survival.[13]

The Bcl 2 gene involved in the mitochondrial pathway of the apoptotic mechanism does play a role not only as a regulator protein for anti-apoptosis, but also as a suppressor of cell growth. It has 3 exons and 3 promoter regions. It is located 1400 bp upstream of the translation initiation site and acts as a negative regulator on P1 (first promoter), which directs the transcription. Bcl 2 not only acts as an anti-apoptosis regulator protein, but also as a proliferation inhibitor. Therefore, Bcl 2 has multiple functional effects in tumorigenesis, probably explaining why Bcl 2 expression is significant in diagnosis and why it differs according to the type of tumor. Polymorphisms altering the function/expression of the Bcl 2 gene affect the apoptotic mechanisms and potentially serve as an important marker to guide targeted treatments.[14,15] In our study, the polymorphisms of the P2 promoter of the Bcl 2 gene which serve as a negative regulator element were explored. In terms of the genotype ratios and allele frequencies of the Bcl 2 -938 C > A (rs2279115) polymorphism, control subjects did not differ significantly from those with PV or ET (p > 0.05). Bcl 2 may be expressed both in normal hematopoietic cells as well as in malignant hematopoietic cells, such as the leukemic blast cells of AML. Accordingly, Moon et al. proposed that a Bcl 2 protein or its gene expression could be used as a diagnostic marker for AML after chemotherapy. Bcl 2 expression is known to be increased upon Bcl 2 induction or selection of cells overexpressing Bcl 2 in patients with AML. Also, AML patients with higher Bcl 2 protein or gene expression have a shorter survival and poor response to chemotherapy. Moon et al. concluded that the Bcl 2 -938 C > A polymorphism was linked to overall survival and remission rates following chemotherapy in patients with leukemia.[16] Nückel et al. in a study involving patients with chronic lymphocytic leukemia (CLL) found that in the Bcl 2 938 C > A polymorphism, the AA genotype was associated with an increased expression of Bcl 2 and that it may be appropriate to use this as a genetic prognostic marker in patients with CLL.[17] Hwan et al. reported that there was a significant association between the Bcl 2-938

Table 1. The frequency of distribution of genotypes and alleles in the patient and control groups

Gene			PV (n : 38) n(%)	ET (n : 55) n(%)	PV + ET (n : 93) n(%)	Control (n : 93) n(%)
Fas-670 G > A rs1800682	genotype	GG	9 (23.7)	13 (23.6)	22 (23.6)	23 (24.7)
		GA	18 (47.4)	23 (41.8)	41 (44.1)	49 (52.7)
		AA	11 (28.9)	19 (34.6)	30 (32.3)	21 (22.6)
	allel	G	36 (47.4)	49 (44.5)	85 (45.7)	95 (51.1)
		A	40 (52.6)	61 (55.5)	101 (54.3)	91 (48.9)
Fas-1377 G > A rs2234767	genotype	GG	28 (75.7)	41 (74.5)	69 (75.0)	68 (73.1)
		GA	8 (21.6)	14 (25.5)	22 (23.9)	24 (25.8)
		AA	1 (2.7)	0 (0.0)	1 (1.1)	1 (1.1)
	allel	G	64 (86.5)	96 (87.3)	160 (87.0)	160 (86.0)
		A	10 (13.5)	14 (12.7)	24 (13.0)	26 (14.0)
FasL IVS2-124 A > G rs5030772	genotype	GG	27 (71.0)	37 (67.3)	8 (8.6)	57 (61.3)
		GA	9 (23.7)	12 (21.8)	21 (22.6)	31 (33.3)
		AA	2 (5.3)	6 (10.9)	64 (68.8)	5 (5.4)
	allel	G	63 (82.9)	86 (78.2)	37 (19.9)	145 (78.0)
		A	13 (17.1)	24 (21.8)	149 (80.1)	41 (22.0)
Bax-248 G > A rs4645878	genotype	GG	29 (76.3)	43 (78.2)	72 (77.4)	78 (83.9)
		GA	8 (21.1)	10 (18.2)	18 (19.4)	13 (14.0)
		AA	1 (2.6)	2 (3.6)	3 (3.2)	2 (2.1)
	allel	G	66 (86.8)	86 (87.3)	162 (87.1)	169 (90.9)
		A	10 (13.2)	14 (12.7)	24 (12.9)	17 (9.1)
Bcl2-938 C > A rs2279115	genotype	CC	8 (21.0)	15 (27.3)	23 (24.7)	20 (21.5)
		CA	21 (55.3)	24 (43.6)	45 (48.4)	49 (52.7)
		AA	9 (23.7)	16 (29.1)	25 (26.9)	24 (25.8)
	allel	C	37 (48.7)	54 (49.1)	91 (48.9)	89 (47.8)
		A	39 (51.3)	56 (50.9)	95 (51.1)	97 (52.2)

polymorphism and disposition to CML, which is a clonal myeloproliferative disorder.[18]

The proapoptotic function of the Bax gene involved in the induction of apoptosis and the regulation of the apoptosis pathway by many other genes, such as Bcl 2 and p53, through their interaction with the Bax gene (at least partially), have given rise to an increased interest in the Bax gene for cancer research. Recent studies have shown that the Bax gene is a tumor suppressor. Deletions of the gene have been shown to be associated with lymphoid hyperplasia and have also been found to possess a significant negative growth function from a hematopoietic aspect.[19]

The genotype ratios and allele frequencies of Bax-248 G > A (rs4645878) polymorphisms, which occur in the 5' untranslated region (UTR) of the Bax gene and which cause a reduction in the expression of the gene, did not differ significantly between control subjects and patients with PV or ET. However, Saxena et al. observed that the Bax -248 polymorphism occurring in the Bax promoter was associated with a decreased expression of Bax in CLL, as well as with the failure to achieve a complete response to conventional therapy.[20] Skogsberg et al., however, concluded that the Bax-248 polymorphism had no role as a marker of survival and prognosis in CLL.[21]

The exact cause of MPNs is unknown. However, molecular-genetic studies suggest that the majority of this type of neoplasm may be the result of acquired clonal genetic events. Therefore, this group of disorders represents a good candidate for molecular diagnostic studies. Particularly in this type of hematological malignancy, a better understanding of the apoptotic mechanisms responsible for homeostasis during the growth and differentiation of hematopoietic blood cells will not only allow us to gain deeper insights into the pathogenesis of these disorders, but will also guide us in our treatment decisions. Our results showed that the Fas -670 G > A (rs1800682), FAS 1377 G > A (rs2234767), Fas L IVS2 -124 A > G (rs5030772), Bax -248 G > A (rs4645878) and Bcl 2 -938 C > A (rs2279115) polymorphisms, which are involved in extrinsic and intrinsic apoptotic pathways, are not associated with the development of PV or ET. Although SNPs in apoptosis-associated genes, and changes in the gene and protein expression have been the subject of extensive research in many cancer types, to our knowledge, no studies examining the polymorphisms of the genes involved in the apoptotic pathway in patients with PV or ET, which are classified as hematological malignant conditions, have been carried out. We believe that further studies involving a larger patient series may better elucidate these associations. Also, the possibility that the occurrence of these diseases may be associated with variants of the genes examined should also be borne in mind. Furthermore, our findings indicate the need to examine other molecular changes of the apoptotic processes in patients with PV or ET.

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Location of the ischemic focus in rehabilitated stroke patients with impairment of executive functions

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Abstract

Background. Executive dysfunctions are part of the clinical symptoms of a stroke and can inhibit the process of rehabilitation. Patients with impaired executive functions may manifest aggression, impulsiveness, impaired thinking and planning.

Objectives. The aim of this study was to assess the effect of the ischemic focus location on the effectiveness of physiotherapy in improving the executive functions in patients after stroke.

Material and methods. Ninety patients after unilateral ischemic cerebral stroke were studied. We studied 45 patients treated at the Department of Rehabilitation and Physical Medicine of the WAM University Hospital of Lodz for 5 weeks. The rehabilitation program included: kinesitherapy, physiotherapy, speech therapy, psychological consultations and psychotherapy. The control group consisted of patients who were waiting for admission to the Department of Rehabilitation. The patients in both groups were divided into three subgroups with different locations of stroke: front, back and subcortical. Executive functions were measured by the Wisconsin Card Sorting Test (WCST), the trail making test (TMT - A, TMT - B), the verbal fluency test (VFT).

Results. Patients rehabilitated in the hospital with the front and subcortical lesion location reported improvement in executive functions in terms of a greater number of the analyzed indicators of the Wisconsin Card Sorting Test (WCST) than those with the back lesion location. Patients rehabilitated at home with the subcortical lesion location did not experience a significant improvement in executive functions in any of the analyzed indicators of the Wisconsin Card Sorting Test (WCST). Most of the indicators, with the exception of the total errors of Wisconsin Card Sorting Test (WCST) and TMT B, have not been modified by the location of stroke.

Conclusions. Executive dysfunction occurs not only in patients with an anterior location of the stroke, but also in the posterior and subcortical locations. Patients with a subcortical location of the stroke require more treatment to mitigate the dysfunction.

Key words: rehabilitation, executive functions, stroke

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Stroke is the main cause of severe and long-term disabilities in the adult population. Each year 50–70% out of about 40,000 patients after stroke require constant treatment and rehabilitation.[1] As a result of a stroke, there are severe motor dysfunctions, such as paralysis, paresis, gait and balance impairment and also cognitive deficits and emotional disorders. Over the five years after the first stroke, 30–40% of patients suffer from recurrent stroke.[2] In this situation, it is important to search for and find out about the factors that may influence the process of treatment and rehabilitation after stroke.

Executive functions are involved in almost every human activity with the exception of automated and learned activities. The individual components of activities such as starting, stopping and shifting are under the executive control. The executive functions integrate and organize various complex cognitive processes by which human behavior is planned, purposeful, conscious and selective.[3,4]

The brain substrate of executive functions both in terms of localization and etiology are a matter of argument. Initially, executive dysfunction was associated mainly with damage to the frontal lobes of the brain and has been reported as frontal dysexecutive syndrome.[5] Intensive development of medical sciences: neurology, psychiatry, neurobiology, psychophysiology has shown a wider spectrum of such disorders.[3] Fuster argued that the limitation of the concept of executive functions to the frontal lobes only simplifies the meta-cognitive-behavioral process which are the essence of the central executive system. Executive dysfunctions occur not only with lesions in the frontal cortex, but also in other subcortical structures such as: basal nuclei, hippocampus, striatum, thalamus, hypothalamus and cerebellum and cortex of other lobes of the brain.[6] Usually the infarction of frontal lobes, the striatum and the thalamus are mentioned among the causes of ischemic disorders of executive functions.[3]

The aim of this study was to investigate the effect of ischemic focus location in people after stroke on the effectiveness of the rehabilitation process for the return of executive functions.

Material and methods

Ninety patients after ischemic stroke were examined. The study group (45 individuals) were patients rehabilitated in the Department of Rehabilitation and Physical Medicine of the WAM University Hospital of Lodz. A rehabilitation program which consisted of physiotherapy, kinesitherapy, speech therapy, psychological consultation and psychotherapy was implemented. Physiotherapy was carried out with the use of modern neurophysiological methods such as Proprioceptive Neuromuscular Facilitation (PNF) and Bobath concepts and methods based on biofeedback. The patients were examined twice – first before the rehabilitation and then at the end of the 5-week rehabilitation program.

The control group consisted of patients waiting for admission to the Department of Rehabilitation, rehabilitated at home. The study included patients after ischemic stroke, from a month to a year after the brain incident. All the patients were right-handed. The studies did not include patients with damage to both hemispheres and diffuse brain damage, previous neurological disorders with total aphasia, and suspected of having dementia or psychotic symptoms.

The patients in both groups were divided into 3 subgroups with regard to the different lesion locations of stroke: front, back and subcortical. All the patients were instructed on the aim of the studies and agreed to participate in them. They were informed about the voluntary participation and the possibility of withdrawal without giving a specific cause. The testing was approved by the Committee on Bioethics (No. RNN/242/10/KB of 18 May 2010).

Executive functions were assessed using the Wisconsin Card Sorting Test (WCST), the trail making test (TMT A TMT - B) and the verbal fluency test (VFT).

The Wisconsin Card Sorting Test (WCST) consists of 2 identical packs of cards (each pack contains 64 cards) and 4 reference cards. Using the feedback provided by the examiner, the subject is trying to lay the card according to the shape, color and number. This test requires retaining information about the currently accepted criterion, potentially possible choices and the implementation plan for solving the problem in the direct memory.

Higher test indicators for total errors, perseverative responses, perseverative errors, percent of perseverative errors, nonperseverative errors, responses trials to complete the first category, failure to maintain set, and lower scores in percentage of conceptual-level responses and categories achieved are indicative of more impaired executive functions.[7]

The trail making test (TMT) consists of 2 parts: TMT A and TMT B. TMT examines psychomotor speed (part A) and visual-spatial working memory as well as the ability to switch to a new criterion after learning a response rule (part B). The result of the test is the time in seconds obtained in parts A and B and the ratio of the time A/B. The parameter B/A greater than 3 indicates a serious disorder of executive functions.[3]

The verbal fluency test (VFT) consists of a letters test and a category test. In the category test, the patients are to provide as many words from each category as they can, and in the letter test, as many words starting with the given letter of the alphabet as they can in 1 min. On average, the persons without brain damage mention 13–14 words beginning with the given letter in 1 min and give 16–17 names from a specific category. The test assesses 3 basic components of executive functions: starting (initiating the task – finding the words starting with the given letter in memory resources), stopping (restraining

previous reactions – the task of administering the words of a given category), and maintaining (sustaining mental focus on the task), and self-monitoring (checking and monitoring commenced operations).[3]

In order to evaluate the effects of rehabilitation, the t-test for dependent data was used to indicate the significance of differences between mean values of the results in different rates in the 2 groups of people after stroke: treated in the hospital and treated at home in different lesion locations (front, back and subcortical) obtained before and after rehabilitation. Descriptive data are presented as means and standard deviations. The level of significance $p < 0.01$.

In order to investigate the union between localization of ischemic focus (front, back and subcortical) with the effects of rehabilitation depending on the kind of rehabilitation (in the hospital, at home), the ANOVA test the Kruskal-Wallis test and the Mann-Whitney test were used to compare differences in location of ischemic focus in groups. Descriptive data are presented as means, standard deviations and medians. The level of significance $p < 0.05$.

The STATISTICA v. 12 was used for data analysis.

Results

The following indicators of the Wisconsin Card Sorting Test (WCST) were used to evaluate changes in the key components of the executive functions of planning activities, their implementation and the control of cognitive rehabilitation following stroke in patients with varying location of stroke: total errors, perseverative responses, perseverative errors, percent of perseverative errors, nonperseverative errors, percentage of conceptual-level responses, categories achieved responses, trials to complete the first category, failure to maintain focus and the results of the trail making test (TMT) part B, and of the category test of verbal fluency test (VFT) and the letter test of verbal fluency test (VFT).

Patients rehabilitated in the hospital with the front and subcortical lesion location reported improvement in executive function in terms of a greater number of the analyzed indicators of the Wisconsin Card Sorting Test (WCST) than those with the back lesion location. Patients rehabilitated at home with the subcortical lesion location did not obtain a significant improvement in executive functions in any of the analyzed indicators of the Wisconsin Card Sorting Test (WCST) (Table 1).

Patients rehabilitated in the hospital, both the front, back and subcortical lesion location and rehabilitated at home with the subcortical lesion location showed a significant improvement in the control of the executive functions measured by the Trail Making Test (TMT B) ($p < 0.01$).

In the case of index B/A the only significant improvement was reported in the group with the front lesion location rehabilitated in the hospital ($p < 0.01$) (Table 2).

The results of category test and letter test of verbal fluency test (VFT) were used to assess the verbal aspect of executive functions in both groups rehabilitated in the hospital and at home with different lesion locations.

Patients after a stroke with front and subcortical lesion locations rehabilitated in the hospital showed an improvement executive functions in the verbal aspect of a larger number of indicators analyzed in comparison to those with the back lesion location.

The location of ischemic focus had no statistically significant effect on the results of the indicators of Wisconsin Card Sorting Test (WCST) in the hospital group ($p > 0.05$). In the group of patients rehabilitated at home statistically significant importance was the indicator of Wisconsin Card Sorting Test (WCST) – total errors ($p < 0.05$).

The indicators of improvement (difference between the results obtained by patient after rehabilitation and the results obtained before the rehabilitation) in total errors in a group of subcortical location of ischemic focus was significantly lower than in the group with the location of the front and back ($p < 0.05$).

The other indicators of Wisconsin Card Sorting Test (WCST) have not been modified by the location of stroke ($p > 0.05$).

In patient rehabilitated at home indicators of improvement in the ratio of trail making test B (TMT B) was significantly lower in patients with back lesion location of stroke than in the group with the location of the front and subcortical ($p < 0.05$).

Indicators of improvement in test categories and test letter of verbal fluency test (VFT) was not modified by the location of stroke.

Discussion

The results of this study confirmed the possibility of occurrence of executive disorders not only in the case of the front location of stroke, but also in the case of back and subcortical locations. Failure within the frontal cortex can lead to dysfunction of planning, controlling operations, disordered control of impulses, affective behavior disorders, as well as sleep disorders resulting in the interference of selectivity and accuracy of mental processes.[8–11]

Patients with lesion location in the frontal lobes sometimes demonstrate impulsivity, aggression, disinhibition, low self-criticism and a tendency to break social norms and rules. The patients also have difficulty with abstract thinking. The connection between the frontal lobes and the striatum create a complex system that is responsible for planning the response and selecting the goals. The striatum is considered part of the anatomical circuit responsible for the processes of controlling the course of cognitive operations. Stroke patients with subcortical location of stroke within the striatum may have problems with stopping commenced operations and abnormal

Table 1. Results of Wisconsin Card Sorting Test (WCST) obtained before and after rehabilitation in patients rehabilitated in the hospital and at home with various localizations of ischemic focus

Variable	Group	Localization of ischemic focus	Time of data collection				t	p < 0.01
			before		after			
			M	SD	M	SD		
Total errors	1	front	66.11	11.93	42.68	18.12	5.671	0.000
		back	61.31	10.40	45.00	12.56	4.067	0.002
		subcortical	63.31	13.40	36.46	17.29	5.026	0.000
	2	front	50.44	6.82	39.63	13.12	3.181	0.006
		back	55.25	9.97	42.83	11.40	3.433	0.006
		subcortical	58.00	13.16	57.24	13.08	0.313	0.758
Perseverative responses	1	front	54.68	23.66	31.53	12.48	3.999	0.001
		back	45.92	20.48	34.08	16.12	1.858	0.088
		subcortical	45.85	25.62	24.62	11.96	2.419	0.032
	2	front	49.13	15.44	34.81	11.20	3.803	0.002
		back	50.42	17.07	39.58	12.28	2.083	0.061
		subcortical	46.88	16.55	43.94	13.01	0.812	0.428
Perseverative errors	1	front	41.58	17.32	23.11	8.79	4.840	0.000
		back	32.62	12.04	23.46	10.56	2.238	0.045
		subcortical	37.08	14.97	17.62	8.17	3.490	0.004
	2	front	28.94	11.77	20.38	7.88	2.692	0.017
		back	33.08	12.00	25.75	8.98	1.837	0.093
		subcortical	28.12	16.40	26.94	11.51	0.452	0.657
Percent of perseverative errors	1	front	37.42	19.68	19.53	5.55	3.981	0.001
		back	25.54	9.35	19.31	7.65	2.056	0.062
		subcortical	29.08	11.63	17.38	5.35	3.100	0.009
	2	front	22.50	9.24	17.00	5.96	2.370	0.032
		back	25.83	9.61	21.67	6.65	1.457	0.173
		subcortical	22.12	12.76	21.29	8.81	0.410	0.687
Non-perseverative errors	1	front	24.47	13.53	19.58	12.54	1.109	0.282
		back	28.69	10.40	21.46	10.44	2.265	0.043
		subcortical	26.23	8.49	19.00	13.20	2.214	0.047

1 – group rehabilitated in the hospital; 2 – group rehabilitated at home; M – mean; SD – standard deviation.

Table 1. Results of Wisconsin Card Sorting Test (WCST) obtained before and after rehabilitation in patients rehabilitated in the hospital and at home with various localizations of ischemic focus (cont.)

Variable	Group	Localization of ischemic focus	Time of data collection				t	p < 0.01
			before		after			
			M	SD	M	SD		
Non-perseverative errors	2	front	22.75	10.17	20.06	12.05	0.924	0.37
		back	21.58	11.36	17.08	9.77	1.855	0.091
		subcortical	29.71	12.65	29.47	11.47	0.118	0.907
Percentage of conceptual-level responses	1	front	30.42	11.21	54.47	16.11	5.911	0.000
		back	37.92	9.22	51.46	16.37	2.925	0.013
		subcortical	33.31	13.19	58.77	16.73	5.456	0.000
	2	front	44.44	6.67	55.44	13.68	3.650	0.002
		back	42.75	9.45	52.42	11.37	2.506	0.029
		subcortical	36.06	12.01	39.47	11.78	1.452	0.166
Categories achieved responses	1	front	1.11	0.83	4.28	1.64	7.504	0.000
		back	1.92	1.61	4.15	1.46	6.512	0.000
		subcortical	1.69	1.38	4.31	1.89	5.226	0.000
	2	front	1.94	1.61	3.38	2.60	3.032	0.008
		back	2.08	1.73	4.00	2.30	3.286	0.007
		subcortical	0.76	0.97	1.41	1.54	2.098	0.052
Trials to complete the first category	1	front	34.11	28.73	19.61	14.63	2.060	0.055
		back	25.92	27.46	21.77	19.16	0.488	0.634
		subcortical	25.08	23.09	12.77	10.86	1.988	0.07
	2	front	29.56	23.66	16.13	15.37	2.053	0.058
		back	32.67	20.57	18.83	17.39	1.402	0.188
		subcortical	12.53	21.04	18.94	22.44	0.797	0.437
Failure to maintain focus	1	front	2.06	1.76	1.44	1.25	1.377	0.186
		back	2.08	1.93	1.46	1.13	1.075	0.303
		subcortical	1.77	1.17	1.69	1.49	0.154	0.88
	2	front	3.31	1.40	2.50	2.34	1.094	0.291
		back	2.50	2.15	1.58	1.83	1.836	0.094
		subcortical	2.94	2.05	2.29	2.42	1.335	0.201

1 – group rehabilitated in the hospital; 2 – group rehabilitated at home; M – mean; SD – standard deviation.

Table 2. Results trail making test (TMT B) and TMT B/A obtained before and after rehabilitation in patients rehabilitated in the hospital and at home with various localizations of ischemic focus

Variable	Group	Localization of ischemic focus	Time of data collection				t	p < 0.01
			before		after			
			M	SD	M	SD		
TMT B	1	front	303.7	172.1	177.7	70.36	4.186	0.001
		back	305.9	247.7	215.3	171.2	3.391	0.005
		subcortical	277.5	110.4	175.6	81.71	6.377	0.000
	2	front	221.6	113.8	182.6	80.41	2.629	0.019
		back	130.1	78.91	116.9	61.40	1.498	0.162
		subcortical	287.8	109.9	239.3	90.77	3.523	0.003
B/A	1	front	2.75	0.86	2.19	0.62	3.005	0.008
		back	3.02	1.98	2.44	1.02	1.752	0.105
		subcortical	2.96	1.46	2.69	1.17	1.379	0.193
	2	front	2.40	0.70	2.50	0.82	0.501	0.623
		back	2.00	0.35	1.97	0.32	0.208	0.839
		subcortical	3.24	1.05	3.00	1.36	0.712	0.487

1 – group rehabilitated in the hospital; 2 – group rehabilitated at home; M – mean; SD – standard deviation.

Table 3. Results of verbal fluency test (VfT) obtained before and after rehabilitation in patients rehabilitated in the hospital and at home with various localizations of ischemic focus

Variable	Group	Localization of ischemic focus	Time of data collection				t	p < 0.01
			before		after			
			M	SD	M	SD		
f No. of words	1	front	6.11	3.09	9.47	2.89	5.113	0.000
		back	7.46	4.43	10.15	4.60	4.815	0.000
		subcortical	7.85	3.02	9.69	3.95	2.984	0.011
	2	front	7.44	4.21	8.69	3.24	2.402	0.03
		back	11.33	3.50	12.08	2.47	0.806	0.437
		subcortical	6.71	4.48	8.59	4.66	0.812	0.001
A No. of words	1	front	5.53	3.47	8.63	3.02	4.572	0.000
		back	6.77	4.19	10.23	5.12	3.304	0.006
		subcortical	7.15	2.58	9.77	3.61	2.339	0.037
	2	front	6.56	3.72	8.63	3.26	3.741	0.002
		back	10.42	2.81	12.58	3.23	2.545	0.027
		subcortical	5.88	4.78	6.88	3.57	1.011	0.327

1 – group rehabilitated in the hospital; 2 – group rehabilitated at home; M – mean; SD – standard deviation.

Table 3. Results of verbal fluency test (VFT) obtained before and after rehabilitation in patients rehabilitated in the hospital and at home with various localizations of ischemic focus (cont.)

Variable	Group	Localization of ischemic focus	Time of data collection				t	p < 0.01
			before		after			
			M	SD	M	SD		
S No. of words	1	front	6.63	3.73	8.68	3.40	3.572	0.002
		back	7.62	4.79	10.00	5.49	1.865	0.087
		subcortical	6.77	2.80	9.62	3.38	4.321	0.001
	2	front	6.25	3.32	7.81	3.23	2.581	0.021
		back	9.67	3.28	12.92	3.32	2.950	0.013
		subcortical	5.76	4.91	6.47	3.62	0.820	0.424
ZW No. of words	1	front	9.11	5.05	10.47	4.54	1.587	0.130
		back	8.77	5.29	11.08	4.65	3.379	0.005
		subcortical	10.38	2.75	13.62	2.93	4.395	0.001
	2	front	9.25	4.80	10.38	4.70	1.140	0.272
		back	13.00	3.13	13.58	5.79	0.466	0.65
		subcortical	9.18	7.15	10.53	5.89	1.743	0.101
O No. of words	1	front	7.79	3.63	10.21	2.92	4.076	0.001
		back	8.54	4.03	10.54	4.86	1.775	0.101
		subcortical	8.69	3.59	12.15	2.44	4.629	0.001
	2	front	8.50	4.20	9.06	2.91	0.753	0.463
		back	11.08	3.53	12.67	4.27	2.130	0.057
		subcortical	7.82	5.66	8.94	4.90	1.924	0.072

1 – group rehabilitated in the hospital; 2 – group rehabilitated at home; M – mean; SD – standard deviation.

attention switching, which means switching between individual aspects of executive actions.[12] However, the location of stroke in the structures of the limbic system often disturbs the execution control of emotions, motivation and drive to action.[13]

With the view to the foregoing, problems of executive dysfunctions due to their different nature should be taken into account when planning the treatment and rehabilitation as it can greatly modify the process. Jodzio et al. noted the most severe executive dysfunctions in patients with the lesion location in the frontal lobes and certain subcortical structures such as the striatum, the thalamus, the internal capsule and the stem.[14] The studies by Goldberg, Summerfield et al. also show a large disproportion in executive dysfunction symptoms between patients with damage to the front of the brain, patients with damage to the back of the brain, always to the disadvantage of the former.[14]

To investigate the influence of stroke location on the effectiveness of the rehabilitation process is a source of new relevant information. Patients rehabilitated in the hospital with front and subcortical lesion location achieved improvement of executive functions of a larger number of indicators of the Wisconsin Card Sorting Test (WCST) than those with the back location. Patients rehabilitated in the hospital with front and subcortical lesion location coped better with the choice of an appropriate strategy for solving the problem (correct sorting criterion) as they could

Table 4. The rating indicators in Wisconsin card sorting test (WCST) in patients rehabilitated in the hospital and at home depending on the location of ischemic focus

Variable	Group	Localization of ischemic focus									Value of the test	$p < 0.05$
		front (1) (N1 = 19 ; N2 = 16)			back (2) (N1 = 13; N2 = 12)			subcortical (3) (N1 = 13; N2 = 17)				
		M	SD	median	M	SD	median	M	SD	median		
No. of errors in total	1	23.42	18.00	20.00	16.31	14.46	14.00	26.85	19.26	23.00	H = 1.78	0.4116
	2	10.81	13.60	12.50	12.42	12.53	13.50	0.76	10.07	3.00	H = 7.75 $Z_{1.2} = 0.14$ $Z_{1.3} = 2.32$ $Z_{2.3} = 2.35$	0.021 0.889 0.020 0.019
Perseverative responses	1	23.16	25.24	32.00	11.85	22.98	8.00	21.23	31.65	20.00	H = 1.35	0.438
	2	14.31	15.05	16.00	10.83	18.01	8.50	2.94	14.93	4.00	H = 4.93	0.085
Perseverative errors	1	18.47	16.64	22.00	9.15	14.75	7.00	19.46	20.11	15.00	H = 2.75	0.254
	2	8.56	12.72	7.50	7.33	13.83	9.00	1.18	10.73	0.00	H = 3.23	0.138
Percent of perseverative errors	1	17.89	19.60	18.00	6.23	10.93	5.00	11.69	13.60	9.00	H = 3.68	0.159
	2	5.50	9.28	5.00	4.17	9.91	5.50	0.82	8.29	0.00	H = 2.16	0.340
Non-perseverative errors	1	4.89	19.24	4.00	7.23	11.51	9.00	7.23	11.78	11.00	H = 0.62	0.735
	2	2.69	11.63	3.00	4.50	8.40	4.50	0.24	8.19	-1.00	H = 1.40	0.497
Percentage of conceptual-level responses	1	-24.05	17.74	-24.00	-13.54	16.69	-11.00	-25.46	16.83	-25.00	H = 4.05	0.132
	2	-11.00	12.06	-12.50	-9.67	13.36	-7.50	-3.41	9.69	-3.00	H = 4.36	0.113
Categories achieved responses	1	-3.17	1.79	-3.50	-2.23	1.24	-2.00	-2.62	1.80	-3.00	H = 2.30	0.316
	2	-1.44	1.90	-1.00	-1.92	2.02	-2.50	-0.65	1.27	0.00	H = 3.71	0.156
Trials to complete the first category	1	19.60	18.00	10.93	9.28	5.00	9.91	19.24	4.00	11.51	H = 1.49	0.474
	2	17.74	-24.00	16.69	12.06	-12.50	13.36	1.79	-3.50	1.24	H = 5.23	0.073
Failure to maintain focus	1	14.50	29.86	14.50	4.15	30.68	0.00	12.31	22.32	4.00	H = 0.74	0.691
	2	13.44	26.18	12.00	13.83	34.17	29.00	-6.41	33.16	0.00	H = 0.22	0.892

H – ANOVA Kruskal-Wallis test; Z – U Mann-Whitney test; 1 – group rehabilitated in the hospital; 2 – group rehabilitated at home; M – mean; SD – standard deviation.

Table 5. The rating indicators in Trail Making Test (TMT B) and TMT B/A in patients rehabilitated in the hospital and at home depending on the location of ischemic focus

Variable	Group	Localization of ischemic focus									Value of the test	p < 0.05
		front (1) (N1 = 19 ; N2 = 16)			back (2) (N1 = 13; N2 = 12)			subcortical (3) (N1 = 13; N2 = 17)				
		M	SD	median	M	SD	median	M	SD	median		
TMTB	1	126.00	131.20	113.00	90.54	96.28	57.00	101.92	57.63	87.00	TMTB	1
	2	39.00	59.33	37.50	13.25	30.65	7.50	48.53	56.80	50.00	H = 6.31 $W_{1.2}$ = 2.09 $W_{1.3}$ = 0.38 $W_{2.3}$ = 2.26	0.043 0.036 0.705 0.024
B/A	1	0.57	0.82	0.30	0.58	1.19	0.60	0.28	0.72	0.30	H = 1.12	0.572
	2	-0.10	0.76	0.01	0.03	0.50	-0.02	0.23	1.36	0.35	H = 0.44	0.799

H – ANOVA Kruskal-Wallis test; Z – U Mann-Whitney test; 1 – group rehabilitated in the hospital; 2 – group rehabilitated at home; M – mean; SD – standard deviation.

Table 6. The rating indicators in Verbal Fluency Test (VFT) in patients rehabilitated in the hospital and at home depending on the location of ischemic focus

Variable	Group	Localization of ischemic focus									Value of the test	p < 0.05
		front (1) (N1 = 19 ; N2 = 16)			back (2) (N1 = 13; N2 = 12)			subcortical (3) (N1 = 13; N2 = 17)				
		M	SD	median	M	SD	median	M	SD	median		
F No. of words	1	-3.37	2.87	-3.00	-2.69	2.02	-2.00	-1.85	2.23	-1.00	H = 2.60	0.271
	2	-1.25	2.08	-1.50	-0.75	3.22	-1.00	0.23	1.36	0.35	H = 0.68	0.713
A No. of words	1	-3.11	2.96	-2.00	-3.46	4.03	-4.00	-2.62	2.21	-2.00	H = 0.70	0.706
	2	-2.06	2.21	-2.00	-2.17	2.95	-2.00	-1.00	4.08	-2.00	H = 0.45	0.798
S No. of words	1	-2.05	2.50	-2.00	-2.38	2.38	-2.00	-2.85	2.42	-3.00	H = 0.88	0.644
	2	-1.56	2.42	-1.50	-3.25	3.82	-3.50	-0.71	3.55	-1.00	H = 3.14	0.204
ZW No. of words	1	-1.37	3.76	-2.00	-2.31	2.65	-2.00	-3.23	3.95	-3.00	H = 2.16	0.340
	2	-1.13	3.95	-2.00	-0.58	4.34	-0.50	-1.35	3.20	-1.00	H = 0.21	0.903
O No. of words	1	-2.42	2.59	-2.00	-2.00	2.70	-2.00	-3.46	2.99	-4.00	H = 2.45	0.294
	2	-0.56	2.99	-1.00	-1.58	2.57	-1.00	-1.12	2.39	-1.00	H = 0.51	0.776
W No. of words	1	-2.16	3.34	-2.00	-1.77	3.91	-2.00	-3.46	3.92	-3.00	H = 1.51	0.469
	2	-1.50	3.92	-1.00	-1.00	2.45	-2.00	-0.06	3.31	-1.00	H = 1.25	0.535

H – ANOVA Kruskal-Wallis test; Z – U Mann-Whitney test; 1 – group rehabilitated in the hospital; 2 – group rehabilitated at home; M – mean; SD – standard deviation.

more effectively use the strategy, thus providing more correct answers and completing a larger number of categories.

This improvement was observed in the basic components of the functions, such as starting operations, stopping the previous reaction, sustaining mental focus on the task and checking and self-monitoring activities.

Despite the fact that the persons with the front and subcortical lesion location showed higher executive dysfunction before the rehabilitation than those with the back location, their recovery indicators after rehabilitation were higher.

Probably the effects of the treatment were modified by the application of modern neurophysiological methods

based on a broad multifaceted stimulation of the nervous system. The strategies based on neurophysiological methods are of particular importance, especially for people with cognitive impairment, including executive dysfunctions, when there are difficulties with the planning, execution and control of the activities.

Patients with subcortical lesion location rehabilitated at home obtained the smallest improvement in executive functions compared to those of other groups.

The total errors indicator showed a significantly lower improvement when compared to the persons with front and back lesion locations. The other indicators of Wisconsin Card Sorting Test (WCST) have not been modified by the location of stroke ($p > 0.05$). (Table 4).

The results of the study show that executive dysfunctions in patients with subcortical lesion location withdraw most slowly in rehabilitation. Therefore, this group of patients should be given special attention in determining the rehabilitation program.

In patients rehabilitated at home, the indicators of improvement in the ratio of trail making test B (TMT B) was significantly lower in patients with back lesion location of the stroke than in the group with the front and subcortical location ($p < 0.05$).

In the group rehabilitated at home, improvement in all the 3 lesion locations was significant only in the single indicator of verbal fluency test (VFT). Aphasia may have affected the results of the verbal fluency test (VFT) obtained in the compared groups.

Indicators of improvement in test categories and test letter of verbal fluency test (VFT) was not modified by the location of the stroke.

Executive dysfunctions may inhibit the process of treatment and rehabilitation after stroke. The patient forgetting or misunderstanding the issued instructions, their lack of insight, impaired awareness of the illness, impulsivity and aggression can reduce the effectiveness of physical therapy and may be the cause of the negative emotions from the physiotherapists.[15]

The problem of the patient's executive dysfunctions is faced by the family and carers who suffer from permanent stress and have to modify the emotional ties, to change their lifestyles and leisure activities. Patients' attempts to return to work usually fail.[3]

The psychosocial consequences of executive dysfunctions are sometimes more dramatic than motor problems and are the cause of permanent disability after stroke. The issue of cognitive dysfunctions should be included in the treatment of patients after stroke.

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The assessment of prosthetic needs of ESRD patients and the general population in Poland on the basis of the Eichner classification and teeth number: A brief, preliminary report

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Abstract

Background. End-stage renal disease (ESRD) patients are considered as a group of high risk of oral cavity diseases. One of the determinants of alveolar bone loss and increased teeth mobility in ESRD patients might be the bone abnormalities associated with chronic kidney disease-mineral and bone disorder (CKD-MBD).

Objectives. The aim of the study was to compare the general health condition, number and location of teeth in a group of ESRD patients with the group of peers from general population and revealing the risk factors of tooth loss.

Material and methods. The ESRD group included 63 patients, 23 females and 40 males, undergoing dialysis with a mean age of 62.4 ± 15.6. The general population sample consisted of 37 people, 20 females and 17 males, applying for general practitioner visit, with a mean age of 65.5 ± 11.1. All the participants were using just public health care insurance. The data analysis was based on anamnesis, history of CKD, selected biochemical parameters of blood tests and clinical examination.

Results. There was no statistical difference in the prosthetic needs of patients undergoing dialysis and the general population. In both groups the situation is alarming.

Conclusions. The new procedures are needed to develop complex health care for ESRD and general population patients, emphasizing prophylaxis of tooth-loss and prosthetic treatment in order to maintain good level of life quality.

Key words: general population, prosthetic needs, Eichner classification, teeth number, ESRD

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End-stage renal disease (ESRD) patients are considered to be a group of high risk of oral cavity diseases.[1] Chronic Kidney Disease-Mineral Bone Disorder (CKD-MBD) often affects this group and is usually related to secondary hyperparathyroidism.[2,3] The bone abnormalities, including the loss of lamina dura and abnormal trabeculation of alveolar bone, may be the cause of tooth loss. The clinical findings are more severe in the group of ESRD patients than in patients with less advanced stadium of chronic kidney disease (CKD), and with time become more noticeable during dialysis.[4–6] As the quality of life strongly depends on oral health, the dental aspects of ESRD patients should be discussed. According to authors' knowledge, the assessment of the prosthetic needs of ESRD patients and the general population in Poland, based on the Eichner classification, has not been published yet.

Methods

The sample consisted of 100 patients. The ESRD group included 63 patients, 23 females and 40 males, undergoing dialysis in the Department of Nephrology and Transplantation Medicine at Wroclaw Medical University. The average age of the participants was 62.4 ± 15.6. The general population group consisted of 37 people, 20 females and 17 males, who applied for general practitioner visit at one of the public ambulatory care units in Wrocław. The mean age of the participants was 65.5 ± 11.1. Both groups were covered by public medical insurance.

The data analysis was based on recollecting the history of CKD, selected biochemical parameters of blood tests and clinical examination. Anamnesis included questions about age, sex, weight and height (in order to assess BMI), place of residence, education, smoking habits and general diseases such as diabetes, hypertension, HBV and HCV infections. The medical history of ESRD considered the cause of CKD, time of renal dialysis and dialysis adequacy parameter (Kt/V). The blood parameters selected in order to evaluate calcium-phosphorus metabolism were: calcium, phosphorus, alkaline phosphatase and parathyroid hormone. The clinical examination focused on the number of teeth and their location in the oral cavity according to the Eichner classification (Table 1).

The statistical analysis was adopted according to Sokal and Rohlf (1995).[7] The normality of distribution in both groups studied was evaluated with the use of the Shapiro-Wilk test. In order to assess the statistical significance in the frequency on observations of variables in nominal scale (e.g. the Eichner classification), the χ^2 test was used. In the case of variables in the interval and absolute scale the Mann-Whitney test was used to compare the statistical significance between the average and median values in the tested and control groups. Linear correlations between variables were checked by the Spearman's rank correlation index. Differences between the average values in many groups were checked by the Kruskal-Wallis multiple comparison test. Statistical significance was defined as $p < 0.05$. Statistical analysis was performed using STATISTICA v. 10 software.

Ethical approval for the study was obtained from the regional ethics committee for human research, Wroclaw Medical University, according to the Helsinki Declaration.

Results

There were no significant differences between ESRD and the general population groups in the mean age of patients (ESRD group: 62.4 ± 15.6; control group: 65.5 ± 11.1), sex (ESRD group: 23 females and 40 males; control group: 20 females and 17 males), place of residence (ESRD group: town 74.6% and countryside 25.4%; control group: town 73% and countryside 27%) and smoking habits (ESRD group: non-smokers 87.3% and smokers 12.7%; control group: non-smokers 81.1% and smokers 18.9%) ($p > 0.05$). The significant differences between both groups concerned education, BMI values and general health. The dialyzed patients more often had vocational training (39.7%) ($p = 0.009$) and less often a primary education (7.9%) ($p = 0.001$) in comparison to the control group: 21.6% and 27%, respectively. The percentage of participants without and with a higher degree in the ESRD group were 27% and 25.4%, respectively. In the control group, the values were: 32.4% and 18.9%, respectively. Moreover, overweight patients (BMI ≥ 25) were significantly more often found in control group ($p = 0.006$). The 54% of dialyzed patients and about 80% of control group patients had BMI ≥ 25. Dialyzed patients significantly more often suffered from diabetes mellitus (33.3%) ($p = 0.024$) and hepatitis B and/or C (27%) ($p = 0.000$) than control group.

The statistical analysis of clinical examination parameters revealed that there were no significant differences in the number of teeth and the frequency of percentage incidence of the Eichner classification groups between dialyzed and general population groups (Table 2). The average number of teeth in ESRD and general population groups were 13.1 ± 10.7 and 13.4 ± 10.4, respectively.

In both groups, a correlation was found between the number of teeth and education ($r = 0.325$, $p < 0.05$; $r = 0.582$, $p < 0.05$ for ESRD and general population groups, respectively) and an inverse correlation for age ($r = -0.518$, $p < 0.05$; $r = -0.674$, $p < 0.05$ for ESRD and general population groups, respectively). In the general population, the additional risk factor of tooth-loss was hypertension. The ESRD group revealed correlations between the number of teeth and dialysis adequacy assessed by Kt/V index ($r = 0.319$, $p < 0.05$) and the level of calcium in blood samples ($r = 0.287$, $p < 0.05$). An inverse correlation was observed between the number

Table 1. Eichner classification

Group A. Intermaxillary contact in four occlusal supporting zones (in the premolars and molars regions)	
A1	Two full dental arches
A2	One full dental arch, one dental arch with interdentally missing teeth
A3	Two dental arches with interdentally missing teeth
Group B. Intermaxillary contact, not in all occlusal supporting zones	
B1	Intermaxillary contact in three occlusal supporting zones
B2	Intermaxillary contact in two occlusal supporting zones
B3	Intermaxillary contact in one occlusal supporting zone
B4	Intermaxillary contact apart from occlusal supporting zones
Group C. No intermaxillary contact	
C1	Two dental arches with residual dentition
C2	One dental arch with residual dentition, one edentulous arch
C3	Two edentulous arches

Table 2. The frequency of observations of patients classified to different groups T (A1–C3) according to Eichner index [number of observations (N) and percentage contribution (%)] in ESRD group (N = 63) and general population group (N = 37) and results of statistical analysis (χ^2, p)

	ESRD group		General population group		χ^2	p
	N	%	N	%		
A1	2	3	1	3	0.00	1.00
A2	5	8	5	14	1.84	0.75
A3	4	6	2	5	0.10	0.76
B1	6	10	1	3	4.03	0.45
B2	8	13	5	14	0.04	0.84
B3	4	6	5	14	3.56	0.06
B4	6	10	2	5	1.80	0.18
C1	2	3	3	8	2.41	0.12
C2	17	27	8	22	0.68	0.41
C3	9	14	5	14	0.00	1.00

of teeth and the concentration of alkaline phosphatase ($r = -0.265$, $p < 0.05$). In addition, there was a correlation between the number of teeth and the cause of ESRD – it was observed that patients with glomerulonephritis had significantly more teeth in the oral cavity than other ESRD patients. However, patients with glomerulonephritis had lower mean age than other patients on dialysis, which may explain their higher number of teeth preserved.

Discussion

The study showed a catastrophic situation in prosthetic needs not just in ESRD group of patients, but also in general population group. Cengiz et al., Brito et al., Castillo et al., Bots et al. and Bayraktar et al. also did not find any statistically important difference in the number of teeth between dialyzed patients and control groups.[8–12] According to Brito et al., the average number of teeth in hemodialyzed patients with the mean age of 50 ± 10 was 17.7 ± 6.4, and in patients undergoing peritoneal dialysis with the mean age of 52 ± 12 was 17.3 ± 6.5.[9] Castillo et al. reported that the mean number of teeth in dialyzed patients with the mean age of 61.5 ± 18.04 was 19.8 ± 8.5.[10] However, Kanjanabuch et al. indicated that patients undergoing peritoneal dialysis had significantly less teeth than those in the control group. According to those authors, the difference in the number of teeth between the two groups is not caused by the changes in the structure of alveolar bone, as in both groups the bone density was comparable.[13]

Teeth maintenance is crucial for maintaining the aesthetics and function of the stomatognathic system. There was no significant difference in the frequency of percentage incidence of the Eichner classification groups, leading to conclusion that number and location of teeth in oral cavity were comparable in test and control groups. However, it should be emphasized that tooth loss is a serious problem. Only 17% of dialyzed patients had 4 supporting zones in the area of premolars and molars. The rest of the patients had missing teeth in the maxilla and/or mandible, which may influence the efficiency of the masticatory organ and life quality of all the patients.[14,15] The very limited amount of available research showed that the frequency of prosthetic reconstructions usage among ESRD patients is very low. Musacchio et al. remarked about the existence of a large group of edentulous patients undergoing dialysis, who do not use dentures.[16] Wilczyńska-Borawska et al. assessed the prosthetic needs of dialyzed females with the mean age of 62 ± 14 and males with the mean age of 67 ± 11 years. The results showed that nearly 70% of ESRD patients do not have reconstructed teeth deficiencies.[17] According to Cunha et al., the percentage of patients undergoing dialysis in need of prosthetic treatment is around 80%.[18]

The oral health state of ESRD and general population patients are equally alarming. The possible reason for the high level of prosthetic needs of patients are definitely not the changes related to the end-stage renal disease, but it might be the inadequate Polish social medical insurance system. As there are no procedures encouraging the patient to consider prevention and attend regular check-up visits at dental offices, providing sufficient oral health care is a daunting task. These system deficiencies might have blurred the impact of ESRD specific features reflected by positive correlations of the teeth number with dialysis adequacy, serum calcium concentration, and inverse with alkaline phosphatase activity. It is crucial to examine the dental health of the Polish population in terms of prosthetic needs in order to create a new algorithm to facilitate prevention.

Conclusions

The new procedures are needed to develop complex health care of ESRD and general population patients in order to maintain a good level of life quality, focusing on prophylaxis of tooth-loss and prosthetic treatment of already existing damage.

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En bloc resection and prosthesis implantation to treat malignant fibrous histiocytoma of the humerus

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

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Abstract

Background. Malignant fibrous histiocytoma (MFH) of the bone is a rare tumor. Most studies comparing limb salvage and amputation have reported that limb salvage had no adverse effect on the long-term survival of patients. This study evaluates the oncological outcomes of limb salvage procedures that were used for 15 patients with MFH of the humerus.

Objectives. The aim of this study was to assess the functional and oncological outcomes of patients with malignant fibrous histiocytoma of the humerus after en bloc resection and prosthesis implantation.

Material and methods. A retrospective review of the charts of 15 patients who had undergone resection of malignant fibrous histiocytoma of the humerus followed by reconstruction with prosthesis was used in this study. A functional evaluation was based on Enneking's modified system of the functional evaluation of surgical management for musculoskeletal tumors. Complications of the procedures were also analyzed.

Results. Eight men and 7 women at an average age of 52.9 years were included in the study. The tumor involved the distal humerus in 3 patients, the proximal humerus in 8 patients and the mid-shaft humerus in 4 patients. Excellent results were achieved in 4 patients, good to fair in 10 and poor in 1. One patient had local recurrence. Pulmonary metastases occurred in 6 patients.

Conclusions. Limb salvage surgery with chemotherapy is a viable treatment option for patients with malignant fibrous histiocytoma of the humerus.

Key words: malignant fibrous histiocytoma, limb salvage, prosthesis implantation

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Malignant fibrous histiocytoma (MFH) is a pleomorphic tumor which is made up of fibroblasts, myofibroblasts and histiocytes. In adults, it is the most common soft tissue tumor. But osseous MFH is an unusual tumor, accounting for less than 2% of all primary malignant osseous tumors.[1,2] Primary osseous MFH is a central disorder detected in the osseous diaphysis or metaphysis that results in invasive bone damage and a soft tissue mass. The main characteristic of osseous MFH is the high local recurrence metastasis. It is recommended to treat MFH with a combination of surgical interference and neoadjuvant chemotherapy.[2] As the principal goal is prolonging survival, osseous MFH that needs surgical removal can be treated by either limb salvage or amputation. Most of the studies have reported that limb salvage had no bad influence on the long-term survival rate of the patients when comparing amputation and limb salvage.[2] This study assessed the efficacy of the limb salvage procedures that were applied for 15 patients with MFH of the humerus.

Material and methods

This study included 15 patients (8 men and 7 women) with a mean age of 52.9 years (range 30–71 years). Assessments were made using local X-ray, magnetic resonance imaging (MRI), chest CT scanning and technetium bone imaging. Tumor staging was carried out according to the Enneking system.[3] The distal humerus was affected in 3 patients, the proximal humerus in 8 patients and the mid-shaft humerus in 4 patients.

All patients before surgery had chemotherapy with 4 cycles of cisplatin and doxorubicin. Then the operation was performed. During the operation, in addition to en bloc of the humerus, an extended wide margin resection was achieved in all patients. The level of bone dissection was determined by examining the roentgenograms, technetium bone scanning, computed tomograms and MRI. The lesion was removed as widely or radically as possible. The margin was at least 7 cm above or below the limit of increased activity of the bone scan. Besides the tumor resection, subfascial dissection with the removal of all of the muscles in the compartment was done. At the level of the bone osteotomy, the muscles were severed. Custom-made prosthesis implantation was done to reconstruct the resected defect. After the operation, all the patients received chemotherapy of doxorubicin and cisplatin for 3–6 cycles. In this study, no patients accepted radiotherapy before or after surgery.

The functional outcome criteria were assessed with the 30-point Enneking scoring system, with 5 representing full function and 0 representing complete disability for each of 6 criteria.[4] All of the quantitative data was assessed using a questionnaire confirmed to possess low intra-observer variability (Table 1). The quantitative score was divided into normal (30 points), excellent (24–29 points), good (19–23 points), fair (12–18 points) and poor (less than 11 points). An assessment of function was done for each patient 6 months after the operation. All of the patients needed to return for follow-up to inspect metastasis, recurrence, or implanted-related complications monthly for the first 6 months, then quarterly after that for the next 3 years. The follow-up study was terminated if the patient died.

Result

The average follow-up period was 28 months (range 6–56 months). At the 6-month follow-up, 4 of the 8 proximal humerus patients had attained excellent functional outcomes. Three distal humerus, 4 of the 8 proximal humerus and 3 of the 4 mid-shaft humerus were good to fair. One mid-shaft humerus was poor.

One patient had local recurrence at 14 months. An amputation was done, but the patient ultimately died of metastatic disease at 20 months following the premier limb salvage procedure. Metastatic tumors of the lung occurred in 6 patients without local recurrence; 2 underwent pulmonary metastasis resection, but all ultimately died of metastatic disease. Table 2 shows patient characteristics and outcomes.

Illustrative case (patient no. 7)

A 56-year-old female presented with a mass in her right upper arm with serious pain. She had been previously diagnosed with soft tissue MFH (storiform-pleomorphic or undifferentiated pleomorphic sarcoma) of her left medial thigh. She had 3 operations to remove the primary and recurrent sarcoma, including the removal and reconstruction of the anterior compartment of the thigh.

The right arm was in the adduction and internal rotation position in the physical examination. The diameter of the upper arm increased from the middle part of the upper arm to the elbow. Abnormal movement and tenderness were also found at the distal 1/3 of the upper arm. The major function of the shoulder and elbow joints was lost. The length of the right upper arm decreased by 1.5 cm compared to the opposite side. However, the function of the wrist and hand were normal. The axillary and supraclavicular lymph nodes were negative. Also, no mass could be found in the left thigh.

X-ray of right humerus showed the complete osteolytic defect reaching from the middle 1/3 to the epicondyle of the humerus, and a “moth-eaten” appearance could be seen proximally (Fig. 1). MRI showed the local cortical defect of right humerus and the mass of soft tissue of irregular and uneven density. Technetium bone scanning showed the radiation aggregation in the right upper arm. The thoracic X-ray, abdomen ultrasound and left thigh MRI proved negative.

The en bloc resection and prosthesis implantation of

Table 1. Enneking functional outcome score

Score	Pain	Function	Emotional acceptance	Hand positioning	Manual dexterity	Lifting ability
5	no pain in arm, no pain medications	not restricted in daily activities, not disabled	enthusiastic about surgery, would recommend it to others	can lift arm over head without difficulty	no limitations in manual dexterity (i.e., button shirt, write, etc.)	can lift as much weight as before surgery
4	occasional discomfort in arm, no pain medications	occasional restrictions in daily activities, not disabled	satisfied with surgery, would recommend it to others	can lift arm over head with some difficulty	minimal limitations in manual dexterity	can lift slightly less than before surgery
3	occasional pain in arm, non-prescription pain medications	occasional restrictions in daily activities, minor disability	satisfied with surgery, would not recommend it to others	can lift arm up to level of shoulder	some loss of fine movements and/or sensation, limited daily activity	can only lift a greatly reduced load than before surgery
2	often pain in arm, non-prescription pain medications	daily occupational restrictions, minor disability	not fully satisfied with surgery, would still try it again	have difficulty lifting arm up to level of shoulder	significant loss of fine movements and/or sensation, inhibited from daily activity	can only lift arm without any weight
1	occasional pain in arm, prescription pain medications	some occupational restrictions, major disability	accept surgery, would repeat it reluctantly	cannot lift arm forward above level of waist	difficulty with basic dexterity such as pinching and/or major loss of sensation	can only use arm to help other arm in activities
0	disabling pain in arm, daily prescription pain medications	total occupational restriction, completely disabled	dislike surgery, would not repeat	cannot move arm	cannot grasp with hand and/or it is completely numb	cannot even use arm to help other arm

Table 2. Patient characteristics and outcomes

Patient no.	Sex/age (years)	Involved site	Tumor stage	Postoperative chemotherapy cycles	Follow-up (months)	Complications	Survival	Functional result (6 months after the operation)
1	M/30	proximal	IIA	6	24	pulmonary metastases	no	excellent
2	M/66	distal	III	4	20	local recurrence	no	fair
3	F/39	proximal	IIB	3	56	no	yes	excellent
4	F/44	mid shaft	IIB	6	12	pulmonary metastases	no	poor
5	M/56	proximal	IIA	2	32	pulmonary metastases	no	good
6	M/51	mid shaft	IIB	3	6	no	yes	fair
7	F/56	mid shaft	III	6	36	no	yes	fair
8	F/71	proximal	IIB	3	44	no	yes	excellent
9	M/48	distal	IIB	4	16	pulmonary metastases	no	good
10	F/46	proximal	IIA	4	20	no	yes	fair
11	F/67	proximal	IIB	3	18	pulmonary metastases	no	good
12	M/59	distal	IIA	2	30	no	yes	good
13	F/46	proximal	IIB	6	53	no	yes	excellent
14	M/62	mid shaft	III	4	26	pulmonary metastases	no	fair
15	M/52	proximal	IIA	4	28	no	yes	fair

Fig. 1. X-ray showing the complete osteolytic defect reaching from the middle 1/3 of the shaft to the epicondyle of the humerus

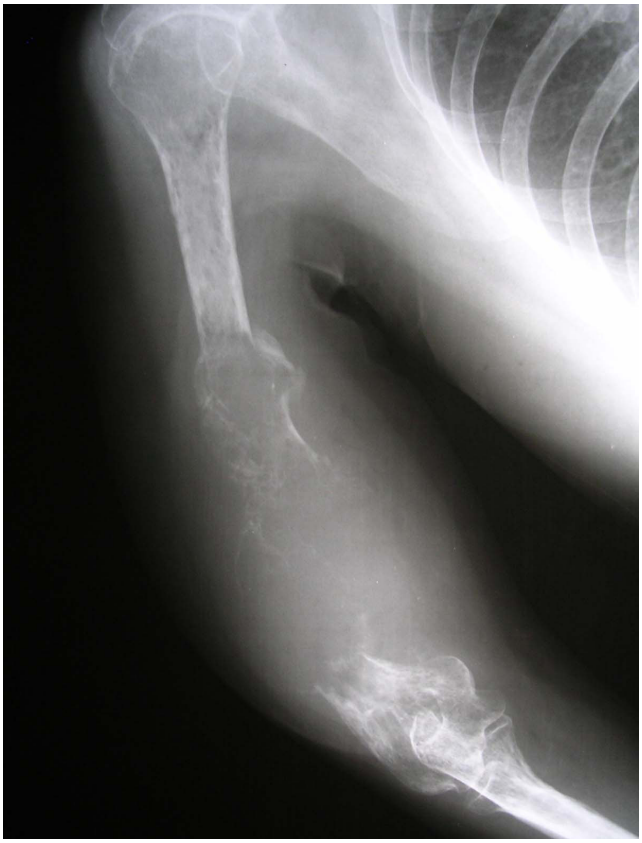
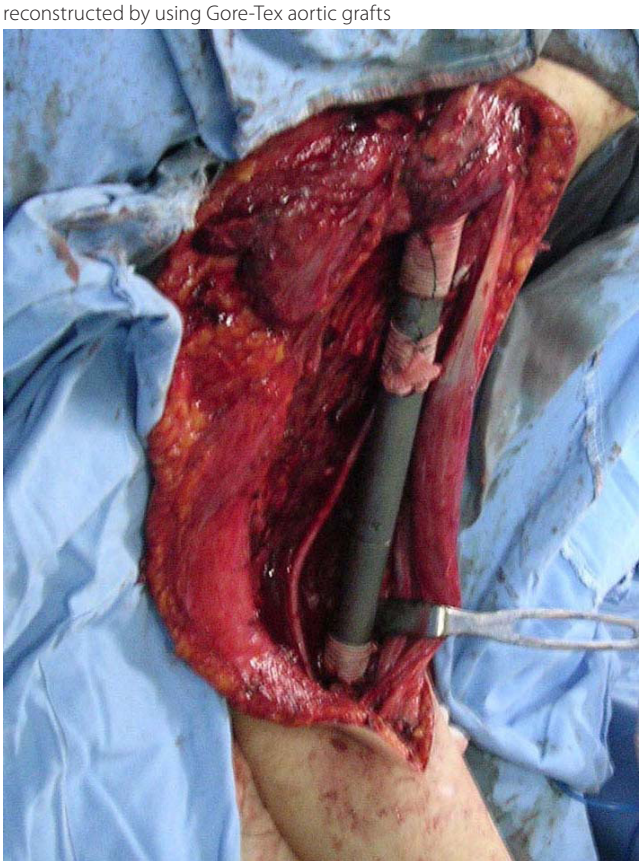


Fig. 2. The whole humerus replacement. The rotator cuff, the insertions of deltoid muscle and origins of forearm extensor and flexor muscles were reconstructed by using Gore-Tex aortic grafts



the whole humerus were performed. 1) En bloc resection of the humerus: The superior shoulder approach of James E, Thompson, Henry was used, and the anteromedial approach to the middle and distal segment of the upper arm was performed. The incision reached 5 cm distally below the elbow transverse line of the forearm. The shoulder joint and the whole humerus were exposed, and en bloc resection was performed through the approach. 2) Prosthesis implantation: The rotator cuff, the insertion of the deltoid muscle and the origins of the forearm extensor and flexor muscles were reconstructed using Gore-Tex aortic grafts. The distal end of the prosthesis was inserted into the proximal ulna (Fig. 2 and 3). After the operation, the shoulder joint was fixed on the abduction brace with the position of 80 degree abduction and 30 degree anteflexion and internal rotation.

The tumor was localized in the distal 2/3 of the humerus in macroscopic findings. The cortex of the humerus was destroyed with a well-circumscribed border and the expansile mass was pseudo-encapsulated. The cross-section of the resected tumor appeared pale and fleshy with zones of hemorrhage, myxoid changes and necrosis (Fig. 4). In histological findings, a storiform growth pattern of tumor cells, nuclear pleomorphism and bizarre tumor giant cells admixed with spindle cells and stromal chronic inflammatory cells were found. For immunohistochemistry, the sections showed Vim(+), Lysozyme(+), Mac387 & CD68(+), DES(-), S-100(-), MyoD1(-).

Postoperatively, the stitches were removed after 3 weeks and the abduction brace was discarded after 4 weeks. The patient received chemotherapy of doxorubicin and cisplatin for 6 cycles. The 36-month follow-up revealed no evidence of recurrence at the left arm and the right thigh. There were no metastases in the lungs. The function of the elbow, wrist and interphalangeal joints was satisfactory, but the abduction range of the shoulder joint was only 10 degrees.

Discussion

It was O'Brien and Stout who first recognized MFH as a histologically distinctive type of sarcoma in 1964.[5] Since the clinicopathologic features of this tumor was defined, MFH has become the most common type of soft tissue sarcoma diagnosed in most cancer centers. The former MFH classification showed a broad range of histological appearances and consisted of 5 subtypes: storiform, pleomorphic, myxoid, giant cell and inflammatory. The 2002 WHO classification recognizes the presence of an undifferentiated, unclassifiable category of pleomorphic sarcoma and defines undifferentiated pleomorphic sarcoma as a group of pleomorphic sarcomas in which any attempt to disclose their line of differentiation has failed.[6,7] It has to be emphasized that this is a diagnosis of exclusion following thorough sampling and judicious use of ancillary

Fig. 3. X-ray showing the prosthesis

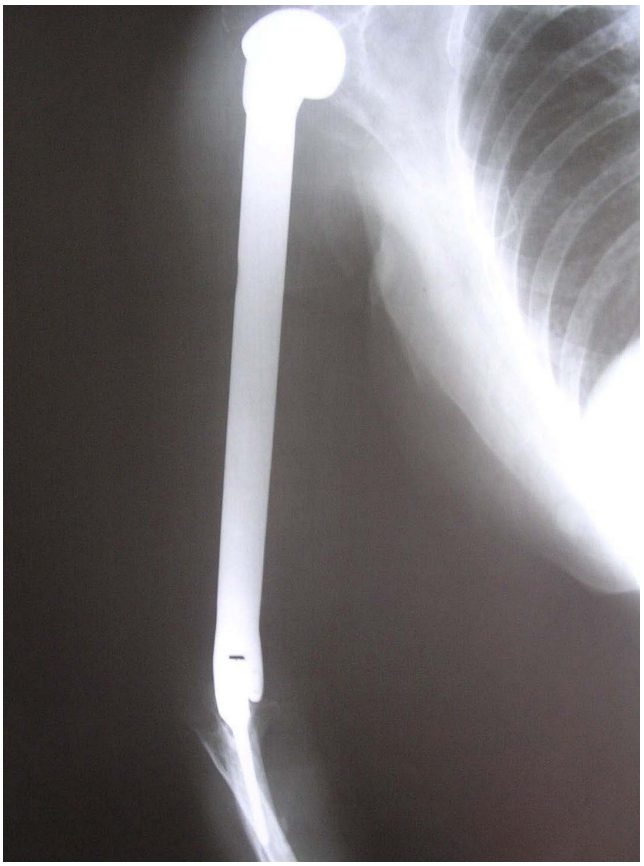
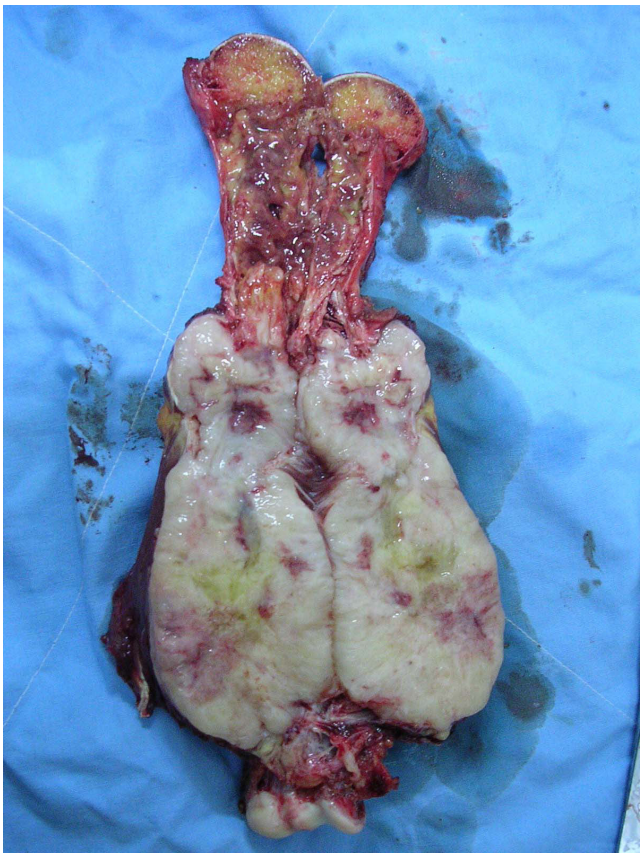


Fig. 2. Cross-section of the resected tumor showing a pale and fleshy appearance with zones of hemorrhage, myxoid changes and necrosis



techniques. Most of those cases in the past have contributed to the category of storiform and pleomorphic MFH.[8]

The principles of osseous MFH treatment are somewhat like those for osteosarcoma. Resection or amputation is the mainstay of therapy. Limb salvage surgery is the treatment of choice for 70–85% of all malignant bone and soft tissue sarcomas in the limb.[9] The decision to undergo amputation or limb salvage surgery is based on the tumor location, the tumor size, patient preferences, the possible complications and multiple reoperations, and the expected functional effect. Limb salvage surgery is generally the preferred treatment unless the functional prognosis is poor. It has been shown that the functional outcome of limb salvage surgery is superior to amputation.[10] Five prognostic factors determine survival outcome: advanced patient age, presence of metastases at presentation, tumor size, tumor grade and tumor depth.[11] It has been established that there has been no statistically significant survival difference found between amputation and limb salvage.[12] Limb salvage surgery patients have better quality of life without compromising survival than those who have undergone amputation.[13–15] Developments in adjuvant therapies, imaging diagnostics and surgical reconstructive techniques have made limb salvage surgery a preferred procedure for most primary sarcomas of the upper limbs with survival rates of 60–70%.[16,17] Today, major amputations are avoidable in most cases if the full potential of reconstructive possibilities is tapped.[8]

Osseous MFH is considered to be a tumor with a higher potential to recur locally.[18] Unplanned biopsies or inadequate surgical procedures are a common problem. It is well accepted that positive histological margins are associated with increased rate of local recurrence.[19,20] Also, it is widely accepted that a wide surgical resection margin is the most important prognostic factor in nearly every type of sarcoma, especially regarding the local recurrence rate.[21] It has been described that recurrence occurred in 64% patients with inadequate margins, 19% with wide margins, and 6.5% with radical surgery.[22] With the achievement of wide surgical margins, no significant difference exists in the recurrence rates between limb salvage and amputated patients.[2]

Negative surgical margins (R0) resection, the removal of the tumor in sano, is nonetheless the prerequisite and foremost oncologic parameter to save the patient from local recurrence.[23] Tumor resection with negative margins (R0) is the goal of surgical treatment. Luetke stated that an amputation would be considered only when the tumor could not be excised with a safe margin.[24] It is recommended that a wide margin could be achieved based on a pre-op MRI. MRI can display the tumor extent, compartment location and adjacent neurovascular structures, which allows the surgeon to know the expected margins and use of adjuvant therapies before the operation. Thus, surgical techniques should be started with careful preoperative planning with MRI to create a map of the desired plane of resection.[25]

The technique most frequently employed for tumor removal is the so-called wide resection. This term means resection of a large amount of surrounding healthy tissue, with safety margins of 4–5 cm to the sides and 1–2 cm deep to the tumor.[26,27] However, other studies suggest different safety margins. Robert recommended that the intention in all resected tumors was to gain clear margins with at least a centimeter of normal tissue around the tumor.[11] Muramatsu considered that there was a need for 2-centimeter-wide margins for high-grade soft-tissue sarcomas and a need for 1-centimeter-wide margins for low-grade bone tumors. If the width of the margin is over 1 cm, major nerves are reserved.[28] Some surgeons considered 2 cm above the tumor as an adequate margin for tumor resection.[10,29] Vasileios recommended that final pathology should confirm a resection margin of at least 2.5 cm at all directions.[30] Nevertheless, the Association of Directors of Anatomical and Surgical Pathology recommends that any margin macroscopically more than 5 cm is believed to be clear.[31,32] In our patients, the lesion was removed as widely or radically as possible. The margin was at least 7 cm above or below the limit of increased activity of the bone scan. The dissection was done subfascially, and all the muscles in the compartment were resected with the tumor. At the level of the bone osteotomy, the muscles were severed.

We know that preserving critical neurovascular and musculoskeletal structures will allow the patient to maintain maximal limb function.[11] Thus, a balance between resection and preservation is of great importance. In general, the main factor leading to amputation is the direct invasion of a major nerve while the vascular structure or bone involvement can often be reconstructed relatively easily after resection.[33] If a single nerve must be removed in order to maintain sufficient margin, the decision depends heavily on the function of the nerve. The sacrifice of a major nerve will result in profound functional deficit after the limb salvage surgery. Functional deficit is often unacceptable to the patient. Evaluation of peripheral nerves, both clinically and with imaging, is very important in the evaluation of patients with limb soft tissue sarcomas.[34] If an essential peripheral nerve is felt to be adjacent to a soft tissue sarcoma but not circumferentially surrounded, it can usually be salvaged by using the technique of epineural dissection. If, however, a critical nerve is circumferentially involved with a tumor, it must be sacrificed for the sake of local control. It is important to inform the patient that normal limb function is not possible. The issue of nerve involvement therefore becomes a critical factor in determining the possibility of limb salvage in borderline cases.[34] In our study, we were well prepared for autologous nerve grafting if a nerve resection was done. We would use the sural nerve as a donor graft for peripheral nerve reconstruction. Luckily, only epineural dissection was needed as a means of preserving the major nerve when it was closely applied to the sarcoma in all cases. Therefore, because of nerve preservation, the influence on limb function was relatively small. Acceptable functional results were relatively easy to obtain.

In patients with a humerus malignant tumor, it is frequently likely to perform curative resection which spares the limb. However, reconstruction including autogenous grafts, allografts and prostheses remains a problem. It has been reported that prosthetic reconstruction has many advantages. Reconstruction with prosthesis offers immediate distal fixation and enables earlier chemotherapy after surgery. It is usually the least time-consuming option available. The prosthesis offers a constant pivot for hand and elbow function and limits pain from traction on the neurovascular bundle.[35] The modular prostheses could regain the length of resected bone easily. Moreover, with the improvement of the materials and design, modern prostheses achieve the primary aim of providing long-term function for some patients with relatively low physical demands.[36] Thus, the objectives of limb reconstruction after oncologic resection are achieved, including providing stability, optimizing the aesthetic outcome and preservation of functional capability with early return to function.

An important factor in the selection method for patients with limited life expectancy is a low rate of complications. With fewer complications and failures, prosthetic reconstruction surgery for metastatic bone disease may be preferable. But oncological reconstruction with the prostheses may appear to have higher complication rates compared with standard total joint arthroplasty due to the extensive nature of the operation, extensive tissue loss and the compromising effects of associated radiotherapy and chemotherapy. The most common complications in prosthetic reconstruction surgery are postoperative infection, prosthetic loosening, periprosthetic fractures and dislocation. The published literature reports a low rate of infection in patients undergoing proximal humerus replacement. Mayilvahanan reported an infection rate of 3.5%.[37] In our series, probably because of the limited number of patients, none of the aforementioned complications occurred. We knew that these patients might be unable to undergo revision surgery due to poor general health with progressive disease. More careful management was done compared with standard prosthesis replacement.

Self-respect, body image, impacts on education and employment opportunities are other important issues which should also be examined in patients who undergo either limb salvage surgery or amputation.[13] The amputation of the upper arm would probably destroy the mental strength of the patient completely. For that reason, the preservation of the upper arm might be more emotionally acceptable than amputation, and the optimal method was en bloc resection and prosthesis implantation. It offered good stability, no pain mobility and overall satisfactory functional outcome in our patients.

Our study is subject to a number of limitations. Firstly, it includes only a small number of patients and lacks a control group. Secondly, our series of tumors was not

homogeneous with respect to tumor location within the humerus, the stage and the adjuvant treatment. Thirdly, different procedures for soft tissue reconstruction were used, according to the specific situation. Fourthly, the follow-up period was too short in some patients to draw long-term conclusions regarding function and survival of the reconstruction.

Conclusion

For patients with humerus MFH, limb salvage procedures with chemotherapy is a practical treatment choice. The therapy not only improves quality of life but also offers a serviceable limb.

Reports of humerus MFH management are limited and contain only a small number of samples. Multicenter studies are needed to acquire statistically significant data that will be of great help for this patient population.

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Total antioxidant capacity in Mediterranean β-thalassemic patients

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Abstract

Background. Beta thalassemia major (BT) is an inherited blood disorder caused by reduced or absent synthesis of the hemoglobin beta chains, associated with profound anemia, jaundice, splenomegaly, expanded bone marrow volume, siderosis and cardiomegaly. Because of repeated blood transfusions, BT patients are subjected to peroxidative tissue injury due to secondary iron overload.

Objectives. The aim of the study was to analyze: 1) the total antioxidant capacity (TAC) value in BT patients (study group) and their healthy controls (control group) from Greece (Central Macedonia) and Italy (Sardinia); correlations between 2) the TAC and ferritin levels of BT patients, and 3) the TAC and ferritin values in BT patients with different chelation therapies;

Material and methods. The studied group consisted of 60 subjects diagnosed with BT (41 female, mean age: 41.5 ± 9.5 years) and 40 healthy controls matched with age and sex (31 female, mean age: 38.5 ± 3.7 years). Desferrioxamine (DFO) was the basic previous chelation regimen for all BT patients. Antioxidant activity was assayed spectrophotometrically, using a TAC Kit (Total Antioxidant Capacity Colorimetric assay kit, produced by Cayman Chemical Co.), and ferritin was assayed by immunoturbidimetry.

Results. Lower levels of TAC were observed in BT patients of both countries when compared with controls (1.83 mmol/L vs 2.7 mmol/L in the Italian study group and controls and 2.42 mmol/L vs 3.2 mmol/L in the Greek study group and controls). There were no significant correlations between plasmatic TAC and ferritin. Furthermore, deferasirox was the only chelation treatment in which TAC showed a correlation in both regions.

Conclusions. Our results potentially suggest that the reduced levels of TAC detectable in BT patients could demonstrate their reduced antioxidant defensive mechanisms.

Key words: oxidative stress, β-thalassemia major, total antioxidant capacity, chelation therapies, Mediterranean countries

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Transfusion practice and chelation therapy are the cornerstones of the modern clinical management of patients with beta thalassemia (BT) major, and have substantially contributed to improving the outcomes in such patients.[1] Nevertheless, iron overload remains a common complication, especially after long-term treatment.[2,3] On the one hand, it has been evident from previous studies that iron overload is the main causative agent responsible for the increased production of free radicals and reactive oxygen species (ROS), and the subsequent oxidative stress which is compensated for by various antioxidants present in the body.[4] Oxidative stress occurs as a result of increased levels of lipid peroxides and free-radical intermediates, along with a decrease in total antioxidant capacity (TAC).[5] On the other hand, the selection of proper iron chelatory agents can be helpful in the regulation of the antioxidant status in patients with BT major. Currently, there are 3 main iron-chelating agents available for continuous use in patients with thalassemia on regular transfusions, i.e. desferrioxamine (DFO), deferiprone (DFP) and deferasirox (DFX), which provide good results in reducing cardiac, hepatic and endocrine toxicity.[6] Historically, the most widely-used chelator is DFO, which demonstrated a significant reduction in the morbidity and mortality of these patients by preventing or reducing the damage to key organs such as the heart, liver and endocrine glands. Two new-generation chelators such as DFP and DFX, which can be administered orally, have substantially improved compliance and quality of life of these patients.[7] Oxidative stress and disturbances in the antioxidant balance have been studied extensively in BT major patients.[8–11] The main purpose of this study was to investigate if there is any correlation between the plasmatic TAC and ferritin blood levels in BT patients of 2 Mediterranean regions such as Central Macedonia and Sardinia. We also evaluated the possible impact of different iron chelators in this setting.

Material and methods

Patients and controls

Table 1 has all the demographical and clinical characteristics of the study and control groups. Sixty β-thalassemic major individuals were included in the study: 30 Italian patients from Sardinia (group A, Servizio Trasfusionale, Ospedale Santissima Annunziata, Sassari), and 30 Greek patients from Central Macedonia (group B, Saint Paul General Hospital of Thessaloniki, Greece). The mean age of the patients was 42 and 41 (range 25–65) years in the 2 groups, respectively. There were 19 (63%) females in group A (Italians) and 22 (73%) in group B (Greeks). All subjects had given informed consent. The diagnosis of β-thalassemia major was made considering the results of hemoglobin electrophoresis and the clinical features of the patients. BT minor and intermediate cases were excluded. In addition, 26.6% of the study group was represented by healthy carriers of HCV. The Italian and Greek patients were receiving an average dosage of iron chelators of 74/5 mg/Kg and 66.6 mg/kg, respectively, while they had been under chelation therapy for an average of 33.5 and 37.1 years, respectively. All the patients who underwent long-lasting transfusion therapy were under chelation therapy with one or more of the following medications: DFX, DFP or DFO. Six subgroups were considered in relation to the treatment modality, group A1 for the Italian study group using DFX (71% female, mean age: 42.8 ± 12.8 years); A2 for those using DFP (85% female, mean age: 39.3 ± 10 years); A3 for the Italian BT patients using DFO (50% female, mean age: 43.7 ± 4.6 years); and A4 for those under DFX + DFO (50% female, mean age: 42.85 ± 7.7 years) chelation treatment. The Greek study group B1 for the patients using DFX (72.7% female, mean age: 44 ± 10.3 years) and B2 for those chelating with DFP (73.6% female, mean age: 37 ± 7.3 years). Forty age- and sex-matched healthy subjects were enrolled in the study: 20 Italians (75% female, mean age: 38.75 ± 3.8 years) from Sardinia and 20 Greeks (80% female, mean age: 38.2 ± 3.65 age) (Central Macedonia). None of the study group and the control group members received any dietary supplementation for at least 6 months before the analysis.

Blood sample collection

Blood samples were obtained in the morning, before transfusional therapy, in EDTA-containing tubes. The average monthly transfusion for group A was 4.4 ± 0.5 times and for group B, 3.2 ± 0.9 times. The samples were centrifuged to divide the cellular components from the plasma, and stored at -80°C until Trolox Equivalence Antioxidant Capacity (TEAC) was carried out.

Measurement of parameters

Antioxidant activity was assayed spectrophotometrically, using a TAC Kit (Total Antioxidant Capacity Colorimetric assay kit, produced by Cayman Chemical Co., Ann Arbor, USA) with the TEAC method in the clinical chemistry laboratory of the Alexander Technological Educational Institute, Thessaloniki, Greece. The levels of ferritin in both groups of patients had been measured previously by immunoturbidimetry. This technique is based on latex bound ferritin antibodies which react with the antigen in the sample to form an antigen/antibody complex, this is then measured turbidimetrically after agglutination. The obtained turbidity is proportional to the ferritin concentration and is determined at 700 nm. Ferritin determinations were carried out in the biochemical laboratory of the Saint Paul General Hospital, Thessaloniki, Greece.

Table 1. Demographical and clinical characteristic of study and control groups

Parameters	Italian study group	Greek study group	Italian controls	Greek controls
Thalassemic major patients	30	30	20	20
Age (average)	42 (± 8.9)	41 (± 10.2)	38.2 (± 3.65)	38.75 (± 3.8)
Sex, female/male	19/11	22/8	15/5	16/4
HCV, positive *	2	14	–	–
Monthly transfusion	4.4 (± 0.5)	3.2 (± 0.9)	–	–
Average dosage of Iron chelators (mg/kg)	74.5 (± 32.1)	66.6 (± 30.9)	–	–
Average years under chelation therapy	33.5 (± 5.8)	37.1 (± 7.07)	–	–

The values represent mean ± standard error of the mean.

Table 2. Mean Total Antioxidant Capacity and ferritin levels of study group and control subjects

Part 1. Mean Total Antioxidant Capacity in control subjects

Study groups	Mean TAC (mmol/L) (n = 20)
Italian controls	2.7 (± 0.7)
Greek controls	3.2 (± 0.5)
Mean controls*	2.95 (± 0.6)

*p = 0.007.

Part 2. Mean Total Antioxidant Capacity and ferritin levels of study group

Study groups	Mean TAC (mmol/L) (n = 30)	Mean ferritin (ng/mL) (n = 30)	r-value	p-value
Italian study group	1.83 (± 0.6)	1817 (± 72.2)	.115	.546
Greek study group	2.42 (± 0.5)	1084 (± 161.15)	.73	.700
Mean study group*	2.125 (± 0.55)	1450 (± 116.675)	–	–

*p = 0.007.

Results

The results of the study are shown in Tables 2 and 3. Plasmatic TAC appeared to be significantly higher in control subjects compared to the study groups (p = 0.007). No correlation existed between TAC and ferritin in either of the study groups (r = 0.115; p = 0.546, r = 0.73; p = 0.700). Interestingly, there were positive correlations in the study subgroups based on their various chelation therapies. We observed positive correlations in the measurements of TAC between Italian study subgroups A1 and A3 (r = 0.682; p = 0.046), and also between A1 and B1 (r = 0.681; p = 0.046). Clearly, patients of both countries that are using deferasirox represent a good model of study because of their statistically significant results. Indeed, significant results were also observed between the ferritin measurements of study subgroups A2 and A3 (r = 0.663; p = 0.036). Furthermore, a notable inverse correlation was seen between the ferritin levels and age of the Italian subgroup A3 (r = -0.775; p = 0.024). Plasmatic TAC measurements of the patients under desferrioxamine showed statistically significant results without any statistically significant correlations with ferritin.

Discussion

Oxidative stress in BT patients activates various antioxidant enzyme systems to protect the body tissues from its damaging effects. Enzymatic and non-enzymatic antioxidants, either endogenous or exogenous, are known to counteract the deleterious effects of ROS (reactive oxygen species) and RNS (reactive nitrogen species), leading to protection against oxidative stress and RNS stress. ROS formation inside red blood cells (RBCs) is almost entirely due to methemoglobin (metHb) formation, but under normal steady-state conditions the RBC antioxidant systems can cope with such a threat.[12,13] In thalassemia, iron mediated ROS formation, originating from imbalanced globin production and regular blood transfusions, can cause systemic tissue damage.[7,14] Antioxidant status can be investigated individually by direct measurement inside the RBC of cytoprotective enzymes such as superoxide dismutase (SOD) and glutathione peroxidase (GSH-Px).[15–18] In our experiments, we chose to measure TAC in plasma, as some studies suggest that assessing total plasmatic antioxidant capacity is more useful than measuring antioxidant individually, since their synergistic interactions could be determined.[9,19,20]

In the present study, we measured TAC in Greek and Italian patients, considering the high prevalence of BT

Table 3. Total Antioxidant Capacity and ferritin levels of BT patients based on their chelation therapy

		DFX (A1) (n = 7)	DFP (A2) (n = 7)	DFO (A3) (n = 8)	DFP + DFO (A4) (n = 8)
Italian study group (A)	TAC (mmol/L)	1.75 (± 0.07)	1.63 (± 0.17)	1.59 (± 0.04)	2.03 (± 0.13)
	FERRITIN (ng/ml)	2061 (± 77)	1627 (± 43)	2202 (± 95)	1396 (± 103)
	p-value	.439	.695	.212	.579
	r-value	.352	.182	.495	.233
		DFX (B1) (n = 11)	DFP (B2) (n = 19)		
Greek study group (B)	TAC (mmol/L)	2.63 (± 0.10)	2.24 (± 0.14)		
	FERRITIN (ng/mL)	1047 (± 298)	1106 (± 194)		
	p-value	277	.071		
	r-value	.360	.496		

The values represent mean ± standard error of the mean.

Part 2. Positive Correlations based on their chelation therapy

	A1+A3 (TAC)	A1+B1 (TAC)	A2+A3 (FERRITIN)
p-value	.046	.046	.036
r-value	.682	.681	.663

in these regions as well as the lack of studies analyzing and comparing the antioxidant status of patients from these Mediterranean countries. A single study by Hamed et al. showed a significant decrease in TAC in Italian BT patients when compared to normal controls.[21] Our study confirmed the decreased levels of TAC not only in Italian but also in Greek BT patients. A different study by Bazvand et al., performed in Iranian patients on serum, showed increased antioxidant status in BT patients. A possible explanation for this unexpected antioxidant status detected in Iranian BT patients could be the different age range (14.7 ± 6.9) and/or chelation regimen.[9]

The reduced TAC of BT patients might be due to an increased utilization of antioxidants in order to counterbalance the effects of ROS, which may be involved in the pathological consequences of BT major and contribute to the gradual development of organ damage.[10] The study would be strengthened if TAC evaluation was used in conjunction with other oxidative stress and antioxidant defense biomarkers, such as F2-isoprostanes.[26] However further investigations are needed in order to gain a better understanding about its biological and therapeutic implications. In particular, prospective studies are needed to validate the use of plasmatic TAC in this clinical setting, as a useful tool to predict the risk of free radical-induced tissue damage.

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Evaluation of the anterior chamber angle in pseudoexfoliation syndrome

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Abstract

Background. Pseudoexfoliation syndrome (PEX) is the most frequently identifiable cause of secondary open-angle glaucoma, known as pseudoexfoliation glaucoma. The exact pathophysiology and etiology of PEX and associated glaucoma remains obscure.

Objectives. The purpose of this study was to determine the differences in the morphology of the anterior chamber angle in people with pseudoexfoliation syndrome and pseudoexfoliation glaucoma compared to a control group. We also evaluated the correlation between intraocular pressure (IOP) and pigmentation of the angle with the amount of exfoliated material in the anterior segment.

Material and methods. The study group was composed of 155 eyes from 103 patients aged between 43 and 86 years. Each patient underwent a complete ophthalmological examination.

Results. Some difference was found in intraocular pressure between the PEX group and the control group and between the pseudoexfoliation glaucoma group and the control group, but no significant difference was found between the 2 study groups. There was a significant difference in the incidence of some degree of pigmentation in the anterior chamber angle and no difference in the widths of the angle between each group. A significant positive relationship was observed between intraocular pressure and the degree of pigmentation of the anterior chamber angle in both the PEX group and the pseudoexfoliation glaucoma group.

Conclusions. The results of this study indicate that the amount of pigmentation and exfoliation material in the anterior segment significantly correlates with the level of IOP and possibly with the degree of trabecular dysfunction. It seems that for clear identification of PEX and pseudoexfoliation glaucoma factors, clinical assessment appears to be insufficient.

Key words: pseudoexfoliation syndrome, PEX, pseudoexfoliation glaucoma, PEXG, morphology of the ocular angle

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Pseudoexfoliation syndrome (PEX) is an age-related systemic disease which is characterized by excessive production and accumulation of amyloid-like protein material caused by the degeneration of elastic fibers of the connective tissue. Exfoliation material (EXM) is produced by pathological cells of the connective tissue. It accumulates in the anterior segment of the eye and in other tissues outside the eyeball, for example in blood vessels, the heart, lungs or kidneys. PEX occurs worldwide, but its geographical distribution is differentiated. It is estimated that approximately 70 million people worldwide suffer from PEX.[1,2]

PEX is the most frequently identifiable cause of secondary open-angle glaucoma, known as pseudoexfoliation glaucoma (PEXG).[3,9] However, the conversion agent from PEX to PEXG still remains unknown—glaucoma is identified in about 30–40% of PEX cases.[3,10,11] The prognosis of glaucoma associated with PEX is generally worse in comparison with other types of glaucoma due to its more dynamic course. It may be characterized by high intraocular pressure, which can imitate an attack of acute angle-closure glaucoma, although the angle is usually open.[3,10] Treatment of PEXG is considered to be difficult and often leads to failure due to an unsatisfactory response to treatment or a large number of cases with higher than expected intraocular pressure (IOP) in glaucoma and its diurnal variation.[11,12]

Despite intensive research and medical progress, the exact pathophysiology and etiology of PEX and the glaucoma which is associated with this syndrome still remain obscure.[3–5] The development of PEXG likely has a multifactorial substrate. The pathological formation of elastosis leads to a disorder in the structure of Schlemm's canal, the trabecular meshwork and the cribriform plate of the ethmoid bone. Moreover, vascular changes in the described rebuilding cause insufficient blood circulation in the front part of the optic nerve. Additionally, mechanical obstruction by the EXMs and pigment grains released from the iris pigment epithelium block the drainage of the aqueous humor during mydriasis.[13]

Due to the fact that PEX is an important indicator of the possibility of glaucomatous optic neuropathy, and that no factor leading to the conversion from PEX to PEXG has been identified so far, finding its symptoms requires regular examination.[3,13] Discovering symptoms which occur before the conversion takes place could be very helpful in deciding whether to examine a patient more frequently or to implement glaucoma treatment earlier.

The purpose of this study was to determine the differences in the morphology of the anterior chamber angle, examined and described through gonioscopy, in people with pseudoexfoliation syndrome and pseudoexfoliation glaucoma compared to a control group. We also evaluated the correlation between the level of intraocular pressure and the pigmentation of the angle with the amount of EXM in the anterior segment.

Another aspect was to determine the differences in the angle obtained by an optical coherence tomography (OCT) test of the anterior segment of the eye after it adapts to darkness.

Material and methods

This prospective, controlled study was approved by the Regional Ethical Review Board and was conducted in accordance with the principles set forth in the Guidelines for Good Clinical Practice and the Declaration of Helsinki (and its amendments).[14]

The study group was composed of 155 eyes from 103 patients (77 women and 26 men) aged between 43 and 86 years (the average age was 68.83 ± 9.68). The patients were consecutively selected and assigned to one of the 3 groups:

1) a control group of healthy patients (C): 35 people, 63 eyes;

2) a group of patients with pseudoexfoliation syndrome (PEX): 38 people, 50 eyes; or

3) a group of patients with pseudoexfoliation syndrome and glaucoma (PEXG): 30 people, 42 eyes.

The groups did not differ significantly in terms of age and gender make-up, but women outnumbered men in all groups. The surface of the optic disc and visual acuity showed no statistically significant differences between the groups (Table 1).

Each patient underwent a complete ophthalmological examination comprising an interview, anterior segment slit lamp biomicroscopy before and after dilation of the pupil and measurement of intraocular pressure (IOP) by Goldmann applanation tonometry – the average of 3 measurements was used for further calculations. Additionally, central corneal pachymetry (Opticon 2000) and optical coherence tomography (OCT) (Visante 1000) were performed. In gonioscopy (Zeiss gonioscopy), the angle was divided into 4 quadrants and classified according to the Spaeth Grading System. The amount of pigment was evaluated according to the following scale:

- 0 – none,
- I – some browning,
- II – firm brown pigmentation,
- III – dark brown or black pigmentation, presence or absence of Sampaolesi line.

The amount of exfoliation material was reported according to the following scale:

- 0 – none,
- I – exfoliation confined to the periphery of the lens and not seen unless the pupil is dilated,
- II – exfoliated material on the edge of the iris or on the surface of the lens capsule or both,
- III – grade II and exfoliated material in the angle.

In order to compare the width of the anterior chamber angle, measured through the use of gonioscopy and OCT, the average value of angular widths on 3. and 9. h was used.

Table 1. Comparative analysis of the study groups: control (C), patients with pseudoexfoliation syndrome (PEX), patients with pseudoexfoliation syndrome and glaucoma (PEXG)

34.	Study groups			p-value
	C	PEX	PEXG	
Number of eyes	63	50	42	
Gender (woman /man)	28(80%) / 7(20%)	27(28.9%) / 11(71.1%)	22(26.7%) / 8(73.3%)	0.15
Visual acuity	0.98 ± 0.04	0.97 ± 0.05	0.92 ± 0.12	0.10
Surface of the optic disc	2.29 ± 0.37 mm^2	2.39 ± 0.44 mm^2	2.43 ± 0.45 mm^2	0.16

Table 2. Incidence of Sampaolesi line in C, PEX and PEXG groups

Group	C	PEX	PEXG
-	63	35	25
%	100.00%	65.00%	60.52%
+	0	15	17
%	0.00%	35.00%	39.48%

Table 3. Occurrence of the specific attachment of the iris

Type	Groups		
	C	PEX	PEXG
D	43	28	28
%	68.25	56.00	66.67
C	17	12	8
%	26.98	24.00	19.05
B	3	10	6
%	4.76	18.00	14.29
A	0	1	0
%	0.00	2.00	0.00

Statistical analysis

The parameters (IOP, degree of pigmentation, AOD 500 and 750, TISA 500 and 750, widths of the angle) were compared using a non-parametric Kruskal-Wallis test and a multiple comparison Kruskal-Wallis test. The qualitative data (incidence of Sampaolesi line, occurrence of pseudoexfoliation material, occurrence of specific attachments of the iris and iris configuration) were compared using a χ^2 test. Specific attachment of the iris and iris configuration were recorded as percentages. A Spearman's rank correlation test was used to examine correlation between variables. The results were considered statistically significant at a p-value of less than 0.05. All calculations were carried out using STATISTICA v. 9.0 software. (StatSoft Inc., Tulsa, USA).

Results

A statistically significant difference ($p < 0.0001$) was found in IOP between the PEX and C groups, and between the PEXG and C groups, while there was no statistically significant difference between the PEX and PEXG groups ($p = 0.1778$)(Fig. 1).

There was a difference in the incidence of some degree of pigmentation of the anterior chamber angle between the PEX and C groups ($p < 0.0001$), the PEXG and C groups ($p < 0.0001$), and between the PEX and PEXG groups ($p = 0.0012$) (Fig. 2). A difference was found in the incidence of Sampaolesi line between the groups ($p < 0.0001$) (Table 2).

A difference was noted in the occurrence of pseudoexfoliation material between the tested groups ($p < 0.0001$). The control group showed an absence of the material, while a similar occurrence was observed in PEX and PEXG groups ($p = 1$) (Fig. 3).

There was no difference in the frequency of specific attachment of the iris between the PEX and PEXG groups ($p = 0.2343$) (Table 3).

Iris configuration differed significantly among the tested groups ($p = 0.0094$): configuration S was the most frequent configuration in the PEX and PEXG groups (14% and 11%, respectively), but the rarest in the control group (approx. 5%) (Table 4).

A significant, strong positive relationship was observed between IOP and the degree of pigmentation of the anterior chamber angle in the PEX group ($p < 0.0001$, $R = 0.729$) (Fig. 4a), as well as in the PEXG group ($p < 0.0001$, $R = 0.598$) (Fig. 4b).

There was a significant, strong positive relationship between IOP and the amount of pseudoexfoliation material on the front surface of the lens capsule and in the anterior chamber angle in the PEX group ($p < 0.0001$, $R = 0.7011$) (Fig. 5a) and a significant positive correlation in the PEXG group ($p < 0.0029$, $R = 0.4479$) (Fig. 5b).

Fig. 1. Distribution of intraocular pressure found in C, PEX and PEXG groups

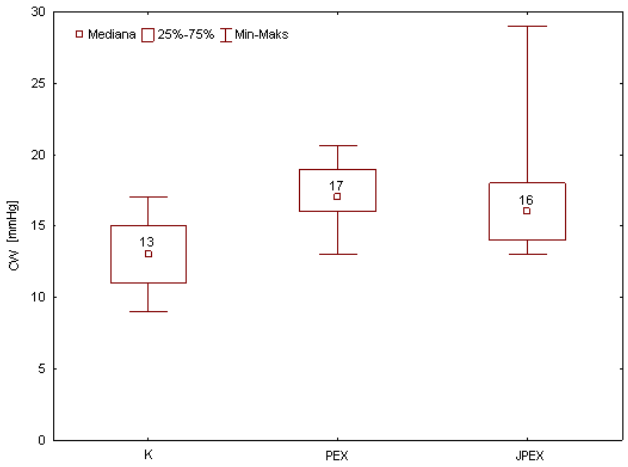


Fig. 2. Distribution of the degree of pigmentation of the angle in C, PEX and PEXG groups

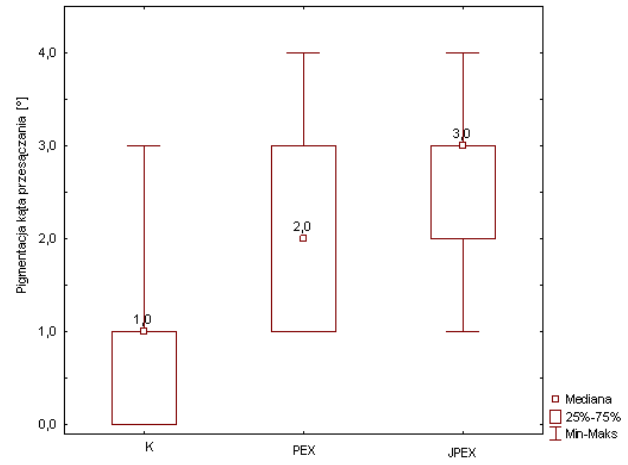


Fig. 3. Comparative summary of the degree of exfoliation in the anterior segment in C, PEX and PEXG groups

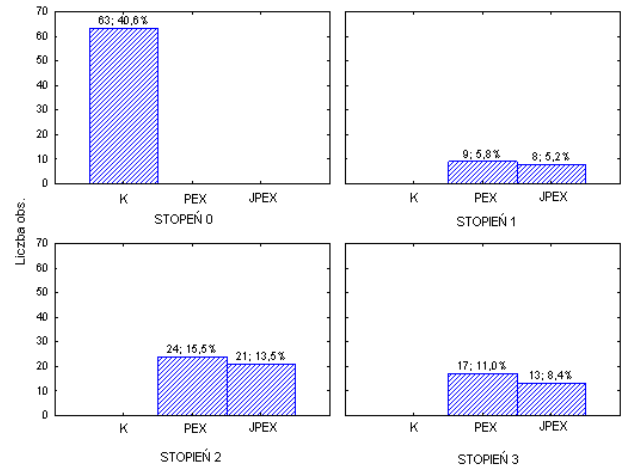


Fig. 4a. Intraocular pressure (IOP) and degree of pigmentation in PEX group

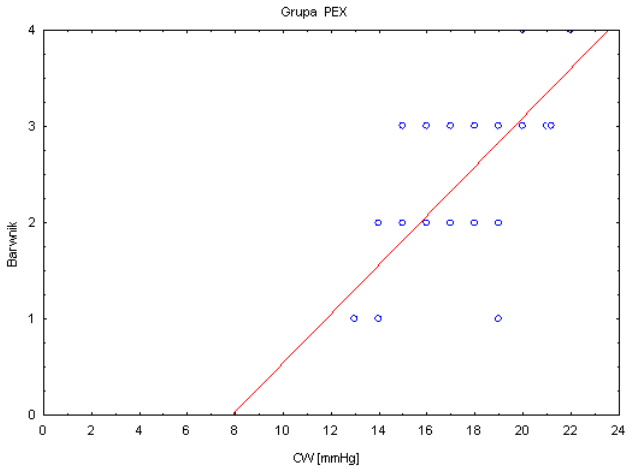
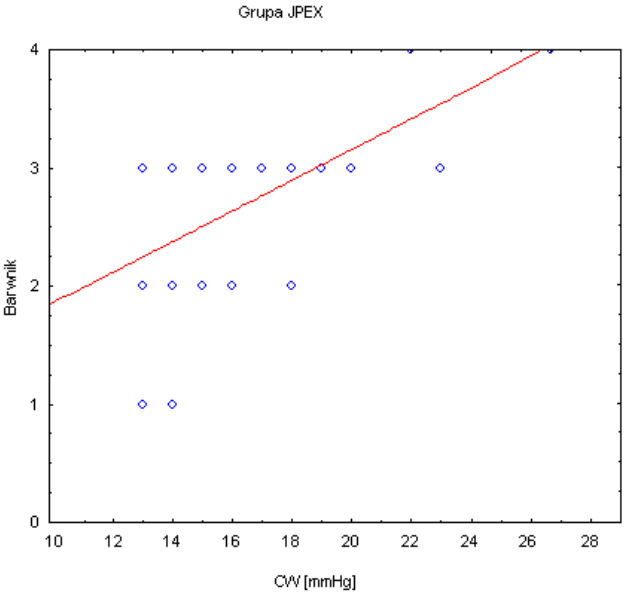


Fig. 4b. Intraocular pressure (IOP) and degree of pigmentation in PEXG group



There was no statistically significant difference in the values of AOD at 500 µm and 750 µm and TISA at 500 µm and at 750 µm, measured from the temple and from the nose by OCT Visante, between the C, PEX and PEXG groups (p = 1).

There was no statistically significant difference between the widths of the anterior chamber angle in the C, PEX and PEXG groups as measured by OCT Visante (p = 0.8081) nor by gonioscopy (p = 0.8469).

Discussion

In our study, no statistical difference was found in the amount of EXM between the PEX and PEXG groups, which may suggest that a higher amount of EXM does

Fig. 5a. IOP and amount of pseudoexfoliation material on the front surface of the lens capsule and in the angle in the PEX group

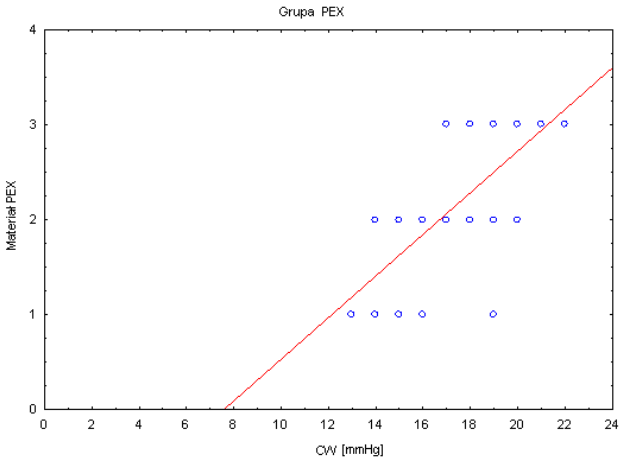
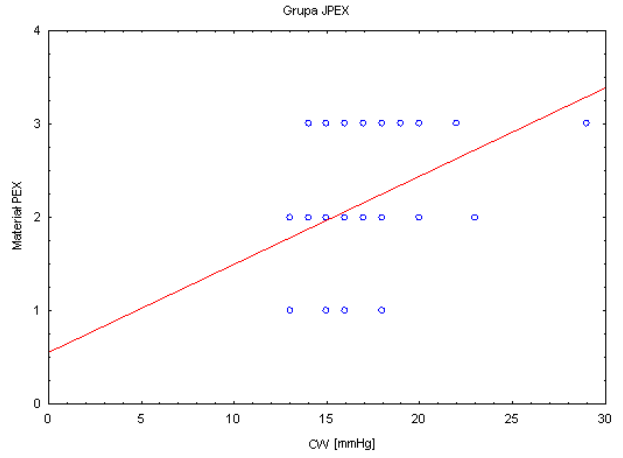


Fig. 5b. IOP and amount of pseudoexfoliation material on the front surface of the lens capsule and in the angle in the PEXG group



not necessarily determine glaucoma conversion. However, a positive correlation between the amount of EXM and the level of IOP was found in both the PEX and PEXG groups. A difference in the frequency of some degree of pigmentation was observed between groups C and PEX, between C and PEXG and between PEX and PEXG. The differences between groups were statistically significant, which suggests the importance of this parameter in distinguishing different groups. A statistically significant correlation also occurred between the amount of pigment and IOP in the PEX and PEXG groups.

One of the main risk factors for the development of both primary angle-closure glaucoma (POAG) and PEXG is an increased level of IOP.[7,16] There were statistically significant differences ($p < 0.0001$) between the PEX and C groups and between PEXG and C. IOP was found to be significantly higher in the groups with PEX and PEXG compared to the control group, whereas there were no statistically significant differences between the study groups ($p = 0.1778$). This finding could suggest quite a good response to treatment in patients from the PEXG group; however, the interview shows that 57.2% of patients with PEXG (24 patients) experienced increases of IOP above 21 mm Hg despite the treatment.

Four patients (11.4%) had IOP higher than 22 mm Hg despite several modifications to the treatment. Many researchers have also observed a statistically significantly higher IOP in patients with PEX compared to the control group.[2,3,15,17,18] Klemetti et al. pointed out that the initial average IOP in eyes with PEX is important in the conversion from PEX to PEXG. They noticed that the eyes with PEX which developed ocular hypertension or glaucoma later in the course of disease had had a significantly higher IOP ($p < 0.001$) compared to the eyes in which there was no development of glaucoma.[19]

Grodum et al. suggested that conversion to glaucoma was twice as fast in patients with PEX and ocular hypertension compared with healthy people, properly selected by gender and age. They also argued that an elevated level of IOP during diagnosis seemed to be one of the strongest risk factors for conversion from PEX to PEXG.[10] Konstas et al. demonstrated the benefits of lowering IOP and suggested that maintaining it below 17 mm Hg in patients with PEX reduced the probability of conversion and could have slowed the progression of glaucomatous optic neuropathy that had already occurred.[19]

A large diurnal fluctuation in IOP seems to be one of the most important risk factors of PEXG.[18,21,22] Nenciu et al. noted that it was much higher in patients with PEX than the physiological variability in healthy individuals.[21]

Another problem related to PEX is the possibility of an increased level of IOP during diagnostic pupil dilation, due to the scattering of grains of pigment and the increase associated with the change of body position.[23,24] These aspects, however, were not examined in this study. The influence of elevated IOP in the case of PEXG may be higher than in the case of POAG because changes in elastosis formation in the cribriform plate of the ethmoid bone and in orbital vessels may cause increased sensitivity to optic nerve damage in comparison with the control group at the same age.[7,13] The increase of IOP and its diurnal variation appears to be an important prognostic factor occurring before the conversion from PEX to PEXG.

The correlation between increased pigmentation and the presence of PEX and PEXG has also been described by others.[25,26] Puska et al. have found that eyes with PEXG show more pigmentation in the angle than eyes with glaucoma but without PEX. In addition, they noted that pigmentation was stronger in eyes with PEXG than in eyes with PEX, although the amount of EXM was similar. Moreover, they showed a positive correlation between IOP and the degree of pigmentation as well as between IOP and the amount of EXM. Because the statistical significance for the latter was smaller, it was concluded that the main risk factor for glaucoma seems to be the degree of pigmentation of the angle glaucoma.[24]

Moreno-Montañés et al. observed a statistically significant relationship between the pigmentation of the anterior chamber angle and IOP, while they found no relationship between the amount of EXM and the level of IOP.[26] On the other hand, it is not known whether the degree of angle pigmentation and the amount of EXM correlates with the severity of glaucoma.[27] Shuba et al. presented a positive correlation between pigmentation of the angle and IOP ($p = 0.047$) and no correlation with the severity of glaucoma ($p > 0.13$). There was no significant correlation between the amount of EXM and the level of IOP, nor a correlation with glaucoma severity indicators ($p > 0.04$).[28] Differences in these correlations may result from the classification system used in this study to determine the amount of EXM. The authors evaluated the amount of material on the front surface of the lens, which may not reflect the actual amount of material that blocks the drainage of the anterior chamber angle.[3,29] Despite the absence of histological examination (clinical only), our results suggest that the amount of pigment and EXM in the angle can be a more important factor in the growth rate of IOP than the presence of PEX material in the anterior lens capsule only. From a clinical point of view, this highlights the importance of gonioscopy in the eyes of patients with PEX.

A Sampaolesi line was observed in both study groups, PEX and PEXG, and the difference between the groups was not statistically significant ($p > 0.05$). This finding confirms that the Sampaolesi line is associated with the presence of pseudoexfoliation syndrome, but does not determine the presence of glaucoma.[27]

PEXG is classified as secondary open-angle glaucoma. However, a higher incidence of narrow- or closed-angle glaucoma in patients with PEX was reported: 18% of patients had narrow-angle, 14% had closed-angle, and 37.83% of patients had angle closure.[30,31] Due to the differences in the width of the angle, we attempted to verify the incidence of narrow angles using the OCT apparatus of the anterior segment because, despite the obvious advantages of gonioscopy in the estimation of the width of the angle, it is quite a subjective examination and the result may be affected, for example, by light intensity, by the force which gonioscopy exerts on the cornea or by the experience of the examiner.[32] There was no statistical difference between the widths of the angle in the 2 groups measured by OCT ($p > 0.1$). In all groups, the angle was open. Although several characteristic features could predispose a patient to angle-closure glaucoma in PEX (instability of the lens zonules, periodic forward movement of the lens or a greater tendency to form rear adhesions), the higher incidence of narrow angles in PEX patients examined by gonioscopy and OCT did not confirm this assumption.[29] Analyzing the parameters of the angle in OCT, there was no statistical difference between the parameters AOD500, AOD750 TISA750 and TISA500. Even though the diameter of the pupil in the dark in the PEX and PEXG groups was similar, it was significantly lower than in the control group ($p = 0.012$). Zheng et al. also found no statistical difference in ACA, AOD500, TIA500 and TISA500 in patients with PEX compared to healthy individuals examined in a dark room.[33] Impairment of mydriasis in the PEX and PEXG groups was apparent, probably associated with an increased firmness of the iris (due to the accumulation of PEX material in the stroma), but the parameters of the angle were not statistically significant. It should be remembered, however, that the image of the angle is affected by the anatomical structures of the anterior segment of the eye. Assessment of the angle was insufficient to fully interpret the results; therefore, further examination of the anterior segment through OCT is needed for patients with PEX.

Conclusions

The results of this study indicate that the amount of pigment and exfoliation material in the anterior segment significantly correlates with the level of IOP and possibly with the degree of trabecular dysfunction. This highlights the importance of performing gonioscopy in patients with PEX. We did not find any differences in the width of the angle or in the parameters measured by OCT among the groups.

It seems that in order to make a clear identification of the factors which would lead to the conversion from PEX to PEXG, clinical assessment, although extremely important, appears to be insufficient.

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Assessment of the primary stability of root analog zirconia implants designed using cone beam computed tomography software by means of the Periotest® device: An ex vivo study. A preliminary report

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

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Abstract

Background. The implant primary stability is a fundamental prerequisite for a success of osseointegration process which determines the prosthetic reconstruction time.

Objectives. The aim of the present study was to assess the quality and precision of modern conical bone computer tomography (CBCT) software in preparing root analog zirconia implants (RAZIs) by measuring its primary stability by means of the Periotest device.

Material and methods. Thirteen pig jaws with proper erupted first premolar (P1) teeth were used in the study. The CBCT examination was conducted in the area of the P1 tooth in each mandible. The 3-dimensional (3D) view of each tooth was designed from CBCT scan. The created 3D images were used to prepare root analog zirconia implants milled from a medical-grade zirconia block by means of laboratory milling. The RAZIs and titanium implants were placed into an alveolar socket after the tooth had been removed. The primary stability of the teeth before their extraction (G1), RAZIs (G2) and titanium implants (G3) were checked by Periotest devices.

Results. The mean results in PTV were: 15.9, 3.35, 12.7 for G1, G2 and G3 group, respectively. RAZIs during immediate loading achieved a significantly higher primary stability (lower Periotest value) as compared to the teeth and implants.

Conclusions. The modern CBCT device allows us to design a precise image of an extracted tooth for the purpose of manufacturing a root analog implant. The additional feature of the surgical protocol using RAZI is the possibility of avoiding the augmentation procedure, which reduces the whole cost of the treatment.

Key words: RAZI, primary stability, root analog zirconia implant, Periotest

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The primary stability of dental implants, determined by the absence of mobility in the bone, corresponds with the mechanical coupling of an implant with the surrounding bone tissue promptly after implant insertion and represents a fundamental prerequisite for osseointegration.[1,2] Poor primary stability results in the formation of fibrous tissues in the interface between the implant and the bone, inducing bone resorption, which is one of the principal reasons for the implant failure.[3] Brunski claims that excessive loads and micromotion may disrupt the process of osteogenesis and endanger implantation success.[3] The development of traditional late-loaded implants in recent years has contributed to fewer problems with regard to primary stability and implant osseointegration.[4] Clinical studies including over 5 years of implant case studies show that 99.1% of implants in the mandible and 84.9% in maxilla undergo osseointegration, which is a part of a fully functional prosthetic reconstruction.[4]

Nevertheless, the situation differs in relation to immediately implantation. Immediate implant placement involves the insertion of implants immediately after surgical extraction of the teeth to be replaced.[2] This approach has several advantages, such as the preservation of the alveolar width and height, reduction of bone resorption and limitation of soft tissue trauma, decrease in the treatment time and avoidance of a second surgical intervention, resulting in cost reduction and improvement of patients' comfort.[2,5–7] However, one of the most important drawbacks related to immediate implant placement is the difficulty in achieving primary stability.[2]

In order to provide sufficient primary stability in immediate or early loaded implants, researchers tried to compile different implantation systems.[5,6,8–11] Hodosh et al. were the first to propose an alternative to the traditional implant systems, i.e. the fabrication of a customized, dental root analog implant (RAI) using polymethacrylate.[6] However, the use of the preceding material caused the root analog to become encapsulated by soft tissue.[8] Therefore, root analog zirconia implants (RAZIs) and root analog titanium implants (RATIs) are being suggested for usage.[5,8] Both types of analogs are closely fitted to the socket but RATI has shown some disadvantages in clinical use.[8] Experimental studies with RATI have yielded successful results with evidence of osseointegration and clinical stability.[6,9] The clinical trial of a 1-month follow-up resulted in 100% primary stability at insertion, but after 9 months this implant system acquired the failure rate of 48%.[10]

In order to assure the implantation success, the researchers developed a novel approach to fabricate a zirconia dental root analogue implant (RAZI) using cone beam computed tomography (CBCT) scan and computer aided designing (CAD)/computer aided manufacturing (CAM) technologies.[8] With those two combined techniques it is possible to manufacture a RAZI with the best primary stability.[8] The combination of CBCT and CAD/CAM enables the fabrication of RAZIs as the perfect copies of the radicular units needed for implantation.[8]

The primary stability of RAZI designed using CBCT scan and CAD/CAM technologies can be measured by a variety of techniques, including: cutting resistance analysis (CRA), reverse torque test (RTT), resonance frequency analysis (RFA), impact hammer method.[12–24]

In the cutting resistance analysis (CRA) a torque gauge incorporated within the drilling unit is used to determine the implant insertion torque and to measure bone quality in various parts of a jaw.[13] The energy required for a current-fed electric motor to cut the bone during implant surgery is measured. This energy was shown to be significantly correlated with bone density and thus determined poor-quality bone, which has been suggested as one of the factors that significantly influences implant stability.[14] The major drawback of this technique is that it does not provide any information regarding bone quality until an osteotomy is performed.[15] Also, CRA cannot identify the lower limit of cutting torque value at which the implantation would end in failure.[15]

The resonance frequency analysis (RFA) is an electronic method for testing implant stability and was developed by Meredith et al.[16] RFA can be used to monitor the changes in stiffness and stability at the implant – tissue interface and to discriminate between successful implants and clinical failures.[17,18] Meredith et al. suggested that this test be executed at the implant placement as a basic reading for future comparison.[16] However, it is tricky to characterize a general standardized range of ISQ readings for a successful implant osseointegration for different implant systems.[19]

The reverse torque test (RTT) was first proposed by Roberts et al. and measures the crucial torque threshold, where bone-implant contact (BIC) was destroyed.[20] Nevertheless, this technique has been criticized as being destructive and it provides only information as to "all or none" outcome (osseointegrated or failed); thus, it cannot quantify the degree of osseointegration. Hence, RTT is mainly used in experiments.[21]

The impact hammer method incorporates the sound generated from a contact between a hammer and an object. The response detection is enhanced by various devices (microphone, accelerometer, strain gauge) and analyzed through fast fourier transform (FFT) in the form of physical properties.[22] This method is used in Periotest (Seimens, AG, Bensheim, Germany) and Dental mobility checker® (DMC).[21] The difference between the two is that DMC applies impact force with a hammer while the Periotest uses a metallic tapping rod in a handpiece, which is electromagnetically driven and electronically controlled. In the Periotest, response to striking is measured by an accelerometer and then analyzed. The signals produced by tapping are then converted to a unique value called the Periotest value (PTV), which depends

Fig. 1. The procedure of preparing the STL file of a tooth from a CBCT scan

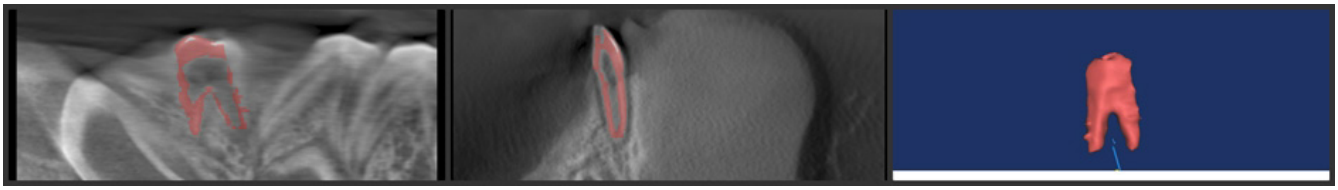
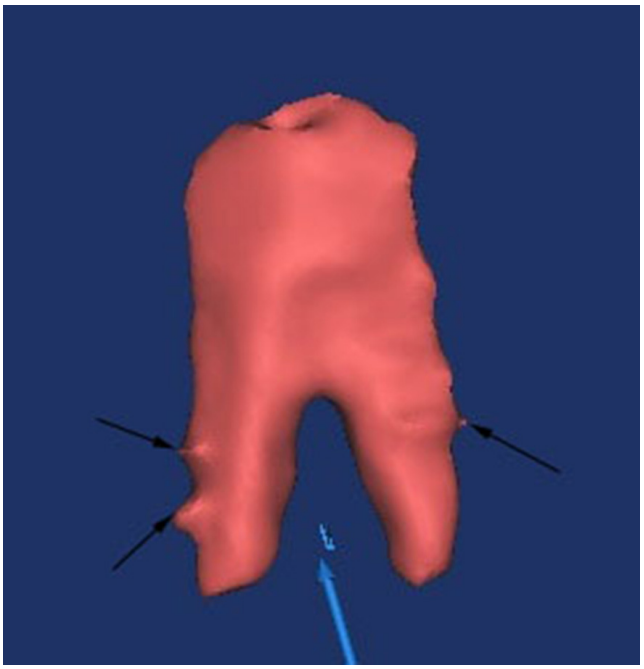


Fig. 2. The 3D view of a tooth. The blue arrow indicates a tooth and black arrows show macro-retentions of the tooth surface



on the damping characteristics of tissues surrounding teeth or implants.[24] According to Dilek et al., immediate loading can only occur if their PTV is in the range of -8–(+9), whereas Atsumi et al. narrowed the range to -4–(-2) and -4–(+2).[21,24]

The aim of the present study was to prove better primary implant stability during immediate placement of the root analog zirconia implants (RAZIs) in comparison to traditional tapered screw implant systems, on the basis of the Periotest values. RAZIs were constructed using the modern cone beam computed tomography (CBCT) scan and 3-dimensional (3D) root surface models.

Material and methods

Samples preparation

The research included 13 mandibles (n = 13) of recently slaughtered pigs, breed: Złotnicka Biała, intended for consumption and which had been obtained from a butcher. The specimens were included in the study due to the proper eruption of a first premolar (P1) tooth in the mandible, then each mandible had been prepared by the front incisors region being cut, washed under tap water and left for 4 h before the research was commenced. The specimens after preparation were placed motionless in a clamp. Ethical approval was not required for this animal ex vivo study.

Design procedure

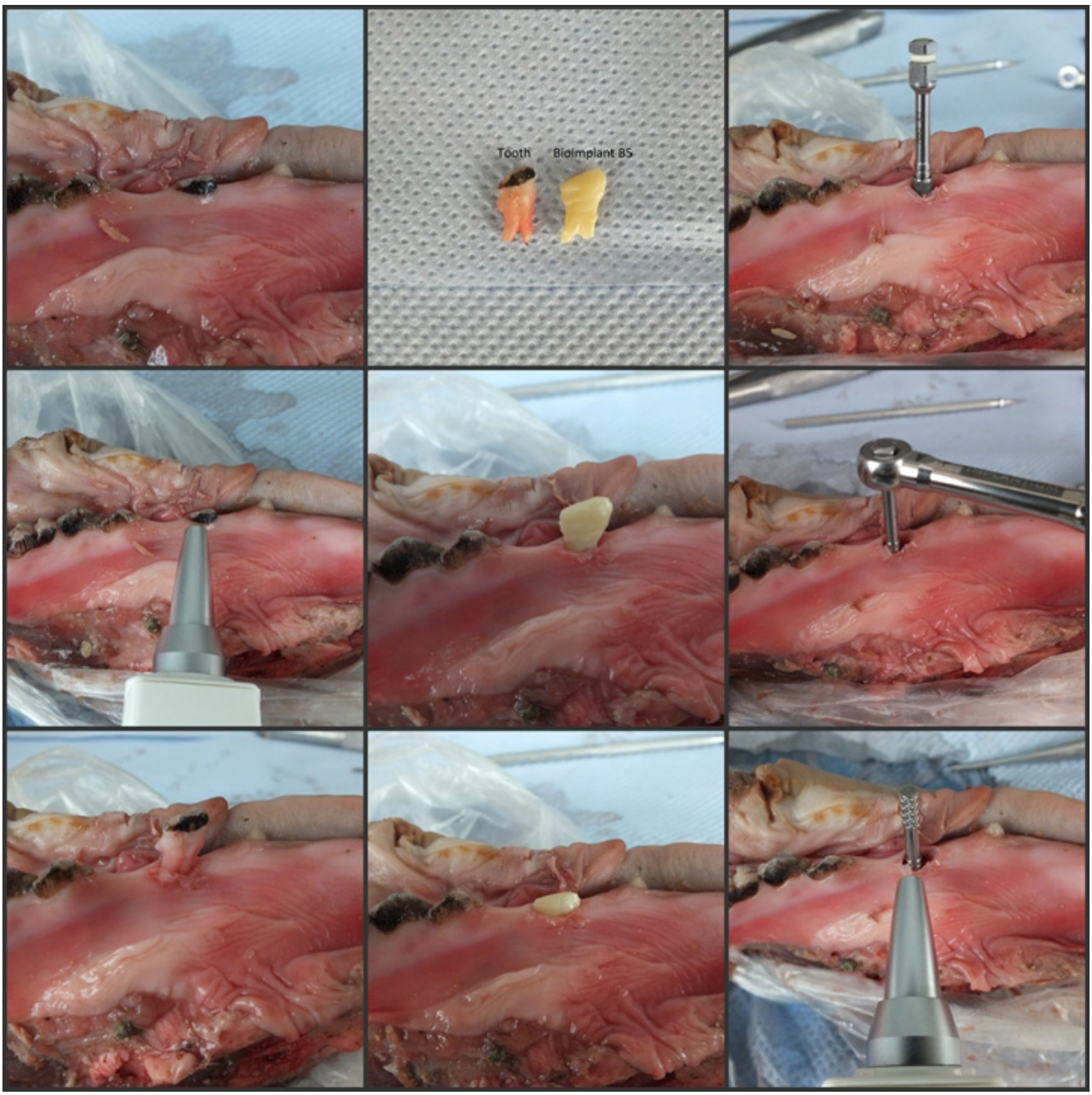
The position of the 1st premolar tooth of each specimen was controlled by conical bone computer tomography (CBCT) examination (Kodak 9000 3D, Carestream/Trophy, Marne-la-Vallée, France), with a field of view (FOV) equal 5 × 4 cm, nominal beam of 73 kV, 12 mA and a voxel size of 90 μm. The 3-dimensional (3D) view of each tooth was designed from CBCT scan by means of computer tomography (CT) software (Mimics, Materialize, Leuven, Belgium) (Fig. 1). The image of the tooth was manually separated in each slice of a conical bone computer tomography by the same operator (J.M.). Next, the surface of the tooth in CBCT was modified to better fit the alveolar socket by remodeling its surface. The macro-retentions were designed only onto mesial and distal part of the root according to the study protocol. The macro-retentions strictly limited to the interdental space, and the buccal and lingual faces were reduced by 0.1–0.2 mm according to Pirker's protocol (Fig. 2).

The created 3D images were exported to STL (Stereo Lithography) file format and were used to prepare a root analog zirconia implants milled from a medical-grade zirconia block by means of a laboratory milling (DMG Sauer Ultrasonic 20, USA). Next, the RAZI was sintered for 8 h to achieve the desired mechanical properties.

Surgical procedure

After the tooth had been cautiously removed with the use of the Periotome instrument, the RAZI was placed into the alveolar socket under finger pressure and then gently tapped with a hammer and a mallet. Next, RAZI was removed and a titanium implant (Superline, Dentium, Korea), 4.0 mm in diameter and 10 mm in length was placed manually using a ratchet, subcrestally into the socket according to factory surgical protocol. The parallel pin was screwed to the implant to control the implant primary stability.

Fig. 3. The surgical protocol and measurement procedure used in the study



Measurement procedure and study design

The primary stability coupled with the Periotest test value (PTV) was measured by means of the Periotest device (Medzintechnik Gulden e K, Modautal, Germany). In practice, the Periotest test values (PTVs) are based on a numerical scale ranging from -8 to +50, determined by mathematical calculations. The lower Periotest values denote higher implant stability and thereby the higher damping effect of the target tissues.

The measurement of PTV was carried out on the tooth before its extraction (G1 group), next on the RAZI, which was placed in the alveolar socket (G2 group), and on the screw titanium implant (G3 group) placed subcrestally in the socket after RAZI removal (Fig. 3).

Statistical analysis

To assess whether the data was normally distributed, the Kolmogorov-Smirnov test was performed at the 95% level. The statistical analysis was performed by means of a one-way ANOVA test with the use of the STATISTICA v. 12 program (StatSoft, Kraków, Poland). Pair comparisons were carried out based on the Tukey post hoc test at significance levels of $p = 0.05$.

Results

An analysis of the primary stability revealed significant differences in PTV results for G2 (RAZIs) as compared to G1 and G3 groups (Table 1). Our findings show that a mean PTV value measured for RAZIs was 4.7 times and 3.8 times lower as compared to the teeth and screw implants, respectively. The lower PTV value means that the primary stability of root analog zirconia implants was significantly higher in comparison with the teeth and the screw implants.

The analysis of the PTV value of the teeth (G1) revealed higher results (lower primary stability) than in the cases when a screw implants (G3) were used. However, there was no significant increase in PTV results when compared this 2 groups (Fig. 4).

Discussion

The use of digital technology in dentistry is becoming more common and presents many advantages. Firstly, due to the CT/CBCT scan banks, the surgical procedure is marked by reduced treatment time and an immediate restoration – whole surgery can end in one initial dental visit, whereas conventional implants take at least 2–3 visits. Thanks to CBCT scans, the root replica is obtained prior to tooth extraction. Therefore, there is no need for subsequent surgery, as the tooth extraction and RAI implantation can take place during the same visit. Other benefits include increased patient comfort as well as better esthetics due to mimicking root features.[8]

The results of research of Moin and Al-Rawi proved the increase in surface details of the 3D surface model compared to the original tooth.[5,25] Through CAD/CAM designing many alterations (macroretentions, corrections of the shape) can be added to the RAI. It is also possible to preoperatively design the abutment form or temporary crown.[8]

Furthermore, Chahine et al. presented many advantages of RAI (specifically RATI) in comparison to the traditional implantation methods.[26] RAI is adjusted to the particular patient's clinical situation, thereby permitting excellent reconstruction of function and esthetics. Since the damaged tooth is extracted using atraumatic methods, the implantation takes almost no site preparation, resulting in enhanced bone response and faster healing. Apart from the aforementioned benefits of RAI, Chahine at al. also list improved bite feel (thanks to micromotion means), patient satisfaction and quality of life, and also reduced treatment cost.[26]

Moin et al. in another study assessed the accuracy of the individually fabricated RAI based on CBCT scan, computer-aided designing (CAD), and computer-aided manufacturing (CAM) technology and measured the discrepancy in congruence with the alveolar socket subsequent to placement of the RAI.[27] The authors showed that the volume of the socket was greater than the root part of the RAI ranging from 0.6% to 5.9%. These results confirmed that preparing macroretensions on the RAI's surface is a key factor in achieving proper initial stabilization of the RAI.

Though RATI presented excellent primary stability sustained up to 1 month, it also showed a failure rate of 48% at a 9-month and 97% at a 1-year follow-up.[10] Therefore, some authors prefer using RAZI instead of RATI.[28]

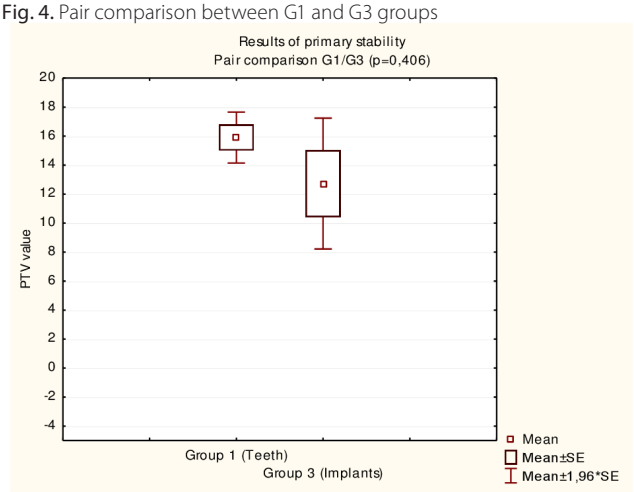
Chen at al. in their research assessed the root analog titanium implants (RATI) and the primary stability and stress distribution in the bone of root analog threaded titanium implants (RATTI).[29] The root analog titanium implants were printed in 3 dimensions (3D) with the selective laser melting technique. The results of their research indicated that better initial stability was obtained with a RATI. The maximum stress between the cortical bone and RATTI interfaces was 41.8%, lower than that of the RATI under immediate loading.[29] Hence, the RATTI design should have an advantage over the conventional RATI because of the beneficial stress distribution in the early stages of healing. The stress in the cortical bone can be reduced by customizing the RAI surface.[29] Pirker et al. proposed to reduce the buccal and lingual face of the root analog im-

Table 1. The mean PTV's values and standard deviation in G1, G2, G3 groups. There were significant differences in PTV results between groups G2 and G1, G2 and G3)

Study groups	Mean PTV	N	SD
G1 (teeth)	15.9000	13	3.2326
G2 (RAZI)	3.3538	13	6.1371
G3 (implants)	12.7231	13	8.3012
All groups	10.6589	39	8.1281

G2 vs G1 p = 0.000151; G2 vs G3 p = 0.00152; PTV – periotest test value; SD – standard deviation; N – number of cases.

Fig. 4. Pair comparison between G1 and G3 groups



plant by 0.1–0.2 mm to diminish stress in the cortical bone and to avoid fracture of the thin alveolar bone wall.[28]

Pirker et al. state better esthetics (no metallic elements showing through the mucosa), enhanced mechanical (high flexural strength, hardness, fracture toughness, high electrical resistance) and chemical properties (superior plaque resistance) of RAZI.[28] A rough root surface topography (micro- and macroretentions) speeds up the osseointegration process. Its excellent biocompatibility is similar to that of titanium implants, which has been demonstrated in several animal investigations.[30]

In the presented study, RAZI was modified according to the Pirker's protocol.[28] The macroretentions were limited only to the interdental space (mesial and distal part of the root) for better primary stability and osseointegration. The buccal and lingual faces were reduced by 0.1–0.2 mm to avoid fracture and bone loss. Moreover, since achieving primary implant stability includes the conservation of the alveolar socket walls, the teeth were extracted using atraumatical techniques. Furthermore, the RAZI fabrication was performed using cone beam computed tomography (CBCT) scan technology of the socket and tooth before the extraction. Fabrication of RAZI may be carried out also by scanning the extracted tooth using the laser scanner.[28] Nevertheless, using just the laser scanner has some disadvantages. If the tooth is damaged before or during the extraction, it becomes difficult to put it together and scan.[8] The implantation is postponed until the dental laboratory prepares the RAZI. Also, the alveolar socket needs to be curetted again before the implantation.[28] CBCT method allows to perform the procedure despite above drawbacks and immediately, since the RAZI is prepared before the extraction.[8]

The presented study showed better values of PTV for RAZI in comparison to the titanium implants and natural teeth. According to Dilek et al., immediate loading can only occur if their PTV is in the range between -8 to +9, whereas Atsumi et al. narrowed the range to -4–(-2) and -4–(+2).[21,24] However, research conducted by Dilek et al. involved mini-implants; therefore, the range of PTV was more extensive.[24] The results of PTV in this study amounted to an average of 3.35, which means that they are the closest to the values that enable primary stability and immediate implantation of RAZI compared to two mentioned studies.

Further studies should be conducted to assess the impact of different macroretentions applied through CAD/CAM method at the root surface on the primary stability of RAZI.

Conclusion

The modern CBCT device allows us to design precise images of an extracted tooth for the purpose of developing a root analog implant. Within the limitations of the present ex vivo study, it is evident that the root analog zirconia implants during immediate loading achieved a good implant primary stability. The additional feature of the surgical protocol using RAZI is the possibility to avoid the augmentation procedure, which reduces the whole cost of the treatment.

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Which organ should be considered a reference in diffusion weighted imaging of the abdomen?: The reproducibility of ADC measurements of the spleen and the renal cortex on a 1.5T MR

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A – research concept and design; B – collection and/or assembly of data; C – data analysis and interpretation; D – writing the article; E – critical revision of the article; F – final approval of article

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Abstract

Background. Diffusion weighted imaging (DWI) is a useful tool for the evaluation of focal lesions in the liver or kidneys, as well as for the diagnosis and assessment of the liver fibrosis process. Some reports show that the spleen and kidneys may serve as reference organs in the staging of liver fibrosis or the evaluation of focal liver lesions.

Objectives. The aim of the study was to determine whether the spleen and renal cortex can be used as reference organs in the DWI technique.

Material and methods. The study group included 36 patients with no liver, spleen or renal pathologies and without any infections or hematologic disease. All the examinations were performed using a 1.5T MR unit with a conventional phased array body coil. Image interpretation and apparent diffusion coefficient (ADC) measurements were done by 3 experienced radiologists.

Results. There was a statistically significant difference between the ADC values noted by 2 of the examiners in the upper/middle and lower part of the spleen parenchyma. There were no statistically significant differences between the ADC values obtained by all 3 examiners in all the parts of each kidney. There were no statistically significant differences between the examiners' ADC values for the spleen and kidneys. The mean ADC values for the left kidney showed the highest measurement reproducibility.

Conclusions. The study showed that the renal cortex seems to be an appropriate region for performing reference ADC measurements. Further studies on a larger group of patients and using various DWI protocols should be performed to ascertain the best conditions for maximizing the reproducibility of ADC measurements.

Key words: magnetic resonance imaging (MRI), diffusion weighted MRI, apparent diffusion coefficient, spleen, renal cortex

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Diffusion-weighted magnetic resonance (DW-MR) is a technique in which image contrast reflects in vivo changes in the motion of water molecules (Brownian motion) in tissues.[1] It has been used for many years in neuroradiology for detecting acute strokes at their early stage, before ischemic lesions are visible on other magnetic resonance (MR) sequences or computed tomography (CT) scans.[2] Over the few last years it has also been successfully applied to breast and prostate imaging to differentiate between benign and malignant tumors.[2-4]

A supplemental tool in diffusion weighted imaging (DWI) is the apparent diffusion coefficient (ADC) map, acquired by post-processing of the DWI images obtained. ADC allows for the definition of diffusion parameters quantitatively (in mm^2/s).[1]

In recent years researchers have tried to find new clinical applications for DWI in diagnosing abdomen pathologies. At present, the usefulness of this method has been established for detection and differentiation within focal lesions in the liver or in kidneys.[5-7] A very promising application of DWI, especially ADC maps, is diagnosing and assessing the liver fibrosis process.[8,9] Recent reports show that the spleen and kidneys can serve as reference organs in assessing the stage of liver fibrosis or evaluation of focal liver lesions.[10,11]

However, the technique has some limitations, such as poor image resolution, including a low signal to noise ratio (SNR), and low reproducibility in ADC value measurements.[5]

The aim of the current study was to determine whether the spleen and renal cortex can serve as reference organs in DWI examinations, including ADC maps.

Material and methods

Study population

MR examinations of the abdomen performed at Independent Public Clinical Hospital No. 1 (Wrocław, Poland) in the years 2013–2014 were retrospectively analyzed. Patients with liver, spleen and renal pathologies, as well as patients with infection or hematologic diseases, were excluded from the study group. Examinations containing motion artifacts were also rejected. Patients with single renal cysts (2 or fewer in one kidney, with a diameter under 10 mm) were included in the study. Based on these criteria a population of 36 patients (Table 1) was selected. Of these 36 patients, 19 were diagnosed with adrenal adenoma; in 7 patients adrenal adenoma was suspected based on ultrasound examination but was not confirmed on MR; 5 patients were suspected of liver focal lesions, excluded on MR; 5 presented an increased level of carcinoembryonic antigen following colon cancer treatment with no pathology on MR.

The study was approved by the ethics committee of Wroclaw Medical University (KB-216/2015).

Table 1. Basic data about the study group

Parameter	Value
Number of participants	36
Number of men*	14 (39)
Number of women*	22 (61)
Age[1]	33.5 (36.75)
Age range	18–84
Men's age[1]	31.5 (29)
Women's age[1]	24.5 (40)

*percentage ratio in brackets; [1]Median; IQR in brackets.

Table 2. The homogeneity of ADC values within the examined organ (Friedman's ANOVA)

Investigator	χ^2	N	df	p-value
Spleen				
Radiologist 1	8.53	36	2	0.014
Radiologist 2	6.08	36	2	0.048
Radiologist 3	3.17	36	2	0.205
Right kidney				
Radiologist 1	2.21	36	2	0.331
Radiologist 2	1.58	36	2	0.454
Radiologist 3	1.06	36	2	0.590
Left kidney				
Radiologist 1	0.18	36	2	0.912
Radiologist 2	2.78	36	2	0.249
Radiologist 3	1.56	36	2	0.459

MR protocol

All the examinations were performed using a Siemens Avanto 1.5T MR unit (Siemens Medical Solutions, Erlangen, Germany) with a conventional phased array body coil.

DWI was performed using a standard protocol, namely single-shot spin-echo-planar imaging (EPI) in the axial plane, with the following parameters: TR 5200–6000 ms, TE 72 ms, voxel size 2 × 2 × 5, Bw 1448 Hz/px, b values 50, 400 and 800, 30 slices, duration time ~ 6 min.

Table 3. The differences between ADC values obtained by the examiners for the spleen

Organ	Radiolgist 1	Radiologist 2	Radiologist 3	ANOVA
Spleen – upper part				
M ± SD	0.75 ± 0.15	0.76 ± 0.12	0.70 ± 0.15	p = 0.180
Me (Q1; Q3)	0.76 (0.68; 0.84)	0.75 (0.67; 0.86)	0.69 (0.61; 0.81)	
Min–Max	0.35–1.14	0.54–0.97	0.31–1.00	
Spleen – middle part				
M ± SD	0.78 ± 0.17	0.77 ± 0.15	0.74 ± 0.13	p = 0.561
Me (Q1; Q3)	0.78 (0.67; 0.87)	0.74 (0.65; 0.91)	0.72 (0.67; 0.85)	
Min–Max	0.45–1.36	0.51–1.07	0.45–1.00	
Spleen – lower part				
M ± SD	0.75 ± 0.15	0.70 ± 0.11	0.68 ± 0.12	p = 0.783
Me (Q1; Q3)	0.76 (0.68; 0.84)	0.68 (0.63; 0.76)	0.69 (0.61; 0.75)	
Min–Max	0.35–1.14	0.52–0.99	0.37–0.92	
Spleen – average value				
M ± SD	0.74 ± 0.10	0.74 ± 0.09	0.72 ± 0.11	p = 0.583
Me (Q1; Q3)	0.73 (0.67; 0.82)	0.74 (0.70; 0.80)	0.73 (0.67; 0.78)	
Min–Max	0.53–0.93	0.53–0.98	0.37–0.95	

M – mean; SD – standard deviation; Me – median; Q1 – lower quartile (25th percentile); Q3 – upper quartile (75th percentile); Min–Max – minimum and maximum.

Table 4. The differences between ADC values obtained by the examiners for the right kidney

Organ	Radiolgist 1	Radiologist 2	Radiologist 3	ANOVA
Right kidney – upper part				
M ± SD	1.74 ± 0.20	1.76 ± 0.18	1.79 ± 0.23	p = 0.659
Me (Q1; Q3)	1.80 (1.58; 1.89)	1.78 (1.61; 1.93)	1.84 (1.55; 1.97)	
Min–Max	1.34–2.17	1.42–2.04	1.41–2.18	
Right kidney – middle part				
M ± SD	1.78 ± 0.16	1.79 ± 0.14	1.81 ± 0.17	p = 0.731
Me (Q1; Q3)	1.80 (1.67; 1.87)	1.82 (1.71; 1.89)	1.84 (1.68; 1.93)	
Min–Max	1.42–2.08	1.47–2.06	1.46–2.13	
Right kidney – lower part				
M ± SD	1.73 ± 0.15	1.76 ± 0.17	1.76 ± 0.17	p = 0.591
Me (Q1; Q3)	1.75 (1.63; 1.82)	1.74 (1.67; 1.84)	1.76 (1.67; 1.84)	
Min–Max	1.37–2.06	1.33–2.16	1.41–2.14	
Right kidney – average value				
M ± SD	1.75 ± 0.14	1.77 ± 0.13	1.79 ± 0.16	p = 0.565
Me (Q1; Q3)	1.77 (1.64; 1.85)	1.79 (1.69; 1.87)	1.80 (1.64; 1.89)	
Min–Max	1.43–2.08	1.51–2.01	1.52–2.12	

M – mean; SD – standard deviation; Me – median; Q1 – lower quartile (25th percentile); Q3 – upper quartile (75th percentile); Min–Max – minimum and maximum.

Table 5. The differences between ADC values obtained by the examiners for the left kidney

Organ	Radiolgist 1	Radiologist 2	Radiologist 3	ANOVA
Left kidney – upper part				
M ± SD	1.78 ± 0.17	1.75 ± 0.18	1.77 ± 0.20	p = 0.853
Me (Q1; Q3)	1.78 (1.63; 1.90)	1.76 (1.60; 1.85)	1.76 (1.60; 1.92)	
Min–Max	1.49–2.16	1.49–2.18	1.44–2.28	
Left kidney – middle part				
M ± SD	1.80 ± 0.14	1.78 ± 0.12	1.79 ± 0.14	p = 0.937
Me (Q1; Q3)	1.79 (1.66; 1.92)	1.77 (1.70; 1.88)	1.76 (1.69; 1.89)	
Min–Max	1.56–2.04	1.57–2.06	1.56–2.18	
Left kidney – lower part				
M ± SD	1.80 ± 0.12	1.81 ± 0.13	1.80 ± 0.16	p = 0.968
Me (Q1; Q3)	1.82 (1.74; 1.86)	1.82 (1.75; 1.89)	1.81 (1.68; 1.92)	
Min–Max	1.56–2.06	1.46–2.14	1.52–2.17	
Left kidney – average value				
M ± SD	1.79 ± 0.12	1.78 ± 0.11	1.79 ± 0.14	p = 0.956
Me (Q1; Q3)	1.81 (1.67; 1.88)	1.78 (1.70; 1.86)	1.78 (1.67; 1.90)	
Min–Max	1.59–2.08	1.60–2.05	1.58–2.08	

M – mean; SD - standard deviation; Me – median; Q1 – lower quartile (25th percentile); Q3 – upper quartile (75th percentile); Min–Max – minimum and maximum.

ADC quantification

To obtain ADC maps using all 3 b values (50, 400 and 800 mm^2/s), dedicated standard software was used on a Syngo workstation (Siemens Medical Solutions, Erlangen, Germany). ADC measurements were performed by 3 experienced radiologists (with 15, 10 and 7 years of experience in abdominal imaging) using the Syngo.via workstation.

Circular regions of interest (ROIs) were placed on the upper, middle and lower parts of the spleen and renal cortex (3 ROIs per organ: the spleen and each kidney). The examiners were allowed to choose the place of the measurement and the size of the ROIs, but they were asked to place the ROIs in the areas of the most homogenous signal intensity in their assessment. Care was taken to avoid areas of artifacts, the major vessels in the spleen, cysts in the kidneys and capsules. The ROIs used in the analysis were from 1 cm to 1.7 cm in size for the spleen, and from 0.4 cm to 0.9 cm for the kidneys.

Statistical analysis

The statistical analysis of the data was conducted using STATISTICA v. 10 software (StatSoft Inc., Tulsa, USA). Friedman's ANOVA test was used to evaluate the homogeneity of the ADC values within the examined organ (spleen or kidney). The ANOVA test was chosen to determine the differences between the ADC values obtained by the 3 examiners.

The reproducibility of the measurements obtained was assessed using Pearson's correlation coefficient and the Bland-Altman method.

Results

Variability in ADC values

There was a statistically significant difference between the ADC values noted by 2 of the examiners in the upper/middle and lower part of spleen parenchyma (Table 2). There were no statistically significant differences between the ADC values obtained by all 3 examiners in all the parts of each kidney.

Difference between mean ADC values among examiners

There were no statistically significant differences between the ADC values noted by the examiners for the spleen and kidneys (Tables 3–5). A diagram analysis revealed lower ADC values noted by the 3rd examiner in each part of the spleen; the difference was not statistically significant, and the same dependence was not observed in the ADC values for the kidneys. Moreover, it was found that the results obtained by the 3rd examiner showed the largest scattering.

ADC reproducibility

Significant Pearson correlation coefficients (for all 3 examiners) were calculated for the ADC values of the left kidney (all parts), for the lower and central parts of the right kidney and for the central part of the spleen. The analysis of the linear correlation coefficient revealed that the most statistically significant results were achieved for the mean ADC values of both kidneys, the ADC values of the central parts of both kidneys and the central parts of spleens (Table 6).

There was no area of measurement in the Bland-Altman plot that showed excellent repeatability (a Bland-Altman

Table 6. Linear correlation coefficient values of ADC measurements obtained by 3 examiners

Organ	Radiologist 1 vs 2	Radiologist 1 vs 3	Radiologist 2 vs 3
Spleen – upper part	**r = +0.662**	**r = +0.352**	r = +0.207
	p < 0.001	**p = 0.035**	p = 0.226
Spleen – central part	**r = +0.798**	**r = +0.419**	**r = +0.501**
	p < 0.001	**p = 0.011**	**p = 0.002**
Spleen – lower part	**r = +0.726**	r = +0.286	r = +0.143
	p < 0.001	p = 0.091	p = 0.404
Spleen – average value	**r = +0.782**	r = +0.159	r = -0.011
	p < 0.001	p = 0.354	p = 0.948
Right kidney – upper part	**r = +0.896**	r = +0.183	**r = +0.355**
	p < 0.001	p = 0.284	**p = 0.034**
Right kidney – central part	**r = +0.799**	**r = +0.747**	**r = +0.641**
	p < 0.001	**p < 0.001**	**p < 0.001**
Right kidney – lower part	**r = +0.783**	**r = +0.385**	**r = +0.514**
	p < 0.001	**p = 0.021**	**p = 0.001**
Right kidney – average value	**r = +0.873**	**r = +0.618**	**r = +0.715**
	p < 0.001	**p < 0.001**	**p < 0.001**
Left kidney – upper part	**r = +0.674**	**r = +0.359**	**r = +0.373**
	p < 0.001	**p = 0.031**	**p = 0.025**
Left kidney – central part	**r = +0.751**	**r = +0.358**	**r = +0.436**
	p < 0.001	**p = 0.032**	**p = 0.008**
Left kidney – lower part	**r = +0.539**	**r = +0.550**	**r = +0.528**
	p = 0.001	**p = 0.001**	**p = 0.001**
Left kidney – average value	**r = +0.800**	**r = +0.494**	**r = +0.503**
	p < 0.001	**p = 0.002**	**p = 0.002**

Correlation coefficient values significantly different than zero, with $p < 0.05$, are in bold.

coefficient less than 5%) for all pairs of examiners. Considering all pairs of examiners, the best ADC measurement reproducibility was in the mean ADC values for the left kidney; the second best was in the ADC values for the lower part of the left kidney (Table 7).

Table 7. Bland-Altman coefficient values for ADC results obtained by 3 examiners

Organ	Radiologist 1 vs 2	Radiologist 1 vs 3	Radiologist 2 vs 3
Spleen – upper part	11.1%	8.3%	5.6%
Spleen – central part	5.6%	11.1%	8.3%
Spleen – lower part	5.6%	5.6%	5.6%
Spleen – average value	**2.8%**	5.6%	8.3%
Right kidney – upper part	**2.8%**	5.6%	8.3%
Right kidney – middle part	8.3%	5.6%	5.6%
Right kidney – lower part	5.6%	5.6%	5.6%
Right kidney – average value	5.6%	5.6%	5.6%
Left kidney – upper part	5.6%	5.6%	8.3%
Left kidney – central	11.1%	8.3%	5.6%
Left kidney – lower part	5.6%	5.6%	**2.8%**
Left kidney – average value	5.6%	**2.8%**	**2.8%**

Results exceeding 5% indicate poor reproducibility (in bold).

Discussion

The results of the statistical analysis in the present study confirm the findings of previous studies pointing out the limitations of the DWI technique and quantitative evaluation of water molecule diffusion using ADC maps. The analysis of the homogeneity of the diffusion in different organs revealed that the spleen may not be a good reference organ. It has been observed that ADC values in each part of the spleen differ significantly. As suggested by Hong et al., the renal cortex seems to be a more accurate area to perform reference measurements.[11] In addition, in the current study, the analysis using Pearson's correlation test showed statistically significant reproducibility of ADC values in all parts of the left kidney, as well as in the central and lower parts of the right kidney. The Bland-Altman test also revealed significant reproducibility of ADC measurements for the kidneys, particularly the left kidney (the mean ADC value as well as the ADC value of the lower part). However, it is often the case that only the upper and central parts of the kidney are visible on echo planar images (EPI), which could make ADC measurement in the lower parts impossible.[10] Due to the statistically significant reproducibility of ADC values in the Pearson's correlation test, the central part of the spleen should be also considered as a reference area. However, the present study included only healthy subjects without spleen pathologies. Patients with liver fi-

brosis usually develop portal hypertension, causing splenomegaly, which can result in increased heterogeneity of the spleen parenchyma on ADC maps as well as changes in water diffusion.[12]

DW-MRI is a non-invasive technique of great usefulness in the detection and differentiation of pathological lesions in the whole body, including the abdomen. However, there are some limitations to this method. One of them is the lack of standardization in DWI sequence protocols (different b-values, different ways of creating ADC maps).[5,13] Another drawback is the absence of explicit cut-off values for the evaluation of different pathologies. As the present study showed, overcoming all these limitations may prove to be very difficult. It seems that further technical improvements are required to maximize the usefulness of DWI sequences. Further studies on a larger group of patients and using various DWI protocols (including 3T MR or higher) should be performed to ascertain the best conditions for maximizing the reproducibility of ADC measurements.

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The effect of desflurane and propofol protocols on preconditioning

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Abstract

Background. Preconditioning is one of the most powerful mechanisms preventing the myocardial ischemic damage that occurs during coronary artery bypass grafting.

Objectives. We aimed to investigate the effects of different propofol and/or desflurane administration protocols in terms of the prevention of ischaemia-reperfusion damage.

Material and methods. Ninety patients, aged > 18 years, American Society of Anesthesiologists (ASA) category III, scheduled to undergo primary elective coronary artery bypass grafting (CABG), were included in the study. During maintenance, the patients in group 1 (n = 30) received a propofol infusion (5–6 mg/kg/h) combined with a fentanyl infusion (3–5 mcg/kg/h); the patients in group 2 (n = 30) also received a propofol infusion (5–6 mg/kg/h) combined with a fentanyl infusion (3–5 mcg/kg/h), but they were also given 6% desflurane inhalation for 15 min both before cross-clamping of the aorta and after removal of the clamp; the patients in group 3 (n = 30) received a propofol infusion (2–3 mg/kg/h) combined with a fentanyl infusion (3–5 mcg/kg/h) and received the continuous 6% desflurane inhalation. Blood samples were drawn in the preoperative period (S1), during cardiopulmonary bypass, before cross-clamping the aorta (S2), after removal of the cross-clamp (S3) and 24 h after the operation (S4).

Results. All groups were similar in terms of age and BMI ($p > 0.05$). TNF-α levels were higher at S3 compared to S1, S2 and S4 ($p > 0.001$). The TNF-α levels at S4 were lower in group 3 than those in group 1 and group 2 ($p < 0.05$). In all groups, h-FABP levels showed an increase in S3 but were significantly lower at S4 ($p < 0.05$). In group 3, h-FABP levels at S2 and S3 were significantly lower than those in group 1 ($p < 0.05$). There was a moderate correlation between h-FABP and TNF-α levels (Spearman's rho = 0.472, $p < 0.001$).

Conclusions. On the basis of the measurement of h-FABP and TNF-α, low-dose propofol and continuous desflurane inhalation provide more effective preconditioning than propofol alone or a short course of desflurane in patients undergoing CABG.

Key words: preconditioning, propofol, desflurane

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Introduction

Ischemic preconditioning has been defined as the reduction of high energy catabolism by producing short periods of ischemia that are accompanied by a decrease in myocardial contractility, arrhythmia and intracellular acidosis. Thus, ischemia-reperfusion-related contractile dysfunction is prevented, which is crucially important in patients with a hypertrophied ventricle. Preconditioning produces short periods of ischemia that help the heart adapt to ischemia-reperfusion compromise.[1,2]

As demonstrated by experimental and clinical studies, producing short periods of ischemia using pharmacological and perioperative volatile anesthetic drugs has a pre-conditioning effect on the myocardium.[3] Propofol was shown to have antioxidant effects and desflurane and sevoflurane were shown to be associated with lower troponin I levels, which may indicate their potential use for preconditioning.[4,5]

Large amounts of reactive oxygen radicals are created during cardiopulmonary bypass, causing an increase in systemic oxidative stress and lipid peroxidation that alters myocardial function.[6] Tumor necrosis factor alpha (TNF-α), which increases during the creation of oxygen radicals, has been shown to increase following cardiopulmonary bypass.[7] Therefore, TNF-α is thought to play an important role in the inflammatory process that causes cardiac dysfunction.[4]

Heart-type fatty acid binding protein (h-FABP) has been shown to be a sensitive marker in the diagnosis of myocardial infarction. Its use in the assessment of preconditioning during cardiac surgical anesthesia was suggested since it may be detected in venous blood within a couple of hours after myocardial ischemia or infarction.[8,9] TNF-α was also suggested to be a useful marker in the assessment of effectiveness of the preconditioning method used in cardiac surgery.[10,11] Another advantage of TNF-α is its stimulation of the acute phase reaction, which may allow the cardiac protective effects of preconditioning to be traced during cardiac surgery.

In light of the above, we sought to evaluate the effects of different propofol and/or desflurane management protocols on preconditioning during coronary artery surgery, with the assessment being based on TNF-α and h-FABP levels.

Patients and methods

The study was approved by our institutional review board (02-2/6, 20.03.2013). All patients were informed about the study protocol and signed procedure-oriented informed consent forms. Patients aged > 18 years of age, American Society of Anesthesiologists (ASA) category III, scheduled to undergo primary elective coronary artery bypass grafting (CABG) were included in the study. Patients with a left ventricle ejection fraction < 50% and those with unstable angina pectoris, diabetes, renal failure (creatinine ≥ 1.2 mg/dL), or acute or recent (< 2 weeks) myocardial infarction were excluded. Patients with a clear indication for combined valve or aortic surgery and those who had cardiogenic shock or low cardiac output syndrome were also excluded. A total of 90 patients were included in the study.

Study protocol and chemical analysis

The patients were pre-medicated with 5 mg oral diazepam on the night before the operation. All operations were performed by the same surgical team. Standard monitoring was performed with 12-lead electrocardiogram and pulse oximetry. A peripheral venous line was introduced via the right antecubital vein. Invasive arterial monitoring was achieved via the right radial artery. After 5 min of pre-oxygenation with 100% oxygen, anesthesia was induced with 1.5–2.0 mg/kg/min of propofol (Lipuro %1, Braun, Melsungen, Germany) and 5–10 mcg/kg of fentanyl (Fentanyl, Mercury Pharma, London, UK). Neuromuscular blockade was achieved with 1 mg/kg of intravenous rocuronium (Curon, Mustafa Nevzat, Istanbul, Turkey). Patients were intubated and were placed on volume-controlled mechanical ventilation. The respiratory rate was set at 12 times per min, positive end-expiratory pressure at 0 mbar, maximum pressure at 30 mbar and tidal volume at 7–10 mL/kg. End-tidal CO_2 was measured using a Nihon Kohden Life Scope 14. Then, a central venous catheter was introduced via the right internal jugular vein and central venous pressure was recorded during and after the operation. Bispectral index (BIS) monitoring was performed in all patients (Aspect Medical Systems BIS VISTA™ Covidien).

The patients were randomly allocated into 3 groups to receive 1 of 3 different anesthetic maintenance regimens. Randomization was achieved using computer-based software. During maintenance, the patients in group 1 (n = 30) received a propofol infusion (5–6 mg/kg/h) combined with a fentanyl infusion (3–5 mcg/kg/h). Patients in group 2 (n = 30) also received a propofol infusion (5–6 mg/kg/h) combined with a fentanyl infusion (3–5 mcg/kg/h) but they were also given 6% desflurane (Suprane, Baxter, Puerto Rico) inhalation for 15 min both before cross-clamping of the aorta and after removal of the clamp. The patients in group 3 (n = 30) received a propofol infusion (2–3 mg/kg/h) combined with a fentanyl infusion (3–5 mcg/kg/h) plus continuous 6% desflurane inhalation. BIS was kept at 40–50.

Body temperature was monitored using a nasopharyngeal probe and patients' body temperatures were cooled down to 32°C. Blood samples were drawn in the preoperative period (S1), during cardiopulmonary bypass, be-

fore cross-clamping of the aorta (S2), after removal of the cross-clamp (S3) and 24 h after the operation (S4). The samples were preserved in a refrigerator at -80°C. TNF-α (USCN Life Science Inc., USA) and h-FABP levels were measured via ELISA. Creatinine kinase (CK), CK-MB, troponin-I, B-type natriuretic peptide (BNP) and lactate dehydrogenase (LDH) levels were measured from samples drawn in the preoperative period and 24th postoperative hour.

Statistical analysis

All analyses were performed using STATISTICS for Social Sciences (SPSS) v. 19.0. For related measurements, normally distributed data was compared using repeated measures analysis of variance and non-normally distributed data was compared using the Friedman test. For independent measurements, normally distributed data was compared using one-way analysis of variance (ANOVA) and non-normally distributed data was compared using the Kruskal-Wallis test. Spearman's correlation analysis was used to test for any linear relationship among the study variables. A p-value of less than 0.05 was considered statistically significant.

Results

The 3 groups were similar in terms of age and body mass index ($p > 0.05$). CK, CK-MB, LDH, troponin I and BNP levels showed a significant increase in the 24th postoperative hour compared to their baseline values ($p < 0.001$). There were no significant differences among the groups either before or after the operation ($p > 0.05$) (Table 1).

In group 1, 2 and 3, TNF-α levels did not differ among S1, S2 and S4 ($p > 0.05$) whereas S3 was significantly higher than S1, S2 and S4 ($p < 0.001$). There was a significant difference between S2 and S4 in group 1 whereas no such difference was observed in other groups ($p < 0.05$) (Table 2). In almost all groups, TNF-α levels showed a significant increase after removal of the cross-clamp but had decreased 24 h postoperatively. In addition, S3 TNF-α levels showed a marked increase compared to other stages in all 3 groups. S3 TNF-α levels did not differ significantly among the 3 groups ($p < 0.05$). S2 TNF-α levels were significantly lower in group 3 compared to group 1 and group 2 ($p < 0.05$). Similarly, S4 TNF-α levels were significantly lower in group 3 than those in group 1 and group 2 ($p < 0.05$) (Table 2). S2 TNF-α levels were significantly lower in group 2 and group 3 (desflurane administered) than those in group 1 (desflurane not administered) ($p < 0.05$). The most profound reduction by the 24th postoperative hour was that seen in group 3 ($p < 0.05$).

Table 1. Comparison of data from patient groups before and after the operation

	Group 1			Group 2			Group 3			Comparison of groups**
	preopmean ± SD	postop mean ± SD	*p	preop mean ± SD	postop mean ± SD	*p	preop mean ± SD	postop mean ± SD	*p	
N (female/male)	30 (11/19)			30 (12/18)			30 (10/20)			
Age, year	64.5 ± 8.8			63.8 ± 6.2			64.0 ± 6.8			> 0.05
BMI, kg/m^2	27.1 ± 2.2			27.4 ± 3.4			26.5 ± 3.1			> 0.05
CK, U/L	93 ± 60	1115 ± 644	< 0.0001	98 ± 61	1048 ± 673	< 0.0001	109 ± 82	1054 ± 678	< 0.0001	> 0.05
CK-MB, U/L	11.8 ± 3.8	66.7 ± 23.7	< 0.0001	13.9 ± 3.7	65.6 ± 27.2	< 0.0001	13.8 ± 4.7	63.8 ± 31.7	< 0.0001	> 0.05
LDH, U/L	270 ± 133	608 ± 202	< 0.0001	257 ± 145	622 ± 320	< 0.0001	262 ± 129	615 ± 234	< 0.0001	> 0.05
Troponin I, µg/L	0.31 ± 0.59	2.47 ± 1.67	< 0.0001	0.30 ± 0.56	2.51 ± 1.48	< 0.0001	0.27 ± 0.66	2.41 ± 1.60	< 0.0001	> 0.05
BNP, pg/mL	233 ± 114	998 ± 545	< 0.0001	239 ± 185	972 ± 631	< 0.0001	244 ± 153	981 ± 658	< 0.0001	> 0.05

*Paired t-test for intra-group comparison; **one-way analysis of variance (ANOVA), comparison of pre-op and post-op values between groups was not significant.

Table 2. Comparison of the TNF-alpha (pg/mL) data of stages belonging to all groups

n		S1		S2		S3		S4		p-value
		30		30		30		30		
Group 1	mean ± SD	20.06 ± 2.50		19.10 ± 11.02		187.16 ± 138.49		39.34 ± 37.46		[b]< 0.0001
	min.–max.	14.97–26.34		6.89–57.71		14.15–510.86		14.01–183.45		
	95% CI from-to	19.12–20.99		14.98–23.21		135.45–238.86		25.36–53.33		
	comparison	S1 vs S2	S1 vs S3	S1 vs S4		S2 vs S3	S2 vs S4		S3 vs S4	
	p-value**	> 0.05	< 0.001	< 0.05		< 0.001	< 0.05		< 0.01	
Group 2	mean ± SD	21.44 ± 3.67		17.93 ± 3.61		188.54 ± 127.66		27.91 ± 9.80		[a]< .0001
	min.–max.	16.09–31.13		8.94–23.88		12.23–447.43		15.88–55.93		
	95% CI From-To	20.07–22.80		16.58–19.28		140.88–236.21		24.26–31.57		
	comparison	S1 vs S2	S1 vs S3	S1 vs S4		S2 vs S3	S2 vs S4		S3 vs S4	
	p-value*	> 0.05	< 0.001	< 0.05		< 0.001	> 0.05		< 0.01	
Group 3	mean ± SD	19.79 ± 3.49		14.25 ± 4.13		122.54 ± 122.12		22.52 ± 8.57		[a]< 0.0001
	min.–max.	12.58–25.93		8.12–26.06		10.31–511.68		14.01–49.44		
	95% CI From-To	18.49–21.10		12.71–15.79		76.94–168.13		19.32–25.72		
	comparison	S1 vs S2	S1 vs S3	S1 vs S4		S2 vs S3	S2 vs S4		S3 vs S4	
	p-value**	> 0.05	< 0.001	> 0.05		< 0.001	> 0.05		< 0.01	
		G1 vs G2		G1 vs G3		G2 vs G3				
Comparison for S1		–		–		–				[c] 0.1164
Comparison for S2**		> 0.05		< 0.05		> 0.05				[c] 0.0260
Comparison for S3		–		–		–				[c] 0.0847
Comparison for S4*		> 0.05		< 0.05		< 0.05				[d] 0.0137

S – stage; G – group; SD – standard deviation; CI – confidence Interval; min.–max – minimum–maximum; [a] – repeated measures ANOVA; [b] – Friedman Test (nonparametric repeated measures ANOVA); [c] one-way analysis of variance (ANOVA); [d] – Kruskal-Wallis Test (nonparametric ANOVA). If p-value obtained by ANOVA is <0.05; *Dunns or **Tukey-Kramer multiple comparisons test (Post-hoc tests) was used to compared all stages (S1,S2 and S3). Post tests were not calculated because the p-value was greater than 0.05.

In group 3, S3 h-FABP levels were significantly higher than S1, S2 and S4 levels ($p < 0.001$) whereas no significant difference was found among S1, S2 and S4 h-FABP levels ($p > 0.05$). In group 1, no significant difference was found between S1 and S2 h-FABP levels whereas the differences among the other stages were statistically significant ($p < 0.001$). In group 2, no significant difference was found between S1 and S2 h-FABP levels whereas the differences among the other stages were statistically significant ($p < 0.001$) (Table 3).

In all groups, h-FABP levels were found to be increased after removal of the aortic cross-clamp and decreased by the 24th hour postoperatively ($p < 0.05$). There was a moderate but significant correlation between h-FABP and TNF-α (Spearman's rho = 0.47, $p < 0.001$).

S1 h-FABP levels did not differ significantly among the groups ($p > 0.05$). S2 h-FABP levels in group 3 were significantly lower compared to group 1 ($p < 0.05$). S3 h-FABP levels in group 3 were also significantly lower compared to group 1 but did not differ significantly from those in group 2 ($p < 0.01$ and $p > 0.05$, respectively). S4 levels in group 3 were significantly lower than those in group 2 ($p < 0.001$) (Table 3).

Discussion

The myocardium is exposed to artificial ischaemia and reperfusion ischaemia during extracorporeal circulation.[12] Myocardial protection against such insults is essential to the success of cardiac surgery. Systemic inflammation plays an important role in the development of reperfusion injury.[13] There is a positive relationship between the degree of systemic inflammation and inflammatory biomarkers.[14] Studies have demonstrated that remote ischemic preconditioning suppresses pro-inflammatory gene transcription in human leukocytes.[15]

Table 3. Comparison of the h-FAPB (ng/mL) data of stages belonging to all groups

n		S1		S2	S3		S4	p-value
		30		30	30		30	
Group 1	mean ± SD	2.24 ± 0.79		3.08 ± 1.77	7.68 ± 3.30		3.76 ± 2.02	
	min.–max.	1.08–3.96		1.16–8.67	1.32 -18.10		1.00–9.86	
	95% CI From-To	1.95–2.54		2.42–3.74	6.45–8.92		3.00–4.51	[b]< 0.0001
	comparison	S1 vs S2	S1 vs S3	S1 vs S4	S2 vs S3	S2 vs S4	S3 vs S4	
	p-value**	> 0.05	< 0.001	< 0.01	< 0.001	> 0.05	< 0.001	
Group 2	mean ± SD	2.51 ± 1.23		2.79 ± 1.20	6.20 ± 3.69		4.60 ± 1.38	
	min.–max.	1.04–5.95		1.04–5.37	2.17–14.74		1.79–6.98	
	95% CI From-To	2.05–2.97		2.34–3.23	4.82–7.57		4.09–5.12	[a]< .0001
	comparison	S1 vs S2	S1 vs S3	S1 vs S4	S2 vs S3	S2 vs S4	S3 vs S4	
	p-value*	> 0.05	< 0.001	< 0.001	< 0.001	< 0.01	< 0.05	
Group 3	mean ± SD	2.10 ± 0.69		2.00 ± 1.26	4.98 ± 2.90		3.06 ± 1.09	
	min.–max.	0.89–3.85		0.74–7.95	1.56 -12.74		1.73–6.39	
	95% CI From-To	1.84–2.35		1.53–2.47	3.89–6.06		2.65–3.47	[a]< 0.0001
	comparison	S1 vs S2	S1 vs S3	S1 vs S4	S2 vs S3	S2 vs S4	S3 vs S4	
	p-value**	> 0.05	< 0.001	> 0.05	< 0.001	< 0.05	< 0.001	
		G1 vs G2		G1 vs G3	G2 vs G3			
Comparison for S1		–		–	–			[b] 0.2295
Comparison for S2**		> 0.05		< 0.05	> 0.05			[b] 0.0126
Comparison for S3		> 0.05		< 0.01	> 0.05			[b] 0.0085
Comparison for S4*		> 0.05		> 0.05	< 0.01			[b] 0.0010

S – stage; G – group; SD – standard deviation; CI – confidence Interval; min.–max – minimum–maximum; [a] – repeated measures ANOVA; [b] – one-way analysis of variance (ANOVA). If p-value obtained by ANOVA is <0.05; **Tukey-Kramer multiple comparisons test (post-hoc tests) was used to compared all stages (S1,S2 and S3).

Landoni et al. reported in their randomized meta-analysis that troponin I levels showed greater reduction with the modern volatile agents desflurane and sevoflurane in patients undergoing cardiac surgery.[16] However, we found no difference in troponin I levels between the groups receiving or not receiving desflurane. This finding may be attributed to the dosage of propofol or desflurane or use of intravenous anesthesia as the anesthetic approach. In addition, the cardio-protective effect propofol produced alone may be another reason why troponin I levels were different.

Moreover, our results are supported by others suggesting that there was no difference between propofol and sevoflurane with regard to postoperative mortality and myocardial infarction in patients undergoing CABG. These results, as reported previously, are due to the antioxidant effects of propofol and preconditioning effects of volatile anesthetics.[17] An inverse relationship was noted between the effectiveness of preconditioning and the amount of reactive oxygen species, whilst propofol is known as a reactive oxygen scavenger. On the other hand, Smul et al. reported in their experimental study on rabbits that propofol inhibits desflurane-related preconditioning.[18] However no conclusive evidence exists to justify the relationship of this effect with free radicals.

In their prospective study on 120 patients, Huang et al. reported that TNF-α showed a significant increase within 5 min after removal of the aortic cross-clamp in all groups whilst TNF-α levels were significantly lower after cross-clamping of the aorta in patients receiving propofol and isoflurane compared to other groups.[8] In line with our data, these authors found that an isoflurane and propofol combination was superior to regimens consisting of isoflurane alone or propofol alone.

In our study, we found that TNF-α levels were significantly lower in patients receiving low-dose propofol and continuous desflurane administration than in other groups after removal of the cross-clamp and by the 24th

postoperative hour, when stress and traumatic events (inflammation) reach their maximum. This may be attributable to the cardio-protective effect of volatile agents and their anti-inflammatory properties.[11,17] Moreover, some studies have reported the anti-oxidant effects of propofol. Such studies demonstrated that, as a pro-inflammatory cytokine that increases with the production of oxygen radicals, TNF-α levels decrease after CPB.[10,11] In light of the above, any increase in TNF-α levels should be considered a negative criterion since it is associated with decreased tolerance of ischemic damage and inflammation.

We found lower TNF-α levels in the propofol combined with continuous desflurane group compared to the propofol alone group before cross-clamping of the aorta, which may be due to the early cardio-protective effects of desflurane. The significant decrease in TNF-α levels in group 3 in the postoperative period highlights the effectiveness of the preconditioning effect of low-dose propofol and continuous desflurane administration. A few studies support these findings.[19] Sayın et al. have reported that propofol inhibits lipid peroxidation.[20] In our study, both the cardio-protective and the anti-oxidant effects of propofol and desflurane might have been observed. Unlike previous studies, the present study demonstrated that the addition of desflurane to propofol reduces TNF-α levels following cardiopulmonary bypass. Desflurane and propofol may potentiate the preconditioning effects of each other.

In the present study, h-FABP levels showed an initial increase after cross-clamping of the aorta but they had decreased by the 24th postoperative hour, especially in group 3. The moderate correlation between h-FABP levels and TNF-α levels may be explained by inflammatory and traumatic processes, supporting the view that they may influence the release of each other. Some studies have suggested that h-FABP may be a marker of early ischaemia.[8,9] Moreover, h-FABP has been shown to have an earlier peak compared to CK-MB or cardiac troponin I. In another study[21], h-FABP was demonstrated to be a marker of long-term mortality following acute coronary syndrome, and is capable of defining high-risk patients.[21] In light of the above, the present study demonstrated that low-dose propofol and continuous desflurane administration was more effective than propofol alone or propofol combined with 15 min of desflurane administration when h-FABP levels were considered as the measure of preconditioning. Lower h-FABP levels were observed in the low-dose propofol and continuous desflurane group compared to propofol alone before cross-clamping of the aorta and more profoundly after removal of the cross-clamp, indicating desflurane's favorable effect on myocardial adaptation to ischaemia. Moreover, the lower h-FABP levels observed in the low-dose propofol and continuous desflurane group at the 24th postoperative hour demonstrate that the longer the duration of desflurane administration, the better prepared the myocardium is against ischaemia and reperfusion.

Tomai et al. found no difference between 15 min of isoflurane administration before cardiopulmonary bypass and control groups with regard to myocardial function and cardiac enzyme levels.[22] We found that troponin levels in the continuous or intermittent desflurane administration and non-desflurane groups were similar. In recent years, there has been no detailed data regarding the combined use of propofol and desflurane or their short-course administration. However, there have been many reports suggesting that these drugs inhibit severe inflammation and reduce TNF-α levels as well as their preconditioning effects. Such studies report that ischemic preconditioning inhibits the local myocardial and systemic inflammatory response.[15,23] However, whether the decrease in TNF-α levels occurs due to the preconditioning effects of these drugs or their effects on inflammation is unclear.

Zhang et al. reported that the antioxidant effect of propofol is due to the phenol group it contains, similar to vitamin E.[24] They found that it causes lower neutrophil activation and a lower increase in C5a levels after CABG.

In conclusion, h-FABP and TNF-α levels may be used to assess the effectiveness of ischemic preconditioning practice. On the basis of the measurement of these pro-inflammatory cytokines, low-dose propofol and continuous desflurane provided more effective preconditioning than propofol alone or short-course desflurane in patients undergoing CABG.

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Usefulness of a new anthropometric indicator – VAI (Visceral Adiposity Index) in the evaluation of metabolic and hormonal disorders in women with polycystic ovary syndrome

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Abstract

Background. Visceral adiposity index (VAI) is a new anthropometric indicator that makes it possible to define the risk of obesity-related cardiometabolic complications even before the diagnosis of metabolic syndrome.

Objectives. Evaluating the usefulness of VAI in order to differentiate metabolically unhealthy women (MU-PCOS) and defining the usefulness of this index in everyday practice.

Material and methods. A prospective study was conducted among 43 women who met the Rotterdam criteria for diagnosing polycystic ovarian syndrome. Body composition was measured using bioelectrical impedance analysis. Statistical analysis was performed using STATISTICA v. 10 and relevant statistical tests. The adopted significance level was $p = 0.05$.

Results. Based on the study results, a significant positive correlation was found between the value of VAI and the concentration of insulin ($r = 0.33$; $p < 0.01$), HOMA index ($r = 0.46$; $p < 0.01$) and FAI ($r = 0.54$; $p < 0.01$). It was also shown that there is a significant negative correlation between VAI and the concentration of SHBG ($r = -0.53$; $p < 0.01$) and HDL cholesterol ($r = -0.88$; $p < 0.01$). In the group of metabolically unhealthy women, the research showed a significantly higher value of free testosterone, SHGB, DHEAS and FAI ($p < 0.05$).

Conclusions. Visceral adiposity index makes it possible to introduce early prevention of metabolic disorders (including cardiometabolic disorders), as well as to evaluate the increase of hyperandrogenemia in women with polycystic ovary syndrome. The use of the cut-off point of VAI = 1.675 is a simple way to evaluate women with MU-PCOS.

Key words: polycystic ovarian syndrome, visceral adiposity index, metabolic disorders

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Background

Visceral adiposity index (VAI) is a recent anthropometric indicator indirectly reflecting the risk of obesity-related cardiometabolic complications. Its application allows early evaluation of such risk, even prior to diagnosing overt metabolic syndrome.[1] VAI can be considered a marker of adipose tissue dysfunction as it provides reliable data when used in the general population and in patients with polycystic ovary syndrome, acromegaly, type 2 diabetes, viral hepatitis C, non-alcoholic fatty liver diseases.[1] This index allows accurate identification of visceral adipose tissue in women with polycystic ovary syndrome, determining intra-abdominal adipose tissue content in a similar way to a computed tomography (CT) scan performed in this group of female patients.[2] VAI is an empirical-mathematical model, gender-specific and based on anthropometric measurements, such as BMI and waist circumference, and biochemical parameters, namely triglyceride (TG) and high-density lipoprotein cholesterol (HDL-C) concentrations.[3]

Fig. 1 illustrates the formula of the visceral adiposity index for women.

Fig.1. Waist circumference (cm), BMI (kg/m^2), TG (mmol/L), HDL (mmol/L)

$$VAI = \left(\frac{\text{waist circumference}}{36.58 + (1.86 \times \text{BMI})}\right) \times \left(\frac{TG}{0.81}\right) \times \left(\frac{1.52}{HDL}\right)$$

Polycystic ovary syndrome (PCOS) manifests itself in menstrual disorders, clinical and biochemical hyperandrogenism and polycystic ovaries on ultrasound. In addition, it is also responsible for higher risk of cardiovascular diseases, impaired glucose tolerance and type 2 diabetes.[4] Insulin resistance and android (abdominal) obesity in PCOS women are not always associated with increased BMI. Excess body weight is not necessary for increased cardiometabolic risk to occur in this group of patients, however it leads to the exacerbation of clinical symptoms of the syndrome in question.[5] Since VAI is a simple and easy method for evaluation of cardiometabolic risk, it can be a useful tool for the assessment of metabolic risk related to visceral obesity both in everyday medical practice and in population studies. Values of VAI > 1.675 make it possible to differentiate women with metabolically unhealthy polycystic ovary syndrome (MU-PCOS) from women with metabolically healthy polycystic ovary syndrome (MH-PCOS). Nevertheless, the index should not be used for individuals who are under 16 years of age, who have morbid obesity (BMI > 40 kg/m^2), who are diagnosed with pendulous abdominal skin folds or hypertriglyceridemia, nor in subjects who are being treated with fibrates and/or are following a low-energy diet.[1]

Objectives

Application of the cut-off point for VAI = 1.675 in order to distinguish women with metabolically unhealthy polycystic ovary syndrome (MU-PCOS) and to determine the usefulness of the index in question among women diagnosed with polycystic ovary syndrome in everyday clinical practice.

Material and methods

The prospective study was conducted among 43 women hospitalized at the Department of Endocrinology and Gynecology of Silesian University, and fulfilling the Rotterdam criteria for diagnosing polycystic ovary syndrome. The diagnosis of the syndrome had to include at least 2 of the following 3 criteria: oligoovulation or anovulation, clinical and/or biochemical hyperandrogenism, polycystic ovaries on ultrasound after exclusion of other causes of the above-mentioned symptoms. The exclusion criteria applied in the study were: diagnosis of androgen excess disorders (congenital or late-onset congenital adrenal hyperplasia, androgen-secreting tumors, Cushing's disease/syndrome, hyperprolactinemia, idiopathic hirsutism), uncontrolled thyroid disorders, mental disorders, morbid obesity (BMI > 40 kg/m^2), pendulous abdominal skin folds, hypertriglyceridemia, low-energy diet, use of hormonal contraception in the past 6 months, taking fibrates or steroids, and being over the age of 40.

Prior to conducting the actual studies, the consent of the Bioethics Committee was obtained, no. KNW/0022/KB1/53/14, dated June 3rd, 2014. Body composition was evaluated by bioelectrical impedance analysis utilizing a Tanita BC-420 MA Body Composition Analyzer (product in accordance with the Medical Device Directive MDD 93/42 EEC). The standard anthropometric measurement method was applied for the circumference of the waist, hips, wrist, shoulder and calf. A stadiometer (Tanita HR-100 Height Rod) was used to measure body height. Waist-to-hip ratio (WHR) was adopted in the study. It can be calculated by dividing the waist circumference (in cm) at the umbilical level by the hip circumference (in cm) at the superior iliac spine level. Values higher than or equal to 0.85 indicate abdominal obesity and lower than 0.85 gluteal-femoral obesity. The value of waist-to-height ratio (WHtR) was obtained by dividing the waist circumference (in cm) at the umbilical level by the body height (in cm). Results higher than 0.5 indicate increased risk of obesity-related cardiovascular diseases.

Blood samples were collected in the morning during routine patient examinations in the ward. The tests were performed by means of the immunoenzymatic method (ELISA) using commercial test kits in an accredited analytical laboratory monitored by the external quality as-

surance programs. Laboratory blood tests included complete lipidogram, levels of glucose, total testosterone, free testosterone, sex hormone binding globulin (SHBG), dehydroepiandrosterone (DHEAS), insulin, homeostatic model assessment of insulin resistance (HOMA-IR), and free androgen index (FAI).

The studied women were divided into 2 groups with metabolically healthy polycystic ovary syndrome (MH-PCOS; VAI < 1.675) and metabolically unhealthy polycystic ovary syndrome (MU-PCOS; VAI > 1.675). The statistical analysis of the obtained results was conducted with STATISTICA v. 10.0. The Shapiro-Wilk test was utilized to verify normal distribution for the analyzed variables. Statistical tests to verify particular study hypotheses were properly selected in order to reveal differences or correlations between the variables. For the differences between the study groups to be determined, the following tests were applied: the Student's t-test (normal distribution) and the Mann-Whitney U-test (abnormal distribution). The results showing normal distribution were expressed as an average value (x) ± standard deviation (SD). The parameters with abnormal distribution were expressed as median and interquartile ranges. For assessment of the correlations between the analyzed variables, the Spearman rank correlation was used. The significance level of p = 0.05 was adopted.

Results

The study was carried out in a group of women at the age of 18–38 (26.52 ± 5.73). The average values of the selected anthropometric parameters were as follows: body weight 73.54 ± 19.07 kg; waist circumference 87.07 ± 16.58 cm; BMI 27.16 ± 6.91 kg/m^2; WHR 0.82 ± 0.08; WHtR 0.53 ± 0.1; fat mass percentage (FM%) 34.01 ± 9.7. Table 1 illustrates the detailed characteristics of the MH-PCOS and MU-PCOS women.

Table 1. Comparison of data from patient groups before and after the operation

Parameters	MH-PCOS (n = 28)	MU-PCOS (n = 15)	p-value
Age (years)	26 (22.5–31)	25.27 ± 5.57	0.3021
Body weight (kg)	61.3 (54.9–70.25)	89.8 (74–103.2)	< 0.01
Waist circumference (cm)	79.52 ± 11.9	100.5 ± 16.03	< 0.01
BMI (kg/m^2)	22.85 (20.7–25.25)	32.61 ± 6.44	< 0.01
WHR	0.78 ± 0.06	0.89 ± 0.08	< 0.01
WHtR	0.47 (0.43–0.52)	0.62 ± 0.09	< 0.01
FM%	29.77 ± 7.98	42.9 (37–46.1)	< 0.01

FM% – fat mass percentage.

Significant differences were revealed between the study groups of women, which concerned the average values of the following anthropometric parameters and indicators: body weight, waist circumference, BMI, WHR, WhtR and FM%. The average values of the analyzed tests were significantly higher in the MU-PCOS group than in the MH-PCOS group (p < 0.01).

The study also revealed significant differences between the MH-PCOS and MU-PCOS groups with regard to the average values of the following biochemical studies: concentrations of triglycerides, HDL cholesterol, SHBG, free testosterone, DHEAS and HOMA-IR (p < 0.05) (Table 2).

Table 2. Analysis of the differences of the average values of the selected biochemical studies between the groups of MH-PCOS and MU-PCOS women

Parameters	MH-PCOS (n = 28)	MU-PCOS (n = 15)	p-value
Testosterone (nmol/L)	2.01 ± 0.73	2.15 (1.63–3.12)	0.1327
Free testosterone (pmol/L)	15.19 (11.13–30.93)	32.66 (17.86–44.83)	< 0.05
SHBG (nmol/L)	60 ± 29.54	27.70 (18.25–52.60)	< 0.05
DHEAS (umol/L)	8.36 ± 3.41	10.82 ± 3.21	< 0.05
FAI	3.3 (3.1–5.5)	6.65 (4.8–12.75)	< 0.05
Glucose (mmol/L)	5.0 (4.67–5.22)	5.25 ± 0.5	0.0647
Insulin (pmol/L)	65.65 (43.05–87.54)	85.31 ± 37.02	0.2082
HOMA	1.66 (1.0–2.5)	2.72 ± 1.21	< 0.05
TC (mmol/L)	4.84 ± 0.63	4.56 ± 0.67	0.1729
HDL-C (mmol/L)	1.83 ± 0.39	1.16 (1.09–1.27)	< 0.01
LDL-C (mmol/L)	2.64 ± 0.66	2.56 ± 0.68	0.7064
TG (mmol/L)	0.81 ± 0.26	1.6 (1.37–2.03)	< 0.01

TC – total cholesterol.

The study involved a correlation analysis between VAI and the studied variables for the whole PCOS group (n = 43). The analysis showed a positive correlation between the VAI value and body weight (r = 0.69; p < 0.01), WHR (r = 0.67; p < 0.01), WHtR (r = 0.72; p < 0.01) and visceral adipose tissue (r = 0.69; p < 0.01), as well as FM% (r = 0.69; p < 0.01). The correlation analysis was also performed for biochemical studies (Table 3).

It was found that, along with an increase in the VAI value, the concentrations of insulin, triglycerides, HOMA-IR and FAI also significantly increased (p < 0.05). A negative correlation was reported between VAI and serum HDL cholesterol and SHGB concentrations (p < 0.01). No significant

Table 3. Evaluation of the correlations between the VAI value and the selected biochemical studies in the study groups of women (n = 43)

VAI	R*	p-value
Glucose (mmol/L)	0.14	0.3690
Insulin (pmol/L)	0.33	< 0.05
TC (mmol/L)	-0.13	0.4016
HDL-C (mmol/L)	-0.88	< 0.01
LDL-C (mmol/L)	0.09	0.5878
TG (mmol/L)	0.94	< 0.01
SHBG (nmol/L)	-0.53	< 0.01
Testosterone (nmol/L)	0.20	0.2053
Free testosterone (pmol/L)	0.30	0.0507
DHEAS (umol/L)	0.30	0.0551
HOMA	0.46	< 0.01
FAI	0.54	< 0.01

*Spearman's rank correlation.

correlations were revealed between VAI and the concentrations of glucose, complete cholesterol, LDL cholesterol, testosterone, free testosterone and DHEAS ($p > 0.05$).

Discussion

VAI, similarly to a computed tomography scan, is an effective indicator of visceral adipose tissue. This was concluded by Jee-Young Oh et al., who had assessed the correlation between VAI and insulin resistance in a group of 180 Korean women with polycystic ovary syndrome. The study revealed a positive correlation between visceral fat area (VFA), measured by a CT scan, and VAI ($r = 0.57$; $p < 0.01$).[2] The authors of this manuscript found significant dependence between VAI and the indicator of visceral adipose tissue measured by the bioelectrical impedance analysis. In another study, which comprised a group of 193 women with PCOS, Androulakis II et al. observed a significant positive correlation between VAI and the concentrations of insulin ($r = 0.57$; $p < 0.01$), SHBG ($r = -0.22$; $p < 0.05$) and HOMA-IR ($r = 0.55$, $p < 0.01$). The same observation was made by the authors of this paper. The authors of the quoted study revealed significantly higher values of VAI and HOMA in the group of PCOS women with menstrual disorders versus the female group with normal ovulation.[7] In the research conducted by the authors of this manuscript, the studied women were not divided according to menstrual disorders.

Amato MC et al., relying on studies conducted in a group of 224 women with PCOS, found that in addition to the fasting plasma glucose level, VAI value and DHEAS concentration can also be considered useful in determining the risk of diabetes in the screening tests for all women with polycystic ovary syndrome.[8] The authors of this manuscript did not reveal a significant dependence between VAI and the fasting plasma glucose level. Such a correlation, however, was found between insulin and HOMA concentrations ($p < 0.05$). Moreover, a correlation analysis between VAI and FAI was performed. On the basis of the results obtained, it can be claimed that the index in question is also an effective tool in assessing the severity of hyperandrogenemia in PCOS women. The correlation results between VAI and FAI have not been published by other researchers as yet. Very few studies have evaluated the usefulness of VAI in PCOS women. Therefore, it is necessary to carry out reliable, large population-based studies among European women diagnosed with polycystic ovary syndrome.

Conclusions

Following the studies conducted by the authors of this manuscript, it may be concluded that utilizing VAI during routine medical examinations makes possible early prevention of metabolic disorders (including cardiometabolic disorders), as well as assessment of hyperandrogenemia severity in women with polycystic ovary syndrome. The cut-off point for VAI = 1.675 is an easy tool for evaluating women with metabolically unhealthy polycystic ovary syndrome (MU-PCOS).

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Orthodontic intrusion of periodontally-compromised maxillary incisors: 3-dimensional finite element method analysis

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Abstract

Background. Loading of the compromised periodontium with orthodontic forces produces different results than those achieved in patients with healthy periodontal support. Determining the force value at a level preventing further deterioration of the patient's periodontal status, thus delivering the most precisely individualized "dose" of loading, seems to be crucial for the successful intrusion of teeth with reduced periodontal support.

Objectives. The aim of the study was to determine the range of force values efficiently intruding maxillary incisors without further compromising the initially-impaired periodontal status. Finite element analysis (FEA), allowing estimation of the stress and strain distribution, was the method of choice.

Material and methods. The CT scans of a periodontally-compromised patient were segmented using InVesalius software. A model – based on NURBS surfaces – was adjusted to the CT scans in order to obtain both smooth and natural curvatures of each model segment. All relevant tissues were modeled as separate volumes. The geometric model was discretized in order to create a numerical model for applying Ansys software (v. 15.07) and using APDL. The central incisors were loaded with external intrusive forces, ranging from 0.1 to 0.4 N.

Results. The simulation, performed iteratively, showed that even the lowest force value – 0.1 N – causes stress changes in the alveolus and on the root surfaces, with a tendency of stress increasing towards the bottom of the alveolus and root apex. It is also notable that during the application of forces of equal magnitude, the stress/strain distribution was significantly higher around tooth 21, which displayed the highest range of PDL reduction. Application of the same force level created a higher stress-strain response around tooth 21, and the characteristics were less homogenous.

Conclusions. A force value of 0.1 N applied in vivo might produce the most effective tooth intrusion and bone modeling which favors bone defect regeneration.

Key words: FEM, intrusion, PDL, bone defect, orthodontic forces

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Introduction

It is assumed that the development of bone defects and attachment loss caused by periodontal disease leads, in 30–50% of patients, to extrusion and proclination of the maxillary incisors, which is also called pathologic tooth migration.[1] Flared maxillary front teeth significantly handicap smile aesthetics and chewing function, therefore they require loading with orthodontic intrusive forces, provided the periodontal disease is stabilized and controlled. In the era of intensive development of multidisciplinary treatment approaches, the application of orthodontic intrusion seems to be beneficial in such cases, provided that the periodontium condition is stabilized. However, despite original studies and quite numerous case reports present in the current literature, intrusion of periodontally compromised teeth remains a controversial movement.[2–5] The intrusive movement is subjected to a large risk of failure, predominantly apical root resorption and reduction of the alveolar bone height.[6,7] The occurrence and range of these failures depend on many issues, but periodontal status as well as force magnitude and direction are the major factors. Loading of the compromised periodontium with orthodontic forces produces different results than those achieved in patients with healthy periodontal support. Determining the force value at a level preventing further deterioration of the patient's periodontal status, thus delivering the most precisely individualized "dose" of loading, seems to be crucial for the successful intrusion of teeth with reduced periodontal support. However, such a protocol demands certain measurements, impossible to be taken in vivo, but requiring in vitro studies and laboratory or numerical techniques, not as yet discussed in the literature.

The aim of the study was to determine the range of force values efficiently intruding maxillary incisors without further compromising the initially-impaired periodontal status. Finite element analysis (FEA) of a model with reduced periodontium, allowing estimation of the stress and strain distribution, was the method of choice.

Material and methods

The CT scans of a periodontally-compromised patient were segmented using InVesalius software (Fig. 1) in order to create a geometric model of the maxilla. This model – based on NURBS (Non-Uniform Rational B-Spline) surfaces – was subsequently adjusted to the CT scans in order to obtain both smooth and natural curvatures of each model segment. All relevant tissues were modeled as separate volumes – cortical bone, spongeous bone, periodontal ligaments (PDL), dentin, enamel and pulp (Fig. 2). The geometric model was discretized in order to create a numerical model for applying Ansys software (v. 15.07) and using APDL (ANSYS Parametric Design Language). The PDL layers of average thickness 0.1 mm were meshed using hexagonal finite elements, whereas the other tissues like bone (spongeous bone and cortical plates) and teeth (enamel and dentin) were meshed using tetragonal finite elements.

The literature review together with our personal experience made it possible to select the most suitable mechanical properties of particular tissues.[8-11] The values displaying the mechanical properties of these tissues are shown in Table 1. As per PDL – considered as a structure displaying strongly nonlinear stress and strain distribution – a hyper-elastic Noe-Hookean material model was proposed. The model was calculated using non-linear elastic PDL properties (Fig. 3). The nominal stress level corresponding to the applied load was below 0.01 MPa, so the region on the strain-stress response of the PDL material was nearly linear, which is shown in Fig. 4.

Fig. 1. Segmentation of CT scan

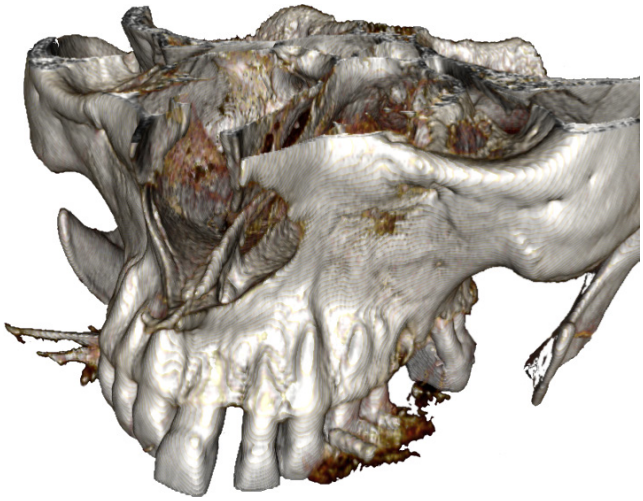


Fig. 2. Modeling of the cortical bone, spongeous bone, periodontal ligaments (PDL), dentin, enamel and pulp

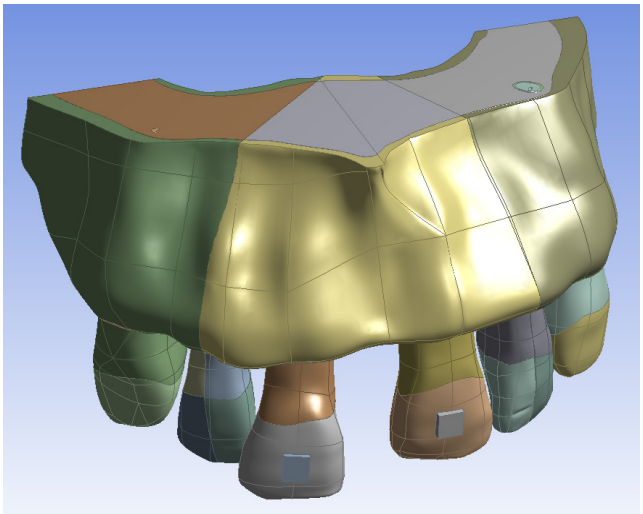


Fig. 3. PDL material properties with marked region of interest with respect to the nominal stress level in the PDL volume

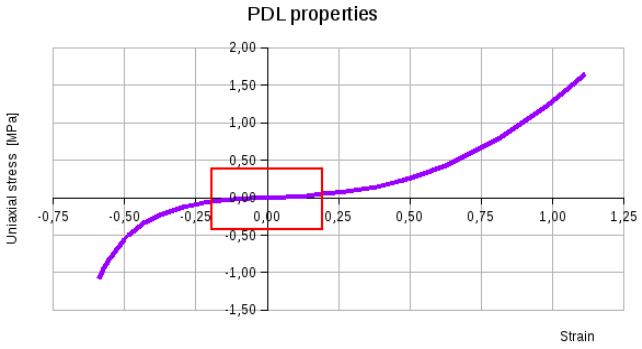
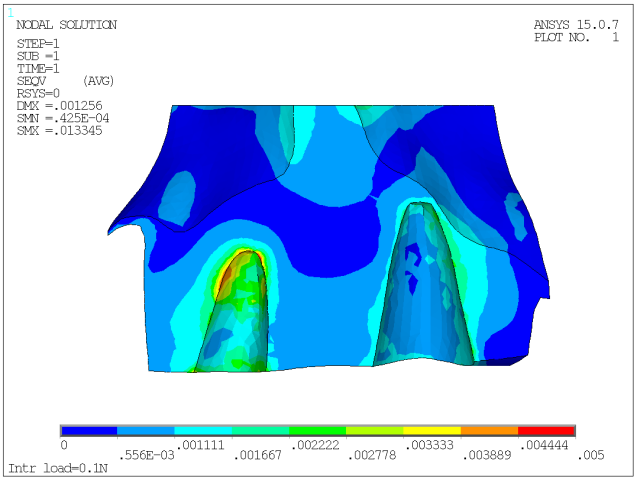


Fig. 4. Changes in the stress-strain distribution



The central incisors were loaded with external intrusive forces, ranging from 0.1 to 0.4 N (with steps of 0.05 N).

Results

The resulting data indisputably proved that a mechanical load, produced by an orthodontic fixed appliance, changes the mechanical properties of the bone tissues. The intrusive orthodontic force changes the stress-strain distribution (Fig. 4 and 5). The simulation, performed iteratively, showed that even the lowest force value, 0.1 N, causes

Table 1. Material properties of evaluated tissues

Material	Elasticity module [MPa]	Poisson ratio [-]
Enamel	85 000	0.3
Dentine	20 000	0.3
Cortical bone	20 000	0.3
Spongeous bone	450	0.42

Fig. 5. Changes in the stress-strain distribution, lateral view

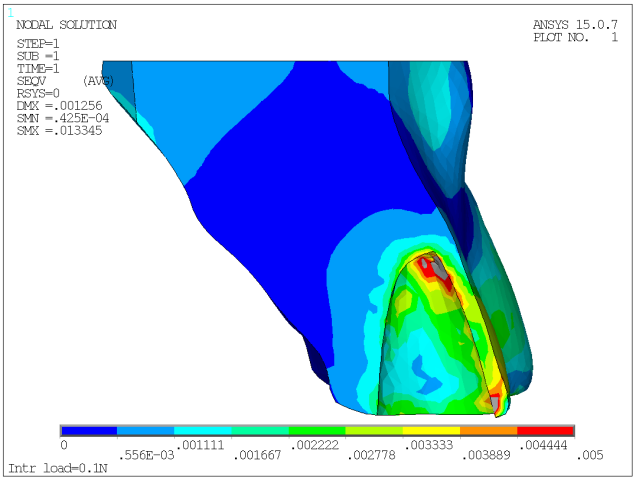
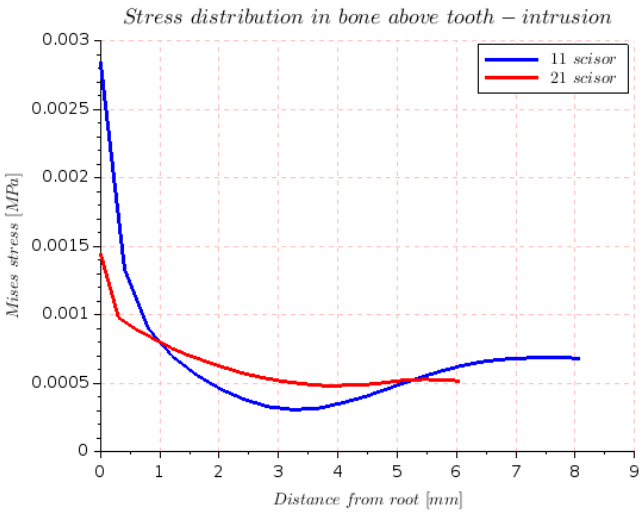


Fig. 6. Changes the stress-strain distribution, lateral view



stress and strain changes in the alveolus and on the root surfaces, with a tendency of stress increasing towards the bottom of the alveolus and root apex. It is also notable that during the application of forces of equal magnitude, the stress/strain distribution was significantly higher around tooth 21, which displayed the highest range of PDL reduction. The periodontal ligaments supporting teeth 11 and 21 were weakened, however the bone defect adjacent to tooth 21 was more pronounced, hence application of the same force level created a higher stress-strain response around tooth 21, of the less homogenous characteristics (Fig. 6).

Discussion

Intrusive movement is laden with a high risk of multiple failures, e.g. root resorption, alveolar bone resorption or pulpal necrosis.[7] Many aspects may contribute to the occurrence of such failures – general factors, e.g. genetic and systematic disorders or gender, as well as local factors

such as orthodontic force direction, its magnitude and the duration of loading.[12–14] Since these failures may be of iatrogenic origin, minimizing the risk of their occurrence should be of major concern to clinicians.

Moreover, intrusion of maxillary incisors that have migrated pathologically due to periodontitis is still controversial.[15] Case reports available in the literature and a limited number of original studies show the positive influence of intrusion on periodontal tissues.[2,3,5,16,17]

Regardless of the direction of the tooth movement, evaluation of the periodontium remodeling influenced by orthodontic forces is impossible in vivo, which is why all of the results reported in the literature follow in vitro studies. Reviewing the literature reveals many measurement methods, e.g. the photo-elastic technique, laser holography and optomechanical set-up.[18–20] However, each of these methods has some limitations; the finite element method (FEM) seems to be the most suitable, especially in a new millennium: the era of intensive development of different digital analysis techniques.

Since the highest resemblance of in vivo conditions in the generated model is of the utmost importance, we used CT scans of a patient to create a fully individualized FEM model of both structures, the jaw bones and teeth. In the literature, one may find papers reporting manual modeling of the particular elements, especially the teeth.[21,22] However, the root shape is a very individual and unique feature and determines tooth behavior during intrusion, which was supported by studies of Levander and Malmgen.[23] That is why individualization achieved due to using the patient's own model seems to be the most suitable.[24]

Moreover, in the present study, during modeling of the bony tissues, the cortical and spongeous bone were distinguished, as well as the dental structures: enamel, dentine and pulp. Even small differences in the anatomical changes affect the behavior of the studied parameters. Therefore it is important to customize the model.[25] Since the PDL were a particular structure, they were given elastic and nonlinear features. It has been proven that bone remodeling is strongly related to PDL characteristics.[26,27] Alveolar bone remodeling influenced by orthodontic forces differs from remodeling influenced by external loads with forces on the other bones. Orthopedic surgeons believe that mechanical forces causing compression stimulate bone formation and tension stimulates resorption, which is contrary to orthodontic bone remodeling.[28] It is assumed that indeed PDL is this unique tissue responsible for different alveolar bone response.

In our study, we began the analysis of the effects produced by the forces at a low level: 0.1 N. We observed that this minimum force already produces stress and strain within the periodontium and on the dental root surface. The predominant stress concentration was found in the alveolus bottom, although discreet changes occurred along the whole alveolus surface. This allows us to conclude that a force of level 0.1 N is already effective in terms of movement of periodontally-compromised teeth. A similar stress and strain distribution was demonstrated by Rudolph et al. and Vikram et al., but they demonstrated the results achieved by forces whose values were more than twice as high and on healthy periodontium.[29,30] During orthodontic intrusion, the stress concentration occurs on a very small surface present on the alveolus bottom and the root apex. That is why it is so crucial to apply the lowest yet still effective forces. The vast majority of studies which evaluate intrusion analyze relatively higher force values acting on healthy periodontium.

Our study proved that there is a relationship between stress and strain distribution and PDL surface. The more extensive the bone defect is, the smaller the PDL surface is loaded with the same force magnitude. This is crucial in periodontally-compromised patients, in which the bone defect ranges vary between individual teeth. During treatment with fixed appliances, it is important to remember that the planned treatment biomechanics should be adjusted to the tooth with the largest bone defect; this tooth is the weakest link and thus the most susceptible to failure.

Conclusions

The bone tissue reaction to load induced by fixed orthodontic appliances and transformed by the tooth is building new internal structures, changed in terms of both their density and mechanical properties. During orthodontic treatment, the forces acting on bone tissue result in both bone formation and bone resorption. As shown in our article, the developed procedure makes possible the adequate planning of orthodontic treatment to obtain bone creation processes significantly prevailing bone resorption, which is crucial in periodontally-compromised patients. Indeed the method is based upon numerical modeling and simulation but, thanks to CT scans, the model obtained is individualized. The data obtained in such a manner allows the conclusion that a force value of 0.1 N applied in vivo might produce the most effective tooth intrusion and bone remodeling which favors bone defect regeneration.

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Serum ICAM-1, VCAM-1 and E-selectin levels in patients with primary and secondary Sjögren's syndrome

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Abstract

Background. Typical features of Sjögren's syndrome (SS) are severe xerostomia and xerophthalmia which are basic diagnostic criteria.

Objectives. The aim of this study was to compare the serum levels of soluble (s) intercellular adhesion molecule 1 (sICAM-1), vascular cell adhesion molecule 1 (sVCAM-1) and sE-selectin between primary (pSS), secondary (sSS) and healthy subjects (HS). We correlated these results with selected clinical parameters of disease activity and parameters of the severity of xerostomia and xerophthalmia.

Material and methods. The serum levels of sICAM-1, sVCAM-1 and sE-selectin were determined by enzyme-linked immunosorbent assay (ELISA) in 16 patients with pSS, 18 with sSS and 15 HS. Eye dryness and xerostomia were assessed by the Schirmer's test, the Fox test and the visual analogue scale (VAS).

Results. The levels of sICAM-1 in pSS and sVCAM-1 in sSS patients were significantly higher when compared to HS ($p = 0.02$ and $p = 0.048$, respectively). There were no differences between pSS and sSS. In pSS, sVCAM-1 correlated positively with VAS ($rS = 0.52$, $p = 0.04$) and the Fox test ($rS = 0.66$, $p=0.01$). In sSS, sE-selectin correlated positively with sICAM-1 ($rS = 0.54$, $p = 0.01$), the duration of the disease ($rS = 0.51$, $p = 0.03$) and negatively with the Schirmer's test ($rS = 0.59$, $p = 0.04$). sICAM-1 correlated positively with the erythrocyte sedimentation rate (ESR) value ($rS = 0.59$, $p = 0.01$).

Conclusions. sVCAM-1 reflects xerostomia in pSS. sICAM-1 and sE-selectin may be additional parameters of sSS activity.

Key words: Sjögren's syndrome, xerostomia, sICAM-1, sE-selectin, sVCAM-1

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Introduction

Recently, the importance of angiogenesis in the pathogenesis of autoimmune diseases related to chronic inflammation such as rheumatoid arthritis (RA), systemic lupus erythematosus (SLE) and Sjögren's syndrome (SS) has been highlighted.[1,2] Observations from many studies support the existence of an important link between inflammation and angiogenesis in the pathogenesis of SS.[1,2] In the labial salivary glands of SS patients, immunocytochemical studies have shown an increased expression of ICAM-1 and E-selectin on endothelial cells and of ICAM-1 on the epithelium.[2,3] The expression and function of ICAM-1, VCAM-1 and E-selectin have been implicated in the pathogenesis of connective tissue diseases (CTD) by the interactions of circulating immune cells with vascular endothelium during extravasation and the interactions between T cells and antigen-presenting cells (APC).[4]

Typical features of SS are severe xerostomia and xerophthalmia, which are basic SS diagnostic criteria. The presence of both dry mouth and dry eyes classified patients with 93% sensitivity and 97.7% specificity.[5] Decreased salivation markedly affects oral health and very often inhibits normal functioning. To determine xerostomia objectively, sialometry is applied to measure the quantity of resting and stimulated saliva secretion over the controlled time. Subjective tests which evaluate the real experience of xerostomia and the discomfort are of similar importance. The VAS and Fox test are carried out for this purpose.

The levels of soluble (s) ICAM-1, sVCAM-1 and sE-selectin in patients with pSS and sSS have not yet been compared. Most of the studies conducted so far have focused on the local expression of ICAM-1, VCAM-1 and E-selectin in salivary glands. Although elevated serum levels have been noticed, our concern is the possibility to correlate their values with basic inflammatory parameters such as the erythrocyte sedimentation rate (ESR) and other selected clinical findings. It seems important to determine how these levels affect the whole body. This would make it possible to use sICAM-1, sVCAM-1 and sE-selectin levels as additional, supporting parameters in the diagnosis and determination of the disease and concomitant inflammation severity.

Thus, the primary aim of this study was to measure and compare the serum levels of sICAM-1, sVCAM-1 and sE-selectin in pSS, sSS and HS groups and to explore possible correlations between the concentrations of the selected molecules and the laboratory and clinical parameters of SS activity, including the severity of xerostomia and xerophthalmia.

Material and methods

Study groups

The study was comprised of 34 women with SS (16 with pSS and 18 with sSS), fulfilling the 2002 American-European Consensus Group classification criteria. In our study, the diagnosis of pSS required 4 of 6 criteria, including antibodies to SSA/SSB. A diagnosis of sSS has not yet been addressed by the American-European Consensus Group. In practice, we required the patients to fulfill the criteria for pSS and, additionally, the American College of Rheumatology criteria for an established CTD such as RA, SLE or mixed CTD (MCTD).[6–9] The patients were recruited consecutively in 2013 from the Department of Rheumatology and Clinical Immunology at Poznan University of Medical Sciences, Poland. Exclusion criteria included: previous radiotherapy to the head and neck, lymphoma, sarcoidosis, graft-versus-host disease, infections of the hepatitis C virus, human T-lymphotropic virus type I and HIV. A routine patient history was taken and physical and dental examinations were performed in each subject. Laboratory assessments included routine measurements of ESR (Westergren) and detection of antinuclear antibodies (ANA) by indirect immunofluorescence on Human Epithelial (HEp)-20-10 cells (Euroimmun, Lubeck, Germany) and their differentiation using ANA Profile3 (antibodies against nRNP/Sm, Sm, SS-A, Ro-52, SS-B, Scl-70, PM-Scl, Jo-1, centromere protein B, PCNA, dsDNA, nucleosomes, histones, ribosomal P-proteins and AMA-M2), (Euroimmun, Lubeck, Germany). Xerostomia (assessed by patients) was measured using the VAS and Fox test. To assess ocular sicca symptoms, the Schirmer's test was carried out. Fifteen age- and gender-matched healthy donors from the Regional Center of Blood Donation and Blood Treatment in Poznań formed the control group (healthy subjects, HS). The protocol for this study was approved by the Bioethics Committee of Poznan University of Medical Sciences, Poland (number 211/2013). This study was performed in accordance with the ethical standards laid down in an appropriate version of the World Medical Association Declaration of Helsinki. Written informed consent was obtained from every subject before any study procedure was carried out.

Sample collection

Peripheral blood samples were collected from the antecubital vein in BD Vacutainer Rapid Serum Tubes (Becton, Dickinson and Company, Franklin Lakes, USA). After 5 min, clots were removed by centrifugation at 4000 rpm for 15 min at room temperature. The sera were stored at -70°C.

ELISA

sICAM-1, sVCAM-1 and sE-selectin levels in serum were determined by commercially available ELISA kits (R&D Systems, Minneapolis, USA) for sICAM-1/CD54 (with a mean minimum detectable dose (MDD) of 0.096 ng/mL), for sVCAM-1/CD106 (with a mean MDD of 0.6 ng/mL) and for sE-selectin/CD62E (with a mean

MDD of 0.009 ng/mL). Absorbance was measured with an ELISA plate reader ELx800 (Bio-Tek Instruments, Winooski, USA) using KCjunior 1.11 analysis software (Bio-Tek, Winooski, USA).

Assessment of xerostomia by Fox test and VAS

Each subject completed a specially prepared questionnaire concerning afflictions, with special attention paid to the presence of oral dryness symptoms. The questionnaire consists of 10 items, 4 of which (1–4) show a significant correlation with reduced salivary flow. These questions concern a feeling of too little saliva in the oral cavity, a sense of oral dryness (xerostomia) while eating, difficulty in swallowing and the need to wash down dry food with water.

Items in the Fox test:

1. Do you need to sip liquids to aid in swallowing dry foods?
2. Does your mouth feel dry when eating a meal?
3. Do you have difficulty swallowing any foods?
4. Does the amount of saliva in your mouth seem to be too little, too much, or you do not notice it?
5. Does your mouth feel dry at night or on awakening?
6. Does your mouth feel dry during the daytime?
7. Do you keep a glass of water by your bed?
8. Do you chew gum daily to relieve oral dryness?
9. Do you use hard candies or mints daily to relieve oral dryness?
10. Do you feel the need for frequent moistening of the oral cavity?

The Fox test score is measured as a proportion of positive to negative answers (yes/no) and expressed as a percentage value.[10,11]

Assessment of the patient's xerostomia on the VAS consisted in the patient marking the severity of dry mouth on a 100 mm line. The VAS is a horizontal line, 100 mm in length, anchored by word descriptors. The VAS score is determined by measuring in mm from the left-hand end of the line to the point of the patient's mark.[12,13]

Assessment of dry eye by Schirmer's test

In 23 patients (11 with pSS and 12 with sSS), the Schirmer's test was carried out to assess the severity of dry eye. Both eyes were tested at the same time. Special paper strips were placed under the lower eyelid of each eye. The patients kept their eyes closed for 5 min. Mechanical irritation resulted in the production of tears. After 5 min, the paper was removed and measured to check how moist it was. More than 10 mm of moisture on the filter paper after 5 min is a sign of normal tear production. Both eyes normally release the same amount of tears. A score between of 6-10 mm is a sign of mild to moderate dry eye. Less than 6 mm of moisture on the filter paper after 5 min is a sign of severe dry eye. To report the severity of dry eye we used the average of both eyes.

Statistical analyses

The calculations were carried out with Microsoft Excel 2010 and STATISTICA v. 10 software (StatSoft Inc., Tulsa, USA). The distributions obtained at each step of data processing were evaluated for normality using the Shapiro-Wilk test. Depending on the number of groups analyzed, the differences between them were tested using the Mann-Whitney U test or the Kruskal-Wallis one-way analysis of variance (ANOVA) by ranks followed by post hoc multiple comparisons of the mean ranks. The Spearman's rank correlation analysis was used to find the associations between the levels of selected cytokines and other laboratory and clinical parameters of SS activity. Unless otherwise stated, the data are expressed as medians with interquartile ranges (IQR). The differences were considered to be statistically significant at $p < 0.05$.

Results

Demography

All the subjects were of Caucasian origin. Tables 1 and 2 present a demographic, laboratory and clinical profile of the SS patients.

Serum sICAM-1, sVCAM-1 and sE-selectin levels

Serum sICAM-1 levels in pSS patients were significantly higher than those of HS ($p = 0.02$). Serum sVCAM-1 levels in sSS were significantly higher than those of HS ($p = 0.048$). Concentrations of sE-selectin in all studied groups were at a similar level. The detailed results are presented in Table 3. In pSS, the serum levels of sVCAM-1 correlated positively with VAS ($r_S = 0.52$, $p = 0.04$), as well as with the scores on the Fox test ($r_S = 0.66$, $p = 0.01$) (Fig. 1A and 1B, respectively). The levels of sE-selectin correlated positively with the levels of sICAM-1 ($r_S = 0.54$, $p = 0.01$) (Fig. 2A) and with the duration of the disease ($r_S = 0.51$, $p = 0.03$) (Fig. 2B). The levels of sE-selectin correlated negatively with the Schirmer's test ($r_S = -0.59$, $p = 0.04$) (Fig. 2C). The levels of sICAM-1 positively correlated with the ESR value ($r_S = 0.59$, $p = 0.01$) (Fig. 2D). No other significant correlations were found between the levels of molecules and the clinical parameters studied.

Discussion

Adhesive molecules such as ICAM-1, VCAM-1 and E-selectin contribute to the pathogenesis of SS in many

Table 1. Characteristics of SS patients

SS patients (n = 34)	pSS (n = 16)	sSS (n = 18)	HS (n = 15)
Age (years)	41.5 (28.5)	56.0 (21.0)	49 (14)
Gender (women/men)	16/0	18/0	14/1
BMI	24.65 (7.60)	23.55 (5.30)	
Disease duration (years)	4.5 (4)	3 (9)	
Connective tissue disease, n (%)			
RA	-	7 (38.89)	
SLE	-	4 (22.20)	
MCTD	-	1 (5.55)	
Others	-	6 (33.33)	
Serological and clinical parameters of SS activity			
Westergren ESR (mm/h)	23.5 (31)	19 (15)	
ANA, n (%)	11 (68.75)	15 (83.33)	
Anti-SAA antibodies	SSA 10 (62.50)	SSA 10 (55.55)	
Anti-SSB antibodies	SSB 7 (43.75)	SSB 4 (22.22)	
Other identified ANA profile 3, n (%)	Ro-52 10 (62.50)	Ro-52 11 (61.11) dsDNA 3 16.67 Sm 1 (5.55) PCNA 1 (5.55) ribosomal-P-protein 3 16.67 centromeres B 1 (5.55) PM-Scl 1 (5.55) histones 1 (5.55) nucleosomes 1 (5.55) RNP 1 (5.55)	
Organ involvement, n (%)			
Arthritis	11 (68.75)	11 (61.11)	
Cutaneous	4 (25.00)	3 (16.17)	
Peripheral nervous system	5 (31.25)	3 (16.67)	
Pulmonary	4 (25.00)	1 (5.55)	
Lymphadenopathy	3 (18.75)	2 (11.11)	
Glandular	1 (6.25)	1 (5.55)	
Current treatment, n (%)			
MTX	0	5 (27.78)	
NSAID	4 (25.00)	4 (22.22)	
Methylprednisolone	4 (25.00)	6 (33.33)	

BMI – body mass index; ESR – erythrocyte sedimentation rate; na – not applicable; RA – rheumatoid arthritis; SLE – systemic lupus erythematosus; MCTD – mixed connective tissue disease; ANA – antinuclear antibodies; MTX – methotrexate; NSAID – non–steroidal anti–inflammatory drugs; unless otherwise stated, the data are expressed as medians (interquartile range).

aspects. In our study, the serum levels of sICAM-1 were elevated in pSS and the serum levels of sVCAM-1 were elevated in sSS as compared with HS. Similar results were obtained by Kapsogeorgou et al. They detected higher spontaneous expression of ICAM-1 in cell lines obtained from SS patients compared to the controls.[3] The epithelial cells are the main target in the pathogenesis of SS and the main site of infiltration formation, which lead to a deterioration in the functioning of the salivary glands and other organs.[5,14] Epithelial cells adjacent to the sites of intense inflammation and lymphocytic infiltrations have been shown to express high levels of ICAM-1, VCAM and E-selectin, which influence the interaction between epithelial cells and immunologic cells. The epithelial cells in SS are activated and act as non-professional APC. ICAM-1 and VCAM-1 are expressed on APC and binding lymphocyte function-associated antigen-1 (LFA-1), and the very late activation antigen-1 (VLA-1) receptors on T cells ensure stabilization of the APC/T cell synapsis.[5] The possible consequence of this interaction is an augmentation of the inflammatory response either by directly activated T cells or by enhancing co-stimulatory and adhesion molecules such as ICAM-1 on the APC.[5] This means that the activation of the epithelial component of salivary glands and other organs could be responsible for the increased levels of ICAM-1 and VCAM-1 in pSS and sSS patients, respectively, and for the positive correlations between the levels of ICAM-1 and the severity of inflammation in sSS patients. SS affects many organs and the similar mechanism of activation of the epithelial cells takes place not only in the salivary and lacrimal glands but in other organs as well, and is responsible for the elevated serum levels of ICAM-1 and VCAM-1.[8]

Furthermore, the presence of proinflammatory cytokines such as interleukin (IL) 1β, tumor necrosis factor alpha (TNF-α), IL-4 and interferon gamma (IFN-γ) in the walls of blood vessels adjacent to the sites of inflammatory infiltrations results in the expression of ICAM-1, VCAM-1 and E-selectin on endothelial cells and on leukocytes.[1,4,14–16] TNF-α and IL-1β also induce de novo VCAM-1 expression.[4] For a comprehensive view of SS, the determination of adhesive molecule-cytokine interactions is necessary. Injuries to the endothelium and its subsequent apoptosis in salivary glands significantly contribute to the pathogenesis of SS.[17] Endotoxins and proinflammatory cytokines released from injuries induce the surface expression of adhesive molecules on endothelial cells, which results in inflammatory destruction.[18] This explains the overexpression of adhesive molecules

Table 2. Oral and ocular characteristics of SS patients

SS patients (n = 34)	pSS (n = 16)	sSS (n = 18)
Xerostomia	9 (56.25)	12 (66.67)
Dysphagia	4 (25.0)	6 (33.33)
PtXer-VAS (mm)	46.5 (52.5)	28.0 (47.0)
Fox test score (%)	45 (40)	55 (30)
Oral symptoms, n (%)		
Cheilitis simplex	4 (25.00)	4 (22.22)
Aphthae	2 (12.5)	1 (5.55)
Cheilitis angularis	1 (6.25)	2 (11.11)
Tongue inflammation	1 (6.25)	2 (11.11)
Stomatitis	1 (6.25)	1 (5.55)
Cheilitis exfoliativa	1 (6.25)	0
Oral paleness	1 (6.25)	0
Non-specific ulcerations	0	1 (5.55)
Ocular symptoms		
Schirmer's test (mm) (pSS n = 11, sSS n = 12)	4 (8)	10.75 (12)
Dryness of the eyes (subjective assessment), n (%)	11 (68.75)	13 (72.22)

PtXer-VAS – patient xerostomia assessment on visual analogue scale; unless otherwise stated, the data are expressed as median (interquartile range).

Table 3. Serum levels of sICAM-1, sVCAM-1 and sE-selectin in the studied groups

	sICAM-1 (ng/mL)	sVCAM-1 (ng/mL)	sE-selectin (ng/mL)
pSS (n = 16)	221.26 (60.95)	576.82 (518.35)	28.03 (7.86)
sSS (n = 18)	216.00 (67.66)	599.35 (509.07)	23.69 (23.07)
HS (n = 15)	168.48 (61.76)	376.44 (194.12)	18.94 (16.27)
p-value#	0.02 (pSS vs HS)	0.048 (sSS vs HS)	ns

The results are expressed as median (interquartile range); # Kruskal-Wallis one-way ANOVA by ranks followed by post hoc multiple comparisons of the mean ranks.

Fig. 1. Correlations between sVCAM-1 and PtXer VAS (A) and the results of the Fox test (B) in patients with pSS. The strength of the correlations was determined using Spearman's rank correlation coefficient; $p < 0.05$ was considered statistically significant

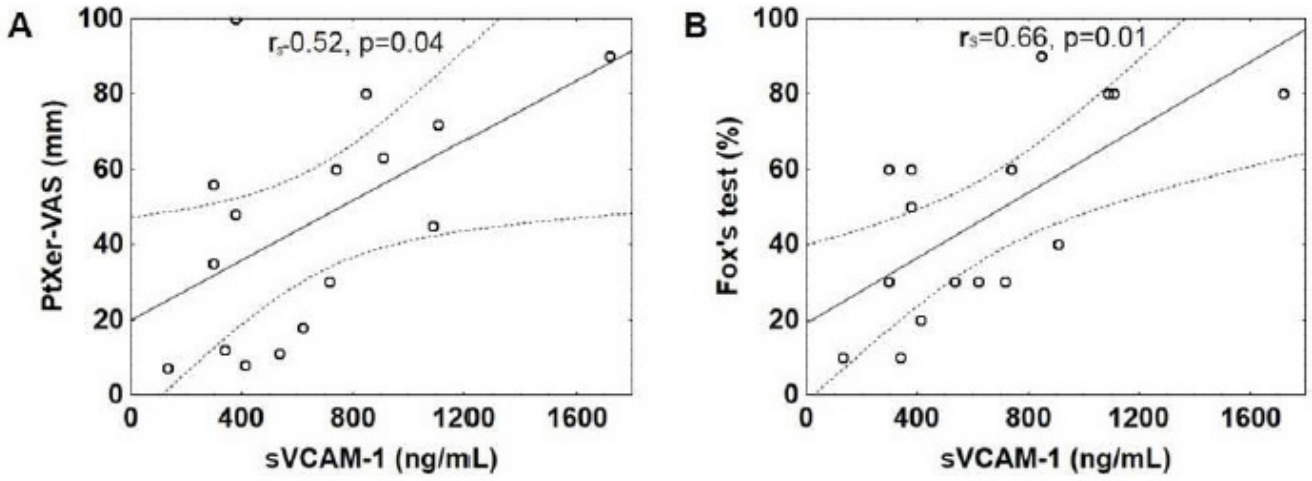
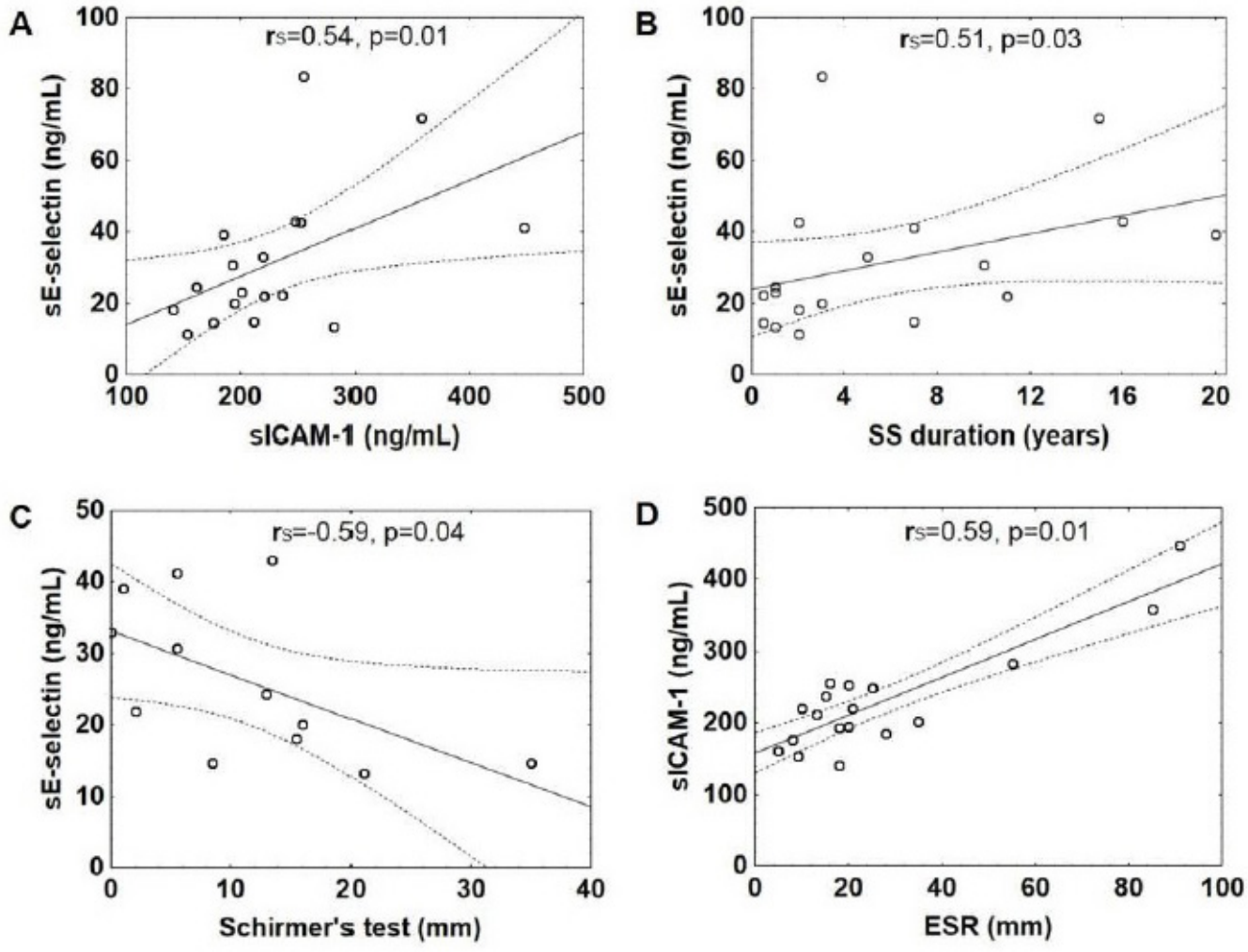


Fig. 2. Correlations between concentrations of sE-selectin and sICAM-1 (A), sSS duration (B) and the results of Schirmer's test (C) and between ESR and sICAM-1 levels (D) in patients with sSS. The strength of the correlations was determined using Spearman's rank correlation coefficient; $p < 0.05$ was considered statistically significant



in damaged salivary glands in SS.[18] Thus, the activation of epithelial cells and endothelial cells reflects the elevated serum levels of sICAM-1 and sVCAM-1 in pSS and sSS compared to HS.

In addition to being caused by aberrant angiogenesis and blood vessel injuries, the increased levels of adhesive molecules can be the result of the VEGF-A/VEGFR2 dependent mechanism of neovascularization.[19] Elevated levels of VEGF appear in many CTDs.[20] Additionally, VEGF promotes the migration of monocytes and lymphocytes into the extracellular matrix and sustains inflammation by endothelial cell expression of ICAM-1 and VCAM-1.[2,19,21] ICAM-1, VCAM-1 and E-selectin function as receptor molecules for circulating neutrophils. After binding, they reduce the blood flow of circulating cells and initiate their migration into the tissue. If the adhesion receptors are no longer needed, they are shed from the endothelial layer and circulate as soluble adhesion molecules. Therefore, the levels of circulating molecules are proportional to their previous activation level and blood vessel destruction. This can also explain the elevated serum levels of soluble adhesive molecules in pSS and sSS patients.

We did not find any differences between pSS and sSS in the levels of sICAM-1, sVCAM-1 and sE-selectin. It was found in recent studies comparing pSS and sSS that the intensity and frequency of some symptoms can vary. There is uncertainty whether sSS is merely a manifestation of the underlying disease or a true overlap of pSS with CTD. According to Salliot et al., subjects with sSS in the setting of SSc suffered from xerostomia, dry eye symptoms, the presence of anti-SSA/SSB antibodies and a classic histological pattern similar to those of subjects with pSS, again suggesting that these two entities are the same.[22] The lack of clear results might arise from the different classification of sSS. The prevalence of sSS has been reported to range from 4% to 31%, depending on the criteria applied, the methodological design and associated CTD.[23] In our study, there were no differences in the levels of adhesive molecules in pSS and sSS patients. In our opinion, similar elevated serum levels of ICAM, VCAM and E-selectin can be a result of the similar histological pattern in pSS and sSS.

In our study, the levels of sVCAM-1 in pSS patients correlated with the severity of xerostomia assessed by VAS and the Fox test. In SS patients, the local environment of the inflamed gland leads to the dysfunction of the residual glandular units owing to the release of cytokines, metalloproteinases and autoantibodies. The earliest changes involve the development of small capillaries into high endothelial venules that secrete chemokines and express adhesive molecules, promoting the migration of immune cells into the glands. The infiltrating cells (T and B cells, dendritic cells) interfere with glandular functions by means of the secretion of IL-1 and TNF-α, which inhibit the release of neurotransmitters such as acetylcholine, and the glandular response to these neurotransmitters ultimately leads to decreased saliva secretion.[8] The blood supply of the salivary glands plays a role in salivation. In earlier studies, it was suggested that VCAM-1 in patients with SS may be related to vasculitis.[4] These mechanisms could explain the correlation between the levels of sVCAM-1, reflecting salivary gland inflammation and glandular dysfunction, and the severity of xerostomia.

The levels of sICAM-1 positively correlated with sE-selectin levels in sSS. This could suggest a similar contribution of these molecules in the pathomechanism of this disease, and their concentrations may be reflected by the activation of endothelial and epithelial cells. For a comprehensive view of sSS, these molecules should be determined together in material more representative for SS.

We also found a positive correlation between sE-selectin and sSS duration. Our result suggests that the higher concentration of this molecule may result from a longer activation of endothelium and epithelium in SS. Therefore, sE-selectin may be considered a more representative parameter for SS progression.

In a study by Gao et al., it has been observed that ICAM-1 levels positively correlated with the progression of dry eye symptoms.[24] We found only a significant correlation between the levels of sE-selectin in sSS patients and the severity of dry eyes assessed by the Schirmer's test. Thus, this molecule seems to be the most representative parameter for the assessment of eye dryness in sSS.

The levels of sICAM-1 correlated positively with the ESR value in sSS patients. Its levels may reflect the previous activation of the epithelium and endothelium. Egerer et al. detected higher levels of ICAM-1 in pSS and SLE patients, as well as in sepsis, indicating a comparable stage of endothelial activation and therefore inflammation and subsequently vascular damage in these diseases. No differences were observed between patients with a localized infection and healthy controls regarding the levels of sE-selectin and sICAM-1. Interestingly, the endothelium in SLE and pSS appears to be chronically activated in contrast to the situation found in sepsis, where a high and constant level of ICAM-1 is usually detectable only for a period of days or weeks.[25] The ICAM-1 level sustained at a constant and increased level can be a useful monitoring parameter of disease severity and the activity of inflammation.[26]

The limitations of this study arise mainly from the small sample size. Thus, it should be considered a pilot study. Our findings need to be verified in a larger SS population. In our study, a few patients were taking methylprednisolone, non-steroidal anti-inflammatory drugs (NSAIDs) and immunosuppressive drugs. There were no naïve patients with regard to both disease-modifying anti-rheumatic drugs and NSAIDs. These drugs may reduce the levels of proinflammatory cytokines and adhesive molecules. Moreover, the drug-cytokine interactions could be responsible for the disparity between the levels

of adhesive molecules found in our study, especially in pSS and sSS patients. Additionally, many drugs applied in the therapy of hypertension, depression and other diseases can reduce salivation. Therefore, current and previous therapy and co-existing diseases and therapies might have interfered with our results but it is not clear whether these drugs can affect serum sICAM-1, sVCAM-1 and sE-selectin levels in SS patients. In our study we correlated the levels of adhesive molecules with parameters of disease activity such as ESR. In our opinion, ESR is a good clinical marker for the assessment of a relationship between inflammation and disease activity in SS. Recently, two disease activity indices have been proposed: The European League Against Rheumatism (EULAR) Sjögren's syndrome patient reported index (ESSPRI) and the EULAR Sjögren's syndrome disease activity index (ESSDAI).[27,28] These indices evaluate the effectiveness of therapy in pSS. In our clinical characteristics of SS patients, we included 6 of 12 domains (organ involvement) from the ESSDAI. Lack of a comprehensive evaluation of organ involvement according to the ESSDAI is a limitation of our study.

The limitations of the assessment of xerostomia and its relationship with the levels of adhesive molecules can arise from a subjective test of xerostomia. In our opinion, the Fox tests and VAS can be representative and helpful tests and they additionally describe the patient's discomfort in SS. Among the selected molecules, sVCAM-1 reflects xerostomia in pSS, and sICAM-1 and sE-selectin may be additional parameters of the activity of sSS. However, they cannot be applied for the differentiation of pSS and sSS.

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Prevalence of cardiovascular disease risk factors among pharmacy students from Wroclaw Medical University (Poland)

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Abstract

Background. Atherosclerotic processes begin in childhood and their development worsens during adolescence. Early prevention of CVD risk factors may have an important impact on the future health of young people. It can be also helpful in reducing the costs of treating CVD later in life.

Objectives. The aim of this study was to assess the prevalence of selected cardiovascular disease risk factors among pharmacy students.

Material and methods. The study group consisted of 1,168 pharmacy students (892 women and 276 men) from Wroclaw Medical University. The average age was 22.9 years among women and 23.2 years among men. This cross-sectional study was conducted between 2004–2012.

Results. 27.5% of men and 7.1% of women were found to be overweight, while visceral obesity was found in 15.2% and in 10.1% of students, respectively. Hypertension was diagnosed in 27.2% of men and in 7.8% of women. Low physical activity was declared by 41.9% of women and by 31.9% of men. There were 22.1% of men and 10% of women who were current smokers. The majority of the study group did not consume enough fruits and vegetables (women 61.8%, men 75%). Body mass index (BMI) was positively associated with waist and hip measurements, waist-to-hip ratio (WHR) and body fat percentage, while blood pressure was positively associated with BMI and waist circumference. It was found that men with high physical activity had lower BMIs, body fat percentage, waist and hip circumferences, WHR, diastolic blood pressure and heart rate than those who declared low physical activity. Comparing women with high physical activity to those with low physical activity, only lower heart rate was observed.

Conclusions. A higher prevalence of cardiovascular risk factors was found more often among men than women. Preventive actions which promote proper nutrition, more physical activity, smoking cessation and regular blood pressure checks and lipid profile tests should be implemented for the students.

Key words: risk factors, cardiovascular disease, lifestyle, hypertension, university students

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The prevalence of cardiovascular diseases (CVD) is notably associated with lifestyle factors such as smoking, unhealthy diet and low physical activity. Other risk factors for CVD, e.g. excessive body weight, high blood pressure, dyslipidemia and high blood glucose level, can also be related to lifestyle.[1] According to the Global Burden of Diseases, Injuries, and Risk Factors Study 2010 (GBD 2010), deaths caused by all non-communicable diseases increased by 30% between 1990 and 2010. Deaths caused by cardiovascular and circulatory diseases increased by 31.2% during those years, while deaths caused by diabetes, urogenital, blood and endocrine diseases increased by 76.5%. In 2010, global CVD mortality among women aged 15–49 years was 10.7% and among men 12.8%.[2] According to the World Health Organization (WHO), the highest mortality rates of CVDs in 2008 were found in Estonia, followed by Hungary and Croatia. The mortality rates of CVDs in Poland placed 7th among the countries which were taken into account.[1]

A healthy lifestyle is one of the major factors in CVD prevention, a fact which is emphasized by experts from the European Society of Cardiology (ESC).[3] Preventive actions should begin even before birth by educating young parents, and should subsequently be continued at pre-school and school age. Therefore, actions aimed at preventing CVD should be part of everyday life beginning with childhood and they should be continued for adolescents, adults and the elderly.[3] Obesity in the first years of life increased the risk of obesity in adulthood and the development of metabolic syndrome and type 2 diabetes in adolescence and adulthood.[4] Juonala et al. observed that obese adults who were overweight in their childhood had an increased risk of dyslipidemia, hypertension, type 2 diabetes and carotid-artery atherosclerosis.[5] Other authors also suggested that the development of atherosclerosis may be increased by the occurrence of CVD risk factors in early life.[6] Therefore, early prevention of risk factors may have a significant influence on CVD development in the future.[7]

The aim of this study was to evaluate the prevalence of selected cardiovascular disease risk factors among pharmacy students from Wroclaw Medical University.

Material and methods

Study group

The study group consisted of 1,168 pharmacy students (892 women and 276 men) from Wroclaw Medical University. This cross-sectional study was carried out between 2004 and 2012. All students who were 4th-year pharmacy students at the time agreed to participate in the study and were recruited into the study. Women who were pregnant were excluded from the study. During the study period, the following number of students were recruited: 154 people in 2004; 141 in 2005; 127 in 2006; 130 in 2007; 124 in 2008; 125 in 2009; 114 in 2010; 139 in 2011; and 114 in 2012. None of the participants declared that they were taking any medications for the treatment of chronic diseases. The average age was 22.9 years among women and 23.2 years among men. The demographics of the study group are presented in Table 1. The study group was asked to complete questionnaires, which included 46 questions about their nutritional habits and lifestyle.

Measurements

To assess the risk factors for CVD among the study group, anthropometrical parameters such as height (rounded to 1 cm), weight (rounded to 1 kg) and waist and hip circumference (rounded to 1 cm) were measured. Weight was measured using an electronic personal scale (Beurer GmbH, Germany) and waist and hip circumferences using non-stretch measuring tape. Based on these anthropometric measurements, body mass index (BMI) and waist-to-hip ratio (WHR) were calculated. BMI classification from the WHO was used.[8] Abdominal obesity was assessed using WHR and waist circumference. The abnormal values for WHR among women and men were ≥ 0.8 and ≥ 0.95, respectively, and those for waist circumference were ≥ 80 cm and ≥ 94 cm, respectively.[9,10] The percentage of body fat was measured using a BF306 Body Fat Monitor (OMRON, Japan). The measurement of body fat percentage was based on electrical resistance and personal data: gender, age, height and weight. Blood pressure was measured using an M6 Blood Pressure Monitor (OMRON, Japan) with a size 3 arm cuff. Measurements were performed twice during 2 visits (4 measurements in total) 1 week apart. Hypertension was diagnosed when systolic blood pressure was ≥ 140 mm Hg and/or diastolic blood pressure was ≥ 90 mm Hg.[11]

Statistical analysis

Continuous variables were summarized by averages, standard deviations (SD) and medians. A non-parametric Mann-Whitney U test was used to compare linear variables and a χ^2 test was used to compare categorical variables. A linear Pearson's correlation test was performed to determine the relationships between data. The criterion for statistical significance was set at the p-value < 0.05. Statistical analyses were made using STATISTICA v. 10.0 PL software (StatSoft Inc., USA).

Results

Anthropometrical parameters, body fat percentage and blood pressure were measured among the study group (Table 1). It was found that height, weight, waist and hip circumferences, BMI, WHR and systolic blood pressure

Table 1. Characteristic of the study group (n = 1168)

Parameter	Women (n = 892)		Men (n = 276)		Women vs men*
	mean ± SD	median	mean ± SD	median	p-value
Age (year)	22.9 ± 1.4	23.0	23.2 ± 1.9	23.0	ns
Height (cm)	166.8 ± 6.0	167.0	180.0 ± 6.3	180.0	< 0.0001
Weight (kg)	57.7 ± 8.7	56.0	76.3 ± 11.3	76.0	< 0.0001
BMI (kg/m^2)	20.7 ± 2.7	20.3	23.5 ± 3.2	23.0	< 0.0001
Waist (cm)	70.7 ± 7.0	69.0	85.3 ± 8.5	85.0	< 0.0001
Hip (cm)	95.2 ± 6.4	95.0	99.2 ± 6.5	99.0	< 0.0001
WHR	0.74 ± 0.05	0.74	0.86 ± 0.05	0.86	< 0.0001
Body fat (%)	27.8 ± 5.4	27.6	18.8 ± 5.9	18.5	< 0.0001
SPB (mm Hg)	122.0 ± 11.1	122.0	134.4 ± 12.9	133.0	< 0.0001
DBP (mm Hg)	76.2 ± 8.3	76.0	75.5 ± 8.7	75.0	ns
Heart rate	80.1 ± 12.9	78.0	76.3 ± 15.8	74.0	< 0.0001

BMI – body mass index; WHR – waist to hip ratio; SBP – systolic blood pressure; DBP – diastolic blood pressure; SD – standard deviation; * – U Mann-Whitney test; ns – no significant differences.

(SBP) were significantly higher among men than among women. The opposite relationship was observed in percentage of body fat and pulse.

The prevalence of selected risk factors for CVD is presented in Table 2. Although the average BMI among both men and women indicated normal body weight, there were 27.5% of men and 7.1% of women with excessive body weight ($p < 0.0001$). However, a notable part of the study group, especially women (17.9%), were underweight. Visceral obesity was observed significantly more often among men than women (15.2% vs 10.1%). Hypertension occurred almost 4-fold more frequently among men than women.

Students were asked to qualify their physical activity by choosing 1 of the 5 following answers: no physical activity; 20–60 min once a week; 20–60 min 2–3 times a week; 20–60 min 4–6 times a week; or 20–60 min every day. Low physical activity, as defined by options 1 and 2, was declared significantly more often by women than by men (41.9% vs 31.9%).

Significantly more men than women were current or former smokers (36.6% vs 19.4%). Almost 50% of the study group had never measured their blood cholesterol level and 27.9% had not measured it in the year prior to the study. About 53% of women and 50% of men had not measured their blood pressure in the month prior to the study. A history of CVD in the family was declared by 34.9% of women and by 32.6% of men.

The majority of the study group did not consume enough fruits and vegetables. Intake of less than the recommended 5 servings of these foods per day overall was declared by about 62% of women and 75% of men. However, when taking into account the recommendation to consume at least 3 servings of vegetables and at least 2 servings of fruit daily, the insufficient intake of these foods was found in about 75% of women and 84% of men (Table 2).

Table 3 summarizes the correlations between BMI, waist circumference, WHR, body fat percentage and other parameters. BMI correlated stronger with waist circumference than with WHR in both men and women. A strong correlation was also found between BMI and body fat percentage. Larger hip circumference, higher WHR and higher body fat percentage were observed with increasing waist circumference. Correlations between blood pressure and anthropometric variables were weak or statistically insignificant.

The comparison between participants with excessive body weight and those with BMI < 25 kg/m^2 is shown in Table 4. It was observed that women with BMI ≥ 25 kg/m^2 had significantly higher SBP, DBP, heart rate, body fat percentage, waist and hip circumferences and WHR compared to those with BMI < 25 kg/m^2. Similar results were obtained in the group of men, with the exception of SBP and DBP, where no significant differences were found.

It was observed that women with abdominal obesity had significantly higher BMI, SBP, DBP, heart rate, body fat percentage, hip circumference and WHR, compared

Table 2. Prevalence of selected risk factors in the study group (n = 1168)

Parameter	Women (W) n = 892		Men (M) n = 276		W vs M*
	n	%	n	%	p-value
BMI < 18.5 (kg/m^2)	161	17.9	9	3.3	< 0.0001
BMI 18.5–24,9 (kg/m^2)	668	74.9	191	69.2	ns
BMI ≥ 25 (kg/m^2)	63	7.1	76	27.5	< 0.0001
Overweight	53	5.9	66	23.9	< 0.0001
Obesity	10	1.1	10	3.6	0.0113
Abdominal obesity by WC (W ≥ 80 cm; M ≥ 94 cm)	90	10.1	42	15.2	0.0187
Abdominal obesity by WHR (W ≥ 0.8; M ≥ 0.95)	120	13.5	13	4.7	0.0001
Hypertension (yes)	70	7.8	75	27.2	< 0.0001
Current smokers	89	10.0	61	22.1	< 0.0001
Former smokers	84	9.4	40	14.5	0.0168
Current + former smokers	173	19.4	101	36.6	< 0.0001
Low physical activity	374	41.9	88	31.9	0.0029
Prevalence of CVD in family (yes)	311	34.9	90	32.6	ns
Low vegetable intake (< 3 portion/day)	623	69.8	218	79.0	0.0031
Low fruit intake (< 2 portion/day)	280	31.4	133	48.2	< 0.0001
Low fruit and vegetable intake (< 5 portion/day)	551	61.8	207	75.0	0.0001
Vegetables < 3 portions AND fruits < 2 portions	671	75.2	231	83.7	0.0034
Was the cholesterol measured last year? (no + don't remember + never)	821	92.0	254	92.0	ns
Not measured last year	249	27.9	77	27.9	ns
Never	430	48.2	131	47.5	ns
Don't remember	142	15.9	46	16.7	ns
Was the BP measured last month? (no + don't remember)	487	54.6	145	52.5	ns
Not measured last month	470	52.7	137	49.6	ns
Don't remember	17	1.9	8	2.9	ns

W – women; M – men; BMI – body mass index; WC – waist circumference; WHR – waist to hip ratio; CVD – cardiovascular diseases; BP – blood pressure; * – χ^2 test; Ns – no significant differences.

to those with waist circumference < 80 cm (Table 5). Similar results appeared among the men, except SBP, where no significant differences were found.

In both groups, participants with hypertension had higher BMI than those with normal blood pressure (Table 6). Women with hypertension also had larger hip circumferences than those without hypertension. Among men with hypertension compared to those without, higher waist circumference and WHR was observed.

Table 7 presents the comparison between participants who declared low and high physical activity. It was found that men with high physical activity had lower BMI, body fat percentage, waist and hip circumferences, WHR, DBP and heart rate than those who declared low physical activity. In the group of women, only lower heart rate was observed among those with high physical activity compared to those with low physical activity.

Discussion

Atherosclerotic processes begin in childhood and their development proceeds during adolescence. Early prevention of CVD risk factors might have an important impact on future health. It can be also helpful in reducing the costs of treating CVD later in life. Regular calculation of BMI and measurement of blood pressure along with screening and counseling for nutritional problems and eating disorders, physical activity and smoking cessation are recommended as prevention and treatment of CVD.[7]

Obesity is one of the major risk factors for CVD and it is a growing worldwide problem. It is related to other risk factors and may cause metabolic disorders.[1] In the Bogalusa Heart Study, the authors concluded that excessive body weight in adolescence and remaining in young adulthood has a strong impact on CVD risk factors, a scenario which requires primary prevention in early life.[12] In the previous study conducted on pharmacy students between 1998 and 2003, the authors observed a slightly lower prevalence of excess weight and obesity than in the present study.[13] Results similar to those obtained from students from Wrocław

Table 3. Pearson correlation between selected parameters in the study group (n = 1168)

Variables	Women (n = 892)		Men (n = 276)	
	r	p-value	r	p-value
BMI				
Waist	0.7683	0.000	0.8283	0.000
Hip	0.8312	0.000	0.8288	0.000
WHR	0.2632	0.000	0.4527	0.000
Body fat percentage	0.6995	0.000	0.7278	0.000
SBP	0.1878	0.000	0.1722	0.004
DBP	0.1810	0.000	0.2086	0.000
Heart rate	0.0049	0.883	0.0355	0.557
Waist				
Hip	0.7262	0.000	0.8029	0.000
WHR	0.7080	0.000	0.7679	0.000
Body fat percentage	0.5781	0.000	0.6982	0.000
SBP	0.1304	0.000	0.1732	0.004
DBP	0.1103	0.001	0.2326	0.000
Heart rate	0.0577	0.085	0.0402	0.506
WHR				
Hip	0.0317	0.344	0.2371	0.000
Body fat percentage	0.1659	0.000	0.4071	0.000
SBP	-0.0036	0.915	0.1484	0.014
DBP	-0.0144	0.669	0.1821	0.002
Heart rate	0.0401	0.232	0.0025	0.967
Body fat percentage				
SBP	0.0850	0.011	0.0976	0.106
DBP	0.1548	0.000	0.2278	0.000
Heart rate	0.0537	0.109	0.0876	0.147

r – correlation coefficient; BMI – body mass index; WHR – waist to hip ratio; SBP – systolic blood pressure; DBP – diastolic blood pressure.

Table 4. Comparison between average values of selected parameters among participants with BMI < 25 kg/m^2 and with BMI ≥ 25 kg/m^2

Parameters	Women (n = 892)		Men (n = 276)	
BMI	< 25 kg/m^2	≥ 25 kg/m^2	< 25 kg/m^2	≥ 25 kg/m^2
Waist (cm)	69.6	85.4[a]	82.0	94.1[a]
Hip (cm)	94.2	109.1[a]	96.7	105.9[a]
WHR	0.74	0.78[a]	0.85	0.89[a]
Body fat (%)	27.1	37.6[a]	16.7	24.3[a]
SBP (mm Hg)	121.4	129.9[a]	133.6	136.5
DBP (mm Hg)	75.7	82.8[a]	74.9	77.3
Heart rate	79.8	83.3[a]	75.5	78.3[a]

BMI – body mass index; WHR – waist to hip ratio; SBP – systolic blood pressure; DBP – diastolic blood pressure; [a] – significant differences (p < 0.5) between participants with BMI < 25 kg/m^2 and with BMI ≥ 25 kg/m^2.

Table 5. Comparison between average values of selected parameters among participants with abdominal obesity and with proper waist circumference

Parameters	Women (n = 892)		Men (n = 276)	
waist circumference	< 80 cm	≥ 80 cm	< 94 cm	≥ 94 cm
Hip (cm)	94.1	105.7[a]	97.7	107.7[a]
WHR	0.73	0.81[a]	0.85	0.92[a]
BMI (kg/m^2)	20.2	25.3[a]	22.7	28.2[a]
Body fat (%)	27.1	34.6[a]	17.4	26.5[a]
SBP (mm Hg)	121.4	128.0[a]	134.1	135.8
DBP (mm Hg)	75.7	80.3[a]	74.9	79.2[a]
Heart rate	79.7	83.1[a]	75.6	80.0[a]

WHR – waist to hip ratio; BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; [a] – significant differences (p < 0.5) between participants with abdominal obesity and with proper waist circumference.

were also observed by other authors assessing the occurrence of excessive weight and obesity among students from other Polish cities.[14,15] In the WOBASZ study (National Multicenter Health Survey in Poland), BMI ≥ 25 kg/m^2 occurred more frequently among participants aged 20–34 than among the examined students from Wrocław (men: 41% vs 27.5%; women: 20% vs 7.1%).[16] A higher prevalence of excessive body weight in the group of students, compared to the current study, was observed also in Greece (men: 39.5%; women: 23.3%) and Lebanon (men: 50%; women: 16.8%).[17,18]

Actions aimed at preventing hypertension should be addressed to those who do not have the disease as well as to those who already have high BP. The development of hypertension can be prevented in particular by changing the patient's lifestyle. According to the recommendations of the Polish Society of Hypertension, annual BP screening should be performed on all adults, regardless of the previous measurement.[11] The WHO emphasizes

Table 6. Comparison between average values of selected parameters among participants with and without hypertension

Parameters	Women (n = 892)		Men (n = 276)	
Hypertension	yes	no	yes	no
Waist (cm)	73.8	70.5	88.0	84.3[a]
Hip (cm)	97.8	95.0[a]	100.5	98.7
WHR	0.75	0.74	0.88	0.85[a]
BMI (kg/m^2)	21.8	20.6[a]	24.4	23.2[a]
Body fat (%)	29.1	27.7	19.7	18.4

WHR – waist to hip ratio; BMI – body mass index; [a] – significant differences ($p < 0.5$) between participants with and without hypertension.

Table 7. Comparison between average values of selected parameters among participants with low and high physical activity

Parameters	Women (n = 892)		Men (n = 276)	
Low physical activity	no	yes	no	yes
Waist (cm)	70.3	71.4	84.1	87.9[a]
Hip (cm)	95.0	95.6	98.6	100.6[a]
WHR	0.74	0.75	0.85	0.87[a]
BMI (kg/m^2)	20.6	20.8	23.1	24.4[a]
Body fat (%)	27.6	28.2	17.5	21.6[a]
SBP (mm Hg)	121.5	122.8	134.8	133.5
DBP (mm Hg)	75.9	76.6	74.6	77.5[a]
Heart rate	78.7	81.9[a]	73.7	81.8[a]

WHR – waist to hip ratio; BMI – body mass index; SBP – systolic blood pressure; DBP – diastolic blood pressure; [a] – significant differences ($p < 0.5$) between participants with low and high physical activity.

that hypertension is a major risk factor not only for myocardial infarction and stroke, but also renal impairment, heart failure and blindness.[1] For the prevention of strokes and heart attacks, hypertension should be detected and treated early.[1] In the present study, more than half of the study group did not have their blood pressure checked during the previous month prior to the study. The prevalence of hypertension in the study group was similar to that observed in the pharmacy students between 1998 and 2003.[13] In the WOBASZ study, the occurrence of hypertension in the group aged 20–34 was lower than that observed in this study.[16] However, hypertension, as in the current study, was diagnosed more frequently among men than among women.[16] Krzych et al. also found that the prevalence of hypertension was higher among young men than women (20.9% vs 9.9%).[19] A similar relationship was observed among Greek students (13.3% vs 6.7%); however, the prevalence of hypertension was lower than among Polish students.[17]

One of the most cost-effective means of preventing the development of CVD among adults is the cessation of tobacco use. It is worth focusing on smoking prevention and cessation during adolescence because of the cost savings associated with smoking cessation and because the costs of quit services are relatively low.[7] According to the SCORE risk chart, the 10-year risk of fatal CVD among smokers is approximately twice as high as among non-smokers.[3] In the European Union at the beginning of the 21st century, the habit of smoking was observed in 39.5% of men and in 30.1% of women aged 20–44; in Poland it was 46% and 31%, respectively.[20] In a comparison of university students from 23 countries, the lowest percentage of male current smokers was in Thailand (14%) and South Africa (15%), while the highest was in Portugal (47%), Greece and Korea (both 44%).[21] Among female university students, the lowest percentage of current smokers was in Thailand (2%), South Africa and Korea (both 4%), while the highest was in Bulgaria and Spain (both 46%), Greece and Portugal (both 42%). Based on the research of Steptoe et al., there were 26% of men and women who were current smokers in Poland.[21] In this study, slightly fewer pharmacy students were current smokers compared to those from earlier years and compared to medical students from Wrocław.[13,22] More current and former smokers were found among students from Lublin than from among the study group from Wrocław (men: 48% vs. 36.6%; women: 34.2% vs 19.4%).[15] 34.4% of male Lebanese students were current smokers and 9.4% were ex-smokers, as well as 27.2% and 5.6%, respectively, of female smokers and ex-smokers.[18] Among university students from Sweden, 20% of women and 24% of men smoked.[23]

Physical inactivity is indicated as one of the risk factors for CVD.[1,3,7] Increased physical activity helps in maintaining proper body weight, controlling blood pressure, preventing and treating lipid disorders, and improving glycemic and insulin sensitivity.[1,7] In the comparison conducted on university students from 23 countries, inactivity during leisure time was observed in 44% of students in developing countries, in 42% of students in the Pacific Asian region, in 39% of those in the Mediterranean region, in 30% of those in Central and Eastern Europe and in 23% in the United States and Northwestern Europe.[24] The highest age-adjusted prevalence of physical inactivity among women was observed in Portugal, South Africa, Korea and Venezuela, and among men in Portugal, South Africa, Romania and Venezuela.[24] Low physical activity was declared more often by pharmacy students in the years 1998–2003 than in the present study (men: 42.9% vs 31.9%; women: 55.1% vs 41.9%).[13] Other results, outside of this study, were obtained from students in Lublin and Warszawa, where

only 5.8% of the respondents declared a low level of physical activity and 72.1% declared a medium level.[25] In the group of students from Sweden, 29% of men and women did not declare regular exercise, while 40% and 30%, respectively, declared a low level of exercise.[23]

Low intake of fruits and vegetables is one of the components of an unhealthy diet, which may increase the risk of CVD development.[1,3] According to the "European Guidelines on cardiovascular disease prevention in clinical practice" it is recommended to consume at least 200 g of vegetables (2–3 servings) daily as well as 200 g of fruit (2–3 servings).[3] The majority of the study group did not eat a sufficient amount of these foods. The average fruit intake among male pharmacy students from Wrocław between 2005 and 2007 was 100.2 g/day and 115.9 g/day among women.[26] The average vegetable intake was 127.7 g/day and 124 g/day, respectively.[26] In the WOBASZ study, the average fruit intake among men aged 20–34 years was 163.9 g/day and 196.8 g/day among women, while vegetable intake amounted to 222.2 g and 185.5 g/day, respectively.[16] In the study of Ślusarska et al., consumption of fresh fruits and vegetables at least twice a day was declared by 44.2% of the respondents.[25] Only 16.3% of the students from Warszawa and Lublin ate these foods at least 3 times a day.[25] Among Lebanese university students, 29.2% of men declared daily consumption of fruit and vegetables and 25.8% and 31.5% of women for fruits and vegetables, respectively.[18]

Conclusions

In conclusion, the risk factors for cardiovascular diseases observed in this study were mainly associated with lifestyle factors, such as low physical activity, insufficient consumption of fruit and vegetables and tobacco use. A higher prevalence of certain cardiovascular risk factors was found more often among men than among women: excessive weight and obesity, abdominal obesity, hypertension, insufficient consumption of fruits and vegetables and smoking. The authors suggested that more attention should be paid to maintaining proper body weight. Physical activity should be promoted among students in view of its beneficial impact on body weight and blood pressure.

The excessive body weight, hypertension, low physical activity, smoking and low fruit and vegetable consumption found in a significant percentage of the study group may contribute to the future development of cardiovascular diseases in these individuals.

The authors concluded that although the study group consisted of young people, there was a high prevalence of cardiovascular risk factors, especially among men, and therefore preventive actions which promote proper nutrition, physical activity and smoking cessation should be implemented for the students.

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The reliability of three psoriasis assessment tools: Psoriasis area and severity index, body surface area and physician global assessment

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Abstract

Background. A wide variety of psoriasis assessment tools have been proposed to evaluate the severity of psoriasis in clinical trials and daily practice. The most frequently used clinical instrument is the psoriasis area and severity index (PASI); however, none of the currently published severity scores used for psoriasis meets all the validation criteria required for an ideal score.

Objectives. The aim of this study was to compare and assess the reliability of 3 commonly used assessment instruments for psoriasis severity: the psoriasis area and severity index (PASI), body surface area (BSA) and physician global assessment (PGA).

Material and methods. On the scoring day, 10 trained dermatologists evaluated 9 adult patients with plaque-type psoriasis using the PASI, BSA and PGA. All the subjects were assessed twice by each physician. Correlations between the assessments were analyzed using the Pearson correlation coefficient. Intra-class correlation coefficient (ICC) was calculated to analyze intra-rater reliability, and the coefficient of variation (CV) was used to assess inter-rater variability.

Results. Significant correlations were observed among the 3 scales in both assessments. In all 3 scales the ICCs were > 0.75, indicating high intra-rater reliability. The highest ICC was for the BSA (0.96) and the lowest one for the PGA (0.87). The CV for the PGA and PASI were 29.3 and 36.9, respectively, indicating moderate inter-rater variability. The CV for the BSA was 57.1, indicating high inter-rater variability.

Conclusions. Comparing the PASI, PGA and BSA, it was shown that the PGA had the highest inter-rater reliability, whereas the BSA had the highest intra-rater reliability. The PASI showed intermediate values in terms of inter- and intra-rater reliability. None of the 3 assessment instruments showed a significant advantage over the other. A reliable assessment of psoriasis severity requires the use of several independent evaluations simultaneously.

Key words: psoriasis, measurement tools, PGA, BSA, PASI

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Psoriasis is a common chronic inflammatory disorder with multiple pathways of pathogenesis that can be associated with metabolic and cardiovascular disease.[1] It is a disease with a high burden and substantial impact on quality of life. Since there are no biomarkers available to assess disease severity, clinical assessment tools are used to measure the disease severity and treatment response in clinical trials and daily practice.[2] The ideal assessment instrument should consistently represent the true degree of disease severity, minimize inter- and intra-rater variability, be responsive to change, use the entire range of the scale (wide response distribution) and be easy to administer.[3–5] A wide variety of scoring systems have been proposed to assess the severity of psoriasis. More than 44 different outcome scoring systems were used in 171 randomized clinical trials of psoriasis therapies between 1977 and 2000, as reported in a review by Naldi et al.[3] Another systematic review performed in 2010 identified 53 separate clinical assessment instruments.[2] The psoriasis area and severity index (PASI) is the most commonly used clinical scoring system in research, but it has several disadvantages. The PASI has been criticized for being resource intensive, complex, lacking sensitivity, being low in accuracy and having a non-linear scale. Despite these limitations, the PASI is the most extensively used psoriasis clinical severity score and is considered the reference scoring system against which other assessments tools are compared. Based on systematic reviews, it appears that no single outstanding instrument has been developed for psoriasis, and none of the severity scores used for psoriasis meet all the validation criteria required for an ideal assessment instrument.[2,6] Therefore, further work is still needed to validate existing instruments and to develop new and better ones.

The aim of this study was to compare and assess the reliability of 3 commonly used measures of psoriasis severity: the PASI, body surface area (BSA) and physician global assessment (PGA).

Material and methods

The subjects

Ten adult patients with plaque-type psoriasis of varying extent were recruited for the study at the Department of Dermatology, Venereology and Allergology, Wroclaw Medical University, Poland. All the subjects voluntarily agreed to participate in the study and written informed consent was obtained from all the subjects before any further procedure. Patients with erythrodermic, guttate or pustular psoriasis were excluded. All the participants were instructed not to apply topical treatment prior to the examination until all the assessments were completed. One patient withdrew consent at the beginning of the experiment and ultimately 9 patients were assessed.

The assessors

Ten practicing dermatologists (7 females and 3 males) were chosen from the Department staff to act as assessors in the study. The physicians' ages ranged from 26 to 36 years, and their duration of dermatology practice ranged from 1 to 11 years. Just before the study, all the participating physicians took part in a training session about using the assessment instruments and recording the data.

The study design

The study was conducted on a single day at Wroclaw Medical University's Department of Dermatology, Venereology and Allergology. In total, 9 subjects were evaluated twice by each of 10 physicians.

On the scoring day each patient was randomly allocated an individual examination room, where s/he remained until the study was completed. The patients received numbers; their names were not used. The physicians rotated from one examination room to the next one in a sequential order, which was precisely controlled. At any given time, only one assessor was assigned to one patient. Each physician independently scored the severity of each patient's psoriasis using the PASI, BSA and PGA, using standardized forms. Each observer used one form for each assessment tool per patient. Each form was collected immediately upon completion and stored until the end of the scoring session. The physicians evaluated all the patients with no time limit for the examinations. In general, the time it took each physician to complete all the assessments for all the patients was about 1 h. Directly after the first assessment, each physician was asked to evaluate each patient again, in the same order as previously, without access to the previous scores. The physicians were not allowed to discuss the assessments with one another until all the study forms were completed. Similarly, no communication between the physicians and the patients was allowed during the examinations.

Scoring methods

Psoriasis area and severity index (PASI)

The PASI was developed in 1978 by Fredriksson and Pettersson to assess the effects of retinoids in psoriasis.[7] The PASI combines assessments of 4 body areas: the head and neck (H), the upper limbs (UL), the trunk (T) and the lower limbs (LL). The percentage of skin affected by psoriasis in each area is given a numerical score (A) representing the proportion involved: 1 (0–9%),

2 (10–29%), 3 (30–49%), 4 (50–69%), 5 (70–89%) or 6 (90–100%). Within each area (H, UL, T, LL) the severity of 3 plaque signs – erythema (E), thickness/induration (I) and desquamation/scaling (D) – is assessed on a 5-point scale: 0 (none), 1 (mild), 2 (moderate), 3 (severe) or 4 (very severe). The final PASI score ranges from 0 to 72, and is calculated using the following formula:

$$PASI = 0.1\ (E_H+I_H+H_H)\ A_H + 0.2\ (E_{UL}+I_{UL}+H_{UL})\ A_{UL} + + 0.3\ (E_T+I_T+H_T)\ A_T + 0.4\ (E_{LL}+I_{LL}+H_{LL})\ A_{LL}$$ [7,8]

Physician global assessment (PGA)

The PGA is an average assessment of all psoriatic lesions, based on erythema, scale and induration. It neither quantifies body surface area nor evaluates individual lesion locations.[9] There are 2 primary PGA forms: a static form, which measures the physician's impression of the disease at a single point, and a dynamic form, in which the physician assesses global improvement from a baseline.[10] The PGA chosen for this study ranged from 0–5 point, and the following categories were used: 0 = clear, 1 = almost clear, 2 = mild, 3 = moderate, 4 = severe, 5 = very severe.

Body surface area (BSA)

The most commonly used method to estimate the BSA of psoriatic lesions is the "rule of nines", which was originally developed for estimating the surface area of burns. It is defined as 9% coverage for the head and neck, 9% for each arm, 9% for the anterior and posterior legs, and 9% for each of 4 trunk quadrants, leaving 1% for the genitalia. The BSA can also be estimated by the number of a patient's hand areas affected, on the assumption that one "handprint" reflects approximately 1% of BSA.[2]

Statistical analysis

All the results were analyzed statistically using STATISTICA v. 12.0 software (StatSoft Inc., Tulsa, USA). Mean values and standard deviations were calculated for all the measurements. Possible differences between the first and second assessments were verified with a paired Student's t-test. Correlations between the parameters studied were calculated with the Pearson correlation test. The intraclass correlation coefficient (ICC) was calculated to analyze intra-rater reliability, with the following ranges: < 0.40 – poor reproducibility; 0.4–0.59 – fair, 0.6–0.74 – good; 0.75–1.0 – very good reproducibility. The coefficient of variation was used to assess inter-rater variability. It was interpreted as follows: 0–20% – slight; 21–40% – moderate; 41–60% – high; > 60% – very high variability. Only p-values less than 0.05 were considered significant.

Results

The mean PASI, BSA and PGA scores given each subject by 10 physicians in 2 assessment sessions are presented in Table 1. There were no significant differences between 2 assessment sessions, except for the 3rd patient's PASI score (p = 0.006).

Table 1. Mean scores for the psoriasis area and severity index (PASI), the body surface area (BSA) and the physician global assessment (PGA) given by 10 physicians in 2 assessments (mean ± standard deviation)

Patients	PASI		p-value	BSA		p-value	PGA		p-value
	assessment 1	assessment 2		assessment 1	assessment 2		assessment 1	assessment 2	
Patient 1	7.5 ± 2.0	7.4 ± 2.8	0.76	8.1 ± 2.9	8.0 ± 2.9	0.89	3.1 ± 0.9	3.2 ± 0.9	0.59
Patient 2	2.3 ± 1.0	2.6 ± 1.2	0.19	5.0 ± 4.7	4.2 ± 3.8	0.4	1.2 ± 0.4	1.2 ± 0.4	1.0
Patient 3	15.0 ± 5.2	18.6 ± 6.2	0.006	40.9 ± 14.3	39.1 ± 13.7	0.54	3.2 ± 0.6	3.5 ± 0.5	0.19
Patient 4	7.4 ± 3.8	6.7 ± 3.7	0.51	20.2 ± 11.4	20.1 ± 16.0	0.98	2.2 ± 1.0	2.6 ± 0.5	0.35
Patient 5	3.2 ± 1.2	3.2 ± 1.0	0.87	3.0 ± 3.5	2.8 ± 3.4	0.58	1.8 ± 0.9	1.9 ± 0.9	0.34
Patient 6	11.8 ± 5.6	13.0 ± 6.7	0.53	39.4 ± 23.5	41.1 ± 20.4	0.63	3.0 ± 1.1	3.1 ± 0.9	0.68
Patient 7	14.2 ± 5.1	16.5 ± 6.5	0.33	28.5 ± 16.5	34.9 ± 25.8	0.5	3.5 ± 0.5	3.4 ± 0.7	0.59
Patient 8	29.6 ± 6.0	27.0 ± 11.1	0.44	90.2 ± 13.4	84.8 ± 24.3	0.62	3.8 ± 0.6	4.0 ± 0.7	0.17
Patient 9	11.4 ± 3.8	12.9 ± 4.8	0.4	23.2 ± 9.3	20.9 ± 8.1	0.09	3.4 ± 0.7	3.5 ± 0.5	0.59

Table 2. Pearson correlation coefficients (r) between the psoriasis area and severity index (PASI), the body surface area (BSA) and the physician global assessment (PGA) obtained by 10 physicians in 2 assessments ($p < 0.05$ for all r values)

Assessment	Scale	Assessment 1			Assessment 2		
		PASI	BSA	PGA	PASI	BSA	PGA
assessment 1	PASI	–	0.84	0.61	0.85	0.77	0.64
	BSA	–	–	0.55	0.68	0.87	0.56
	PGA	–	–	–	0.53	0.53	0.85
assessment 2	PASI	–	–	–	–	0.78	0.66
	BSA	–	–	–	–	–	0.61

Significant correlations were observed among the scales in both assessments (Table 2). The Pearson correlation coefficient between the PASI and BSA for all 10 physicians indicates that the evaluations were very strongly correlated in the 1st assessment (0.84) and strongly correlated in the 2nd assessment (0.78). The correlation between the PASI and PGA was strong in both the 1st and 2nd assessments (0.61 vs 0.66). The correlation between the PGA and BSA was moderate in the 1st assessment (0.55) and strong in the 2nd assessment (0.61).

Table 3 summarizes the intra-rater reliability of the PASI, BSA and PGA for all the physicians. In all 3 scales the ICCs were > 0.75, indicating very good reliability. The highest ICC was for the BSA (0.96) and the lowest one was for the PGA (0.87). The ICC for all components of the PASI were also > 0.75, indicating very good reliability, except for the ICC for scaling (0.72), indicating good reliability (Table 3). Among the components of the PASI, the highest ICC was observed for the area score (0.97).

Table 4 shows the inter-rater variability of the PASI, BSA and PGA for all the physicians. The coefficients of variation (CVs) for the PGA and PASI were 29.3 and 36.9, respectively, indicating moderate variability. The CV for the BSA was 57.1, indicating high variability. The CVs for the separate components of the PASI showed the highest variability for the head and neck (117.8) and the lowest 1 for the area score (26.8) (Table 4).

Table 3. Intra-class correlation coefficients (ICCs) for the psoriasis area and severity index (PASI), each of the PASI components, the body surface area (BSA) and the physician global assessment (PGA)

Scale		Intra-class correlation coefficient (ICC)	Intra-rater reliability (interpretation)
PASI	total scoring	0.91 ± 0.06	very good
	erythema	0.81 ± 0.16	very good
	induration	0.77 ± 0.14	very good
	scaling	0.72 ± 0.21	good
	area score	0.97 ± 0.02	very good
	head and neck	0.85 ± 0.13	very good
	trunk	0.86 ± 0.18	very good
	upper limbs	0.84 ± 0.11	very good
	lower limbs	0.89 ± 0.06	very good
BSA		0.96 ± 0.05	very good
PGA		0.87 ± 0.07	very good

Table 4. Coefficients of variation (CVs) for the psoriasis area and severity index (PASI), each of the PASI components, the body surface area (BSA) and the physician global assessment (PGA)

Scale		Coefficient of variation %	Inter-rater variability (interpretation)
PASI	total scoring	36.9 ± 9.8	moderate
	erythema	33.2 ± 7.6	moderate
	induration	35.9 ± 13.9	moderate
	scaling	59.6 ± 10.3	high
	area score	26.8 ± 10.3	moderate
	head and neck	117.8 ± 115.4	very high
	trunk	59.7 ± 43.0	high
	upper limbs	49.2 ± 16.7	high
	lower limbs	42.9 ± 9.7	high
BSA		57.1 ± 31.8	high
PGA		29.3 ± 12.4	moderate

Discussion

The PASI, BSA and PGA are the most frequently used psoriasis assessment tools. While these scales are commonly used to assess psoriasis severity in both clinical research and clinical practice, they have not been fully evaluated in terms of their validity and reliability, and no consensus exists on the best way to monitor psoriasis severity.[6,8] Despite its widespread use, the PASI has never been formally validated. Its grading has never been properly defined or standardized.[11] Therefore, this study was undertaken with the aim of assessing the reliability of the PASI, BSA and PGA.

In the present study a significant correlation was found between all 3 of the psoriasis assessment tools tested. The correlation between the PASI and BSA was higher than between the other scales, whereas the lowest correlation was noted between the BSA and PGA. The lack of assessment of the area affected as a component of the PGA, in contrast to the PASI, is probably responsible for the lower correlation between the PGA and other scales. However, Langley and Ellis reported that the sum of the area scores using PASI was more highly correlated with PGA (r = 0.8) than the summed scores for erythema, induration or desquamation (r = 0.3 to 0.6), which suggests that physicians do incorporate the extent of involvement when using the PGA, even though it is not stated in the PGA.[13] However, it must be mentioned that many different versions of the PGA are described in clinical trials, and this variation has the potential to produce non-standardized results. The scales used may range from 4 points to 10 points and the definitions used for the specific points on the scale vary slightly.[9]

The present study demonstrated very good intra-rater reliability for all 3 scales, which can be ranked in the following order from the highest to the lowest In terms of intra-rater reliability: BSA > PASI > PGA. Intra-rater reliability for the BSA has also previously been described as very good.[14,15] Two studies conducted by Berth-Jones et al. have demonstrated substantial intra-rater reliability for the PASI and PGA, and higher reproducibility for the PASI than for the PGA.[8,12] However, the data presented by Langley and Ellis revealed over 12 times higher intra-rater variation for the PASI than for the PGA.[13] The difference between these studies might be due to the statistical methodology.

In the present study, all of the PASI components had very good reproducibility, except for scaling, which had good reproducibility. As compared to the BSA, the PASI area score demonstrated the highest intra-rater reliability among all the PASI components. In terms of intra-rater reliability, the psoriatic plaque features are ranked from the highest to the lowest as follows: erythema > induration > scaling. The body areas are ranked in the following order: lower limbs > trunk > head and neck > upper limbs.

In the present study, the BSA demonstrated high inter-rater variability. A systemic review performed by Puzenat et al. also showed that inter-rater variation was unacceptably high for the BSA.[6] For this reason, the BSA was not recommended for assessment of the clinical severity of psoriasis. The BSA could be calculated either by the "rule of nines" method or by the number of patient's hand areas affected. The findings of a meta-analysis performed by Rhodes et al. demonstrated that the widely accepted value of hand surface area as 1% of total BSA is inaccurate.[16] If hand surface area is to be used for adults, the estimate should be 0.9% for men and 0.85% for women. However, these values will be influenced by BMI and ethnic origin. This may explain why the BSA is often overestimated and why this assessment tool shows high inter-rater variability.

In the present study, the PASI and PGA achieved moderate inter-rater variability. In 2 previous studies inter-rater reliability was substantial for the PASI and moderate for the PGA.[8,12] In contrast to the present study, the PASI was suggested as a more reliable assessment instrument than the PGA. However, in another study the PASI showed more variability than the PGA.[13]

The data from the present study suggests that the head and neck area is the most difficult component of the PASI to be assessed properly, as it demonstrated very high inter-rater variability. This fact was not surprising, because scalp assessment is time-consuming and difficult due to the presence of hair. All other body areas demonstrated high inter-rater variability. It was surprising that, in contrast to the BSA, the body area score showed the lowest intra-rater variability among all the PASI components. The different methods of evaluating the affected area used in the PASI and BSA may be an explanation for this discrepancy. In the BSA the area is normally expressed as a percentage, whereas in the PASI it is given a value on a scale of 1–6 for 4 separate body areas.

Assessments of erythema and induration in the PASI revealed moderate inter-rater variability, whereas scaling showed high variability. There is no agreement on how scaling should be scored. Although the original publication[7] is used as the standard reference source, it is not desquamation (the shedding of skin scales) but scale thickness that is generally scored.[11] Scaling is a very unstable sign; applications of topical treatment and emollients have an influence on it. This was eliminated in the present study, because patients were asked not to apply any topical treatment prior to the examination. Patients rate degree of scaling as a strong indicator of disease severity, so appropriate evaluations of scaling by physicians is important in assessing the severity of psoriasis.[2]

The relatively high inter-rater variation of the components of the PASI suggests that this scoring method is not suitable for follow-up of isolated signs of intensity and body areas.

The main limitation of this study is its small sample size (9 patients). However, in this study each assessor had to re-

cord over 50 measurements for each patient. Thus, a larger number of patients in a relatively short time period would fatigue the assessors and yield inaccurate results.

In general, comparing the PASI, PGA and BSA showed that the PGA had the lowest (i.e. moderate) inter-rater variability among 3 scales, whereas the BSA had the highest intra-rater reliability. The PASI showed intermediate values in terms of inter- and intra-rater reliability. The PGA is a quick and easy method to perform, making it a suitable tool in everyday practice, where there are different physicians performing each assessment. The BSA is the most reproducible method, which means it should be chosen in situations where repeated assessment are performed by the same physician. In conclusion, none of the 3 scoring methods showed a significant advantage over the others. A reliable assessment of psoriasis severity requires the use of several independent evaluations simultaneously.

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Hydronephrosis in the course of ureteropelvic junction obstruction: An underestimated problem? Current opinions on the pathogenesis, diagnosis and treatment

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Abstract

Ureteropelvic junction obstruction (UPJO) causes a reduction in the urine flow from the renal pelvis into the ureter. Untreated UPJO may cause hydronephrosis, chronic infection or urolithiasis and will often result in progressive deterioration of renal function. Most cases of UPJO are congenital; however, the disease can be clinically silent until adulthood. Other causes, both intrinsic and extrinsic, are acquired and include urolithiasis, post-operative/inflammatory/ischemic stricture, fibroepithelial polyps, adhesions and malignancy. In the past, the most frequent symptom of UPJO in neonates and infants was a palpable flank mass. Nowadays, thanks to the widespread use of maternal and prenatal ultrasound examinations, asymptomatic hydronephrosis is diagnosed very early. In adults and older children symptoms may include intermittent abdominal or flank pain, nausea, vomiting and hematuria. In addition to high specificity and sensitivity in detecting UPJO, modern technologically advanced equipment such as ultrasound, magnetic resonance imaging and computed tomography provides a lot of information about the function of the affected kidney and the anatomy of the surrounding tissues. Treatment options for UPJO include a wide spectrum of approaches, from active surveillance or minimally invasive endourologic techniques to open, laparoscopic or robotic pyeloplasty. The main goal of therapy is to relieve symptoms and maintain or improve renal function, but it is difficult to define treatment success after UPJO therapy.

Key words: hydronephrosis, ureteropelvic junction obstruction, pyeloplasty

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Ureteropelvic junction obstruction (UPJO) causes a reduction in the urine flow from the renal pelvis into the ureter. The narrowing of the ureter can be partial or complete. Untreated UPJO may cause hydronephrosis, chronic infection or urolithiasis and will often result in progressive deterioration of renal function. The main mechanism triggering renal parenchymal damage is increased pressure in the pelvicalyceal system. Most cases of UPJO are congenital, but the disease can remain clinically silent until adulthood. Other causes, both intrinsic and extrinsic, are acquired, and include urolithiasis, post-operative/inflammatory/ischemic stricture, fibroepithelial polyps, adhesions and malignancy.[1] Congenital UPJO is typically caused by the presence of an aperistaltic segment of the ureter, which does not allow the development of an effective peristaltic wave. In this pathological section, spiral musculature is replaced by longitudinal muscle and/or fibrous tissue. Other pathologies, such as abnormal secretion of compounds like transforming growth factor-β, epidermal growth factor and other cytokines, nitric oxide and neuropeptide Y have a proven role in the pathogenesis of UPJO.[2]

The importance of interstitial Cajal cells in UPJO is controversial. The results of trials investigating distributional difference in Cajal cells in obstructed and unobstructed UPJ are contradictory in different publications.[3] A case of UPJO caused by Kawasaki disease and vasculitis of the ureter vessels has been reported.[4]

The role of crossing vessels in the pathogenesis of UPJO is also controversial. They have been observed in 30% of the general population and in up to 63% of UPJO cases; however, despite this association, the relationship between crossing vessels and UPJO is unclear. In some cases, because of a lack of demonstrable histopathological changes, it appears that crossing vessels cause only a mechanical obstacle. In other patients, crossing vessels lead to UPJ inflammation, fibrosis and smooth muscle hypertrophy, which subsequently result in obstruction. Finally, UPJO occurs due to other tissue pathologies, and is not associated with coexisting, clinically silent crossing vessels. To demonstrate the presence of crossing vessels, Doppler ultrasound (USG) scanning, endoluminal sonography, magnetic resonance imaging (MRI), and computed tomography (CT) can be used with nearly 100% accuracy.[5]

Symptoms

In the past, the most frequent symptom of UPJO in neonates and infants was a palpable flank mass. Nowadays, thanks to the widespread use of maternal and prenatal ultrasound examinations, asymptomatic hydronephrosis is diagnosed very early. Occasionally, UPJO can be diagnosed during extended diagnostics of other congenital abnormalities.

In adults and older children, symptoms may include intermittent abdominal or flank pain, nausea, vomiting, hematuria or features of urinary tract infection. Laboratory findings can reveal microhematuria or pyuria. In same rare cases, hypertension is possible.

Diagnosis

Classically, excretory urography was the most commonly used diagnostic option for UPJO. However, in addition to high specificity and sensitivity in detecting UPJO, modern technologically advanced equipment such as MRI, USG or CT provides a lot of information about the function of the affected kidney and the anatomy of the surrounding tissues. There are attempts to use CT imaging to estimate renal function based on renal parenchymal thickness. This non-invasive method does not require the injection of contrast, but further studies on larger groups of patients are needed to determine its role in UPJO diagnostics.[6] Another imaging method widely used in UPJO diagnosis and in estimating pre- and postoperative kidney function is nuclear renography (RN). RN involves intravenous administration of a radioisotope and observation by gamma camera as the radioisotope is excreted by the kidneys and passes through the urinary tract. Renal function is assessed between 1 and 2 min after the radioisotope injection and is expressed in terms of extraction value. A normal level of extraction is between 45 and 55%. Resistance to urine flow from the renal pelvis into the ureter is evaluated by T1⁄2 (the time taken for the renal unit to excrete half of the radioisotope). In cases of ureter obstruction the radioisotope will not pass beyond the level of the obstruction, or its transit time will be delayed. When T1/2 is shorter than 10–15 min, there is no obstruction; T1/2 longer than 20 min indicates significant obstruction; and when T1/2 is between 15 and 20 min, the result is equivocal.[7] RN can be also used to differentiate between UPJO and multicystic dysplastic kidneys. Multicystic dysplastic kidneys rarely reveal a concentration of isotope, while kidneys with UPJO usually demonstrate good concentrations of isotope. Despite the strong position of RN in UPJO diagnosis, there are publications indicating that it may not be necessary to perform routine RN to assess drainage and assess renal function in patients who become pain-free after UPJO operative treatment.[8]

Ultrasonography ranks first among the diagnostic procedures used to detect UPJO. However, ultrasonography does not provide information on the renal function of the hydronephrotic kidney. As dilatation of the pelvicalyceal system does not always result from UPJO, an ultrasound scan may turn out to be a false positive. Therefore, modifications of this imaging test have been introduced to improve the detection and diagnosis of UPJO. Diuretic ultrasonography comprises an ultrasound scan performed

before and after an injection of a diuretic. Duplex Doppler ultrasonography has been used for more than 20 years to evaluate UPJO. Renal obstruction causes a change in the Doppler waveform detected by means of the resistive index (RI). An RI value greater than 70 indicates obstruction. In adults, this diagnostic test is reported as showing a sensitivity of 92% and specificity of 88%.[5]

The multivariate scoring system introduced by Garcia-Pena et al. is yet another attempt to improve ultrasound sensitivity in diagnosing UPJO.[9] Those authors wrote: "The seven variables associated with a significantly higher risk of urinary tract obstruction were increased echogenicity, parenchymal rims 5 mm or less, contralateral hypertrophy, resistive index ratio 1.10 or greater, resistive index difference with diuresis of 70% or greater, ureter diameter 10 mm or greater and aperistaltic ureter".[9] The sensitivity of the multivariate scoring system in high risk children was reported as 91%.

In some patients other diagnostic methods like retrograde pyelography are needed to confirm the diagnosis and to pinpoint the exact position of the obstruction. In cases with associated infection or reduced renal function, placement of a percutaneous nephrostomy tube is necessary to allow decompression of the collecting system and to allow antegrade pyelogram and dynamic pressure perfusion studies. The Whitaker test, an invasive diagnostic examination, is rarely recommended. It is indicated for patients with nephrostomy or when other diagnostic tests yield equivocal results. The test entails measuring renal pelvic pressure during an infusion of saline into the collecting system at a fixed rate of 10 mL/min. Intrapelvic pressure of less than 15 cm H_2O is considered normal; greater than 22 cm H_2O is indicative of obstruction; and between 15 and 22 cm H_2O is indeterminate.[10]

It should be kept in mind that transitional cell carcinoma symptoms can sometimes mimic UPJO symptoms. Any case of UPJO, especially in an elderly patient and/or one with coexisting hematuria, warrants increased oncological vigilance.

To grade UPJO in both children and adults, the Society of Fetal Urology's grading system based on ultrasound examination may be used. It has 5 stages: Grade 0: normal findings; Grade I: separation of the renal hilum; Grade II: Grade I + pelviectasis; Grade III: Grade II + caliectasis; Grade IV: Grade III + 50% or more thinning of the renal cortex relative to normal.[11] The degree of hydronephrosis can be also estimated on the basis of diuretic intravenous urography (IVU), and presented using a 4-grade scale; it has been shown that Grades I and II have no clinical significance in a large groups of patients.[11]

Infants with hydronephrosis on postnatal ultrasound with an anterior posterior diameter > 7 mm should undergo evaluation with a voiding cystourethrogram (VCUG). This fluoroscopic method provides a visualization of the anatomy of the bladder and urinary system, allowing the diagnosis of vesicoureteral reflux, posterior urethral valves, ureteroceles and other anatomic abnormalities.[12]

Additionally, some findings in blood examinations can indicate UPJO. It has been shown that transforming growth factor-beta (TGFβ), epidermal growth factor (EGF), endothelin-1 (ET-1) and urinary tubular enzymes such as N-acetyl-beta-D-glucosaminidase (NAG), gamma-glutamyl transferase (GGT) and alkaline phosphatase (AKP) can be useful as non-invasive auxiliary examination tools in diagnosing UPJO in children. However, these biochemical markers are not yet used in routine clinical practice, but mainly in research settings.[13]

Treatment

The first open pyeloplasty was reported by Trendelenburg in 1886, but the patient died during the procedure. The first successful open pyeloplasty was performed by Kuster in 1891. Nowadays treatment options for UPJO include a wide spectrum of approaches, from active surveillance or minimally invasive endourologic techniques to open, laparoscopic or robotic pyeloplasty. The main goal of therapy is to relieve symptoms and maintain or improve renal function; however, it is difficult to define treatment success after UPJO therapy. Various criteria such as relief of symptoms, stable or improved renal function or improvement in the RN/intravenous urogram are used either alone or (more often) in combination. Indications for UPJO treatment include cases in which extraction is reduced to less than 40% renal function in unilateral hydronephrosis with a normal other kidney, symptomatic patients with fast deterioration of renal function because of hydronephrosis, or Grade IV bilateral hydronephrosis. An extraction fraction of 10% or less usually indicates a non-salvageable hydronephrotic kidney.[14]

Non-invasive approaches

In some children with congenital UPJO, the problem resolves spontaneously. Predicting which patients with UPJO will not require surgery is difficult. It has been shown that levels of urinary enzymes NAG, AKP and GGT were significantly higher in patients with hydronephrosis in whom operations were necessary when compared to those not requiring surgery. It has therefore been suggested that the levels of these enzymes can be used to identify patients requiring pyeloplasty. Additionally, it is known that the level of AKP falls significantly after UPJO treatment, so the concentration of this enzyme can be used as an adjunctive marker of improvement in drainage after pyeloplasty.[15]

In children, close follow-ups are required in the first 2 years of life to identify the subgroup of children with

obstruction that requires prompt surgery. That form of management appears to be a safe and recommended approach for neonates with primary bilateral ureteropelvic junction type hydronephrosis.[16] According to some authors each patient should be maintained on prophylactic antibiotics for the first year of life or until there was significant improvement in hydronephrosis.[17]

In adults with mild asymptomatic UPJO, careful observation with cyclic RN studies may be an appropriate approach. However, it is unclear how long the follow-up period should last. Only a few studies concerning this problem have been conducted and most of them were based on small group of patients. It seems reasonable to discharge patients with minimally symptomatic and/or asymptomatic UPJO from further evaluation 2 years after the diagnosis if they do not have symptomatic or renographic deterioration.

Patients with persistent pain secondary to UPJO can pose a management challenge. Analgesic drugs and physical interventions such as autonomic sympathetic blocks can be used to relieve the pain. There are reports of spinal cord stimulation as auxiliary treatment for chronic renal pain secondary to UPJO.[18]

The endoscopic approach

Endoscopic endopyelotomy was firstly introduced by Ramsay et al. in 1984. The advantages of this procedure include shortened hospital stays and faster post-operative recovery. On the other hand, the success rates of endopyelotomy are worse than that of open, laparoscopic or robotic pyeloplasty. The available studies of antegrade endopyelotomy show success rates from 65 to 93%.[19] Outcomes could be improved with careful patient selection. It has been shown that patients with less advanced hydronephrosis (Grade II) have better antegrade endopyelotomy outcomes than more advanced cases do (Grade IV). Likewise, it has been shown that patients with better renal function have better outcomes than those with poor renal function. Stricture length is also an important factor. For strictures longer than 2 cm endopyelotomy results are unfavorable.[20]

Endopyelotomy is a lateral full-thickness incision made from the side of ureteral lumen. The incision is made laterally to avoid damage to possible crossing vessels. A double-J stent left after procedure to promote healing. The incision can be performed using a cold knife, a hot knife or a holmium laser in either antegrade and retrograde fashion, under vision or fluoroscopic control. Alternative endoscopic methods include balloon dilatation and Acucise endopyelotomy. This second procedure uses 2.8 cm long, 150 μm wide electrosurgical cutting wire mounted on an 8-mm inflatable balloon catheter to incise the pelviureteric junction under fluoroscopic control. There are several reports, mainly in animal models, on Heineke-Mikulicz and Anderson-Hynes endopyeloplasties performed through percutaneous tracts.[21]

The most common complication after every type of endopyelotomy is hemorrhage, with an incidence of 2–4%. Other complications include ureteric injury, infection, ureteral spasms and scarring.[22]

Open pyeloplasty

For years open pyeloplasty was considered the gold standard in UPJO treatment. Open pyeloplasty can be performed through an anterior extraperitoneal approach or posterior lumbotomy. The length of the incision and the morbidity associated with the procedure are the main disadvantages of open pyeloplasty.

Laparoscopic pyeloplasty (LP)

Laparoscopic treatment of UPJO was first described in 1993, by Schuessler et al. as well as by Kavoussi and Peters. Because of the fact that laparoscopy entails less pain and blood loss, has comparable efficacy and better cosmetic results than open surgery and significantly better results than endourologic approaches, it became the new gold standard in UPJO treatment in both children and adults. It has been shown that LP is a good treatment option for both very young children (< 1 year) and for elderly people (70 years or older).[23,24] LP has also been shown to be adequate treatment for patients with UPJO in horseshoe kidneys.[25] In large studies the outcomes of LP are very good, ranging from 85 to 100%.[26] However, compared to open pyeloplasty or endopyelotomy, laparoscopic pyeloplasty requires advanced laparoscopic skills, mainly because of the complexity of the intracorporeal suturing. This means that LP is performed only at centers specialized in laparoscopy. LP is also a longer procedure than open pyeloplasty in the majority of published studies; however, some authors report that retroperitoneal LP can be shorter than an open operation.[27] Unfortunately, most of the available data comparing various methods come from non-randomized studies. Since the methods are significantly different in terms of invasiveness, it is difficult to persuade patients to accept a random procedure selection. In adult populations, only one randomized controlled trial has been conducted so far, including 62 patients, and the results were similar to those from retrospective studies.[28]

LP can be performed through a transperitoneal, anterior extraperitoneal or retroperitoneal approach. Because of the larger working space and more familiar anatomy, the retrocolic and transmesenteric transperitoneal routes are more frequently chosen. In a prospective randomized trial comparing trans- and retroperitoneal approaches, there was no difference in terms of functional outcomes,

but there was a longer operative time for the retroperitoneal approach.[29] Retrospective studies conducted on large cohorts confirm those results.[30] Comparing retrocolic and transmesenteric approaches, it seems that the latter should be considered whenever possible because it offers a significantly shorter operative time at no increased risk. The decision should be made during the operation, depending on intraoperative findings.

There is a controversy among surgeons what type of suture (running or interrupted) should be used while anastomosing the UPJ. Experimental studies performed on laboratory animals have shown that using running sutures could cause vascular compromise, deficiencies in collagen regeneration and more tissue ischemia and necrosis.[26] Additionally, continuous sutures in laparoscopy are associated with difficulty in maintaining appropriate tension during the procedure. However, in clinical studies, it has been shown that using the running suture method is associated with higher surgical efficiency and lower complication rates.[31] Based on the existing research, it can be concluded that continuous suturing reduces the overall cost of the procedure by shortening the time of procedure, the duration of drainage and thus the duration of hospitalization. Recently a novel mini-laparoscopic approach for management of UPJO in adults was reported, with good outcomes and better cosmetic results compared to classic LP.[32]

Alternative methods include one-trocar-assisted pyeloplasty (OTAP), first presented by Lima et al. in 2007. OTAP has been shown to be safe, feasible, and efficacious, with operative times similar to or even shorter than those of other minimally invasive techniques; the complication rates are similar to those of other laparoscopic UPJO treatments.[33]

Complication rates for LP range from 4 to 12.7%. One of the most common complications is anastomotic leakage, often related to double-J stent obstruction. It seems that most failures after LP occur in the first 2 years after the procedure; however, it is difficult to estimate the exact long-term results of LP because there are few reports with long-term follow-up of patients who have undergone pyeloplasty.[34]

Robot-assisted pyeloplasty

Robotic systems reduce the limitations of laparoscopy through a 3-dimensional magnified view, full mobility of the instruments and the elimination of any tremor. However, there is no tactile feedback and total time of a robotic procedure and its total costs are higher. Additionally, in the hands of an experienced laparoscopic surgeon, a robot does not provide a significant clinical advantage compared with conventional laparoscopy. Nevertheless, it seems that whenever the technology is available, robotic pyeloplasty is likely to become the favored approach. Clinical trials have shown that robotic pyeloplasty is a safe, feasible and effective procedure for treating UPJO.[35] A review and meta-analysis of the literature demonstrated that robotic pyeloplasty was associated with a 10-min reduction in operative time and significantly shorter hospital stays compared with the LP approach. However, there were no differences between the techniques concerning the rates of success and complications.[36] It has also been shown that the total cost was lower among patients undergoing open pyeloplasty as opposed to robotic pyeloplasty; however, the direct costs of robot-assisted surgery were lower than open surgery. This cost difference was largely attributable to the costs of robotic equipment.[37] Recent meta-analyses comparing robotic, laparoscopic and open pyeloplasty in children showed that robotic pyeloplasty might offer shorter hospital stays, lower analgesia requirements and lower estimated blood loss. The post-operative success rate was comparable in the 2 groups, but there was a significantly higher complication rate and higher costs in the robotic group.[38] Additionally, it has been shown that with 2 years of follow-up, only 5% of patients who underwent robotic pyeloplasty require a secondary procedure, compared with 13% of those who underwent a standard laparoscopic pyeloplasty.[39] Moreover, robot-assisted laparoscopic reoperative repair after failed primary treatment is safe and effective, and is less technically demanding than open repair for UPJO.[40] Robotic procedures are typically performed via a transperitoneal approach, although there are reports of a retroperitoneal approach being used in a small group of patients.[41]

UPJ reconstruction techniques

Dismembered pyeloplasty

Dismembered pyeloplasty is also called the Anderson-Hynes operation. Because dismembered pyeloplasty involves complete disconnection of the ureter and excision of the pathological segment, it is the most versatile form of operation. It allows reduction of a redundant pelvis and transposition of the UPJ when crossing vessels are the cause of obstruction. This method though, is not appropriate for long or multiple strictures, or for small intrarenal pelvis (Fig. 1–2).

Flap procedures

Flap procedures include multiple techniques such as the Foley Y-V plasty, the Culp-DeWeerd spiral flap or the Scardino-Prince vertical flap. The most frequently used is the Foley Y-V plasty. The advantages of this procedure include significantly reduced operative time and decreased risk of devascularization of the UPJ. Flap procedures are suitable for long ureteral strictures with high insertion of the ureter into the renal pelvis. In cases with crossing

Fig. 1. Dismembered pyeloplasty

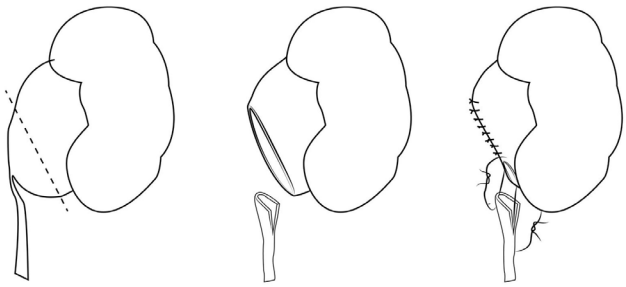


Fig. 2. Dismembered pyeloplasty when crossing vessels are present: dorsal transposition of the vessel

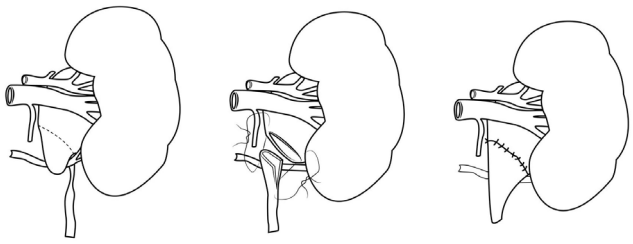


Fig. 3. Foley Y-V plasty

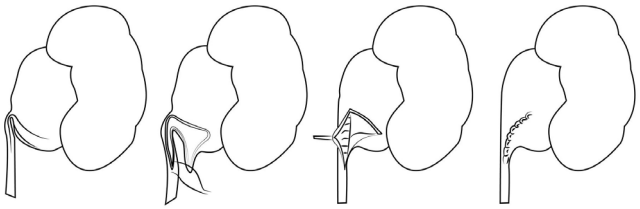
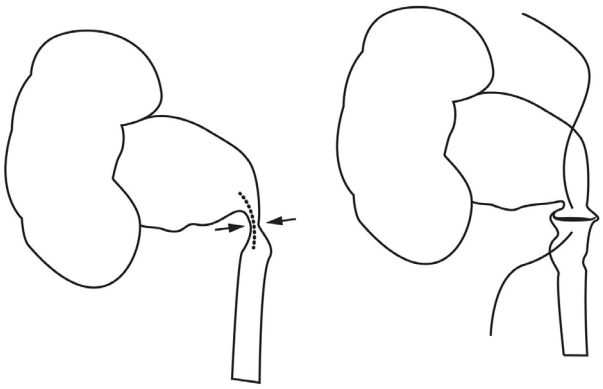


Fig. 4. Fenger plasty



vessels, cephalad translocation of the vessels should be performed instead of posterior transposition.[42] In a study comparing laparoscopic Anderson-Hynes pyeloplasty with laparoscopic Y-V pyeloplasty, it was shown that in the Y-V plasty group operative time was significantly shorter and intracorporeal suturing was easier with similar outcomes.[43] In another study that prospectively compared success rates in Anderson-Hynes and Y-V plasty, the former resulted in a higher success rate than Y-V pyeloplasty, but the difference was not statistically significant (Fig. 3).[44]

Fenger plasty

Fenger plasty is the first nondismembered pyeloplasty introduced, and also the simplest approach that involves a Heineke-Mikulicz longitudinal incision through a narrowed segment and transverse closure. Laparoscopic suturing and knot tying are reduced to the placement of a few interrupted sutures. This procedure can be applied only to short strictures in the absence of high insertion of the ureter. This technique often causes shortening of the suture line on one side, resulting in buckling or kinking of the UPJ (Fig. 4).[45]

Ureterocalicostomy

Ureterocalicostomy is a rarely used procedure for primary UPJO. Indications include proximal ureteral stricture associated with a small intrarenal pelvis. It is also used as a salvage technique for failed pyeloplasties.

Nephrectomy

In some rare cases a nephrectomy can be performed. It may be indicated in symptomatic patients with diminished function or a non-functioning kidney, or after repeated repair failures. Nephrectomy should be considered only in cases with a normally functioning contralateral kidney.

Crossing vessels

Cases with coexisting crossing vessels represent a therapeutic dilemma. There is no consensus on whether the transposition of the crossing vessels is necessary. The outcomes of operations with and without relocation seem not to differ. Therefore, transposition is indicated in cases with difficult anatomical relations. The decision should be made during surgery. The translocation technique, also called a vascular hitch, entails mobilizing, moving and fixing the vessel to a higher position on the renal pelvis. Laparoscopic pyelopyelostomy, another transpositioning option, has also been shown to be effective in a small cohort of patients.[46]

The use of ureteral stents

Another dilemma concerns anastomosis stenting. There is no agreement on what type of stenting (retrograde or antegrade) should be used, and whether there is a need for stenting at all. Since the retrograde stenting procedure involves a number of technical inconveniences

(the need for patient repositioning, renal pelvis collapsing), attempts have been made to introduce stents in an antegrade fashion. Studies comparing the effectiveness of the 2 methods have reported conflicting results.[47]

Ureteral stenting, despite reducing the frequency of urinoma formation, can cause complications, especially in children. Trials comparing both stentless and stented techniques have shown that stentless pyeloplasty is a feasible and safe procedure. Still, some authors recommend stent use in patients with a solitary kidney, difficult anastomosis, significant bleeding during the procedure, and in patients with a thick noncompliant ureter due to chronic inflammation.[48]

Conclusions

UPJO is a pathology of the upper urinary tract which may be diagnosed at any age. Untreated UPJO leads to deterioration and eventual loss of renal function. Current diagnostic methods make it possible to detect the disease early and to implement minimally invasive surgical procedures that are capable of enhancing renal function at best or, at least preventing its deterioration.

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MicroRNA in cardiovascular biology and disease

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Abstract

MicroRNAs (miRNAs) are members of a non-coding RNA family. They act as negative regulators of protein translation by affecting messenger RNA (mRNA) stability; they modulate numerous signaling pathways and cellular processes, and are involved in cell-to-cell communication. Thus, studies on miRNAs offer an opportunity to improve our understanding of complex biological mechanisms. In the cardiovascular system, miRNAs control functions of various cells, such as cardiomyocytes, endothelial cells, smooth muscle cells and fibroblasts. The pivotal role of miRNAs in the cardiovascular system provides a new perspective on the pathophysiology of disorders like myocardial infarction, hypertrophy, fibrosis, heart failure, arrhythmia, inflammation and atherosclerosis. MiRNAs are differentially expressed in diseased tissue and can be released into circulation. Manipulation of miRNA activity may influence the course of a disease. Therefore, miRNAs have become an active field of research for developing new diagnostic and therapeutic tools. This review discusses emerging functions of miRNAs in cardiogenesis, heart regeneration and the pathophysiology of cardiovascular diseases.

Key words: microRNA, cardiovascular disease, heart regeneration, heart development

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MicroRNAs (miRNAs) are a class of single-stranded, non-coding RNAs, about 22 nucleotides in length, which negatively regulate gene expression at the post-transcriptional level. They bind to messenger RNA (mRNA) in a complementary way, and cause gene silencing through the inhibition of translation and/or degradation of mRNA.[1] MiRNAs play a role in regulating various biological processes including embryogenesis, cell proliferation and differentiation, apoptosis or tumorigenesis.[2] In the cardiovascular system, miRNAs control cardiomyocyte growth and contractility, the development and maintenance of cardiac rhythm, plaque formation, lipid metabolism and angiogenesis.[2–5] Altered miRNA expression can be found in the blood of patients with various cardiovascular diseases, which makes them attractive candidates for noninvasive biomarkers.[6,7] It has been estimated that miRNAs control the activity of 30–50% of protein-coding genes.[7] Unlike transcriptional regulators, which have a turn-on-and-off function in controlling gene expression, the varied profiles of miRNAs appear to fine-tune the level of protein expression to changes in environmental conditions.[2] A single miRNA may influence the expression of hundreds of genes in a cell, and each mRNA molecule may be regulated by multiple miRNAs that interact or compete with each other.[8] The first miRNA, *lin-4*, was discovered in the nematode *Caenorhabditis elegans* in 1993.[1] To date, about 2500 miRNAs have been identified in the human genome. All known sequences of miRNAs are available in the database at the website www.mirbase.org.

MicroRNA biogenesis and mechanisms of action

MiRNA genes are an evolutionarily conserved integral part of the cell genome. They can be transcribed as independent transcription units in intergenic regions or in the introns and exons of protein-coding genes. MiRNA genes can exist individually or form polycistronic clusters containing multiple miRNA components.[1] MiRNAs are transcribed in the nucleus by RNA polymerase II (RNA Pol II) to primary miRNAs (pri-miRNAs), which can be a few kilobases long. Pri-miRNAs are cleaved by a protein complex containing the RNase III endonuclease Drosha into approximately 70-nucleotide precursor miRNAs (pre-miRNAs) with a hairpin structure.[1] Next, a GTP-dependent protein, exportin-5, recognizes a short stem of 2-3 nucleotides overhanging at the end of the pre-miRNAs and transports them from the nucleus to the cytoplasm.[1] Alternatively, pre-miRNAs can be processed independently of the Drosha complex through the direct splicing of introns.[6]

In the cytoplasm, pre-miRNA is cleaved by the RNase III endonuclease Dicer to approximately 22-nucleotide double-stranded miRNAs. One strand, called a guide strand, is loaded onto the RNA-induced silencing complex (RISC) and becomes mature miRNA, while the other strand, called a passenger strand, is degraded or incorporated into microvesicles and released from the cell.[9] Both mature and pre-miRNAs can be found in microvesicles.[9] The formation of the RISC effector, which contains Argonaute 2 (Ago2) protein, allows miRNAs to bind to target mRNAs.[1,10]

The RISC binding sites are complementary sequences present mainly in the 3'-untranslated region (3'-UTR) of mRNAs. In cases of perfect complementarity of the miRNA-mRNA sequences, Ago2 protein, which has endonuclease activity, cleaves the mRNA, leading to its degradation. Mismatches in the sequence inhibit translation (Fig. 1).[1]

Most miRNAs are localized intracellularly, but some of them are released into the blood in association with proteins (e.g. Ago2, nucleophosmin 1 and HDL) or as a component of cell-derived microvesicles (e.g. exosomes or apoptotic bodies). MiRNAs may be released in response to cell activation stimuli, injury or after cell death.[9] In the circulation, miRNAs are transported to distant sites and interact with cells by fusing with the cell membrane or through receptor-mediated binding, which suggests that miRNAs play a role in cell-to-cell communication.[9,10] For example, in response to tissue damage, miR-126 is transported in endothelial cell-derived apoptotic bodies to vascular smooth muscle cells (VSMCs), where it mediates the synthesis of the CXCL12 chemokine to recruit progenitor cells and provide vascular protection.[5]

The nomenclature of microRNAs

With the exception of a few miRNAs that were discovered early (such as the let family), the nomenclature of mature miRNAs consists of the prefix "miR" and the identifying number, e.g. miR-499. Pre-miRNAs are indicated by italics and the lower case prefix *"mir"*. Three- or four-letter prefixes indicate the species, e.g. hsa-miR-101 in *Homo sapiens*. An additional lower case letter is appended to miRNAs with similar sequences, differing by only one or two nucleotides, e.g. miR-123a or miR-123b.[6] If two pre-miRNAs that are located at different sites in the genome lead to an identical mature miRNA, the miRNA is annotated with an additional hyphen and number, e.g. miR-194-1 or miR-194-2. Two different miRNAs that originate from the same precursor are named according to their location on the hairpin: miR-17-5p (5' arm) or miR-17-3p (3' arm), or based on their level of expression: miR-123 or miR-123*. An asterisk indicates the miRNA strand that is expressed at a lower level.[6]

Controlling microRNA activity

The activity of miRNAs can be modulated by two different approaches based on mimicking miRNA functions

Fig. 1. A schematic representation of the biogenesis of miRNAs and their mechanism of action: a miRNA gene is transcribed by RNA polymerase II (RNA Pol II) to stem-loop primary miRNAs (pri-miRNAs). Within the nucleus, pri-miRNA is processed by the RNase III endonuclease Drosha into hairpin-like precursor miRNAs (pre-miRNAs). Alternatively, pre-miRNAs are processed independently of the Drosha complex, through direct splicing of introns, to form pre-miRNAs called mirtrons. The pre-miRNAs/mirtrons are then transported to the cytoplasm by a GTP-dependent protein transporter, exportin-5. Within the cytoplasm, pre-miRNAs are cleaved by the RNase III endonuclease Dicer to approximately 22-nucleotide double-stranded miRNAs. After unwinding, both miRNA strands can be functional; however, usually one of them, termed the guide strand, is incorporated into the RNA-induced silencing complex (RISC). MiRNA-RISC complexes containing Argonaute 2 (Ago2) protein bind to the 3'-untranslated region (3'-UTR) of target mRNAs and causes gene silencing by inhibiting translation and/or through mRNA degradation

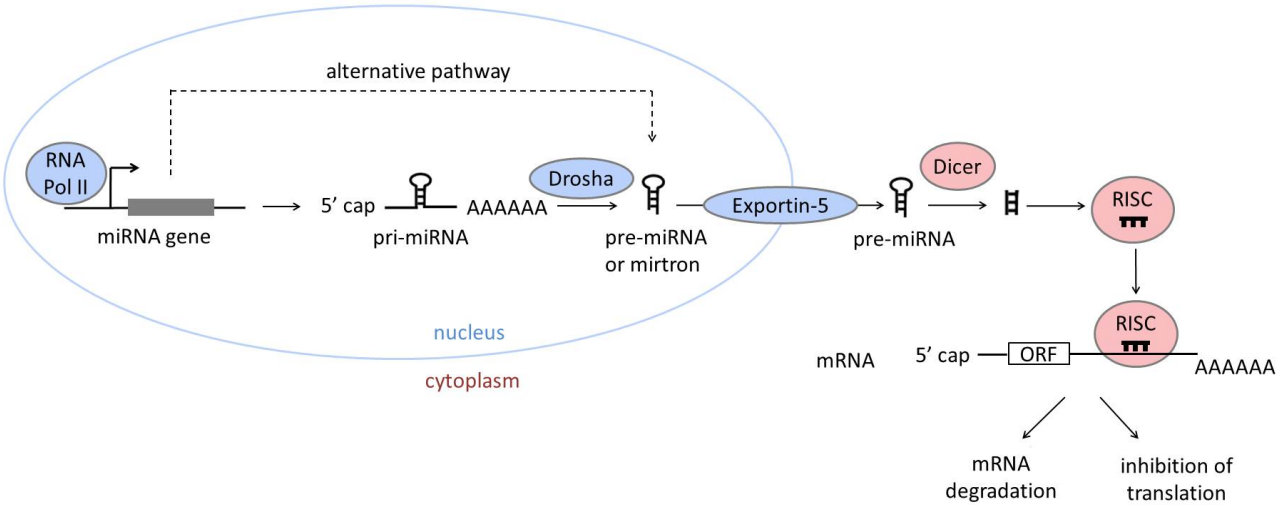
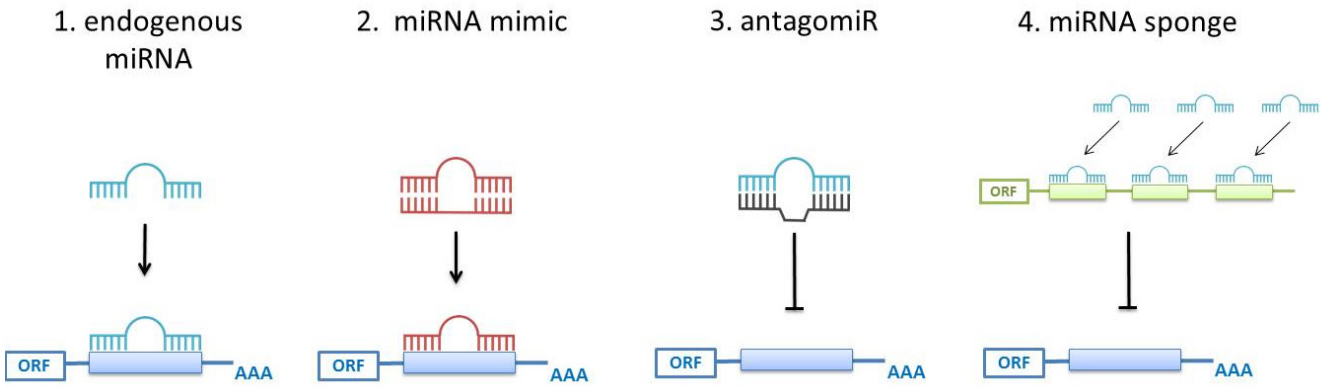


Fig. 2. Different approaches to targeting miRNA activity. (1) Endogenous miRNA (blue) binds to a complementary sequence known as a seed sequence (blue box) present in the 3'-untranslated region (3'-UTR) of mRNA. (2) A miRNA mimic (red) is a chemically synthesized double-stranded RNA molecule. This structure imitates endogenous miRNA and targets complementary mRNA. (3) An antagomiR (black) is a synthetic oligonucleotide that is complementary to a particular miRNA. This oligonucleotide binds to miRNA and inhibits its action. (4) MiRNA sponge (green) contains several seed sequences for a particular miRNA. Delivery to the cell results in binding with the target miRNAs and reduces the number of free and active miRNAs



or silencing its action (Fig. 2).[3] In cases of compromised miRNA levels, exogenous miRNAs can be administrated in vivo. MiRNA mimics are small, chemically synthesized double-stranded RNAs that imitate endogenous miRNAs and cause gene silencing. One strand of this molecule is identical to the native form of miRNA, the other is complementary. The double-stranded structure is required so that the RISC can recognize miRNA mimics accurately.[11]

AntagomiRs are synthetic RNA molecules used to silence aberrantly expressed miRNAs. AntagomiRs function by binding to miRNAs and inhibiting their actions. They are complementary to the sequence of a full-length sequence or a seed sequence (a 28-nucleotide-long mRNA binding site) of a specific miRNA. They are chemically modified to improve cellular uptake, in vivo stability and affinity to miRNA.[11] MiRNA sponges are another approach to reducing miRNA levels. MiRNA sponges are transcripts that contain multiple complementary regions for miRNAs that have the same target site. Delivering miRNA sponges to a cell results in their binding with the target miRNAs and reduces the number of free and active miRNAs.[11]

The highly conserved miRNA sequence facilitates the adaptation of results from studies in animal models to

clinical settings. However, the use of miRNA-targeting drugs still remains challenging. To enter the cell, oligonucleotides must pass the lipid bilayer of the cell membrane. They are therefore chemically modified to improve cellular uptake and in vivo stability. Changes include modifications in 2' sugar (e. g. 2'-OMe, 2'-MOE, 2'-F) or conjugation with cholesterol. Bicyclic nucleotides called locked nucleic acid (LNA) have enhanced affinity to miRNAs and stability.[11] Another method is delivering antisense nucleotides by means of supramolecular nanoparticles, such as liposomes or polymeric nanoparticles.[12] Unlike conventional drugs, which are specific for one cellular target (e.g. an enzyme or a receptor), one miRNA can modulate a number of target genes in different cells in a pathway, which can provide greater therapeutic effects. However, this feature could also be a disadvantage, because it can lead to undesired effects. Understanding this effect is an important step in developing miRNA-based therapies.[12]

MicroRNAs in heart development

The importance of miRNAs in heart biology was revealed by blocking the expression of all the miRNAs in the cardiovascular system.[2] This effect was obtained through tissue-specific deletion of genes essential to miRNA biogenesis, such as *Drosha*, *DGCR8* (coding for a protein which forms a complex with Drosha), *Ago2* or *Dicer*. Deletion of these critical genes in mice resulted in death during early gestation due to severe developmental defects of the heart and blood vessels. However, deleting genes for individual miRNAs is not lethal.

Different types of cells are characterized by their specific profile of miRNA expression. Interestingly, only 18 miRNA families account for approximately 90% of cardiac miRNAs.[13] Among cardiomyocytes, miR-1 is the most abundant one. MiR-1 and miR-133 arise from the same bicistronic transcript, but miR-133 expression is lower. MiR-1 and miR-133 cooperatively promote mesoderm differentiation in embryonic stem cells (ESCs). Later in their development, they play opposite roles: miR-1 promotes and miR-133 inhibits differentiation of mesoderm into cardiomyocytes.[14] MiR-1 influences cardiogenesis by regulating the expression of transcription factors Irx5 and Hand2.[15] The Hand2 protein is involved in the development of the outflow tract and right ventricle.[16] Targeted deletion of miR-1 results in ventricular septal defects (VSDs).[15] The Irx5 protein regulates the expression of potassium channel genes like potassium voltage-gated channel subfamily D member 2 (Kcnd2) and determines the cardiac ventricular repolarization gradient.[15] MiR-133 influences the activity of serum response factor (SRF) and cyclin D2, the transcription factors involved in cell cycle progression. SRF regulates the genes responsible for cardiac muscle and smooth muscle differentiation and growth; it also activates transcription of miR-133/1. Cyclin D2 controls cardiomyocyte proliferation by acting on the phosphorylation of retinoblastoma protein in the G1 phase of the cell cycle.[17]

Contractile protein expression is strictly regulated during heart development. Abnormal synthesis of myosin genes underlies pathological cardiac remodeling. Myosin genes remain under the control of miR-208a, miR-208b and miR-499, which are encoded in the introns of *Myh6* (alpha-myosin heavy chain, α-MHC), *Myh7* (beta-myosin heavy chain, β-MHC) and *Myh7b*, respectively.[14] In rodents, β-MHC (a slow ATPase) expression occurs during embryonic development, α-MHC (a fast ATP-ase) after birth, while *Myh7b* expression occurs at both stages.[18] The fact that miR-208a and miR-208b have identical seed sequences suggests that they regulate common target genes at different stages of development. In the adult heart, β-MHC synthesis is re-expressed in cardiomyocytes under stress conditions such as hypoxia or hypothyroidism.[14] Deletion of miR-208a results in ectopic expression of fast skeletal muscle genes and impaired postnatal stress response.[19]

The miR-15 family consists of miR-15a/b, miR-16-1/2, miR-195 and miR-497, which have identical seed sequences. MiR-195 is upregulated immediately after birth and halts cardiomyocyte proliferation; it controls numerous cell cycle genes, including checkpoint kinase 1 (*Chek1*). Overexpression of miR-195 results in VSDs and ventricular hypoplasia.[20] MiR-15b controls ATP level in cardiomyocytes by targeting Arl2, a component of the ADP/ATP exchanger in mitochondria.[21]

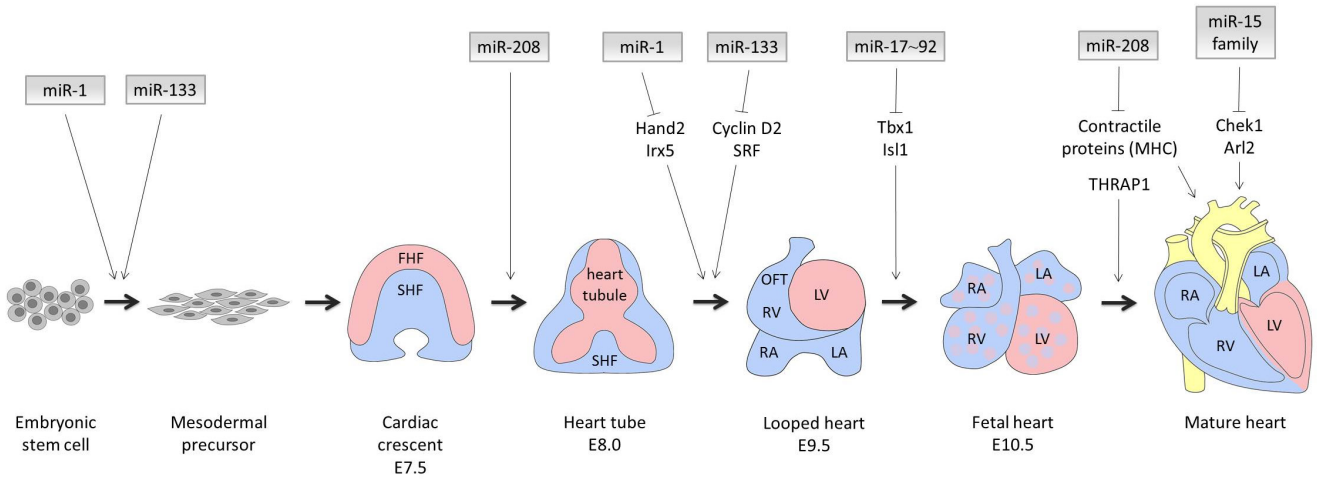
The miR-17~92 cluster consists of miR-17, miR-18a, miR-19a/b, miR-20a and miR-92. MiR-17~92 transcription is activated by the bone morphogenetic protein (BMP) signaling pathway. Through downregulation of *Isl1* and *Tbx1*, miR-17~92 promotes the development of the cardiac outflow tract and the differentiation of the second heart field (SHF) progenitors into right ventricle myocytes.[22] Deletion of miR17~92 leads to VSDs and lung hypoplasia, and consequently to death. These effects are attributed in part to the upregulation of pro-apoptotic proteins like Bim, which is a target gene of this miRNA cluster.[22]

The role of miRNAs during various stages of heart development is schematically represented in Fig. 3.

MicroRNAs in heart regeneration

Neonatal murine hearts have been demonstrated to regenerate following infarction. Ligation of the left anterior descending (LAD) artery in a one-day-old mouse results in necrosis of about 75% of the heart muscle. Within several weeks, spontaneous regeneration of the anterior wall leads to the recovery of the systolic function. Overexpression of miR-195 has been shown to impair myocardial regeneration and cause massive cardiac fibrosis.[23]

Fig. 3. A schematic representation of mouse cardiac morphogenesis and the role of miRNAs in this process. The colors represent the contribution of different precursor pools to the forming chambers. The heart is of mesodermal origin. At embryonic day 7.5 (E7.5), 2 populations of cells, termed the first heart field (FHS) and the second heart field (SHF), build up the cardiac crescent. Subsequently, the cells migrate and the linear heart tube is formed at E8.0. Shortly thereafter, the heart tube starts spontaneous contractions and supports the blood supply of the developing embryo. Finally, the process of cardiac looping and a series of morphological changes contribute to the formation of the four-chambered heart by E10.5. The progressive septation of the atria, ventricles and the common outflow track then takes place



Inhibition of the miR-15 family promotes myocyte proliferation and ameliorates cardiac functions in adults after myocardial infarction.[23] MiR-133 is another molecule regulating the cell cycle of cardiomyocytes. Following resection of 20% of the zebrafish ventricular apex, proliferation of the remaining cardiomyocytes occurs, which leads to complete heart regeneration. Studies have noted reduced expression of miR-133 in regenerating zebrafish heart. Upregulation of miR-133 diminishes its regenerative potential, whereas decreasing the level of miR-133 with specific miRNA sponges promotes regeneration.[24] To sum up, the miR-15 and miR-133 families attenuate the regenerative capacity of the heart by inhibiting cardiomyocyte proliferation.

MiR-199 and miR-590 have been found to induce cell cycle re-entry in cardiomyocytes. Intracardiac administration of these molecules into the infarct border zone stimulates cardiomyocyte proliferation in adult mice. MiR-199 and miR-590 have been shown to promote regeneration of the myocardium and to improve cardiac function.[25]

Stem cell-based therapies represent an attractive approach for treating cardiovascular diseases. However, regenerative therapy based on delivering stem cells into the heart has not yet been successful in clinical trials. Nowadays, many studies aim to improve the effects of this therapy by increasing the survival of cells transplanted into the infarcted region and enhancing the differentiation of stem cells into cardiomyocytes.[26] MiR-1 and miR-133 have been found to induce the differentiation of stem cells and heart progenitor cells into cardiomyocytes.[14] Moreover, transplanting stem cells overexpressing miR-1 into the infarcted zone increases cardiomyocyte differentiation, promotes regeneration and improves cardiac function.[27] Similar results are achieved by transplanting c-kit+ cardiac progenitor cells overexpressing miR-499.[28]

Cardiac fibrosis following myocardial infarction, resulting from excessive fibroblast activation, reduces regeneration, leads to pathological remodeling, impairs systolic function and increases susceptibility to arrhythmias. It has been shown that the expression of a suitable combination of transcription factor genes (*Gata4*, *Mef2c*, *Tbx5*) could convert resident cardiac non-myocyte fibroblasts into contractile cells by direct reprogramming.[29] A similar effect can be obtained when miRNAs are used. Lentiviral-mediated delivery of miR-1, miR-133, miR-208 and miR-499 into the infarct border zone has been found to induce direct fibroblast reprogramming into cardiomyocytes in situ.[30] Reprogrammed cardiomyocytes express cardiac markers and sarcomeric organization, and have electrophysiological properties characteristic of mature ventricular cardiac myocytes. Moreover, reprogramming is associated with an improvement in fractional shortening, suggesting the functional recovery of the damaged myocardium.[31]

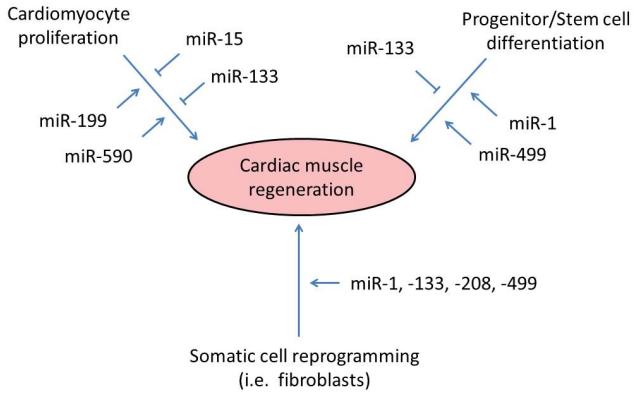
Fig. 4 summarizes the role of miRNAs in cardiac regeneration: regulation of cardiomyocyte proliferation, stem or progenitor cell differentiation and direct reprogramming of fibroblasts.

MicroRNAs in heart diseases

Myocardial infarction and cardiac remodeling

Ischemia/reperfusion injury associated with myocardial infraction leads to remodeling in the myocardium, which is regulated by various miRNAs. Activation of

Fig.4. MiRNA activity in the process of myocardial regeneration. Three approaches to cardiac muscle regeneration are presented: regulation of cardiomyocyte proliferation; stem or progenitor cell differentiation; and direct reprogramming of fibroblasts



stress signaling pathways triggers changes in miRNA expression; miR-24, miR-320 and miR-29 are downregulated in myocardial infarction. MiR-24 inhibits translation of Bim, a proapoptotic protein. Restoration of miR-24 to physiological levels by specific miRNA mimics attenuates apoptosis and decreases scar size.[2] Proapoptotic properties are attributed to miR-320, which negatively regulates heat shock protein 20 (HSP20), which functions as a cell protector after an ischemic injury.[2] MiR-29 controls genes encoding collagen (*COL1A1*, *COL1A2*, *COL3A1*) and extracellular matrix proteins, including fibrillin (*FBN1*) and elastin (*ELN1*). Low expression of miR-29 after myocardial infarction results in scar formation.[32] In addition, miR-199 is downregulated in cardiac myocytes during oxygen deprivation. This induces its target genes: hypoxia-inducible factor-1α (*HIF-1α*) and sirtuin 1 (*Sirt 1*), and subsequent activation of hypoxia-triggered pathways. Restoration of physiological miR-199 levels inhibits *HIF-1α* expression and its stabilization of p53, a tumor supressor responsible for sustaining the genome integrity, which leads to a reduction in apoptosis.[33]

Expression of the miR-15/16 family and miR-499 increases after myocardial infarction.[4,34] The miR-15/16 family regulates cardiomyocyte proliferation and survival in response to injury, and its inhibition protects cardiomyocytes from apoptosis.[34] MiR-499 influences cardiomyocyte apoptosis by downregulating calcineurin and dynamin-related protein 1 (Drp1), which are involved in mitochondrial fission. According to the literature, the upregulation of miR-499 reduces apoptosis and infarct size, while miR-499 knockdown has the opposite effect.[4] In contrast, another report showed that miR-499 overexpression in the heart can lead to cardiomyocyte hypertrophy and cardiomyopathy, suggesting discrepancies that may be caused by acute or chronic modulation of miRNA.[4] MiR-214, which increases in mouse and human tissue after myocardial infarction, exerts a protective effect during ischemia/reperfusion. It reduces calcium overload and promotes cardiomyocyte survival through inhibition of the sodium/calcium exchanger (NCX1) and Bim. Deletion of miR-214 increases injury and mortality following myocardial infarction.[4]

At the molecular level, cardiac remodeling is accompanied by a gene expression switch from the adult α–*MHC* isoform to the fetal β-*MHC*. MiR-208a, which is encoded in the intron of the α-*MHC* gene, has been shown to be involved in this process, inducing cardiomyocyte hypertrophy, fibrosis and increasing β-*MHC* expression. Deletion of miR-208a protects the heart from pathological remodeling under stress conditions. Thyroid-hormone-receptor-associated protein 1 (*THRAP1*) is considered a target gene for miR-208a.[3,34]

MiR-21 promotes myocyte hypertrophy and fibrosis by repressing the Sprouty2 transcription factor, which controls the pro-fibrotic extracellular signal-regulated kinase-mitogen-activated protein kinase (ERK-MAPK) pathway. Specific antagomiR-mediated inhibition of miR-21 blocks this cascade and results in a reduction in both hypertrophy and fibrosis.[13] However, genetic deletion of miR-21 does not alter the pathological cardiac response to pressure overload.[34] This discrepancy indicates that miR-21 plays a complex role in the pathophysiology of heart diseases, which requires further investigation. Another possibility may be the existence of a compensatory mechanism revealed under a permanent miR-21 knockdown.[34]

Heart failure

Impaired cardiac contractile function caused by disrupted calcium handling is a hallmark of heart failure. Recently, Cai et al. demonstrated that miR-765 is overexpressed in failing hearts and is involved in contractile regulation. MiR-765 contributes to increased protein phosphatase 1 (PP-1) activity and the subsequent dephosphorylation of key calcium cycling proteins by silencing its endogenous inhibitor-1.[35] Likewise, miR-25 is upregulated in failing hearts and controls myocyte contractile function by repressing the sarcoplasmic reticulum calcium uptake pump, SERCA2a. Anti-miR-25 delivery restores cardiac function and improves survival.[36] Also, miR-24 regulates calcium homeostasis through Junctophilin-2 repression, which results in decreased efficiency of excitation-contraction (E-C) coupling in cardiomyocytes.[37]

In a recent paper, Melman et al. provided evidence that increased cardiac miR-30d expression has a significant impact on responses to cardiac resynchronization therapy (CRT), and that plasma levels of miR-30d may correlate with responsiveness to CRT in heart failure patients. MiR-30d is regulated by mechanical stretch, and is released in exosomes by cardiomyocytes. It protects cardiomyocytes from TNF-α-elicited inflammation and cell

death by targeting the MAP4K4 protein or, possibly via other indirect pathways, resulting in beneficial cardiac remodeling.[38]

Potous et al. revealed the role of miR-126 in right ventricle (RV) failure associated with pulmonary arterial hypertension (PAH). Due to the methylation process, miR-126 is downregulated in RV failure in PAH patients, causing decreased capillary density. MiR-126 is expressed in endothelial cells and directly targets Sprouty-related EVH1 domain-containing protein 1 (SPRED-1), a negative regulator of the vascular endothelial growth factor (VEGF) signaling pathway. Administration of miR-126 mimics ameliorates microvascular density, improves RV function and diminishes fibrosis, whereas antagomiR-mediated miR-126 downregulation exacerbates RV failure.[39]

Recent research by Halkein et al. proposed the 16-kDa N-terminal fragment of the nursing hormone prolactin (16K PRL) as a potential factor initiating and driving peripartum cardiomyopathy (PPCM). Expression of miR-146a is induced in endothelial cells (ECs) by 16K PRL. By targeting the *NRAS* gene, miR-146a reduces the proliferation and viability of ECs and leads to the destruction of the cardiac microvasculature. Moreover, 16K PRL promotes the transfer of miR-146a-loaded exosomes from ECs to cardiomyocytes, which results in diminished overall metabolic activity and increased vulnerability to apoptosis. In cardiomyocytes, miR-146a also downregulates *Erbb4*, *Notch1* and *Irak1*. Post-natal knockout of ErbB2 in mice leads to dilated cardiomyopathy, which indicates that ErbB signaling plays an essential role in the physiological status of the adult heart. Pharmacological inhibition of miR-146a or 16K PRL attenuates ErbB4 downregulation and improves cardiac function. Increased miR-146a and decreased ErbB4 expression has been reported in the heart muscle of PPCM patients, suggesting that increased miR-146a and decreased ErbB4 expression contributes to the development of PPCM in humans.[40]

Arrhythmias

MiR-1 and miR-133 play an important role in the pathophysiology of arrhythmias. MiR-1 is upregulated in ischemic myocardium, and contributes to the slowdown of cardiac conduction by and depolarization of the cytoplasmic membrane. The arrhythmogenic properties of miR-1 include repression of *GJA1* and *KCNJ2*, which encode the connexin43 and Kir2.1 subunits of the I_{K1} channel, respectively.[41] Moreover, overexpression of *HCN2* and *HCN4*, which are regulated by miR-1 and miR-133, support arrhythmia-prone mechanisms. *HCN2* and *HCN4*, which belong to the hyperpolarization-activated cyclic nucleotide-gated channel (*HCN*) gene family, are found in pacemaker, atrial and ventricular cells. Age-associated low levels of miR-1 and miR-133 contribute to the overexpression of *HCN2* and *HCN4*, and this results in abnormal cardiac electrical activity.[42]

Hypertension

MiRNAs target genes in the renin-angiotensin-aldosterone system (RAAS), which is crucial in blood pressure regulation. MiR-155 has been found to regulate expression of the angiotensin II type 1 receptor, AGTR1. *AGTR1* correlates negatively with miR-155 and positively with blood pressure. Inhibition of miR-155 in Chinese hamster ovary cells resulted in upregulation of AGTR1 and ERK1/2 activation.[43] Furthermore, the miR-155 gene is located on chromosome 21, and trisomy 21 is associated with decreased blood pressure. An analysis of monozygotic twins revealed low AGTR1 protein expression and upregulation of miR-155 in patients with trisomy 21. An *AGTR1* allele with a single nucleotide polymorphism (SNP) in the 3'-UTR region (+1166A/C) is not recognized by miR-155. This SNP is associated with an increased risk of essential hypertension.[44] Moreover, blood pressure alterations have been linked with SNPs located on the miRNA-binding site of other RAAS genes: the arginine vasopressin 1A receptor (*AVPR1A*), bradykinin receptor 2 (*BDKRB2*) and thromboxane A2 receptor (*TBXA2R*).[4,44]

Pressure overload induces overexpression of miR-23a through activation of the nuclear factor of activated T cells (NFAT) transcription factor NFATc3. This results in inhibition of the anti-hypertrophic molecule-muscle specific ring finger protein 1 (MuRF1). MuRF1 in turn halts NFATc3, establishing a positive feedback loop. Downregulation of miR-23a with a specific antagomiR prevents cardiac hypertrophy. NFATc3 has also been shown to induce miR-199 expression. MiR-199 targets the kinase Dyrk1a, which inhibits NFATc3. It is noteworthy that miR-199 inhibition does not only prevent pathological hypertrophy, but also reverses cardiac remodeling.[34]

MicroRNAs in vascular homeostasis and diseases

As components of vessel walls, ECs and smooth muscle cells (SMCs) take part in maintaining vascular homeostasis. MiR-10a controls a pro-inflammatory EC phenotype by regulating the expression of adhesion molecules, and miR-10a expression is decreased in atherosusceptible regions of the aorta.[3] MiR-10a knockdown enhances the synthesis of such pro-inflammatory mediators as monocyte chemotactic protein-1 (MCP-1), interleukin-6 (IL-6), IL-8, vascular cell adhesion molecule-1 (VCAM-1) and E-selectin. Other molecules involved in regulating inflammation include miR-17-3p, miR-31, miR-126 and miR-181.[3] Recent evidence suggests that miRNA Let-7g has a pleiotropic effect on ECs. Let-7g targets genes in the transforming growth factor (*TGF*-β) signaling pathway and *Sirt 1*, a protein involved in cell senescence. Let-7g has been demonstrated a key anti-inflammatory and anti-aging molecule. Downregulation of Let-7g leads to en-

dothelial activation and subsequent vessel injury. Moreover, low serum levels of Let-7g have been associated with increased circulating plasminogen activator inhibitor-1 (PAI-1).[45]

Vascular injury triggers phenotypic changes (de-differentiation) in VSMCs. An altered phenotype is characterized by improper contractility, increased proliferation and migration, which result in restenosis.[13] Upon arterial injury, miR-143 and miR-145 are downregulated, whereas miR-21 is upregulated. Restoration of physiological miRNA levels protects VSMCs from de-differentiation and restenosis.[13] MiR-145 controls neointimal lesion formation by silencing Kruppel-like factor 5 (KLF5) and its downstream molecule, myocardin.[3]. MiR-21 function is mediated by a tumor suppressor known to negatively regulate Akt/PKB signaling pathway PTEN and anti-apoptotic protein Bcl-2. MiR-221 promotes VSMC proliferation by repressing the cyclin-dependent kinase inhibitor $p27^{Kip1}$ and reduces expression of contractile genes α-smooth muscle actin (SMA), smooth muscle calponin (CNN), and SM22α ($p27^{Kip1}$-independent mechanism). Similar effects are caused by miR-26, which downregulates extracellular signal transducers Smad1 and Smad4 in the bone morphogenetic protein (BMP) signaling pathway.[2]

Zhao et al. reported the miR-143/145 expression in SMCs is induced by ECs. MiR-145 targets TGF-β receptor II (TGFBR2) and regulates TGF-β signaling in a selective manner: MiR-145 diminishes expression of matrix genes, while smooth muscle differentiation genes remain unaffected.[46] On the other hand, when stimulated by cell-to-cell contact, EC-derived TGF-β mediates miR-143 and miR-145 transfer from SMCs to ECs through membrane protrusions known as tunneling nanotubes. This decreases the ability of ECs to form capillary-like structures and lowers their proliferation index, which leads to vessel stabilization. MiR-143 and miR-145 target hexokinase II (*HKII*) and integrin β 8 (*ITGβ8*) genes, respectively.[47]

MiR-145 has been also implicated in the pathophysiology of atherosclerosis due to its effect on VSMC proliferation and phenotypic changes. In endothelial cells miR-143/145 expression is mediated by shear-responsive transcription factor - Kruppel like factor 2 (KLF2). ECs release extracellular vesicles (EVs) containing miR-143/145, which are absorbed by SMCs to control target genes and act as atheroprotective molecules. Administering EC-derived EVs enriched with miR-143/145 results in a reduction in atherosclerotic lesion formation in the aortas of apolipoprotein E-deficient mice.[4] An increase in miR-145 expression reduces plaque size in the aortic sinuses, diminishes the necrotic core and promotes collagen synthesis; these phenomena lead to plaque stabilization.[3] On the other hand, neoangiogenesis and atherosclerotic plaque hemorrhage increase the subject's susceptibility to plaque rupture and clot formation, and miR-222/221, the miR-155 family and the miR-17~92 family are involved in the process.[3] Macrophages are the main effector cells in atherogenesis, as they promote inflammatory response, degrade lipoproteins and phagocyte cell debris. Cholesterol-loaded macrophages produce VEGF, a proangiogenic cytokine, and the miR-155 family, the miR-17~92 family and miR-222/221 regulate this process. Moreover, miR-342--5p activates macrophages by inhibiting Akt1 kinase.[3]

Considering the important role of cholesterol in the pathophysiology of atherosclerosis, it is worth mentioning miR-122 and miR-33, which have been described as regulators of lipid homeostasis.[3] MiR-122 is highly expressed in the liver, where it is involved in fatty acid oxidation and lipid synthesis. Downregulation of miR-122 results in decreased levels of both HDL and LDL cholesterol.[4] MiR-33a and miR-33b are encoded in the introns of the sterol regulatory element-binding protein genes *SREBP1* and *SREBP1,* respectively. MiR-33a targets ATP-binding cassette transporter A1 (*ABCA1*) and inhibits cellular cholesterol export. Because 3'UTR mouse and human *ABCA1* genes possess several miR-33a binding sites, mRNA repression is strong. In addition, miR-33a/miR-33b regulate *NPC1* and *ABCG1*, which are also involved in cholesterol trafficking[48]; as well as *CROT, CPT1a*, *HADHB* and *AMPKa,* which are engaged in fatty acid oxidation.[4] Inhibition of miR-33a/b upregulates ABCA1 expression in hepatocytes and macrophages, and leads to increased total cholesterol and HDL levels in serum.[4] Interestingly, both strands of the miR-33 locus act together in lipid metabolism regulation, since miR-33a* and miR-33b* repress genes similar to those targeted by miR-33a/b.[48]

Conclusions

The discovery of miRNA has changed our understanding of the regulation of gene expression. In the cardiovascular system, miRNAs control the proliferation and differentiation of stem and progenitor cells, and the function of cardiac myocytes, pacemaker cells, endothelial cells and smooth muscle cells. MiRNAs play a crucial role in cardiac development and regeneration. They are involved in cardiovascular pathophysiology and their expression is altered in various cardiovascular diseases. Modulation of miRNA expression may indeed change the course of a disease. The encouraging results of miRNA applications in experimental settings and reports of negligible toxicity to healthy tissues suggest that these molecules have considerable therapeutic potential.[3]

Currently, only two chemically modified oligonucleotides have been used in clinical trials. An antagomiR directed against miR-122 has completed the second phase of clinical trials. This oligonucleotide is used to treat hepatitis C virus (HCV). MiR-122 is specific to liver cells and is required for HCV replication. Delivering the antisense inhibitor of miR-122 reduces the number of viral copies

without evidence of treatment resistance.[49] Another molecule, MRX34, which mimics miR-34, has recently (at the time of writing) entered phase I clinical trials for the treatment of primary liver cancer. MiR-34 inhibits multiple oncogenic pathways and induces apoptosis in tumor cells.[50]

MiRNA studies represent an attractive and promising field of investigation. Identifying and understanding the role of miRNAs is an important step in the development of new therapeutic and diagnostic tools.

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Systemic sclerosis sine scleroderma

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Abstract

Systemic sclerosis is a rare generalized disease with scleroderma, i.e. skin thickening as one of the most common symptoms. The disease has 2 main subsets, diffuse and limited forms. The subset known as systemic sclerosis sine scleroderma (ssSSc) is a very rare subset characterized by the total or partial absence of cutaneous manifestations of systemic sclerosis with the occurrence of internal organ involvement and serologic abnormalities. The classification of ssSSc into 3 groups was proposed. Type I (complete) is characterized by the lack of any cutaneous changes typical for the disease at least until systemic sclerosis-related insufficiency of any internal organ occurs. Type II (incomplete) is characterized by the absence of sclerodactyly, but other cutaneous involvements (e.g. calcifications, telangiectasies, pitting scars) can be found. Type III (delayed) is characterized by clinical internal organ involvement typical for systemic sclerosis that has appeared before skin changes (complete or incomplete). In general, the demographic and clinical characteristics of the ssSSc patients suggest that they are similar to those with diffuse or limited form of the disease. Diagnosis of ssSSc still remains difficult and this disease form should be considered in all cases of unexplained fibrotic involvement of the internal organs.

Key words: systemic sclerosis, scleroderma, sclerodactyly, internal organ fibrosis

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Systemic sclerosis is a rare generalized connective disorder of unknown etiology and unclear pathogenesis. It is generally accepted that the disease is characterized by widespread fibrosis of the skin and internal organs, vascular abnormalities and immune disturbances. On the other hand, systemic sclerosis is considered as a heterogeneous disorder (scleroderma spectrum disorders) and has a broad spectrum of clinical features.[1] The main subsets of systemic sclerosis are limited cutaneous form and diffuse form. There are several differences between the subsets, including the most important, which is that internal organ involvement is significantly delayed and relatively minor in patients with limited cutaneous form of systemic sclerosis. Other differences include different distributions of cutaneous changes and the common occurrence of different autoantibodies (against topoisomerase I, Scl-70 in patients with diffuse form and anticentromeric antibodies, ACA in those with limited form).[2,3]

Skin involvement is considered a cardinal feature of systemic sclerosis, and the name "scleroderma" means hardening of the skin. Cutaneous manifestations are also crucial in the initial diagnosis. Although the disease is a heterogeneous disorder, skin involvement usually develops at the early stage of the disease and is preceded by Raynaud's phenomenon. Skin changes begin with nonpitting edema that is followed by the thickening of the skin leading to sclerodactyly. Tapering of fingertips and digital ulcers are common features seen in patients and they result from ischemia due to vasculopathy. Vascular insufficiency may cause atrophy and auto-amputation of the distal parts of the fingers. Cutaneous manifestations include typical facial features, teleangiectasias, abnormal calcium depositions in the subcutaneous tissue, altered pigmentation ("salt and pepper skin"), loss of hair follicles and sebaceous glands with resultant dryness.[4] Skin alterations may be localized in different parts of the body. A small number of the patients with systemic sclerosis suffer from systemic sclerosis sine scleroderma (ssSSc), a disease subset characterized by the presence of visceral involvement occurring in the absence of skin manifestations. The subset, sometimes called "visceral scleroderma", was described for the first time in detail by Rodnan and Fennel in 1962, who also coined the term "progressive systemic sclerosis sine scleroderma".[5] The only earlier case was reported by Abrams et al.[6] They published in 1954 a description of a patient with visceral scleroderma, but without detectable skin involvement. ssSSc is a rare form of the disease and its real prevalence is difficult to estimate. It is believed that some ssSSc cases remain underdiagnosed and are considered to be various forms of idiopathic fibrosis of internal organs. In 2013, joint groups of the American College of Rheumatology and European League Against Rheumatism elaborated new classification criteria for systemic sclerosis.[7] Three major hallmarks of systemic sclerosis were included in the list of criteria: skin and organ fibrosis, signs of vasculopathy, and autoantibodies. Internal organ involvement is included in the criteria as the interstitial lung disease resulted from the pulmonary fibrosis. A sufficient criterion is skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints. The point scoring system is applied in the classification criteria, and individuals with a score ≥ 9 are classified as having systemic sclerosis. According to the criteria, score 9 can be achieved in a patient without skin thickening when systemic sclerosis-related autoantibodies, Raynaud's phenomenon, interstitial lung disease or pulmonary arterial hypertension and abnormal nail fold capillaries are detected. On the other hand, it is clearly stated that the criteria are designed for the inclusion of patients in studies and are not diagnostic criteria per se. Diagnosis of systemic sclerosis can be based on other symptoms and signs, including those related to internal organ involvement, and only a part of the patients classified as having systemic sclerosis are being diagnosed as having the disease. This is of importance, especially in patients with ssSSc.

Prevalence

Limited epidemiological data on ssSSc is available. Marangoni et al. retrospectively analyzed 947 consecutive patients with systemic sclerosis treated in two academic medical centers in Brazil.[8] They were able to identify a very high rate of the ssSSc patients, i.e. 79 cases (8.3%). Poormoghim et al. compared patients with limited cutaneous systemic sclerosis and ssSSc, who were evaluated at the University of Pittsburgh Division of Rheumatology during 24 years (1972–1996), and they were able to identify 48 cases of ssSSc and 507 patients with limited cutaneous systemic sclerosis, 9 and 91%, respectively.[9] It can be roughly estimated that ssSSc patients account for about 5% of all patients with systemic sclerosis (this calculation was based on the rate of patients with diffuse subset, i.e. 40% of all ones with systemic sclerosis). The low rate of ssSSc subset was reported in the German and Spanish nationwide registry analyses, i.e. 1.5 and 7.5%, respectively.[8,10] Most of the literature is limited to descriptions of isolated cases, usually containing additional rare features, complications or associated conditions. According to Marangoni et al., the total number of 139 cases was reported until 2012.[8] In 2014, Diab et al. analyzed data from 1,417 subjects with systemic sclerosis in the Canadian Scleroderma Research Group registry.[11] Twenty-seven subjects (1.9%) were considered as ssSSc with a mean follow-up of 2.4 years. The initial diagnosis that had been made at the first visit was more common, and had been set in 57 subjects (4.0%), but more than a half of them were reclassified as limited systemic sclerosis within 1.9 years. Moreover, the ssSSc patients had no sclerodactyly (as per definition) but had some

other cutaneous involvements typical for systemic sclerosis (e.g. calcinosis 15.4%, telangiectasias 69.2%, digital pits 11.1%). Additionally, nail fold capillary abnormalities were detected in 74.1% of ssSSc patients. Thus, the ssSSc subgroup was not without any cutaneous involvement.

Definition and classification

It is important to specify the term "ssSSc" because it is used in medical literature to describe patients with at least 2 variants systemic sclerosis. The 1st variant described as ssSSc consists of patients who have no cutaneous involvement during the whole period of clinical observation but suffer from sclerodermatous internal organ involvement. The remaining variant also termed ssSSc consists of patients who suffer from visceral manifestation typical for systemic sclerosis that was diagnosed when they had no cutaneous manifestation but later characteristic skin features are visible.

The problem of skin manifestation should also be specified. Some reports considered patients with visceral involvement and some cutaneous manifestations (e.g. teleangiectasias) but without skin thickening (i.e. "true" scleroderma) as those belonging to ssSSc subset, while other reports consider ssSSc patients only when they have no cutaneous features typical for systemic sclerosis. All these discrepancies, together with the scarce data on ssSSc in the literature, are the main difficulty for a comprehensive analysis of the problem.

In this paper, we propose a detailed classification of ssSSc based on 2 features. The 1st feature is the lack of sclerodactyly in patients with evidence of sclerodermatous internal organ involvement detectable during the whole period of clinically overt disease, at least until any internal organ insufficiency related to systemic sclerosis occurs. Despite the lack of sclerodactyly, the patients can have other cutaneous involvements typical for systemic sclerosis (e.g. telangiectasias). The absence of any sclerodermatous skin involvement is a basis for classifying those patients as "complete" (type I), while the lack of thickening coexisting with other typical cutaneous features is named "incomplete". The 2nd feature included in the classification is a sequence of skin and internal organ manifestations. We propose type III of ssSSc comprising patients with "delayed cutaneous involvement", i.e. those whose cutaneous involvement becomes detectable later than clinically overt internal organ manifestations. Patients with type III of ssSSc may suffer from a "complete" or an "incomplete" form of the disease as well. Based on these factors, the following classification of patients with ssSSc is proposed:

ssSSc type I: complete visceral scleroderma – patients with evidence of sclerodermatous involvement of an internal organ or organs and without any sign or symptom of skin involvement typical for systemic sclerosis. The Raynaud's phenomenon as a vascular abnormality can be presented. There is no skin involvement at least until systemic sclerosis-related insufficiency of any internal organ is detectable.

ssSSc type II: incomplete visceral scleroderma – patients with evidence of sclerodermatous internal organ involvement with cutaneous manifestations typical for systemic sclerosis other than skin thickening, i.e. without sclerodactyly.

ssSSc type III: with delayed cutaneous involvement – patients with evidence of internal organ involvement typical for the disease and no sign of cutaneous sclerodermatous involvement, at least when internal organ manifestation becomes overt (complete form), or no skin thickening signs, at least when internal organ manifestation becomes overt (in complete form).

In most cases of ssSSc with delayed cutaneous involvement, the clinical picture of the disease is indistinguishable from diffuse systemic sclerosis. Analysis of the sequence of appearance of the disease manifestations is the only way to distinguish this type of ssSSc from diffuse systemic sclerosis with early development of skin involvement.

A distinction of the above-described types of ssSSc is based on the stage of the disease, when internal organ and cutaneous abnormalities become overt. Due to the variability of internal organ involvement, it is difficult to establish the exact necessary period of observation. The proposed classification is based on detectable (and to some extent measurable) organ insufficiency. Practically, all internal organ involvement has well-known criteria for organ insufficiency or failure. The time point is proposed to establish the period defining the lack of skin abnormalities before the appearance of at least 1st stage of organ insufficiency. The term "complete" or "incomplete" was introduced to separate cases of ssSSc without any sclerodermatous cutaneous manifestations from those with some involvement of the skin but without evidence of thickening. Raynaud's phenomenon that is primarily vascular abnormality is not considered as a skin abnormality.

Analysis of almost 1,500 patients revealed that female to male ratio in ssSSc patients is about 9 : 1 and is rather similar to the total group of systemic sclerosis patients. Forty per cent of the ssSSc patients had autoantibodies against topoisomerase I (55% in diffuse form of the disease) while 35% had anticentromeric antibodies (62% in those with localized form of systemic sclerosis). This suggests that, at least immunologically, ssSSc cannot be considered as a subset of any "major" form of the disease. Almost all internal organ involvements were found to be more common in ssSSc patients as compared with the total group and this is related to the role of visceral fibrosis in the diagnosis of ssSSc. There was no difference between patients with ssSSc and all the patients with systemic sclerosis at the age of the onset of Raynaud's phenomenon or at the age of organ involvement.[10]

Clinical picture

The study conducted by Diab et al. is the largest reported cohort of ssSSc patients and is a very valuable tool for comparing those patients with the ones with diffuse systemic sclerosis and limited systemic sclerosis.[11] Baseline demographic and clinical characteristics of subjects with diffuse, limited and ssSSc disease were found to be similar. They included the proportion of women to men, duration of overt disease and age at disease onset. Clinical signs and symptoms reflecting the involvement of a few internal organs were less prevalent in ssSSc than in diffused or limited subsets of the disease, including esophageal dysmotility, inflammatory polyarthritis, myositis and other than esophageal gastrointestinal symptoms. Intestinal lung disease was as common in ssSSc patients as in those with limited systemic sclerosis, and was detected in about 1/4 of the patients. This was reported earlier by Fischer et al.[13] Also, pulmonary hypertension was present in about 11% of the patients with ssSSc, similarly to other subgroups of systemic sclerosis patients.

The slightly lower prevalence of internal organ involvement at the baseline characteristics of the study is an indication of high awareness of ssSSc in the Canadian Scleroderma Research Group registry. In most of the cases reported in the literature, only severe internal organ manifestations attracted the attention of physicians because of the possibility of diagnosing ssSSc

The study conducted by Diab et al. once again confirmed that ssSSc is a rare form of the disease, especially in the rare cases without any skin involvement typical for systemic sclerosis.[11] The general profile of ssSSc patients is similar to the others, especially those suffering from limited systemic sclerosis. The lack or atypical picture of cutaneous manifestations is the key differential feature.

The clinical picture of ssSSc is similar, and gastrointestinal and pulmonal manifestations are the most common in these patients. The involvement of respiratory and alimentary systems seems to be relatively highly symptomatic and detectable, and this finding may explain their common occurrence both in reported series of the patients and in the majority of individual case reports. Poormaghim et al. found in ssSSc patients gastrointestinal involvement in 79% (31/39), pulmonary in 68% (32/47) and cardiac in 9% (4/45) only.[9] The large group of ssSSc patients reported by Marangoni et al. confirmed that esophageal involvement was the most frequent visceral manifestation (83%) and was followed by pulmonary involvement (68%).[8] Similar findings were described in the German and Spanish groups of ssSSc patients.[8,10] Esophageal dysmotility was shown in 73% and 45% of the patients, while interstitial lung disease was found in 73% and 64%, respectively. Gastrointestinal manifestations include swallowing disturbances, crampy abdominal pain, nausea or vomiting. Gastroesophageal reflux disease is a frequent diagnosis in systemic sclerosis patients of all subsets, including those with ssSSc. Pulmonary involvement symptoms include predominant dyspnea at early stages of exertion. Interstitial lung disease and pulmonary hypertension are the most common pulmonary manifestations.

Toya and Tzelepis carried out an analysis of cardiopulmonary involvement in 108 cases of ssSSc described in the literature.[12] Cardiac involvement was documented in 25% of the patients. The most common manifestations were chronic heart failure, pericardial effusions, conduction disturbances or arrhythmias. It is suggested that myocardial fibrosis is the underlying cause of cardiac manifestations.

Diab et al. suggested that the prevalence of myosistis in patients with ssSSc is lower than in those with other subsets of the disease.[11] This was confirmed by Sánchez-Montalya et al., despite a rather common occurrence of anti-SSA/Roi52 autoantibodies in patients with ssSSc.[14] In 132 consecutive patients with SSc and anti-SSA/Ro52 autoantibodies patients with ssSSc constituted 22.7%. It is also suggested that acute phase reactants are more commonly elevated in patients with ssSSc than in other subsets.[10]

Renal involvement

There are discrepancies in the estimation of frequency of renal involvement in patients with ssSSc. Poormoghim et al. showed that the kidneys are uncommonly involved in the patients and this finding supported the suggestion that ssSSc patients are at least similar or even constitute a subgroup of patients with limited cutaneous systemic sclerosis.[9] On the other hand, Hunzelmann et al. reported that renal involvement is twice as common in patients with ssSSc than it is in patients with systemic sclerosis.[10] Additionally, renal insufficiency was also more than twice as common in the patients as compared to the total group of systemic sclerosis patients. An analysis of the literature revealed a relatively high rate of case reports describing patients with renal failure due to systemic sclerosis without or with delayed cutaneous manifestations.[15–18]

Renal involvement in ssSSc patients manifests in 2 different forms: scleroderma renal crisis and chronic forms leading to renal failure. Scleroderma renal crisis is characterized by the acute onset of severe hypertension, acute left ventricular failure and retention of nitrogen bodies due to rapid progressive renal failure. Few patients with renal crisis due to systemic sclerosis but without skin involvement at the onset of the crisis were reported.[15–18] In most of the cases, rapid development of skin changes typical for the disease occurred after renal failure. Less common is the slow development of renal failure. Chung et al. reported nephritic syndrome in women with ssSSc akin to those seen in patients with other forms of systemic sclerosis.[19]

Prognosis

Retrospective analysis of internal organ involvement in ssSSc patients is limited, due to variety of criteria used in investigations. Some studies were based on clinically overt symptoms, while others applied more and less sensitive laboratory or imagine methods. It is well known that the evaluation of the cardiopulmonary and gastrointestinal systems in patients with systemic sclerosis with functional or imagine test revealed a higher rate of involvement than the analysis of clinically overt symptoms. The same applies to ssSSc patients. This is probably the source of discrepancies between published series and individual reported cases.

Special situations

A search of literature revealed some descriptions of an atypical course of ssSSc. It is impossible to conclude from these anecdotal reports, but due to the rarity of the disease at least a few of them should be mentioned.

A case of ssSSc with multiple intracerebral hemorrhages and significantly high antitopoisomerase-I autoantibodies is akin to an earlier description of limited systemic sclerosis and vasculitis overlap syndrome.[20,21] Recently, Anand et al. reported a case of ssSSc with overlap syndrome and characterized by ANCA associated vasculitis.[22] The main clinical abnormality was pulmonary fibrosis. A case of ssSSc with anterior uveitis has also been recently reported.[23]

Scleroderma-like disease exposed to epoxy resin polymerization was also reported in a form of ssSSc.[24] It is an absolutely unique case of such a form of the disease.

Concluding remarks

Diagnosis of ssSSc is difficult. From a practical point of view, it is important to consider the diagnosis of ssSSc in patients with multisystemic internal organ abnormalities that cannot be attributed to other diseases. The occurrence of Raynaud's phenomenon or antibodies typical for systemic sclerosis is useful for establishing a diagnosis. Vascular abnormalities detectable with capillaroscopy are included in the new classification criteria. Capilaroscopic evaluation is a valuable tool in the diagnostics of ssSSc.[23]

Systemic sclerosis is still a very enigmatic disease. Pathomechanism of fibrosis and its localization remains unknown. The elucidation of this phenomenon may be a key to understand the sequence and grade of skin and internal organ involvement. The current classification and distinction of ssSSc as a separate subset is based exclusively on clinical symptomatology and the course of the disease.[25,26] Unfortunately, a diagnosis of ssSSc has no substantial effect on the management of the patients and the general rules of organ-oriented therapy are to be applied in those patients.

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The Notch signaling pathway in head and neck squamous cell carcinoma: A meta-analysis

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Abstract

Background. The Notch signaling pathway has been associated with the regulation of self-renewal capacity, cell cycle exit, and survival. However, the relationship between the Notch signaling pathway and HNSCC remains controversial.

Objectives. A meta-analysis was conducted to evaluate the role of Notch signaling pathway in HNSCC.

Material and methods. Relevant studies published until March 31, 2015 were identified by searching the PubMed, EMBASE and Ovid database.

Results. A total of 9 articles were eligible for this meta-analysis. The meta-analysis results showed that the expression of Notch1, Notch3 and NICD was significantly higher in HNSCC as compared with control tissue. There was no significant difference in Jagged1 and HES1 expression between HNSCC and control tissue. Stratified analysis results showed that the expression of Notch1 was significantly higher in poor differentiation, III and IV stage and positive lymph node metastasis patients. Additionally, over-expression of Notch1, NICD, HES1 and DLL4 significantly predicted poor OS in HNSCC patients.

Conclusions. The Notch signaling pathway plays an important role in tumor development of HNSCC. Inhibition of the Notch signaling pathway is a potential therapeutic method of HNSCC.

Key words: prognosis, Notch, head and neck squamous cell carcinoma, systematic review and meta-analysis

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Head and neck squamous cell carcinoma (HNSCC) is one of the most frequent cancers with significant morbidity and mortality around the world. Despite the advancements in surgery and radiochemothreapy, the 5-year survival rate of patients with advanced HNSCC has only marginally improved.[1] Therefore, it is urgent to identify the biomarkers to screen out high risk patients and evaluate the prognosis of HNSCC.

The Notch signaling pathway has been associated with the regulation of self-renewal capacity, cell cycle exit, and survival.[2] In mammals, this signaling is comprised of 4 receptor isoform (Notch1, Notch2, Notch3, Notch4) and 5 ligands: Delta-like 1 (DLL1), Delta-like 3 (DLL3), Delta-like 4 (DLL4), Jagged1, Jagged2.[3] The pathway is activated when one cell expressing the appropriate ligand interacts with another cell expressing a Notch receptor. Next, the transmembrane Notch receptor is subsequently cleaved by a disintegrin and metalloprotease (ADAM) metalloprotease and γ-secretase complex. Then, the cleaved product, Notch intracellular domain (NICD), translates into the nucleus to regulate the expression of target genes.[4,5] The hairy/enhancer of split (HES) and hairy/enhancer of split related with YRPW motif (HEY) families are the most prominent targets proteins of the Notch pathway.[6]

Mechanism studies demonstrated that dysfunctional Notch signaling can lead to numerous diseases, such as breast cancer, T-cell leukemia, diabetic nephropathy.[7–9] In an osteogenic sarcoma mouse model, the activation of Notch signaling was verified as a common triggering mechanism in mesenchymal cells original carcinoma.[10] Nevertheless, in a tissue-specific inducible gene-target mice model, Notch1 deficiency resulted in a skin tumor by increasing and sustaining the expression of GLI family zinc finger protein 2 (Gli2).[11] In HNSCC, the Notch signaling pathway has been implicated in many facets of cancer biology, including angiogenesis, cancer stem cell and drug resistance.[12–14] A recent exome sequence demonstrated that Notch1 mutations occur in approximately 15% of HNSCC.[15] Moreover, Notch1 mutations indicate a loss of function mutation and they play a tumor suppressive role through reduced target gene expression (HES1 and HEY1).[16]

The dual biological effect of Notch signaling pathway playing either a cancer-promoting role or a tumor-suppressing role has been debated. Therefore, we performed a meta-analysis to assess the association of the Notch signaling pathway with HNSCC and the predicted role of Notch signaling pathway in HNSCC patients.

Material and methods

Search strategy

We searched the electronic databases PubMed, EMBASE and Ovid by using the search terms: (head and neck squamous cell carcinoma or HNSCC or oral cancer or laryngeal cancer or pharyngeal cancer or tongue cancer or oropharyngeal cancer) and (notch or NICD or notch intracellular or DLL or delta or delta like or jagged or HES1 or HEY1 or notch mutation). The search was limited to English language papers. The search was updated until March 31, 2015.

Inclusion and exclusion criteria

The papers were included in the meta-analysis if they met the following criteria: (1) studies containing patient case of head and neck squamous carcinoma (HNSCC), including: oral cancer, tongue cancer, larynx cancer, oropharynx cancer and nasopharyngeal carcinoma; (2) studies measuring the expression of Notch signaling pathway proteins; (3) studies containing sufficient data to estimate the odds ratio (OR) or hazard ratio (HR) of overall survival (OS) or disease-free survival (DFS); (4) studies published as peer-reviewed and original research; (5) if there were more than one study based on a similar population, the largest or the most recent study was included. Meanwhile, studies were excluded according to any of the following criteria: (1) the study was published as a review or letters; (2) the study lacked sufficient information to calculate the OR or HR. If patient specimens were involved in different studies, the most recently published reports were included in this study.

Data extraction

The studies were independently searched and assessed by 2 reviewers (Zhao YY and Yu GT), and the inclusion of a study was decided by consensus. The following items were extracted: first author, publication year, patient distribution, specimen size, assessment method of Notch signaling pathway, the expression of Notch signaling pathway, OS and/or DFS and follow-up period.

Statistical analysis

Data statistical analysis was performed using STATA (v. 12.0, STATA Corporation, College Station). OR and 95%CI were used to assess the relationship between the Notch signaling pathway and HNSCC, including gender, clinical stage, tumor differentiation and lymph node metastasis status. The HR was used to assess the association between the Notch signaling pathway and OS or DFS. The Q test and p-value was used to evaluate the heterogeneity. When $p < 0.1$ or $I^2 > 50\%$, a random effect model was applied. Otherwise, the fixed effect model was used. The sensitive analysis was applied to measure whether the heterogeneity of these studies is from one single study. Publication bias was assessed by Begg's rank correlation method. The value of $p < 0.05$ was considered statistically significant.

Table 1. Characteristics of all eligible studies

Ref.	Country	Year	Technique	Detected markers	No. of patients	No. of control	Follow-up (month)
Gokulan et al.[17]	India	2014	IHC	NICD/HES1	44	8	14–50
Sun et al.[16]	USA	2014	IHC	Notch1/HES1/HEY1	56	11	na
Yoshida et al.[18]	Japan	2013	IHC	Notch1/NICD/Jagged1	54	12	na
Zhang et al.[19]	China	2012	IHC	DLL4	311	na	1–120
Gokulan et al.[20]	India	2011	IHC	Notch1	62	na	9–36
Zhang et al.[21]	China	2010	IHC	Notch1/Notch3/Jagged11/Jagged2	74	74	na
Lin et al.[22]	China	2010	IHC	Notch1/Jagged1	59	na	60
Joo et al.[23]	Korea	2008	IHC	Notch1/Notch3	51	5	na
Zhang et al.[24]	China	2008	IHC	Notch1	25	25	na

Table 2. Subgroup analysis of the Notch1 expression of HNSCC

Stratification of HNSCC	No. of patients	OR (95%CI)	Statistical method	p-value	No. of Ref
Gender: male vs female	128	1.069 (0.517–2.209)	fixed	0.858	2
Clinical stage: I–II vs III–IV	128	0.174(0.069–0.439)	fixed	0.000	2
Lymph node: mestastasis: positive vs negative	125	4.205(1.767–10.007)	fixed	0.001	2
Differentation: well vs moderate	112	1.102(0.482–2.519)	fixed	0.819	2

Results

Description of study

The literature search retrieved 448 related references. After browsing the title and abstract, 38 potential studies were selected. Further, 9 studies were eligible to be included in this meta-analysis after the full text articles had been read against the inclusion and exclusion criteria (Fig. 1).[16–24] An immunohistochemical (IHC) method was used to assess the Notch signaling in HNSCC. These studies were carried out in India, America, Japan, Korea and China. Among these studies, 5 focused on acceptor Notch1, 2 focused on acceptor Notch3, 2 focused on NICD, 2 focused on ligand Jagged1 and 2 focused on target protein HES1. Only 1 study focused on Jagged2 and HEY1, respectively. The detailed characteristics of these studies were shown in Table 1.

Meta-analysis of Notch signaling in HNSCC

Notch1: Five studies evaluated the expression of Notch1 in HNSCC and in control tissue. The combined results showed that Notch1 expression was significantly higher in HNSCC than in control tissue (OR = 10.17, 95%CI: 2.31–44.85; a random effects analysis, p = 0.002) (Fig. 2A). Given the I^2 = 69.4% and p = 0.011, we next assessed the heterogeneity of these 5 studies by sensitive analysis (Fig. 2B). We found that the article of Zhang et al. was the main source of heterogeneity. Further, the combined results based on the remaining 4 studies also demonstrated that Notch1 had a significantly high expression in HNSCC as compared with control tissue (OR = 16.86, 95% CI: 6.27–45.33; a fixed effects analysis, p = 0.000) (Fig. 2C). Moreover, there was no obvious heterogeneity in these 4 studies (I^2 = 47.7%, p = 0.125). This data indicated that Notch1 was up-regulated in HNSCC.

As shown in Table 2, we analyzed the relationship of Notch1 expression with clinical features. We found that there was no difference in Notch1 expression between males and females (OR = 1.069, 95%CI: 0.517–2.209). Similarly, there was also no difference in Notch1 expression between well differentiation and poor differentiation (OR = 1.102, 95%CI: 0.482–2.519). When stratifying for clinical stage, we found that Notch1 expression was significantly increased in III and IV stage patients as compared with I and II stage patients (OR = 0.174, 95%CI: 0.069–0.439). And when patients presented lymph node

Fig. 1. Flow chart of articles selection

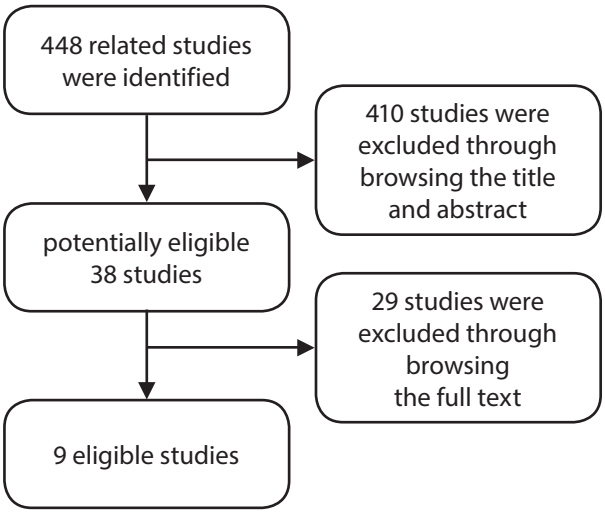
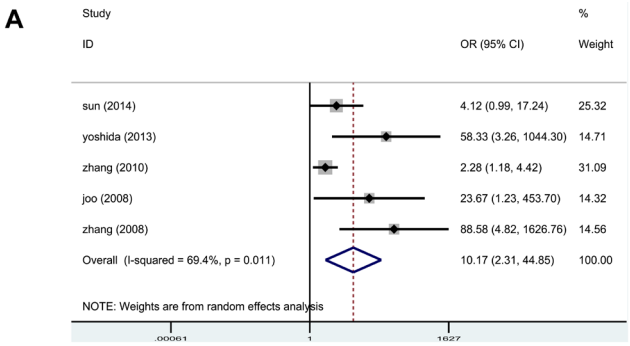
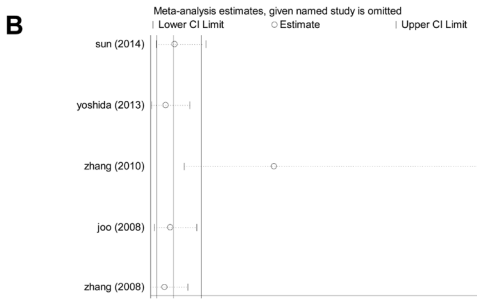
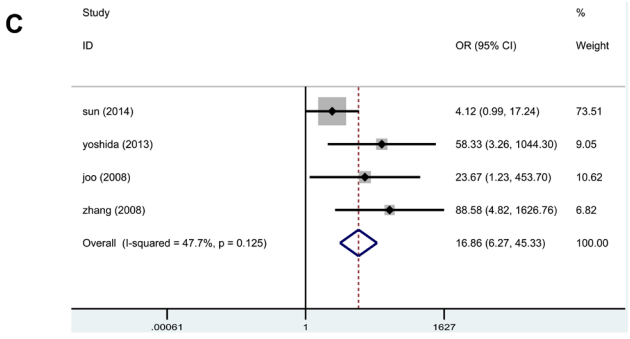


Fig. 2. (A) Forest plot for the association between Notch1 expression and HNSCC. (B) Sensitive analysis for the assessment of heterogeneity of included studies. (C) Once again forest plot for the association between Notch1 expression and HNSCC by excluding a heterogeneous study







metastasis, the expression of Notch1 was also significantly up-regulated (OR = 4.205, 95%CI: 1.767–10.007).

Notch3: The combined results based on 2 independent studies showed that Notch3 has a significantly higher expression in HNSCC than in control tissue. The OR was 3.19 (95%CI: 1.73–5.89, p = 0.000), without any heterogeneity between studies (I^2 = 0.00%, p = 0.458, a fixed effects analysis) (Fig. 3A). When stratifying for age, gender, lymph node metastasis and histology, no statistically significant difference was found in HNSCC patients. When stratifying clinical stage, we found that Notch3 expression was higher in III and IV stage patient as compared with I and II stage patients.[21]

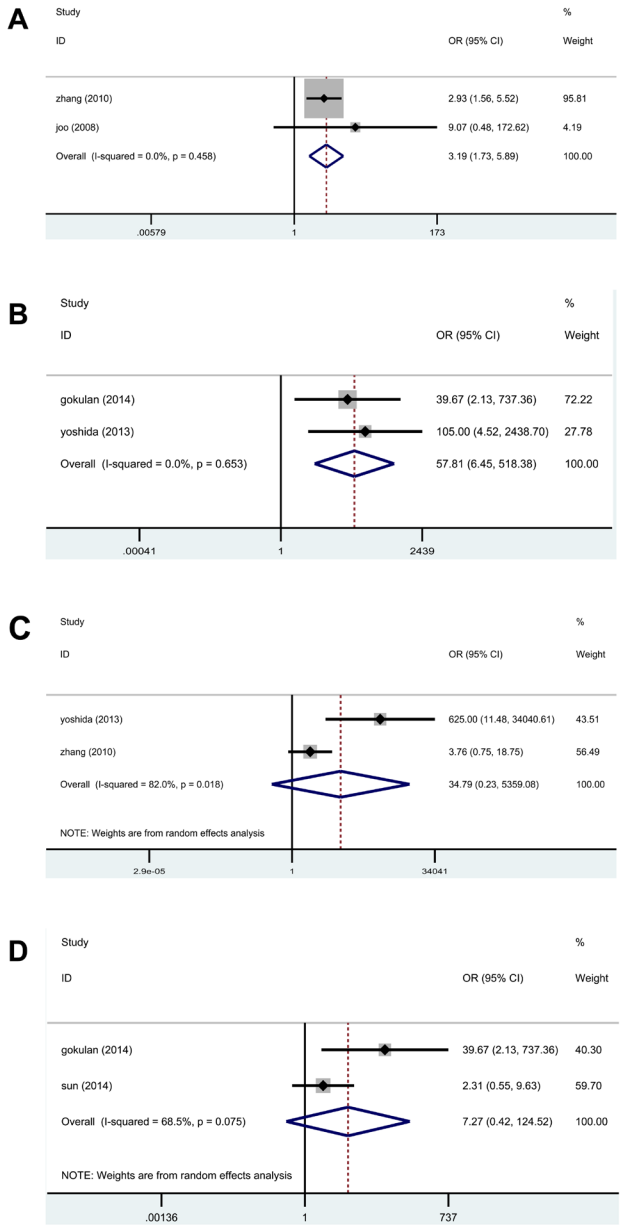
NICD: The combined results based on 2 independent studies showed that NICD has significantly higher expression in HNSCC than in control tissue. The OR was 57.81 (95%CI: 6.45–518.38, p = 0.000), without any heterogeneity between studies (I^2 = 0.00%, p = 0.653, a fixed effects analysis) (Fig. 3B). When stratifying for age, gender and histology, no statistically significant differences were found in HNSCC patients. When stratifying clinical stage and lymph node metastasis, NICD has significantly higher expression in III + IV stage patients and positive lymph node metastasis patients.[17]

Jagged1: The combined results based on 2 independent studies showed that there was no statistically significant difference in Jagged1 expression between HNSCC and control tissue. The OR was 34.79 (95%CI: 0.23–5359.08, p = 0.167). But the heterogeneity was detected between studies (I^2 = 82%, p = 0.018, a random effects analysis) (Fig. 3C). When stratifying for age, gender, clinical stage and histology, no statistically significant differences were found in HNSCC patients. When stratifying for lymph node metastasis, NICD has significantly higher expression in positive lymph node metastasis patients than in the case of negative lymph node metastasis.[21]

HES1: The combined results based on 2 independent studies showed that there was no statistically significant difference in HES1 expression between HNSCC and control tissue. The OR was 7.27 (95%CI: 0.42–124.52, p = 0.171). However, heterogeneity was detected between studies (I^2 = 68.5%, p = 0.075, a random effects analysis) (Fig. 3D). When stratifying for age, gender and histology, no statistically significant differences were found in HNSCC patients. When stratifying clinical stage and lymph node metastasis, HES1 has a significantly higher expression in III + IV stage patients and positive lymph node metastasis patients.[17]

Other components of Notch signaling pathway: The expression of Jagged2 was not statistically different in HNSCC and control tissue (p = 0.157). When stratifying clinical stage, Jagged2 has a significantly higher expression in III + IV stage patients as compared with I + II stage patients.[21] The expression of HEY1 was also not statistically significant different in HNSCC and control tissue (p = 0.11).[16]

Fig. 3. (A) Forest plot for the association between Notch3 and HNSCC. (B) Forest plot for the association between NICD and HNSCC. (C) Forest plot for the association between Jagged1 and HNSCC. (D) Forest plot for the association between HES1 and HNSCC



The role of prognosis of Notch signaling pathway

Four studies reported the prognostic of the Notch signaling pathway. Two studies focused on Notch1. Meta-analysis results revealed that over-expression of Notch1 was associated with poor prognosis (HR = 0.74, 95%CI: 0.12–1.35; a fixed effects analysis, p = 0.019) (Fig. 4). The Kaplan-Meier and log-rank analysis revealed that a high expression of NICD and HES1 was inclined to poor survival.[17] Zhang et al. reported that elevated DLL4 expression independently predicts poor prognosis.[19]

Publication bias

Publication bias analysis was performed among studies based on Notch1 expression. The funnel plots were asymmetric (Figure not shown). This may be due to the number of eligible studies.

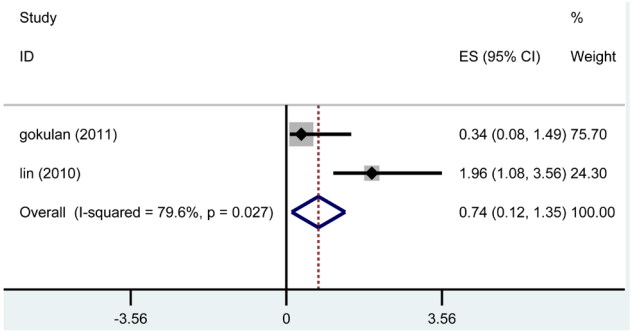
Discussion

Notch phenotypes were first identified 100 years ago in Drosophila melanogaster. Accumulating evidence indicates that Notch signaling plays a vital role in cancer stem cell, cell fate determination and differentiation and tumor angiogenesis.[25] However, whether Notch signaling acts as an oncogene or a tumor suppressor is still controversial. In lung cancer, the activation of Notch signaling promotes hypoxia induced epithelial mesenchymal transition.[26] In melanoma, activated Notch signaling enhances the adhesion and metastasis of melanoma cell by up-regulating N-cadherin.[27] On the contrary, inhibition of Notch signaling can promote skin squamous cell carcinoma formation.[28] Similarly, the role of Notch signaling is controversial in HNSCC. In this meta-analysis, we investigated the association between Notch signaling pathway and HNSCC. We found that the expression of Notch1, Notch3 and NICD was increased in HNSCC as compared with control tissue. And there was no statistically significant difference in Jagged1 and HES1 expression between HNSCC and control tissue. Further, we searched the relationship between Notch signaling and prognosis of HNSCC patients. Notch1 can be used as an independent predict biomarker in HNSCC patients.

In the current meta-analysis, we retrieved 5 studies focused on Notch1 expression in HNSCC and 2 studies showed the correlation between Notch1 expression and prognosis in HNSCC patients. According to the results of the meta-analysis, Notch1 expression was significantly up-regulated in HNSCC. However, Notch1 mutations were detected in approximate 15% of HNSCC. Moreover, Notch1 mutations were loss-of-function mutation and played a tumor suppressive role in HNSCC development.[16] Sakamoto et al. also reported that the expression of Notch1 was decreased in OSCC as compared with control tissue.[29] Although, there was a litter subset of HNSCC exhibiting loss-of-function mutations, the activation of Notch1 is found in major HNSCC. This is consistent with our results of this meta-analysis. Additionally, Notch1 was found to be more preferably expressed in HNSCC patients who had lymph node metastasis and poor clinical stage. This suggested that the activation of Notch1 might promote epithelial-mesenchymal transition and sustain the cancer stem cell.

Irregular Notch3 activity has been associated with some solid tumors, such as breast cancer and lung cancer.[30,31] Studies revealed that Notch3 expression is lim-

Fig. 4. Meta analysis of Notch1 expression and survival of HNSCC patients. Forest plot of RR for overall survival



ited to the mammal's vascular system. In a mouse model, knockout Notch3 results in vascular smooth vessel defects, suggesting a relationship of Notch3 with angiogenesis.[32] Given the role of Notch3 in angiogenesis, the activation of Notch3 probably promotes tumor development by increasing angiogenesis in cancer. In this meta-analysis, we found that Notch3 had a significantly high expression in HNSCC as compared with control tissue. Notch3 expression also had a significant correlation with clinical stage in HNSCC patients.

The Notch1 ligand Jagged1 was demonstrated to be involved in tumor progress.[33] Blockage of Jagged1-mediated Notch signaling reduced tumor growth by decreasing angiogenesis.[34] In this meta-analysis, we found that the expression of Jagged1 was up-regulated in HNSCC, especially in positive lymph node metastasis patients. Besides, Jagged1 inhibition of Notch1 ligand Jagged2 similarly decreased tumor growth by reducing angiogenesis.[34] However, Zhang et al. reported that the expression of Jagged2 did not have a statistically significant difference in HNSCC tissue and control tissue. NICD acting as Notch intracellular fragment was involved in tumor cell proliferation, apoptosis and angiogenesis.[35] The activation of NICD translating into the nucleus can regulate the expression of target genes, such as HES1 and HEY1.[5] Mechanism research showed that HES1 plays a key role in stemness, metastasis and multi-drug resistance.[36] The meta-analysis revealed that NICD had a higher expression in HNSCC than in control tissue. As only 2 studies focused on HES1 expression, there was no statistically significant difference in HES1 expression between HNSCC and control tissue. The data from the Cancer Genome Atlas (TGGA) database suggested that the expression of HES1 is enhanced in HNSCC.[16] Ravindran reported that both NICD and HES1 were correlated with prognosis in HNSCC.[17] This data indicated that the Notch signaling pathway plays a critical role HNSCC development.

There are several limitations in current meta-analysis. Firstly, the number of eligible studies and the number of studies including HNSCC patients are too scarce. Secondly, there are differences in the criteria for Notch signaling evaluation. Although IHC method was applied in all included studies, the cutoff values of positive ranges from 5 to 15%. Thirdly, the funnel plot analysis showed publication bias and is still a concern in these studies.

Conclusion

Taken together, our meta-analysis indicated that the Notch signaling pathway was activated in HNSCC. The Notch signaling pathway was associated with partial clinicopathological characteristics of HNSCC, such as clinical stage, lymph node metastasis status and differentiation. Notch signaling may be used as a poor prognostic biomarker for HNSCC. Inhibition of Notch signaling pathway is a potential therapeutic method of HNSCC. Since the number of studies is relatively limited, further research should be performed to investigate the precise role of the Notch signaling pathway in HNSCC.

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