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Advanced glycation end products derived from serum albumin modification by glucose (AGE-1) reflect clustering of lipid-associated metabolic abnormalities and are decreased in patients treated with acarbose: A cross-sectional study

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

Background. Advanced glycation end products (AGEs) are formed during protein modification by a reduction of sugars or reactive aldehydes. Depending on the pathology, various AGEs may be formed. They are stable compounds and are considered as potential diseases markers.

Objectives. The objective of this study was to assess glucose-mediated albumin modification that yields non-standard epitopes of AGEs (AGE-1) in diabetes and in associated metabolic abnormalities.

Material and methods. The AGE-1, expressed as median AGE-1 level and AGE-1 positivity, was determined in 246 individuals (198 with prediabetes/diabetes) using a new slot-dot-blot method (allowing for detection of barely traceable analytes) and related to the presence of diabetes-associated metabolic abnormalities and complications, and treatment.

Results. The AGE-1 level was higher in patients with prediabetes/diabetes than in controls. Its elevation was associated with metabolic syndrome (MetS), obesity, hyperlipidemia, and non-alcoholic fatty liver disease (NAFLD) but not with diabetic control or micro- and macroangiopathy, except for atherosclerotic plaques formation in carotid arteries. The AGE-1-positive patients had higher triglycerides and lower high-density lipoprotein (HDL)-cholesterol. In patients untreated with aspirin, AGE-1 positivity was associated with higher C-reactive protein (CRP) level. Treatment with aspirin, sulfonylureas and gliptins was associated with higher AGE-1 level and with dyslipidemia medications with higher AGE-1 positivity. In patients with abnormal glucose metabolism, acarbose treatment was associated with lower AGE-1 positivity. Multivariate analysis showed MetS, carotid artery plaques, NAFLD, and treatment with aspirin and acarbose to be independently associated with AGE-1 positivity.

Conclusions. Unlike standard AGEs, AGE-1 is more tightly associated with abnormalities in lipid than glucose metabolism, and lower in patients treated with acarbose but not with other antidiabetics.

Key words: metabolic syndrome, diabetes, atherosclerosis, acarbose, advanced glycation end products

Introduction

Diabetes is increasingly viewed as a spectrum of disorders, such as prediabetes, type 1 (T1DM) and type 2 (T2DM) diabetes, gestational and neonatal diabetes, maturity onset diabetes in young people, and latent autoimmune diabetes in adults.[1] The common denominator of these disorders is abnormal glucose level or tolerance, accompanied by lack of or inadequate response to insulin. With the current estimates of 8.5% of adult population worldwide being affected, the prevalence of diabetes is still on the rise and parallels the increasing prevalence of overweight/obesity. Diabetes is one of the top causes of mortality for both women and men. If not controlled, it leads to complications, such as cardio- and cerebrovascular disease, further increasing the risk of premature death.[2] Obesity and abnormal glucose constitute the core features of metabolic syndrome (MetS). The remaining components include atherogenic dyslipidemia and hypertension. Metabolic syndrome is viewed as a cluster of metabolic risk factors for diabetes and cardiovascular disease with the risk prediction increasing with the number of co-existing metabolic abnormalities.[3]

Glycation is a non-enzymatic reaction occurring between reducing carbohydrates or reactive aldehydes and amino groups of macromolecules. The accumulation of reaction products, that is, advanced glycation end products (AGEs), is particularly evident during aging. However, AGEs formation is accelerated in diabetes, facilitated by disease-associated hyperglycemia, oxidative stress and low-grade inflammation.[4]

Enhanced accumulation of AGEs and resulting modifications of macromolecules contribute to cardio- and cerebrovascular complications.[5] Accordingly, intervention studies have shown that low-AGE diet improves insulin sensitivity and thus may reduce diabetes and cardiovascular disease risks.[6] Advanced glycation end products, in turn, further exacerbate oxidative stress and inflammation. Additionally, AGEs form covalent crosslinks with extracellular matrix proteins, contributing to cardiac fibrosis, stiffening of the arteries and abnormal vasodilator response to nitric oxide.[5] Moreover, glycoxidation-caused alterations in the protein conformation may lead to the formation of neo-epitopes and the rise of autoantibodies, causing adverse immunological responses.[7]

The causative role for AGEs in the pathogenesis and progression of metabolic diseases is widely accepted but to what degree particular AGEs are harmful and thus clinically relevant, needs to be elucidated. Advanced glycation end products are an extremely diverse group of compounds displaying distinct physicochemical and immunogenic properties.[8] The type of AGE formed depends not only on the modified macromolecule and glycating agent but also on the reaction conditions. Despite their enormous variety, only the structures of epitopes of several AGEs have been identified thus far.[9] Consequently, quantification of AGEs and studies on their association with disease pathology either concern the so-called "total AGEs" or are focused on a few specific AGEs, such as N^{ε}-carboxymethyllysine (CML), N^{ε}-carboxyethyllysine (CEL) or imidazolones. The contribution of other AGEs remains largely unknown.[10] Moreover, in easily accessible biological material, such as blood or urine, specific AGE epitopes are present in trace amounts and are consequently difficult to detect and quantify. Sophisticated laboratory techniques that are required to assess the majority of specific AGE epitopes[11] reduce the applicability of AGEs as biomarkers in clinical practice. In turn, available immunoassays mostly use polyclonal antibodies and thus are not suitable for detection of individual AGEs. Moreover, CML modified proteins appear to be the main epitope for anti-AGE antibodies.[12] Yet, CML-modification has been shown to better reflect lipid peroxidation than glycation.[13] Therefore, the need to use the antibodies against non-CML AGEs in studies of the effects of glycation has been emphasized.[14]

This study was designed to assess, using a newly developed immunoassay based on monoclonal, commercially available antibodies, the glycation of serum albumin by glucose with the formation of epitopes other than typical ones (AGE-1) in diabetes and associated metabolic abnormalities, with reference to treatment.

Material and methods

Patients

The study population consisted of 246 individuals: 198 with and 48 without abnormalities in glucose metabolism. Patients with deregulated glucose metabolism were recruited from the Department of Angiology, Diabetes and Hypertension of Wroclaw Medical University, Poland. The group consisted of individuals with prediabetes (n = 10) or with diabetes mellitus (n = 188; 14 with T1DM, 163 with T2DM and 11 with a secondary diabetes (T3DM) – the main cause of secondary diabetes was pancreatitis).

Table 1. Characteristics of study population

Variables	Controls	Patients with abnormal glucose metabolism	p-value
Number of cases	48	198	–
Sex (F/M), n	26/22	121/77	0.414[F]
Age [years], mean (range)	62 (59.7–63.2)	63.5 (61–65)	0.249[M]
AGE-1 positivity, n (%)	17 (35.4)	109 (55.1)	0.016[F]
AGE-1 [AU], mean (range)	0 (0–572)	4,888 (0–13,061)	0.004[M]

Continuous data presented as medians accompanied with 95% confidence interval (95% CI). [F] – Fisher's exact test; [M] – Mann–Whitney U test; F/M – female-to-male ratio; AGE – advanced glycation end products. AGE-1-positive samples were defined as samples with measurable AGE-1 (AGE-1 > 0); n – number of observation; AU – arbitrary units.

The control group, without known abnormalities in glucose metabolism, consisted of patients with atherosclerosis (n = 18) and apparently healthy blood donors (n = 30), recruited from the Regional Center of Blood Donation and Therapy in Wrocław. The inclusion criteria were the following: age >50 years, no known systemic disease, dementia, depression or ongoing inflammation, and fasting glucose <100 mg/dL.

Demographic, clinical and laboratory data was collected prospectively. The characteristics of the study population are given in Table 1, while data on treatment and the coexistence of other abnormalities is presented in Tables 2–5.

Definitions

Prediabetes was defined as impaired fasting plasma glucose (100–125 mg/dL) or glucose tolerance (2-hour plasma glucose in the 75-gram oral glucose tolerance test = 140–199 mg/dL).[2] The World Health Organization (WHO) criteria for diabetes were applied. Diabetes was considered controlled if the percentage of glycation of the hemoglobin A_1c chain (HbA_1c) was ≤6.4%.[2]

The WHO classification of adult weight according to body mass index (BMI) was applied with BMI 25–30 kg/m^2 indicative of overweight (pre-obese) and BMI ≥ 30 kg/m^2 indicative of obesity (class I: 30–35 kg/m^2, class II: 35–40 kg/m^2, class III: ≥40 kg/m^2).[15]

Metabolic syndrome was defined according to the International Diabetes Federation (IDF) criteria[16] as the coexistence of central obesity plus any of the 2 following: hypertriglyceridemia (≥150 mg/dL) or receiving treatment (RT), low level of high-density lipoprotein (HDL)-cholesterol (<50 mg/dL in women and <40 mg/dL in men), hypertension (≥130 mm Hg or ≥85 mm Hg for systolic or diastolic, respectively) or RT, glucose ≥100 mg/dL, or diagnosed with diabetes and/or RT.

Hyperlipidemia was defined according to the guidelines of the European Society of Cardiology (ESC) and the European Atherosclerosis Society (EAS)[17] as elevated total cholesterol (≥190 mg/dL), and/or low-density lipoprotein (LDL)-cholesterol (≥115 mg/dL) and/or triglycerides (≥150 mg/dL) or RT.

Hyperuricemia definition was based on the NHANES-III criteria[18] as a serum urate level of 7.0 mg/dL in men and 5.7 mg/dL in women.

Macroangiopathy (atherosclerosis of arteries) was diagnosed in patients who had a myocardial infarction or stroke, percutaneous coronary intervention or coronary artery bypass graft, acute coronary syndrome (defined as treatment in a hospital as a consequence of 1 or more episodes of ischemic discomfort at rest and characterized by electrocardiogram changes and/or elevation of a cardiac serum marker to an extent not indicative of a myocardial infarction), objective evidence of coronary artery disease (defined as positive exercise test, angiography with at least 1 stenosis >50), symptomatic peripheral arterial obstructive disease (confirmed by an ankle/brachial pressure index <0.90 or an amputation), stenosis of carotid artery, or cardiovascular death.

Microangiopathy was diagnosed in patients with diabetic nephropathy (albuminuria >30 mg/g, creatinine and estimated glomerular filtration rate (eGFR) assessed using Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) creatinine equation), diabetic retinopathy (microaneurysms, dot-blot hemorrhages, flame-shaped hemorrhages, retinal edema and hard exudates, cotton-wool spots, venous loops and venous beading, intraretinal microvascular abnormalities, hemorrhage into the vitreous, traction retinal detachments, macular edema etc., detected using ophthalmoscopy, fundus fluorescein angiography or optical coherence tomography), or polyneuropathy (diagnosed after excluding other possible causes with the assessment of pain sensation according to the pinprick test (absent or decreased), electromyography, and/or clinical evaluation using, e.g., Neuropathy Impairment Score).

Ethical considerations

The study protocol was approved by the Medical Ethics Committee of Wroclaw Medical University (approvals No. KB-303/2010 and No. KB-384/2012) and was in accordance with the ethical standards formulated in the Helsinki Declaration of 1975. Informed consent was obtained from all subjects.

Analytical methods

Serum samples were obtained from clotted (15 min, room temperature) and centrifuged (15 min, 400 × g) blood drawn using venipuncture into serum-separator tubes following a 12-hour overnight fast.

The AGE-1 level was assessed in sera using monoclonal anti-AGE-1 antibodies (clone No. 7C1; CosmoBio, Tokyo, Japan) and a slot-dot-blot method, recently developed in our laboratory (manuscript submitted). The antibodies react selectively with glucose-modified (AGE-1) bovine serum albumin (BSA) and are confirmed to not cross-react with methylglyoxal-modified BSA (AGE-4), glyceraldehyde-modified BSA (AGE-2), glycolaldehyde-modified BSA (AGE-3), glyoxal-modified BSA (AGE-5), 3-DG-imidazolone-modified BSA (AGE-6), carboxymethyllysine-modified BSA (CML), carboxyethyllysine-modified BSA (CEL), or native BSA.[19] Briefly, diluted sera were applied into methanol-activated PVDF membranes in Bio-Dot® SF Microfiltration Apparatus (BioRad, Hercules, USA). Membranes were blocked with 5% skimmed milk and incubated with primary antibodies (1:1,000), followed by secondary antibodies (goat anti-mouse IgG; 1:2,000; Jackson ImmunoResearch Europe Ltd., Cambridgeshire, UK) conjugated with horseradish peroxidase. 3-amino-9-ethylcarbazole (Sigma-Aldrich, St. Louis, USA) was used as a peroxidase

substrate and the density of developed color, proportional to AGE-1 concentration, was determined using densitometry and expressed in arbitrary units (AU).

Data on biochemical indices, determined using standard automated procedures, were collected at the time of blood sampling for AGE-1 determination.

Statistical analysis

Data distribution was tested using Kolmogorov–Smirnov test. Data on AGE-1 is presented as medians with 95% confidence interval (95% CI) and analyzed using Kruskal–Wallis H test or Mann–Whitney U test. Advanced glycation end products distribution was compared using one-way analysis of variance (ANOVA) and high-sensitive C-reactive protein (hsCRP), and HbA_1c levels were analyzed using t-test for independent samples. Frequency analysis was conducted using Fisher's exact test or χ^2 test. Correlation was analyzed using Spearman's test. Logistic regression (stepwise method) with $p < 0.05$ as an entrance criterion and $p > 0.1$ as a removal criterion was applied to identify independent predictors of AGE-1 positivity. Odds ratios (ORs) with 95% CI were calculated for significant variables.

All calculated p-values were two-sided; $p \leq 0.05$ was considered statistically significant. Statistical analysis was conducted using MedCalc Statistical Software v. 17.9.6 (MedCalc Software bvba, Ostend, Belgium; http://www.medcalc.org; 2017).

Results

AGE-1 and abnormal glucose metabolism

Individual distribution of AGE-1 in the study population is depicted in Fig. 1. A total of 120 out of 246 study participants had undetectable AGE-1 level and there was substantial variability among the AGE-1-positive samples (range: 141–348,864 AU). Therefore, the data was analyzed by comparing both AGE-1 levels and the proportions of AGE-1-positive samples (defined as samples with measurable AGE-1 levels), referred to as AGE-1 positivity.

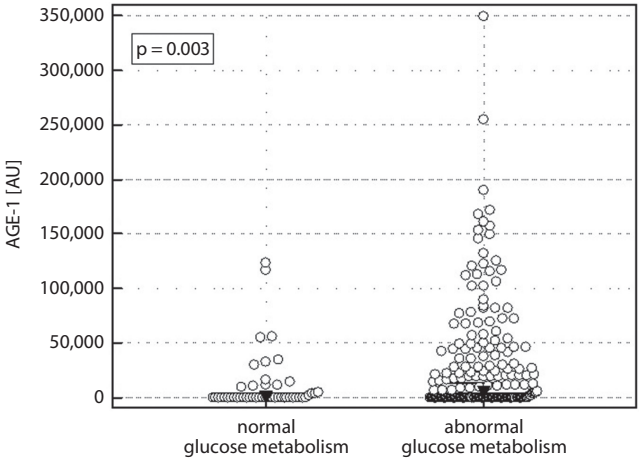

Fig. 1. AGE-1 in individuals with normal and abnormal glucose metabolism. Open circles represent individual AGE-1 levels, black triangles represent median values, whiskers represent 95% confidence interval (95% CI) around median. Data analyzed using the Mann–Whitney U test

The AGE-1 positivity and median AGE-1 levels were significantly higher in patients with abnormal glucose metabolism than in controls (Table 1).

Detailed analysis showed that patients with prediabetes, T1DM and T2DM had comparable AGE-1 positivity, higher than T3DM patients or controls, with the difference between T2DM and controls being statistically significant. Patients with uncontrolled diabetes had higher AGE-1 level than those with controlled disease, but the difference did not reach statistical significance (Table 2).

We additionally compared AGE-1 and HbA_1c levels in patients with prediabetes and diabetes (regardless of its type). The AGE-1 level in prediabetes was comparable to diabetes (10,629 AU (0–130,721 AU) vs 4,699 AU (0–12,411 AU), $p = 0.541$) whereas HbA_1c level was significantly lower (5.5 g/dL (5.3–5.6 g/dL) vs 7.3 g/dL (6.3–8.8 g/dL), $p < 0.001$). Also, AGE-1 positivity was similar between patients with prediabetes and diabetes (60% vs 54.8%, $p = 0.748$).

Patients treated with metformin, sulfonylureas or gliptins had higher AGE-1 level, significantly so in the case of sulfonylureas and gliptins, without a significant difference in AGE-1 positivity (Table 3). When the analysis was restricted to patients diagnosed with abnormal glucose metabolism, only the difference in median AGE-1 level between patients treated and untreated with gliptins remained significant (26,251 AU (0–49,783 AU) vs 2012 AU (0–10,452 AU), $p = 0.028$). In turn, AGE-1 positivity tended to be lower in acarbose-treated patients (Table 3). The difference gained significance when analyzed exclusively in patients with abnormal glucose metabolism (34.8% in treated vs 57.7% in untreated, $p = 0.046$).

AGE-1 and obesity

Underweighted/normal weight patients had significantly lower AGE-1 level than patients with overweight or obesity (Table 2). There was also a clear tendency towards a difference in AGE-1 positivity, with the differences between underweight/normal weight and overweight patients significant in paired analysis, and the differences between underweight/normal weight and obese or morbidly obese patients with $p \leq 0.06$.

AGE-1 and dyslipidemia

The AGE-1 level was significantly higher in patients with hyperlipidemia and in patients with non-alcoholic fatty liver disease (NAFLD; Table 2).

Table 2. AGE and metabolic abnormalities

Variables	AGE-1 level [AU] median (95% CI)	p-value^{M}	AGE-1 positivity n (%)	p-value^{F}
Type of deregulation				
normal (controls; n = 48)	0 (0–572)[1]	0.044	17 (35.4)[1]	0.088
prediabetes (n = 10)	10,629 (0–130,721)		6 (60)	
T1DM (n = 14)	7,146 (0–18,710)		9 (64.3)	
T2DM (n = 155)	4,945 (0–18,964)[2]		90 (55.2)[2]	
T3DM (n = 19)	0 (0–45,705)		4 (36.4)	
Diabetes control*				
controlled (n = 52)	0 (0–12,096)	0.266	25 (48.1)	0.258
uncontrolled (n = 136)	7,970 (0–19,186)		78 (57.4)	
Atherosclerosis				
no (n = 33)	0 (0–13,072)	0.355	15 (45.5)	0.331
yes (n = 183)	6,141 (0–14,739)		100 (54.6)	
Weight**				
underweight/normal (n = 54)	0 (0–1,452)[3]	0.008	21 (38.9)†	0.067
overweight (n = 71)	14,619 (0–25,178)		42 (59.2)†	
obesity class I/II (n = 83)	4,830 (0–18,856)		46 (55.4)	
obesity class III (n = 8)	33,356 (0–52,711)		6 (75)	
Weight (dichotomized)**				
underweight/normal (n = 54)	0 (0–1,452)	0.001	21 (38.9)	0.018
overweight/obesity (n = 162)	10,655 (144–20,232)		94 (58)	
Hypertension				
no (n = 17)	0 (0–37,992.5)	0.477	7 (41.2)	0.322
yes (n = 199)	4,568 (0–11,765)		108 (54.3)	
Hyperuricemia				
no (n = 121)	14,620 (386–2,0213)	0.119	72 (59.5)	0.179
yes (n = 71)	0 (0–9,707)		35 (49.3)	
Hyperlipidemia				
no (n = 24)	0 (0–1,646)	0.046	7 (29.2)	0.016
yes (n = 192)	6,478 (0–14,677)		108 (56.2)	
NAFLD				
no (n = 170)	0 (0–9,093)	0.035	84 (49.4)	0.032
yes (n = 46)	14,580 (1,049–29,852)		31 (67.4)	
Metabolic syndrome				
no (n = 75)	0 (0–2,686)	0.002	30 (40)	0.006
yes (n = 141)	10,815 (637–20,460)		85 (60.3)	
Albuminuria				
no (n = 98)	0 (0–7,678)	0.117	45 (45.9)	0.059
yes (n = 118)	9,498 (184–19,589)		70 (59.3)	
Chronic kidney disease				
no (n = 197)	2,013 (0–10,839)	0.191	103 (52.3)	0.472
yes (n = 19)	18,993 (0–48,799)		12 (63.2)	

[1] – significantly different from T2DM; [2] – significantly different from controls; [3] – significantly different form other groups; * – assessed in person with diabetes, n = 188; ** – assessed in clinical patients (without blood donors), n = 216; NAFLD – non-alcoholic fatty liver disease; [M] – Mann–Whitney U test; [F] – Fisher's exact test; † – comparison of proportion between those 2 groups showed significant differences (p = 0.0025); bold – statistically significant results; n – number of observations; AU – arbitrary units; T1DM – type 1 diabetes mellitus; T2DM – type 2 diabetes mellitus; T3DM – secondary type 2 diabetes mellitus; AGE-1 – advanced glycation end products; AGE-1 positivity – samples with measurable AGE-1 level (AGE-1 > 0).

The AGE-1-positive patients had higher concentrations of triglycerides (153 mg/dL vs 140 mg/dL, p = 0.031), but not of total (p = 0.666) or LDL cholesterol (p = 0.418), and lower concentrations of HDL cholesterol (44.5 mg/dL vs 48 mg/dL, p = 0.020).

Patients treated with lipid-lowering medications had significantly higher AGE-1 positivity than untreated patients (Table 3). However, the difference lost its significance when the analysis was restricted to treated against untreated patients diagnosed with hyperlipidemia (58% vs 48.6%, p = 0.349).

AGE-1 and hypertension, hyperuricemia and kidney function

There was no significant difference in AGE-1 levels or AGE-1 positivity with respect to hypertension, hyperuricemia or the kidney function except for a tendency towards higher AGE-1 positivity in patients with albuminuria (Table 2). There was no correlation between AGE-1 and GFR index (ρ = 0.03, p = 0.644).

Treatment with anti-hypertensive medications did not affect AGE-1 level or positivity (Table 3).

Table 3. Effect of AGE-1 treatment on AGE-1

Variables	AGE-1 level [AU] median (95% CI)	p-value[M]	AGE-1 positivity n (%)	p-value[F]
Antihypertensive drugs no (n = 32) yes (n = 184)	 0 (0–32,799) 4,888 (0–12,313)	0.663	 14 (43.7) 101 (54.9)	0.256
Dyslipidemia medications no (n = 59) yes (n = 157)	 0 (0–8,417) 6,814 (104–16,832)	0.123	 24 (40.7) 91 (58)	0.032
Aspirin no (n = 75) yes (n = 141)	 0 (0–6,643) 10,408 (167–18,971)	0.020	 32 (42.7) 83 (58.9)	0.031
Anticoagulants no (n = 188) yes (n = 28)	 1,584 (0–10,364) 19,289 (0–32,988)	0.336	 98 (52.1) 17 (60.7)	0.424
Clopidogrel no (n = 188) yes (n = 28)	 4,068 (0–14,447) 0 (0–16,606)	0.486	 102 (54.3) 13 (46.4)	0.543
Insulin no (n = 130) yes (n = 86)	 4,069 (0–15,947) 2,744 (0–13,397)	0.574	 70 (53.8) 45 (52.3)	0.889
Metformin no (n = 74) yes (n = 142)	 1,584 (0–10,894) 4,888 (0–19,331)	0.164	 38 (51.4) 77 (54.2)	0.774
Acarbose no (n = 193) yes (n = 23)	 6,141 (0–13,753) 0 (0–13,207.5)	0.214	 107 (55.4) 8 (34.8)	0.077
Sulfonylureas no (n = 144) yes (n = 72)	 0 (0–9,434) 12,069 (44.5–24,259.5)	0.048	 71 (49.3) 44 (61.1)	0.113
Gliptins no (n = 184) yes (n = 32)	 263 (0–9,015) 26,251 (0–49,783)	0.016	 94 (51.1) 21 (65.6)	0.179

[M] – Mann–Whitney U test; [F] – Fisher's exact test; bold – statistically significant results; AGE-1 – advanced glycation end products; AU – arbitrary units; n – number of observations; AGE-1 – advanced glycation end products.

AGE-1 association with micro- and macroangiopathy and atherosclerosis

Neither median AGE-1 level nor AGE-1 positivity differed significantly between patients with and without microangiopathy (Table 4). Also, there was no significant difference in AGE-1 level and AGE-1 positivity between patients with carotid arteries plaques and without them (Table 5).

Treatment with clopidogrel had no significant effect on AGE-1 level or positivity (Table 3).

AGE-1 and inflammation

The AGE-1-positive patients tended to have higher concentrations of hsCRP than patients with undetectable AGE-1 level (2.58 mg/dL (2.0–3.3 mg/dL) vs 2.07 mg/dL (1.6–2.7 mg/dL), p = 0.227, n = 187). The difference in hsCRP between AGE-1-positive and -negative patients was significant when the analysis was limited to patients not treated with aspirin (3.55 mg/dL (2.0–6.3 mg/dL) vs 1.44 mg/dL (0.98–2.10 mg/dL), p = 0.008). Also, there was a positive correlation between AGE-1 and hsCRP in these patients ($\rho = 0.28$, p = 0.029, n = 61).

Two-way ANOVA was applied to co-examine the effect of AGE-1 and aspirin treatment on hsCRP and showed that AGE-1 positivity (p = 0.048) but not aspirin (p = 0.687) was significantly associated with hsCRP.

Treatment with aspirin was associated with higher AGE-1 levels and frequency of AGE-1 positivity (Table 3).

Multivariate analysis

Logistic regression was applied to discern the independent predictors of AGE-1 positivity in patient cohort (n = 216). All the variables significantly associated with AGE-1 positivity in univariate analysis, that is, hyperlipidemia, obesity (dichotomized), MetS, NAFLD, carotid artery plaques, and treatment with aspirin or lipid-lowering drugs, were entered into the analysis as explanatory variables. When analyzed exclusively in clinical patients, the effect of abnormal glucose metabolism did not reach statistical significance (p = 0.088) and was not included.

Table 4. AGE-1 association with microangiopathy

Variables	AGE-1 level [AU] median (95% CI)	p-value[M]	AGE-1 positivity n (%)	p-value[F]
Microangiopathy no (n = 110) yes (n = 106)	 6,478 (0–16,077.5) 1,584 (0–12,172)	0.952	 59 (53.6) 56 (52.8)	1
Retinopathy no (n = 186) yes (n = 30)	 4,069 (0–11,770) 2,316 (0–41,540)	0.876	 99 (53.2) 16 (53.3)	1
Nephropathy no (n = 178) yes (n = 38)	 4,069 (0–13,666) 1,584 (0–19,253)	0.976	 94 (52.8) 21 (55.3)	0.859
Polyneuropathy no (n = 136) yes (n = 80)	 648 (0–11,586) 5,533 (0–19,643)	0.398	 69 (50.7) 46 (57.5)	0.397

[M] – Mann–Whitney U test; [F] – Fisher's exact test; AU – arbitrary units; n – number of observation; AGE-1 – advanced glycation end products; AGE-1-positive – samples with measurable AGE-1 level (AGE-1 > 0).

Table 5. AGE-1 association with macroangiopathy

Variables	AGE-1 level [AU] median (95% CI)	p-value[M]	AGE-1 positivity n (%)	p-value[F]
Macroangiopathy no (n = 48) yes (n = 168)	 648 (0–17,730) 4,069 (0–12,309)	0.884	 25 (52.1) 90 (53.6)	0.871
Ischemic heart disease no (n = 136) yes (n = 80)	 2,316 (0–12,393) 6,731 (0–19,721)	0.723	 72 (52.9) 43 (53.7)	1
Acute coronary syndromes no (n = 171) yes (n = 45)	 1,155 (0–11,516) 9,126 (0–21,277)	0.685	 89 (52) 26 (57.8)	0.508
Ischemic stroke no (n = 191) yes (n = 25)	 1,155 (0–11,299) 10,408 (0–21,599)	0.728	 99 (51.8) 16 (64)	0.291
Arteriosclerosis obliterans no (n = 132) yes (n = 84)	 4,888 (0–16,672) 0 (0–13,080)	0.440	 74 (56.1) 41 (48.8)	0.329
Carotid artery plaques no (n = 73) yes (n = 143)	 0 (0–3,984) 9,126 (242–18,976)	0.053	 31 (42.5) 84 (58.7)	0.030

[M] – Mann–Whitney U test; [F] – Fisher's exact test; 95% CI – 95% confidence interval; AU – arbitrary units; n – number of observations; AGE-1 – advanced glycation end products; AGE-1-positive – samples with measurable AGE-1 level (AGE-1 > 0).

When co-examined, MetS (p = 0.001), carotid artery plaques (p = 0.006) and treatment with aspirin (p = 0.018) contributed significantly to the prediction of AGE-1 positivity with the following ORs: 2.8 (95% CI = 1.5–5.1), 2.4 (95% CI = 1.3–4.3) and 2.1 (95% CI = 1.1–3.7), respectively. The effect of obesity, hyperlipidemia, NAFLD, and treatment with lipid-lowering drugs lost its significance.

To address the issue whether the effect of treatment with acarbose or gliptins on AGE-1 was independent from disparity in occurrence of metabolic abnormalities between treated and untreated patients, we repeated the analysis on a cohort of patients with abnormal glucose metabolism (n = 198), introducing treatment with acarbose and gliptins as additional independent variables.

When co-examined, MetS (p = 0.017), carotid artery plaques (p = 0.007), NAFLD (p = 0.030), and treatment with aspirin (p = 0.016) and acarbose (p = 0.008) contributed significantly to the prediction of AGE-1 positivity with the following ORs: 2.3 (95% CI = 1.2–4.6), 2.5 (95% CI = 1.3–4.8), 2.5 (95% CI = 1.1–5.6), 2.2 (95% CI = 1.2–4.1), and 0.25 (95% CI = 0.09–0.7), respectively. The effect of obesity, hyperlipidemia and treatment with lipid-lowering drugs or gliptins lost its significance.

Discussion

Studies on albumin glycation have recently gained momentum with the focus of clinical research divided between the potential of glycated albumin as a biomarker and the effect of glycation on albumin affinity to drugs.[20] Albumin glycation yields a plethora of diverse early and

advanced products, frequently measured with methods using general properties of some of them, such as fluorescence, and referred to as "total AGEs". Analysis of specific products is less common and mostly limited to CML, CEL and the most abundant AGEs in human plasma[21] – imidazolones – or their derivatives.[20] To our knowledge, this is the first report on specific AGE-1 in patients with diabetes and related disorders, measured using commercially available monoclonal antibody directed against glucose-modified albumin, other than typically assessed glycation products. Similarly to other glucose-derived AGEs,[22] our AGE-1 was elevated in patients with diabetes as compared to healthy individuals; however, probably due to group size, the difference was significant exclusively for T2DM.

The formation of AGEs is heavily dependent on glucose concentration and reaction time; therefore, their accumulation is classically associated with diabetic complications.[23–26] However, not all epidemiological studies have confirmed an independent association of AGEs with the incidence of diabetic nephropathy.[27] Also, our AGE-1 was associated neither with polyneuropathy, nephropathy nor retinopathy. Still, AGE-1 accumulation was significantly increased in the presence of carotid artery plaques. Moreover, carotid plaques were found to be an independent predictor of AGE-1 positivity. Therefore, our observation corroborates the notion on other glucose-derived AGEs that they concentration is associated with the development of atherosclerosis in persons with diabetes.[22,28]

Glycated albumin in all its forms is considered a promising biomarker for the prediction not only of diabetic complications but also of prediabetes.[29] Accordingly, we demonstrated equally elevated AGE-1 levels, but not HbA_1c levels, in prediabetes and diabetes. This seems to imply that the accumulation of our AGE-1 is an earlier event than that of HbA_1c, despite the fact that formation of Amadori products, to which HbA_1c belongs, precedes the synthesis of AGEs by months. It is possible that, in a manner similar to oxidative modification, albumin glycation may serve as a protective mechanism, slowing down the glycoxidation of more vital proteins. Corroborating our findings, Brunvand et al.[30] reported that methylglyoxal-derived hydroimidazolone-1, but not HbA_1c, was associated with early indications of diabetic cardiomyopathy. Yamagishi et al.[31] found AGEs to contribute to the initial stages of diabetic nephropathy, while Heier et al.[22] discovered that they redound to early steps of atherosclerosis. It should be mentioned, however, that pentosidine has been found to contribute to late events, such as coronary artery calcification.[28]

Diabetes is frequently accompanied by dyslipidemia, which contributes to micro- and macrovascular complications. Interestingly, we showed AGE-1 to be more tightly allied with abnormalities in lipid metabolism than hyperglycemia. With control group excluded, neither abnormal glucose metabolism was a predictor of AGE-1 positivity, nor AGE-1 correlated with glucose or HbA_1c. In turn, AGE-1 was consistently associated with all lipid parameters. Moreover, MetS, encompassing 3 lipid-associated components, that is, reduced HDL cholesterol level, increased triglycerides and central obesity, was an independent predictor of AGE-1 positivity. This observation corroborates findings on CML and pentosidine, significantly associated with MetS or hyperlipidemia.[23,32] Our results also agree well with a causative role attributed to AGEs in lipid accumulation. Yuan et al.[26] showed that CML increased the expression of 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, directly facilitating ectopic accumulation of lipids. Moreover, albumin modification alters its affinity towards fatty acids and translates into their increased blood concentration.[20] Furthermore, a tight association between AGE-1 and lipid abnormalities is in line with findings showing that AGE inhibitors improve lipid profiles in persons with diabetes.[33] Counterintuitively, treatment with lipid-lowering drugs in our cohort was associated with higher AGE-1 positivity. However, this might reflect the association of AGE-1 with dyslipidemia, since the effect was not observed in hyperlipidemic patients. Also, treatment with lipid-lowering drugs lost significance when co-analyzed with other variables.

Although not considered a diagnostic criterion for MetS, low-grade inflammation belongs to its satellite conditions. C-reactive protein, a marker of systemic inflammation, is elevated in MetS and increases gradually with the number of accumulated metabolic abnormalities. It is considered an independent predictor of diabetes and cardiovascular events that adds prognostic information to lipid screening.[3] Low-grade inflammatory response and glycation are tightly intertwined.[5,19] Indeed, with the anti-inflammatory effect of aspirin accounted for, the positive association between glycation and low-grade inflammation could be observed in our cohort as well. Not only CRP levels were significantly higher in AGE-1-positive patients, but also AGE-1 directly correlated with CRP. Similarly, Heier et al.[22] observed increased levels of methylglyoxal-derived hydroimidazolone-1 in the association with low-grade inflammation in T1DM.

Owing to its anti-inflammatory and anti-thrombotic activity, aspirin therapy is applied in primary and secondary prevention of cardiovascular events.[34] Aspirin has been reported to inhibit glycation in vitro[35] and lower tissue content of AGE,[33] whereas its effect on circulating AGE has not been determined. Counterintuitively, there was a positive association between treatment with aspirin and AGE-1 in our cohort and aspirin treatment was a predictor of AGE-1 positivity, independent from MetS and plaque formation. Therefore, further mechanistic studies exploring aspirin effect on this particular AGE are warranted.

Formation of AGEs in people with diabetes is inhibited by rigorous glycemic control or metformin treatment.[33] Accordingly, pentosidine, but not CML, has been significantly reduced in metformin-treated patients.[32] However,

AGE-1 in our cohort did not differ significantly with respect to the effectiveness of diabetes control and there was no correlation between AGE-1 and HbA_1c. Moreover, except for acarbose, any treatment intended to improve glucose metabolism was associated with higher AGE-1 levels, significantly so in the case of gliptins. Similarly, Haddad et al.[32] found a strong tendency towards higher CML in MetS patients treated with metformin. In our cohort, this unexpected observation might be explained by the fact that treated patients had a significantly higher frequency of MetS, obesity or NAFLD. Indeed, when co-examined in multivariate analysis, the undesirable effect of gliptins on AGE-1 accumulation lost its significance. Nevertheless, altered drug effectiveness may contribute to the positive effect of treatment on AGE-1 accumulation. Albumin is a suitable transporter for a number of drugs and metabolites.[36] However, its glycoxidation reduces its ability to prolong plasma circulatory time for low-molecular drugs and to assure their intracellular delivery.[36] Moreover, any modification affects albumin–drug interaction, with the strength and the direction of the effect depending on the type and conditions of albumin modification as well as of the drug.[20,36] Reduced availability of free, biologically active drug fraction may translate into improper control of glycemia[20] and accelerate the formation of AGEs. Of note, fatty acids, frequently elevated in patients with metabolic abnormalities, have also been shown to affect the overall binding and affinity of native and glycated albumin towards sulfonylurea drugs.[37]

Unlike other evaluated glucose-lowering drugs, acarbose seems to be independently associated with reduced AGE-1 level. However, Tsunosue et al.[38] reported that treatment with acarbose had no significant effect on postprandial plasma glucose. Instead, it decreased serum levels of glyceraldehyde-derived AGEs as well as those of free fatty acids. Acarbose has also been found to delay the progression of intima media thickening and reduce the incidence of cardiovascular disease.[31] Observations made by our team and by Tsunosue et al.[38] indicate that the beneficial effect of the drug might, at least partially, be mediated by AGE-lowering activity of acarbose.

Antibodies used in this study were directed against modified bovine and non-human serum albumin, which might be considered a limitation of our study. However, there is a great degree of homology between human and bovine albumin, and AGE-bovine albumin has been highly toxic when tested against human cell lines.[31] Even more importantly, antibodies against mammal albumins are highly cross-reactive.[39] The uncertainty as to which particular AGE epitopes are recognized by the antibodies used here is yet another limitation. The advantage of the method is, in turn, the monoclonal character of the antibodies. The producer claims no cross-reactivity towards the most typical AGEs, but there is no information concerning potential cross-reactivity towards others, e.g., pentosidine. However, taking into account that we did not find an association between our AGE-1 and diabetic microangiopathy or hypertension or that we have observed no metformin-associated reduction of AGE-1 accumulation reported for pentosidine in the literature,[23,32] it is rather unlikely that antibodies used in our study recognize this particular AGE-1.

Conclusions

The dot-blot-slot method allows for the detection and quantification of non-standard AGE-1 epitopes, present in serum at low concentrations. The AGE-1, unlike typical AGEs, was more closely associated with the abnormalities in lipid than glucose metabolism. This finding confirms the diverse effects AGEs may have on the metabolism and stresses the need for their individual evaluation. Interestingly, only acarbose treatment was accompanied by decreased AGE-1 accumulation. The possible AGE-1-lowering effect of acarbose might be taken into account when planning treatment with antidiabetics to prevent lipid-associated complications.

This finding substantiates the notion on the diversity of the effects AGEs may have on the metabolism and stresses the need for their individual evaluation. Interestingly, only acarbose treatment was accompanied by decreased AGE-1 accumulation. The possible AGE-1-lowering effect of acarbose might be taken into account when planning treatment with antidiabetics to prevent lipid-associated complications.

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Relations between circulating and myocardial fibrosis-linked microRNAs with left ventricular reverse remodeling in dilated cardiomyopathy

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Abstract

Background. Left ventricular reverse remodeling (LVRR) determines clinical status and outcomes in dilated cardiomyopathy (DCM). The extent of myocardial fibrosis is connected to the systolic function of the heart. The recent discovery of the contribution of microRNAs (miRs) to the regulation of cardiac remodeling, LVRR and fibrosis warrants exploration.

Objectives. The aim of the study was to examine the predictive value of circulating and myocardial miR expression for LVRR in DCM.

Material and methods. Seventy consecutive DCM patients (age 48 ±12.1 years, 90% male, ejection fraction (EF) 24.4% ±7.4%) were included in the study. At baseline, all patients underwent clinical assessment, echocardiography, venous blood sampling, and right ventricular endomyocardial biopsy. Circulating and myocardial miRs (miR-21, -26, -29, -30, -133a, and -423) were measured with quantitative real-time polymerase chain reaction (qRT-PCR). LVRR was defined as an increase in EF ≥ 10%, accompanied by a decrease in left ventricle end-diastolic diameter (LVEDd) ≥10% or LVEDd ≤ 33 mm/m^2 between baseline and 3-month follow-up.

Results. At the 3-month follow-up, 4 patients had died and 3 patients had incomplete data. The remaining patients were divided according to the presence of LVRR into LVRR-present (n = 32, 51%) and LVRR-absent (n = 31, 49%) groups. Out of all the circulating and tissue miRs under study, only myocardial expression of miR-133a significantly differed between the LVRR-present and LVRR-absent group (1.22 (0.47–1.90) vs 0.61 (0.25–0.99) ΔCq, respectively, $p < 0.01$). miR-133a was found to be a significant LVRR predictor in unadjusted (odds ratio (OR) = 2.81 (1.23–6.40), $p < 0.05$) and adjusted for duration of disease, left ventricle end-diastolic (LVED) volume (LVEDvol), hs-troponin-T, and NT-proBNP (OR = 5.20 (1.13–24.050, $p < 0.05$) models.

Conclusions. From all of the circulating and tissue miRs, only myocardial miR-133a showed increased expression in LVRR-present patients and was found an independent LVRR predictor. This indicates a link between miR-133 and cardiac remodeling in DCM.

Key words: microRNA, dilated cardiomyopathy, left ventricle reverse remodeling

Introduction

Heart failure (HF) is a worldwide problem with a prevalence of 1–2% in the general population.[1] In a younger population, between 20 and 40 years of age, the most common HF etiology is dilated cardiomyopathy (DCM).[2] It is a progressive myocardial disease, characterized by ventricular wall thinning and dilation accompanied by gradual functional impairment.[3] During the progression of the disease, the left ventricle (LV) undergoes profound adverse morphological and functional changes, termed remodeling. This process has a crucial role in the transition from the compensated and oligo-symptomatic DCM phenotype to overt and symptomatic HF. Cardiac fibrosis contributes to adverse remodeling.[4]

Following optimal medical therapy, in approx. 30–50% of DCM patients, the heart – and the LV in particular – can undergo LV reverse remodeling (LVRR). This is a broad term that describes beneficial changes in LV morphology (decrease of LV size) and function (improvement of LV systolic function).[5,6] The current recommendations of the European Society of Cardiology (ESC) on HF management take the possibility of the occurrence of LVRR into account and emphasize the need for regular monitoring of cardiac morphology and function.[1] Since LVRR is one of the strongest prognostic predictors in HF and DCM, many investigations have been carried out to determine LVRR predictors.[5] Despite huge efforts being made towards LVRR prediction as well as the treatment of HF through LVRR induction, HF is still one of the most common causes of mortality worldwide.

The recent discovery of the contribution of microRNAs (miRs) to the regulation of cardiac remodeling and fibrosis suggests their potential diagnostic and therapeutic role. In mice, up- and downregulation of specific miRs were found to be related to various aspects of cardiac pathology.[7,8] From previous studies, miR-21, miR-26, miR-29, miR-30, miR-33a, and miR-133a are known to be related to myocardial fibrosis, cardiac remodeling and cardiac hypertrophy.[9,10] These miRs are believed to be valuable biomarkers of cardiac diseases; however, their relations with LVRR in DCM is currently unknown.

Methods

Study population

Seventy consecutive DCM patients with a clinical and echocardiographic diagnosis of DCM (63 men and 7 women, with an average age of 48 ±12.1 years), admitted to the cardiology clinic between July 2014 and October 2015, were included in the study. All patients were willing to participate in the study. During the study, 4 patients died and 3 patients had incomplete data before the 3rd month of follow-up. For the purposes of this study, we analyzed 63 of the 70 patients (84%) with available laboratory and echocardiographic data at baseline evaluation and after 3 months.

Dilated cardiomyopathy was diagnosed by the presence of LV dilation (>117% of predicted LV end diastolic diameter (LVEDd)) and impaired LV systolic function (ejection fraction (EF) <35%) shown on detailed echocardiogram, after the exclusion of significant coronary artery disease, arterial hypertension, congenital heart disease, and primary heart valve disease – in line with the current ESC 2017 recommendations.[3] Moreover, patients were excluded from the study based on the presence of chronic liver insufficiency, neoplasms, peripheral atherosclerosis, as well as bone and joint diseases affecting collagen metabolism and circulating levels of procollagens. All patients had optimal HF therapy with regards to drug type and dosage, and stable HF symptoms (according to the New York Heart Association (NYHA) scale) for at least 2 weeks before inclusion in the study.

This study was approved by the Jagiellonian University Ethics Committee (protocol No. KBET/164/B/2014, date of approval: June 30, 2014). Written informed consent was obtained from all participants prior to inclusion in the study. All procedures performed were in accordance with the ethical standards of the institutional research committee and the 1964 Helsinki declaration and its later amendments, or comparable ethical standards.

Study design

All patients underwent detailed clinical assessment, laboratory tests, echocardiography, electrocardiography, cardiopulmonary exercise, and right ventricular endomyocardial biopsy at the baseline of the study. All subjects were also evaluated at the 3rd month of follow-up and underwent clinical and echocardiography examination.

Right ventricular biopsy

Endomyocardial biopsy procedures were performed by experienced operators via a femoral or jugular vein approach. Long, flexible biopsy forceps (7 French size) with small jaws (Cordis®; Johnson & Johnson Co, Miami Lakes, USA) were used. Up to 5 myocardial samples were obtained from the RV septum and then stored in formalin for light microscopic examinations of fibrosis (2–3 samples) or frozen in OCT-embedding medium and then stored at –80°C for further studies (2 samples; Fig. 1).

Laboratory measurements

At baseline, venous blood samples were drawn in a fasted state in the morning after 30 min of supine rest. All venous blood samples, after centrifuge, were stored at –20°C until assay. Before examination, small tissue samples from the right ventricular biopsy from the baseline were homogenized. Levels of circulating and tissue miRs were established with quantitative polymerase chain reaction

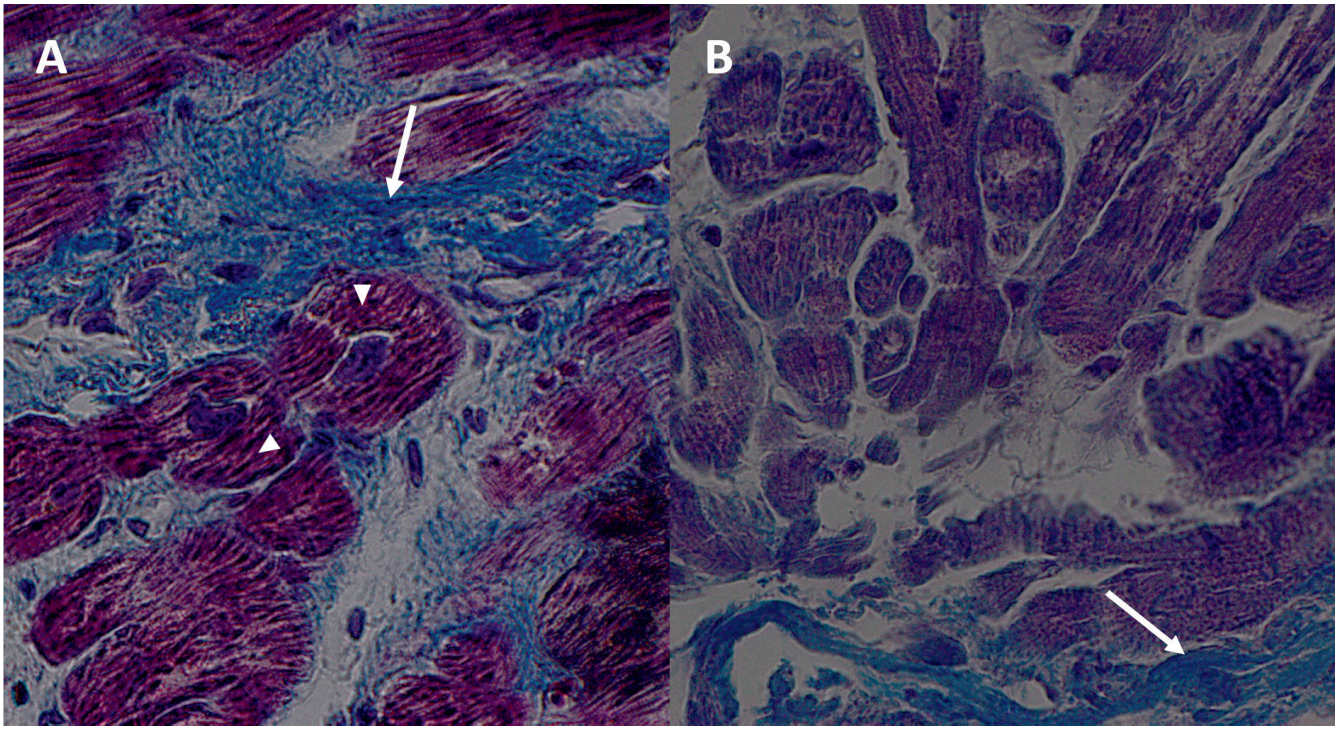

Fig. 1. Right ventricular bioptates. Biopsy specimens stained with collagen specific Masson's trichrome. Panel A – massive diffuse (interstitial) fibrosis (arrowheads). Compression of the adjacent myocytes (arrows). Panel B – widespread endocardial fibrosis (arrowheads)

(qPCR).[11] With a MirVana kit (Life Technologies, Carlsbad, USA) and according to the manufacturer's protocol, RNA was extracted from 100 µL of sample. Then, with a TaqMan Advanced MicroRNA cDNA Synthesis Kit (Life Technologies), reverse transcription was performed on 2 µL of extracted RNA. After ×10 dilution of the cDNA samples, the qPCR reaction was conducted with TaqMan Advanced MasterMix and TaqMan Advanced Assays on 384-well plates. The qPCR targeted hsa-miR-21-5p, hsa-miR-26a-5p, hsa-miR-29b-3p, hsa-miR-30a-3p, hsa-miR-30c-5p, and hsa-miR-133a-3p. Fifteen-microliter reactions were prepared with the Bravo pipetting station (Agilent Technologies, Santa Clara, USA) and the real-time qPCR reaction (RT-qPCR) was run and read on the CFX384 Real Time PCR Detection System (Bio-Rad Laboratories, Hercules, USA). We tested miR-423, selected on the basis of our previous experience with qPCR in plasma samples. Mean quantitation cycle (Cq) values were normalized to the geometric mean of hsa-miR-423, which was selected as a relatively stable control in pilot experiments. Data was expressed for each sample as ΔCq, which is the difference between the Cq value, the miRNA of interest and the geometric mean of miR-423 for a particular sample.

Echocardiography

All echocardiographic examinations were performed in accordance with the recent European and American recommendations.[12] Volumetric and EF measurements were performed in apical 4- and 2-chamber views, utilizing the method of disk summation – modified Simpson's rule. All echocardiographic parameters were calculated as the mean of 3 measurements for patients with sinus rhythm, and of 5 measurements for patients with atrial fibrillation. Chamber diameters, areas and volumes were normalized for body surface area (BSA). All examinations were made on commercially available equipment (Vivid 7 GE Medical System, Horten, Norway) with a phased-array of 1.5–4 MHz transducer by experienced echocardiographers.

Definition of LVRR

Left ventricle reverse remodeling was defined as an absolute increase in EF of minimum 10%, with a simultaneous decrease in LVEDd of at least 10% or an indexed LVEDd under 33 mm/m^2.[5] Left ventricular reverse remodeling was calculated between the baseline and 3rd month of follow-up measurements (Fig. 2).

Statistical analysis

Tissue and circulating levels of miRs are presented as median and quartiles, and other parameters as mean ± standard deviation (SD). All variables were tested for normal distribution of the data with the Shapiro–Wilk test. Comparisons of clinical and echocardiographic parameters between the LVRR-present and LVRR-absent groups were conducted with t-tests when normality was confirmed, or with a Mann–Whitney test for parameters without a normal distribution. The impact of circulating and

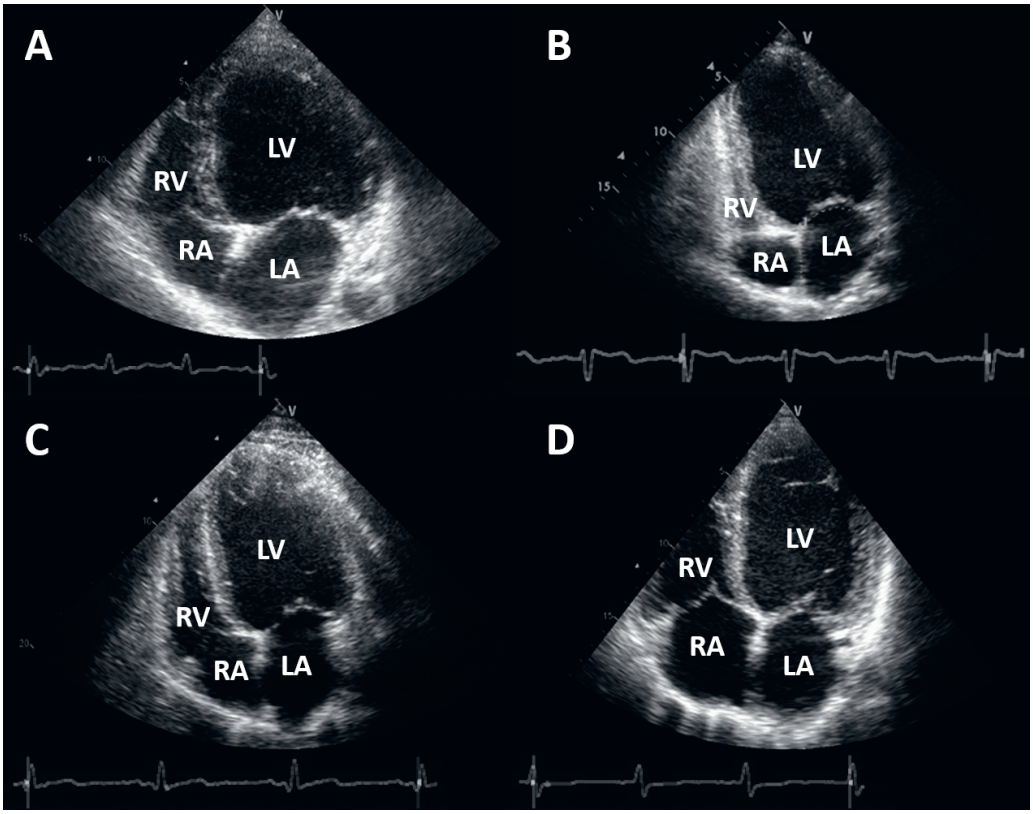

Fig. 2. Apical four-chamber echocardiographic projections. Panel A and B – patient with LVRR (A – at baseline echo, showing grossly enlarged, thin-walled LV with severely impaired LV systolic function, EF – 22%; B – substantial decrease of LV cavity size alongside improvement of LV systolic function, EF – 42%). Panel C and D – patient without LVRR (C – baseline echo; D – lack of improvement of LV size and systolic function)

LV – left ventricle; LA – left atrium; RV – right ventricle; RA – right atrium; LVRR – left ventricular reverse remodeling.

myocardial tissue miRs on the probability of LVRR was analyzed with uni- and multivariate logistic regression methods. Areas under the receiver operating characteristics (ROC) curves (areas under the curve (AUC)) were used to establish the most optimal cut-off values of the analyzed parameters and their diagnostic accuracy. For regression analysis, log10 of the NT-proBNP level was used. All results were considered statistically significant when p-value was <0.05. The STATISTICA package v. 13.0 (StatSoft, Inc., Tulsa, USA) was used for the statistical analysis.

Results

Baseline characteristics

Sixty-three patients with complete data were divided according to the presence of LVRR: an LVRR-present group (n = 32, 51%) and an LVRR-absent group (n = 31, 49%). Compared with the LVRR-absent individuals, LVRR-present patients were characterized by a shorter duration of HF symptoms ($p < 0.01$), a smaller LV dimension (LVEDd, $p < 0.001$) and volume (LVEDvol, $p < 0.001$), as well as lower serum levels of troponin ($p < 0.05$) and NT-proBNP ($p < 0.01$) (Table 1).

Comparison of miRNA plasma and tissue levels

Expression of all circulating miRs were similar in both groups. However, myocardial expression of miR-133 was significantly higher in patients with LVRR ($p < 0.01$) (Table 2).

The predictive value of clinical, echocardiographic and laboratory parameters for LVRR

All parameters, including symptom duration time, LVEDvol, troponin, NT-proBNP and myocardial miR-133a, that differentiated LVRR-present and LVRR-absent were found to be LVRR predictors in ROC analyses and univariate regression. The respective results are presented in the Table 3 and Fig. 3A–E. Finally, only LVEDvol and miR-133 were found to be significant predictors in multivariate analysis, adjusted for symptom duration time, and troponin (hs-TnT) and NT-proBNP levels (Table 4). The combined model was found to be superior compared to models with only a single variable (AUC = 0.93, 95% CI = 0.85–1.00; specificity 85% and sensitivity 87.5%) (Fig. 3F).

Table 1. Baseline characteristics of the study population, divided into groups with and without LVRR

Parameter	LVRR-present group (n = 32, 51%)	LVRR-absent group (n = 31, 49%)	p-value
Age [years]	48.9 ±9.9	46.9 ±9.9	0.53
Sex [male]	29 (90.6%)	28 (90.3%)	0.97
BMI [kg/m^2]	28.3 ±4.8	25.9 ±5.5	0.07
NYHA class	2.56 ±0.84	2.48 ±0.57	0.74
HF symptoms' duration [ms]	14.7 ±29.9	35.8 ±40.8	0.005
QRS [ms]	102.8 ±30.0	114.5 ±36.0	0.24
LVEDd/BSA [mm/m^2]	31.7 ±4.1	39.5 ±6.6	0.02
LVEDvol/BSA [mL/m^2]	101.5 ±36.3	151.6 ±72.0	0.02
EF [%]	25.8 ±7.6	22.3 ±6.7	0.07
LAA [mm]	31.1 ±8.3	30.4 ±9.2	0.77
PA mean [mm Hg]	21.0 ±9.3	24.2 ±12.2	0.33
VO_2peak [mL/kg/min]	15.7 ±4.5	16.4 ±7.0	0.78
Hb [g/dL] (14.0–18.0)	14.8 ±1.6	14.3 ±1.4	0.21
CK-MB [U/L] (UNL < 24)	12.9 ±4.9	13.5 ±5.5	0.52
hs-TnT [ng/mL] (UNL < 0.014)	0.016 ±0.01	0.028 ±0.02	0.02
hs-CRP [mg/dL] (UNL < 3.0)	11.9 ±29.2	7.7 ±18.9	0.07
NT-proBNP [pg/mL] (UNL < 125)	2,606 ±5,041	4,242 ±6,268	0.009
TGF-β [pg/mL] (4,639–1,4757)	2,867 ±1,472	2,358 ±975	0.22
CTGF [ng/mL] (2.3–42.5)	5.63 ±4.56	4.68 ±2.96	0.55
Galectin-3 [ng/mL] (8.6–10.9)	13.8 ±4.49	14.4 ±4.20	0.55
CRT	5 (15.6%)	15 (48%)	0.005
β-blocker	31 (96.9%)	31 (100%)	0.32
ACE-I	31 (96.9%)	29 (93.5%)	0.54
MRA	31 (96.9%)	28 (93.1%)	0.29
Furosemide	19 (59.4%)	19 (61.3%)	0.88

Data is presented as mean ±SD or n (%). BMI – body mass index; NYHA – New York Heart Association class; LVEDd/BSA – indexed LV end-diastolic diameter; LVEDvol/BSA – indexed LV end-diastolic volume; EF – ejection fraction; LAA – left atrium area; PA mean – mean pulmonary artery pressure; VO_2peak – peak oxygen uptake; Hb – hemoglobin, CK-MB – myocardial fraction of creatine kinase; hs-TnT – high sensitivity troponin T; hs-CRP – high sensitivity C-reactive protein; NT-proBNP – amino-terminal pro B-type natriuretic peptide; TGF-β – transforming growth factor β; CTGF – connective tissue growth factor; CRT – cardiac resynchronization therapy; ACE-I – angiotensin converting enzyme inhibitor; MRA – mineralocorticoid receptor antagonist; UNL – upper normal limit.

Table 2. Comparison of plasma and cardiac tissue levels of selected microRNAs in patients with and without LVRR

microRNA	LVRR-present group (n = 32)	LVRR-absent group (n = 31)	p-value
miR-21 [ΔCq]	0.19 (–0.12–0.50)	0.02 (–0.41–0.77)	0.77
miR-26 [ΔCq]	–0.25 (–0.62–0.14)	0.33 (–0.64–0.94)	0.12
miR-29 [ΔCq]	2.87 (2.49–3.18)	2.72 (2.40–3.08)	0.81
miR-30a3 [ΔCq]	7.98 (6.73–8.78)	7.77 (6.82–9.43)	0.86
miR-30c5 [ΔCq]	4.31 (3.86–4.81)	4.03 (3.73–4.82)	0.65
miR-133a [ΔCq]	6.99 (6.31–8.94)	7.60 (5.73–9.07)	0.59
miR-423 [ΔCq]	–0.25 (–0.62–0.14)	0.33 (–0.64–0.94)	0.12
Tissue miR-21 [ΔCq]	1.33 (0.73–1.92)	1.01 (0.40–1.47)	0.09
Tissue miR-26 [ΔCq]	–1.33 (–1.77––0.92)	–1.41 (–1.72––0.97)	0.54
Tissue miR-29a [ΔCq]	1.49 (0.87–1.91)	1.16 (0.64–1.51)	0.17
Tissue miR-29b [ΔCq]	1.38 (0.72–2.07)	1.07 (0.65–1.63)	0.21
Tissue miR-30c [ΔCq]	–0.51 (–1.01–0.19)	–0.73 (–1.03––0.18)	0.26
Tissue miR-133a [ΔCq]	1.22 (0.47–1.90)	0.61 (0.25–0.99)	**0.006**

Data is presented as median and 95% confidence interval (95% CI).

Table 3. Odds ratios (OR) and ROC analysis for LVRR prediction from univariate logistic regression analysis

Parameter	Univariate logistic regression		ROC analysis		Cut-off value (specificity, sensitivity)
	OR (95% CI)	p-value	AUC (95% CI)	p-value	
Duration of disease [months]	0.98 (0.97–0.99)	0.049	0.70 (0.57–0.83)	0.002	3 (68%, 63%)
LVEDvol [mL/m^2]	0.993 (0.987–0.999)	0.02	0.68 (0.55–0.82)	0.009	187 (84%, 50%)
hs-TnT [ng/mL]	<0.0001 (0–0.022)	0.03	0.69 (0.55–0.83)	0.007	0.011 (79%, 50%)
NT-proBNP [pg/mL]	0.29 (0.11–0.78)	0.02	0.70 (0.56–0.83)	0.005	826 (90%, 48%)
miR-133a [ΔCq]	2.81 (1.23–6.40)	0.015	0.71 (0.56–0.85)	0.003	1.016 (77%, 63%)

Duration of disease, LVED volume (LVEDvol), troponin (hs-TnT), NT-proBNP and myocardial miR-133a levels as LVRR predictors in univariate logistic regression

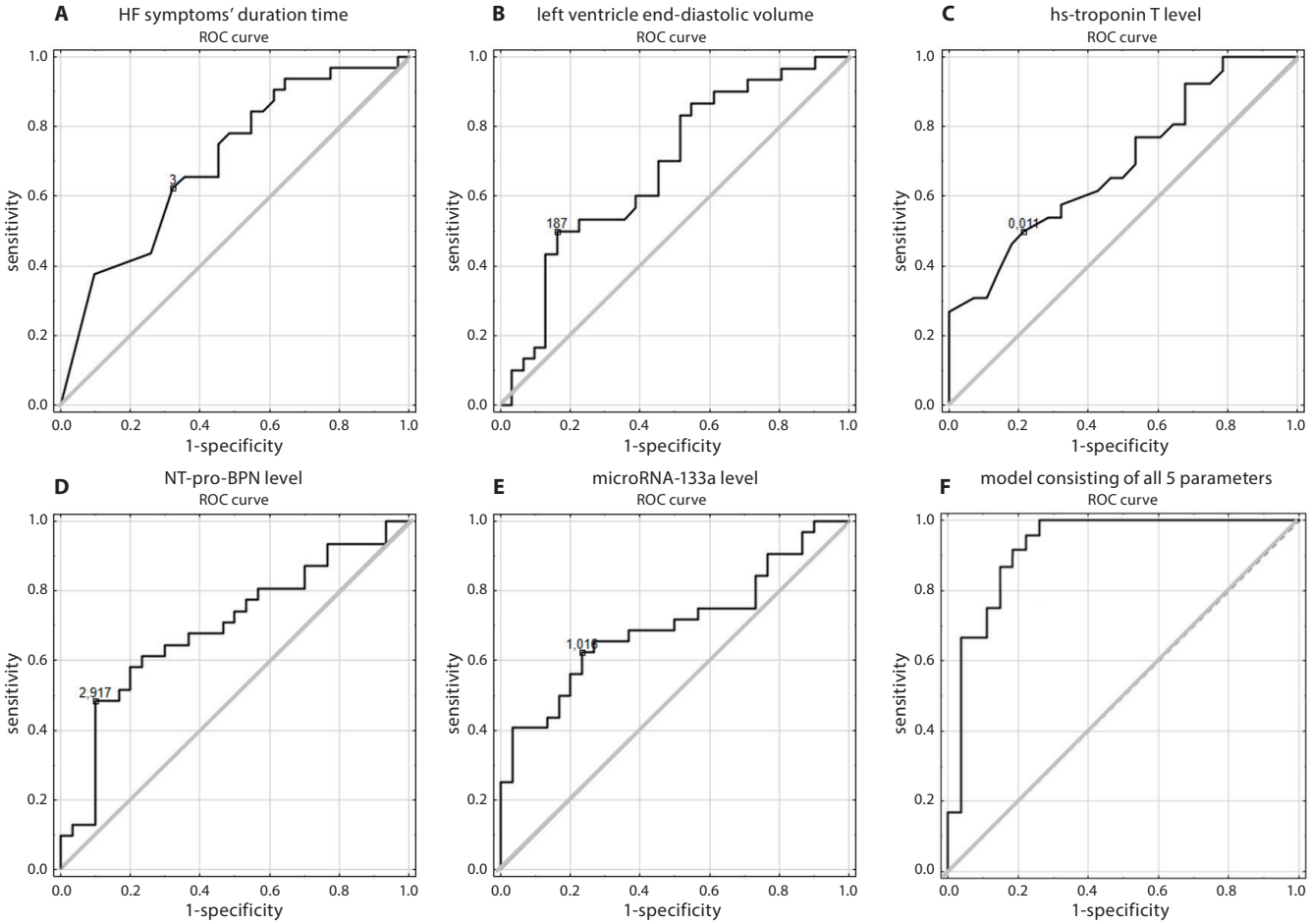

Fig. 3. ROC curves for LVRR prediction based on: HF symptom duration (A), LVED volume (B), troponin (C), NT-proBNP (D), and miR-133a levels (E), and ROC curve based on a model comprised of all parameters (F)

ROC – receiver operating characteristics; LVRR – left ventricular reverse remodeling; HF – heart failure.

Table 4. The predictive value of duration of disease, LVEDvol, hs-TnT, NT-proBNP, and myocardial miR-133a for LVRR prediction – multivariate logistic regression analysis

Parameters	OR (95% CI)	p-value
Duration of disease	0.97 (0.94–1.01)	0.09
LVEDvol	0.99 (0.98–1.00)	0.03
hs-TnT	<0.0001 (1*10^{-7}–4*10^{12})	0.22
NT-proBNP	0.25 (0.03–1.98)	0.19
miR-133a	5.20 (1.13–24.05)	0.03

Discussion

Study findings

The main findings of the study can be summarized as follows: firstly, LVRR was present in half of DCM patients. Secondly, of all the circulating and myocardial miRs under study, myocardial miR-133a alone differentiated LVRR-present and LVRR-absent groups. Thirdly,

myocardial miR-133a was found to be a significant LVRR predictor. The multivariate regression model, including HF symptom duration, myocardial mir-133a, LVEDvol, troponin and NT-proBNP levels were found to be highly accurate for LVRR prediction in DCM.

Predictors of left ventricle reverse remodeling in DCM

Left ventricular reverse remodeling is of paramount importance in DCM. The rate of LVRR differs between studies and depends on many factors, such as the definition of LVRR, time of assessment, management strategy, etc. In our cohort, LVRR was present in half of the patients, which is in line with the literature.[5,13] The division of DCM patients by the presence of reverse remodeling is dictated by its significance in prognosis and strong correlation with survival in DCM.[5,14] Improvement in myocardial function can occur spontaneously and in response to HF therapy (drugs, cardiac devices).[15,16] In our group, nearly 100% of patients were receiving adequate therapy as recommended by the ESC (95% were on β-blockers, ACE-I and MRAs, and nearly 1/3 had received cardiac resynchronization therapy (CRT)).[1,13]

The topic of LVRR has been intensively investigated and several LVRR independent predictors have been identified such as: longer symptom duration and higher NYHA class, larger LVEDd and LVEDvol and left atrium (LA) size, higher troponin and NT-proBNP levels, late gadolinium enhancement in magnetic resonance, left bundle branch block and longer QRS duration, and higher grade of mitral regurgitation.[5,15,17] In our analysis, symptom duration, LVED volume, and troponin and NT-proBNP levels were significant LVRR predictors in DCM patients.

Circulating and cardiac tissue miRs in cardiac remodeling and reverse remodeling

Certain miRs under study, including miR-21, -26, -29, -30, -33a, -133a, and -423, are known to be related to myocardial fibrosis, which leads to cardiac remodeling.[9,18] Previously, we reported associations between circulating miR-26 and miR-30, and collagen volume fraction, which is a quantitative expression of myocardial fibrosis in DCM.[10] However, in this study, we found no connection between miR-26 or -30 and reverse remodeling.

Little is known about the role of miRs in cardiac reverse remodeling and LVRR, especially in DCM. Recently, it was shown that circulating miR-208a and miR-29a, which regulate hypertrophy, fibrosis and inflammation, were associated with LVRR.[19,20] Furthermore, circulating miR-16, miR-27a, miR-101, and miR-150 were related to cardiac function improvement after myocardial infarction. In another study, higher expression of circulating miR-208, -208b and -499 were found in DCM patients when compared with healthy controls.[21–23] Both Sucharov et al. and Shah et al. have recently reported dynamic changes in miRNA expression during LVRR, including an increase in miR-21, especially in response to β-blockers.[24,25] However, we did not confirm any differences in miR-21 levels between DCM patients with and without LVRR. Moreover, Szczerba et al. found a significant decrease of miRs during pregnancy as protection from myocardial fibrosis during volume overload in pregnancy.[26]

Out of all the circulating and myocardial miRs under study here, we report for the first time the association between myocardial miR-133a and LVRR. Of note, none of the other circulating and tissue miRs, including circulating miR-133a, were related to LVRR. Based on previous laboratory studies, miR-133a has proven to have an important role in heart physiology and pathology, including cardiac development, hypertrophy, remodeling, and fibrosis.[27–30] In a murine HF model, miR-133a was found to play an important role in the hypertrophic pathway. Interestingly, decreased levels of miR-133a cardiac tissue expression were linked with adverse cardiac remodeling, whereas increased miR-133a expression was related to a reduction of cardiac fibrosis and favorable ECM remodeling.[31–33] Moreover, miR-133a was found to protect myocardial tissue and decrease apoptosis of cardiomyocytes after myocardial infarction.[34] On the other hand, in recent studies, we did not observe any relations between either circulating or myocardial miR-133a with fibrosis, expressed either qualitatively or quantitatively.[6] This is in line with the findings from other authors, who reported a lack of straightforward relations between particular miRs, including miR-133a, and cardiac fibrosis.[33,35]

Furthermore, several authors have reported close links between miR-133a and outcomes in various cardiac conditions. Circulating miR-133a was found to be a marker in the prediction of all-cause mortality and major adverse cardiovascular events in patients with coronary artery disease.[22,36–39] Moreover, Besler et al. showed a connection between myocardial miR-133 levels and clinical outcomes in inflammatory cardiomyopathy.[32] Widera et al. and Keller et al. showed an improvement in risk stratification with a panel consisting of circulating miRs, including miR-133, outperforming troponin level.[37,40] In line with this, we have recently reported that myocardial miR-133a was an independent predictor of survival in DCM.[6] Bearing this in mind, the present observation that myocardial miR-133a is independently associated with LVRR further reinforces the aforementioned findings that indicate that LVRR is one of the strongest factors influencing outcomes.

Study limitations

There are several study limitations which we would like to comment on. The study population is relatively small, but, on the other hand, the utilization of biopsy and tissue qRT-PCR to study miRs makes our study one of the largest

in the field. Another limitation is the utilization of qPCR, which allows one to measure only selected miRs, whereas next generation sequencing enables identification of virtually all mRs. Thus, we concentrated on only a few miRs which were previously linked with fibrosis, and by doing so excluded hundreds of other miRs that may also have a role in fibrosis and LVRR.

Conclusions

Among circulating and tissue miRs: miR-21, -26, -29, -30, -133a, and -423, only myocardial miR-133a expression was increased in patients with LVRR. Moreover, myocardial miR-133a was found to be an independent predictor of LVRR, and has been previously reported as a mortality predictor in DCM. This may indicate that miR-133a has a potential role in the reverse remodeling process in DCM and its potential role in anticipating LVRR in DCM. This might be further exploited by miR-133a expression level restoration with miRNA mimics.

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Ubiquitin-specific protease 22 is associated with poor prognosis in neuroblastoma

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Abstract

Background. Ubiquitin-specific protease 22 (USP22) alters histone ubiquitination and is considered to be an oncogenic factor involved in tumor progression. The USP22 aberrance has been implicated in several malignancies, but whether USP22 plays a role in neuroblastoma (NB) remains unclear. To the best of our knowledge, the clinicopathological significance of USP22 expression in NB has not been previously reported in the English-language medical literature.

Objectives. The aim of this study was to investigate the role of USP22 and its association with potential targets in patients with NB.

Material and methods. The potential clinicopathological significance of USP22 expression in NB was studied using immunohistochemistry, immunohistochemical staining assessment and statistical analyses.

Results. Based on the immunohistochemical analysis, the USP22 protein was detected more manifestly in NB tissues than in healthy peritumoral tissue. Furthermore, an association between USP22, lymph node metastasis and NB clinical stage was observed, whereby the level of USP22 protein was higher in stage III–IV specimens than in stage I–II specimens ($p < 0.05$). Furthermore, tumors expressing USP22 were associated with poorer patient prognosis than the USP22-negative tumors. The multivariate Cox regression analysis suggested that the level of USP22 protein is a predictive factor for survival ($p < 0.05$).

Conclusions. Our results indicate a significant association between USP22 level and poor prognosis in NB. Thus, USP22 represents a valuable biomarker for predicting the outcome of patients with NB.

Key words: neuroblastoma, ubiquitin-specific protease 22, poor prognostic

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Introduction

Neuroblastoma (NB) is a rare type of cancer that develops in children and infants at an annual incidence rate of between 6 and 10 per million.[1] Originating in primitive precursors of the sympathetic nervous system, NB is mostly observed in the adrenal medulla and sympathetic nerve ganglia.[2] In addition, NB is characterized by rapid growth and shows a strong capacity for invasion and metastasis, which often cause the advanced expansion and outward invasion of the tumor into neighboring tissues. Despite substantial progress in the treatment of NB, including surgical techniques and chemotherapy, the 5-year survival rate remains low, especially in patients in clinical stages III and IV.[3] Thus, identifying new biomarkers for the effective diagnosis of NB and predicting its therapeutic outcome is highly valuable.

Ubiquitin-specific protease 22 (USP22) is an element of the 11-Polycomb/cancer stem cell signature that is involved in the alteration of histone ubiquitination, which makes it a valuable marker for predicting the therapeutic response of individual patients with cancer.[4] Elevated USP22 expression is related to a poor prognosis in colorectal, gastric, liver, lung, and breast cancers.[5–9] USP22, as a component of the human Spt-Ada-Gcn5-acetyltransferase (hSAGA) complex, is at the core of several physiological and pathological processes and is directly implicated in cell-cycle progression and transcriptional regulation.[10] As a multi-subunit complex, hSAGA is organized into several functional submodules, including the deubiquitinating module (DUB), the histone acetyltransferase (HAT) module, and the suppressor of Ty (SPT) and TATA-binding protein-associated factor (TAF) modules. The USP22 is the essential protein linked to the DUB module. It modifies histones H2A and H2B, which facilitate a variety of cellular events, including gene regulation. Therefore, upregulation of USP22 expression will lead to abnormal activation of multiple pathways to promote cell survival, while downregulation of USP22 expression can induce cell cycle arrest at the G0/G1 phase in different types of cancer cells. More specifically, USP22 modulates growth and oncogenic transformation by regulating transcription factors, such as BMI-1, c-MYC, p53, TRF1, and SIRT1.[11–16] These findings provide evidence that USP22 is vital in tumor progression and advancement; thus, it is a valuable target as an NB marker.

Nevertheless, no reports have been published on the clinicopathological consequences of USP22 in NB. Therefore, this study aimed to evaluate USP22 expression in tissues from representative patients with NB using an immunohistochemical analysis, and to determine whether there is any association between USP22 and NB clinicopathology, including prognosis.

Material and methods

Patients

A total of 68 specimens were acquired from patients (29 boys and 39 girls) at Harbin Children's Hospital, China, between March 2005 and August 2013. The age of the patients ranged from 36 days to 11 years (median age: 4.2 years). A histological analysis established that 48 patients (70.5%) had lymph node metastases. The pathological diagnosis indicated that all patients had NB, while none had received any treatment prior to diagnosis. The tumor stages were assessed based on the International Neuroblastoma Staging System (INSS). The pathological data were retrieved from the patients' medical histories, and the follow-up records were acquired by phone interviews and outpatient clinical databases. The survival analysis was performed by postoperatively examining the patients with NB periodically until September 2016 or until death (median follow-up time: 52 months). In the histological analysis, the negative control samples consisted of tissue from the same tissue slides, 1 cm from the tumors. It was necessary to obtain a broad area because it would otherwise be difficult to find enough controls. Thus, the adjacent tissue from the patients served as the negative control samples. The Ethical Committee of Harbin Children's Hospital approved our retrospective study, and written informed consent was provided for each patient's participation from their parents or legal guardians.

Immunohistochemistry

The USP22 immunohistochemical analysis was performed based on previously published methods.[15] Briefly, the tissues were post-fixed with formalin, embedded in paraffin and cut into 4-micrometer sections. Subsequently, the sections were deparaffinized in xylene, rehydrated in ethanol solutions of descending concentration and submerged in ethylenediaminetetraacetic acid (EDTA; pH 8). Antigenicity was retrieved by autoclaving the sections at 121°C for 5 min. After quenching the endogenous peroxidase in 3% H_2O_2 for 15 min and washing with phosphate-buffered saline (PBS), the sections were incubated with a primary antibody against USP22 (1:200, ab4812; Abcam, Cambridge, UK) overnight at 4°C. A 30-minute peroxidase-conjugated streptavidin incubation and diaminobenzidine incubation were subsequently performed, followed by counterstaining with a commercially available hematoxylin to stain the nuclei (H9627; Sigma-Aldrich, St. Louis, USA).

Immunohistochemical staining assessment

The immunolabeled sections were evaluated independently under a light microscope (Olympus CX41; Olympus, Tokyo, Japan) by 2 experienced pathologists who were

blinded to the patients' outcomes. A total of 200 cells from 5 randomly selected areas per section were evaluated to examine the expression of USP22 (a semi-quantitative assessment), and the percentage of USP22-positive cells was subsequently estimated. In general, USP22 was mainly detected in the nuclei. The extent and intensity of the immunolabeling were estimated. The extent of the staining was evaluated on a 5-point scale, where 0 indicated no positive cells, 1 indicated ≤25% positive cells, 2 indicated 26–50%, 3 indicated 51–75%, and 4 indicated ≥76% positive cells. Similarly, the intensity was scored using a 5-point scale as follows: 0 (no staining), 1 (very weak), 2 (weak), 3 (moderate), and 4 (strong). The final score was determined by combining the proportion and intensity scores, and the results were categorized as follows: 0–1 (negative), 1–3 (low) and ≥4 (high).

Statistical analyses

We used SPSS v. 12.0 software package (SPSS Inc. Chicago, USA) for all the statistical analyses, with the significance level set at $p < 0.05$. To compare the clinical features according to USP22 expression, χ^2 test was used, while the Kaplan–Meier method and a log-rank test were used to measure overall survival.

Results

Expression of USP22 in NB tissues

To evaluate the contribution of USP22 to oncogenesis and tumor progression/growth, we examined its expression level in 68 NB specimens from patients between the age of 36 days and 11 years. Based on our results, 61.76% of the analyzed samples were positive for USP22. Strong USP22 staining was detected mainly within the nuclei, while only weak cytoplasmic staining was observed. Furthermore, no obvious expression of USP22 was detected in the tumor-adjacent tissues (Fig. 1).

Association between USP22 and NB clinical hallmarks

Table 1 summarizes the comparisons of the results between the USP22 immunoreactivity and NB clinicopathological parameters. Based on these results, positive correlations were observed between the expression of USP22 and lymph node metastasis and the NB clinical stage (both $p < 0.05$). In particular, USP22 was more enriched in patients at stages III–IV than stages I–II ($p < 0.05$). However, the results indicated no significant correlation between USP22 expression and age, sex or tumor size and site. In addition, when the study population was divided into 2 groups by age (<2 years and ≥2 years, instead of 3 years as the group cut-off age), the results also indicated that there was no significant association between USP22 and age.

Expression level of USP22 is correlated with a poor disease prognosis

We assessed the overall survival rate in our patients based on the USP22 expression level. The median overall survival for all 68 specimens was 57.5 ±2.1 months, while the 5-year survival rate was 32.5% ±1.9%. However, the survival rate was 23.6% ±2.1% in the patients with

Table 1. Relationship between USP22 expression and the clinicopathological features of NB

Variables	Number of patients (n = 68)	USP22 expression			Positive rate (%)	χ^2	p-value
		high	low	negative			
Gender							
male	29	12	5	12	58.6	0.38	>0.05
female	39	14	11	14	64.1		
Age							
≥3	30	12	6	12	60.0	0.29	>0.05
<3	38	14	10	14	63.2		
Tumor size							
≥5 cm	37	14	8	15	59.4	0.11	>0.05
<5 cm	31	12	8	11	64.5		
Tumor site							
adrenal gland	35	13	9	13	62.9	0.57	>0.05
retroperitoneal	28	11	6	11	60.7		
neck	3	1	1	1	66.7		
thoracic and mediastinal	2	1	0	1	50.0		
Clinical stage							
high stage group (stage III–IV)	41	25	8	8	80.5	6.73	<0.05
low stage group (stage I–II)	27	2	9	16	40.7		
Lymph node metastasis							
absent	20	2	5	13	35.0	8.27	<0.05
present	48	24	11	13	72.9		

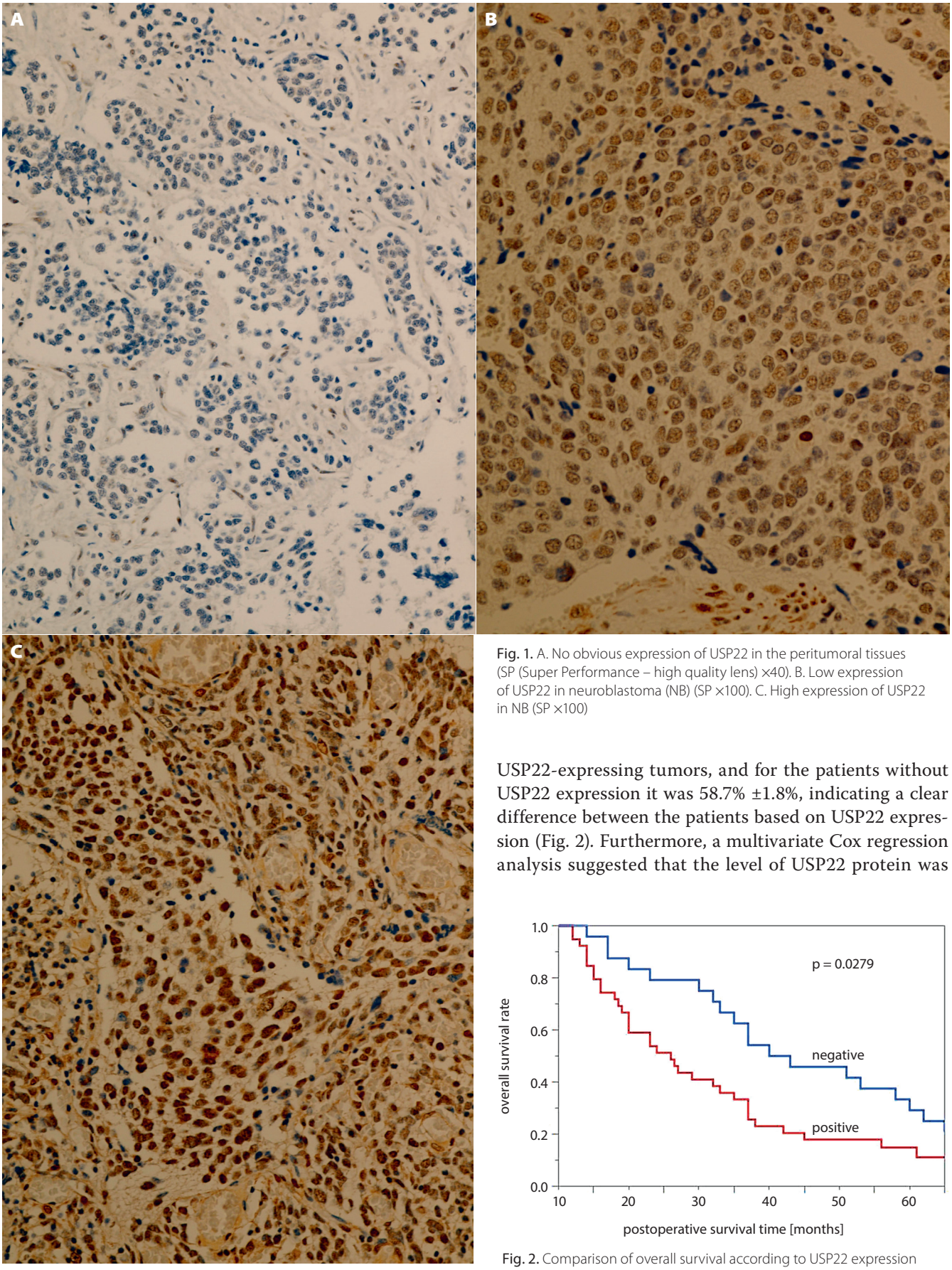

Fig. 1. A. No obvious expression of USP22 in the peritumoral tissues (SP (Super Performance – high quality lens) ×40). B. Low expression of USP22 in neuroblastoma (NB) (SP ×100). C. High expression of USP22 in NB (SP ×100)

USP22-expressing tumors, and for the patients without USP22 expression it was 58.7% ±1.8%, indicating a clear difference between the patients based on USP22 expression (Fig. 2). Furthermore, a multivariate Cox regression analysis suggested that the level of USP22 protein was

Fig. 2. Comparison of overall survival according to USP22 expression

a predictive factor for survival ($p < 0.05$). These findings demonstrated that detecting USP22 in patients with NB is a valuable biomarker for poor disease prognosis.

Discussion

One of the most common pediatric extracranial tumors is NB, which originates from the precursor cells of the sympathetic nervous system. The clinical presentation of NB varies greatly between patients, with some tumors undergoing spontaneous remission/regression, and others displaying malignant behavior. The rapid tumor progression, high degree of malignancy, metastasis and drug resistance, and its association with a poor prognosis are all factors which contribute to the challenges in treating NB. Although significant progress has been achieved in NB therapeutic strategies, 5-year event-free survival remains limited, with a rate of below 31% in children with high-risk NB.[17] Thus, in determining new biomarkers for the development of personalized therapies, the elucidation of the mechanisms underlying metastasis and therapy resistance and the prediction of the disease prognosis are fundamental to improving the therapeutic outcomes of NB.

Although USP22 is considered a promising biomarker for NB diagnosis and prognosis,[5–9,18–20] little is known about its effect in NB. Indeed, it is involved in the effective prediction of the response to therapy in individual patients with cancer. Furthermore, it is demonstrated to be centrally implicated in the pathogenesis of epithelial cancers and other solid tumors.

It is well-known that USP22, as part of hSAGA, deubiquitinates H2A and H2B, thus editing the histone code.[21] However, the mechanism by which USP22 is implicated in cancer progression and metastasis is unclear. Liu et al.[21] demonstrated an oncogenic role of USP22 via the Polycomb complex protein BMI-1-mediated INK4a/ARF pathway and the protein kinase B (Akt) pathway. Furthermore, the alteration of the transcriptional regulator far upstream element binding protein 1 (FBP1) ubiquitination has also been proposed as a mechanism by which USP22 modulates tumorigenesis.[22] Another critical mechanism contributing to the invasion and metastasis of various cancers is the epithelial–mesenchymal transition.[23] Yang et al. demonstrated the implication of USP22 in spinocerebellar ataxia and its progression.[24] Additional research is warranted to elucidate the connection between these USP22 functions and other chromatin-modifying systems in order to provide more insights into chromatin remodeling and USP22 gene activation.

To evaluate whether or not USP22 activation is implicated in NB initiation and/or progression, we acquired tissues from representative patients. Our results indicated a significantly increased expression of USP22 in the NB tissues compared with the peritumoral tissues, with approx. 61.76% of the NB samples tested positive for USP22. Furthermore, our findings suggested a significant correlation between USP22 and clinicopathological parameters, including INSS stage and lymph node metastasis.

As evidenced by the Kaplan–Meier survival analysis, the patients with USP22-positive tumors displayed a worse overall survival rate than the patients with USP22-negative tumors. Thus, the increased expression of USP22 may be valuable in predicting poor prognosis, while suppressing USP22 may be a potential target for therapeutic intervention in NB. Furthermore – given that it might not just be the pure presence or absence of USP22 that is indicative – further, broader studies should examine the results as they correlate to the intensity or proportion of USP22 expression in the patients.

To our knowledge, this is the first study to indicate a specifically increased expression of USP22 in clinical NB samples and positive correlations between USP22 expression and NB progression and poor prognosis. These findings support USP22 as a potential candidate for identifying patients with NB with a poor prognosis and as a predictor of their therapeutic outcomes. However, this study had several limitations, including the small study population and short follow-up period. Therefore, further studies with a larger sample size and an extended follow-up period are still required in order to confirm presented observations.

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Can laparoscopic cystectomy become the method of choice in the treatment of invasive urothelial urinary bladder cancer?

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Abstract

Background. Radical cystectomy with pelvic lymphadenectomy is the method of choice for muscle-invasive urothelial cell cancer (UCC) treatment and provides the best cancer-specific survival. It can be performed as an open radical cystectomy (ORC), laparoscopic radical cystectomy (LRC) or robot-assisted surgery (RARC).

Objectives. The aim of this study was to compare laparoscopic and open radical cystectomy in terms of perioperative and oncological results.

Material and methods. This retrospective study included 260 patients who underwent surgery due to invasive bladder cancer. A laparoscopic radical cystectomy (LRC) was performed on 131 patients and an open radical cystectomy (ORC) on 129 patients. Group was stratified according to the urinary diversion. Oncologic results expressed as perioperative variables were analyzed, adjusted to the type of urinary diversion.

Results. The LRC patients were in worse perioperative condition according to the American Society of Anesthesiologists (ASA) score than the ORC group (3.1 and 2.52, respectively; $p = 0.001$). The serum protein level was significantly lower in the ORC group, with no difference in body mass index (BMI) between the groups. The median operation time was significantly shorter in the LRC group with ileal conduit and uretero-cutaneostomy than in the same groups operated using open approach (252.5 min and 180 min vs 290 min and 225 min, respectively), as was the hospital discharge time (8.18 days and 11.63 days, respectively; $p = 0.004$). In both LRC groups, median blood loss was lower, compared with corresponding ORC groups (325 mL and 400 ml vs 800 mL and 1,100 mL, respectively; $p < 0.001$ in both cases). The level of complications was significantly lower in both LRC groups than in the ORC groups ($p < 0.001$ and $p = 0.001$, respectively). The lymph node yield was 12 in the LRC group and 10 in the ORC group. The LRC group had a lower positive surgical margins ratio.

Conclusions. The laparoscopic approach should be a valid option for radical cystectomy, given the fewer complications, smaller blood loss, and shorter operating and hospitalization times experienced by patients who underwent a laparoscopic cystectomy.

Key words: laparoscopy, cystectomy, urinary bladder neoplasms, cystectomy methods

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Introduction

Urothelial cell cancer (UCC) is one of most common neoplasms that invade the genitourinary tract.[1] There are 380,000 new cases each year worldwide and more than 150,000 deaths per year.[2] Radical cystectomy (RC) with pelvic lymphadenectomy, together with neoadjuvant or adjuvant chemotherapy for advanced cases, is the method of choice for muscle-invasive UCC and provides the best cancer-specific survival.[3] After a cystectomy, the 10-year recurrence-free survival rate is 50–59%, and the overall survival rate is around 45%.[2,4] Radical cystectomy can be performed as an open surgery (ORC), a laparoscopic surgery (LRC) or a robot-assisted surgery (RARC).

Open radical cystectomy can be associated with a clinically significant number of perioperative complications and a prolonged recovery time.[5] Since it is a highly morbid procedure, a minimally invasive approach was proposed.[6] Laparoscopic radical cystectomy was introduced in 1993 by de Badajoz et al.[7] who performed it for the first time for an oncologic indication. This method reduces blood loss, analgesic consumption and postoperative complications; it also allows the patient to recover bowel function earlier and to return to normal activity.[8] On the other hand, LRC has a long learning curve and requires a large amount of effort for it to become an established method. More recently, Menon et al. reported the first series of RARC, a procedure that has since gained wide popularity.[9] A recently published meta-analysis showed an even significantly lower rate of complications 30 days and 90 days after RARC than ORC. When using this minimally invasive surgical approach, the number of complications was significantly lower in groups with grade 4 and 5 complications. The RARC group had a longer operating time but the advantages were lower blood loss and a shorter hospital stay than the ORC group.[10]

The RARC is a demanding procedure and has a long learning curve. It is associated with high costs, which secondary or tertiary urological centers cannot afford. On the other hand, the equipment required for LRC is less expensive and can be afforded by most hospitals worldwide.[10] The benefits and lower costs associated with LRC suggest that it is a valid alternative that should be developed, despite the existence of a more modern robotic approach.

There are not many head-to-head trials comparing LRC and ORC. Since it is more widely used,[11] it seems that LRC is slowly becoming an acceptable alternative to ORC. In this study, we explored the safety and efficacy of LRC compared to ORC in terms of perioperative and oncological results.

Material and methods

In this retrospective study, we analyzed the data from 260 consecutive patients from 2 institutions who received RC between 2012 and 2016. Of these, 129 received an ORC and 131 received an LRC. The choice of approach (open or laparoscopic) was based on surgeons' choice and their confidence in laparoscopic surgery. The surgical procedure included the removal of the prostate gland in male patients and the reproductive system in female patients. The obturator, external, internal, common iliac, and presacral lymph nodes were dissected for pathological analysis according to the procedures described in *Campbell–Walsh Urology*.[12] Indications for cystectomy were, according to the guidelines from the European Association of Urology (EAU), urothelial cT2N0M0-cT4aN0M0 disease or noninvasive papillary cancer that could not be controlled with transurethral resection.

This research project was carried out according to the Declaration of Helsinki.

All patients underwent a preoperative examination, which included routine laboratory tests, a chest radiogram, an abdominal ultrasonography (USG), and a computed tomography (CT) scan or magnetic resonance imaging (MRI). Age, gender, body mass index (BMI), comorbidities, surgical history, and laboratory test results were collected. The operating time (defined as the duration of anesthesia), estimated blood loss and transfusion rates were also collected. Anesthesia risk was assessed and scored according to the American Association of Anesthesiology (ASA) Physical Status Classification System. The nutritional status of patients was assessed using the Nutritional Risk Screening 2002 scale.[13] Oncologic variables and results were noted, and neoplasm staging was done according to the TNM (tumor-nodule-metastasis) classification system of the Union Internationale Contre le Cancer.[14] The clinical outcomes were analyzed according to the following definitions: 1) perioperative mortality: any death within 30 days after the surgery, 2) early complications: occurring within 90 days after cystectomy and 3) late complications occurring >90 days after cystectomy.[15] Complications were classified according to the Clavien–Dindo classification system.[16] Both groups were divided into 3 subgroups according to the urine derivation (ileal conduit, orthotopic neobladder according to Studer, and simple uretero-cutaneostomy). All perioperative data was analyzed according to those variables.

Statistical analysis

The results were expressed as median, mean and standard deviation (±SD). Comparisons between groups of nonparametric data were performed using the Mann–Whitney U test and the Kruskal–Wallis test as appropriate. Dunn's test was used for post-hoc comparisons, with the Benjamini–Hochberg method for false discovery rate correction. Statistical significance was set at $p \leq 0.05$ for all tests.

Results

Preoperative status evaluation

There was a significant difference in the risk of anesthesia between the LRC and ORC groups (ASA score 3.1 and 2.52, respectively; p = 0.001; Fig. 1). No statistical difference was found in the BMI between the LRC and ORC groups (26.21 kg/m^2 and 25.65 kg/m^2, respectively; p = 0.11), but the serum protein level was statistically different (7.10 g/L and 5.56 g/L, respectively; p = 0.001).

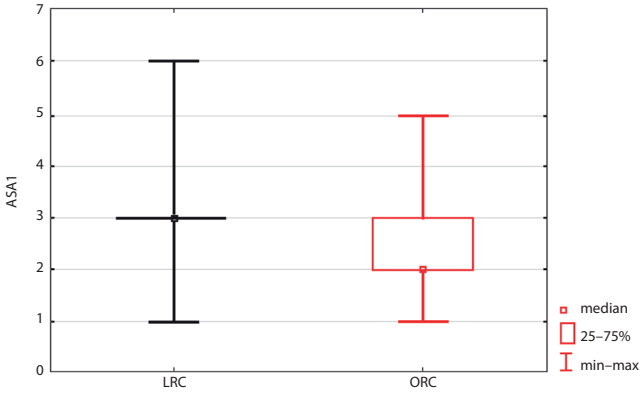

Fig. 1. Mean ASA score in the LRC vs ORC group

Surgical procedure evaluation

Uretero-cutaneostomy was performed in older and compromised patients, while younger ones received an ileal conduit or Studer neobladder (Table 1).

Table 1. Urine derivation after radical cystectomy

Group	Urine diversion			
	ileal conduit	orthotropic neobladder	uretero-cutaneostomy	other
LRC	36	0	93	2
ORC	71	31	26	1

LRC – laparoscopic radical cystectomy; ORC – open radical cystectomy.

The median operating time was significantly shorter in the LRC group than in the ORC group (190 min vs 290 min; p = 0.001). In the case of both groups operated using laparoscopic approach (group with urine derivation using uretero-cutaneostomy and ileal conduit), the median time of surgery was shorter compared to the same groups operated using open approach. It was significantly shorter in the laparoscopic (LAP) group with uretero-cutaneostomy (p = 0.0016) than in the group in which the open technique was used, and almost reached significance in the LAP group with ileal conduit (p = 0.067) compared to the group where the open technique was used (Table 2).

The median blood loss was significantly lower in the LRC group (350 mL) than in the ORC group (850 mL; p = 0.0001). In the case of both groups operated using laparoscopic approach (group with urine derivation using uretero-cutaneostomy and ileal conduit), the median blood loss was significantly lower compared to the same groups operated using open approach (p < 0.001 in both cases).

The mean hospital stay for the LRC group was 8.18 days and for the ORC group 11.63 days. Patients from the LRC group required a statistically significant shorter time before being released home (p = 0.004).

Complications

Most perioperative complications were classified as level 1 and 2 in the LRC group and as level 2 in the ORC group. The mean Clavien–Dindo score was significantly lower in the LRC group than in the ORC group (2.24 vs 2.65; p = 0.0001).

Only 1 patient in the LRC group required conversion to open surgery as a result of neoplasm infiltration of the rectal wall that required resection of the rectum. Both groups included patients that required reoperation. In the ORC group, 8 patients required reoperation: 2 because of urine leakage, infection and peritonitis; 4 due to eventration; 1 due to ileal conduit leakage and re-anastomosis; and 1 due to extensive bleeding. In the LRC group, 9 patients required reoperation: 1 due to eventration; 1 due to incidental loss of urethral drainage; 3 due to ileus and

Table 2. Surgical results adjusted to urine derivation after radical cystectomy

Variable	LRC+ ileal conduit	LRC+ uretero-cutaneostomy	ORC+ ileal conduit	ORC+ orthotopic neobladder	ORC+ uretero-cutaneostomy	p-value*
Number of patients	36	93	71	31	26	
Operation time [min] (median (IQR))	252.50 (237.50, 290.00)	180.00 (155.00, 200.00)	290.00 (260.00, 315.00)	325.00 (300.00, 360.00)	225.00 (180.00, 275.00)	<0.001
Blood loss [mL] (median (IQR))	325.00 (200.00, 400.00)	400.00 (250.00, 600.00)	700.00 (500.00, 1000.00)	800.00 (600.00, 900.00)	1,100.00 (500.00, 1,500.00)	<0.001
Complication rate (median (IQR))	1.00 (1.00, 2.00)	2.00 (1.00, 2.00)	2.00 (2.00, 2.00)	2.00 (2.00, 2.00)	2.00 (2.00, 2.50)	<0.001

* Kruskal–Wallis test; IQR – interquartile range; LRC – laparoscopic radical cystectomy; ORC – open radical cystectomy. Complication rate according to Clavien–Dindo scale.

urine leakage followed by infection; and 3 due to urine leakage and ileal conduit re-anastomosis.

Patient deaths in the perioperative period occurred in both groups: 5 in the LRC group and 4 in the ORC group. Four of them were due to intestinal complications (ileus) that led to more serious complications and reoperations. One was due to acute cholecystolithiasis, which required an operation and then had surgical complications. Another death was a result of urine leakage and an infection, followed by sepsis and septic shock. One other case was a result of excessive bleeding with serious cardiovascular complications. Finally, 2 deaths were due to cardiovascular complications.

In the case of both groups operated using laparoscopic approach (group with urine derivation using ureterocutaneostomy and ileal conduit), the median Clavien–Dindo complication rate was significantly lower, compared to the same groups operated using open approach ($p = 0.001$ and ($p < 0.001$, respectively).

Oncologic results

Pathologic evaluation revealed urothelial carcinoma in different stages of the disease. In the LRC group, 60% of patients were diagnosed with pT3-4 disease (Fig. 2). In the ORC group, the percentage of patients with high- and very high-risk disease was similar (59%; Fig. 3).

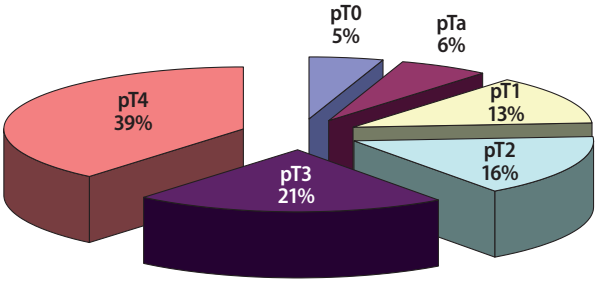

Fig. 2. Pathological stage of disease in patients operated using LRC

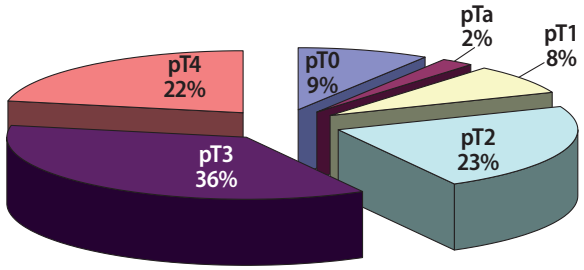

Fig. 3. Pathological stage of disease in patients operated using ORC

Nodal involvement was observed in 26% of patients from the LRC group and 36% from the ORC group, and the mean number of nodes taken per patient was 10 and 12, respectively. There was no statistical difference between the groups.

Positive surgical margins (PSM) were observed in both groups, without significant differences in most instances. The exception was ureteral PSM, with 10 instances in the ORC group and 4 in the LRC group ($p = 0.04$; Table 3).

Discussion

The classical treatment for urothelial muscle-invasive bladder cancer (MIBC) is ORC. According to the 2016 EAU guidelines, this approach should be proposed to all patients, since the cure rate is highest compared with other methods used alone (radiotherapy, chemotherapy).[2] Open radical cystectomy provides the longest cancer-specific survival and is considered the standard method of treatment. Since the end of the 20th century, LRC has gained wide popularity. It is less invasive and causes less blood loss and a lower number of perioperative complications. It provides good cancer control and similar oncological outcomes to ORC.[8,11] There are very few comparative prospective trials comparing ORC and LRC. Up to now, mostly comparative retrospective analyses have been conducted, which have showed no inferiority of LRC vs ORC. A report from a comparative study indicates a recurrence-free survival of 77% after LRC compared with 80% after ORC in a period of 3–4 years, but the difference was not statistically significant ($p = 0.2$).[17] Unfortunately, the analyzed series was small, consisting of only 55 patients.

In our study, we included 260 patients, which seems to be the highest number of patients analyzed so far. Only Huang et al. included a comparable number of patients in their report (108).[18]

Local recurrence, PSM, lymph node yield, and recurrence-free survival are the most important oncologic variables. In available studies, the local recurrences, lymph node yield and cancer-free survival were similar in LRC and ORC patients. This indicates that, from an oncologic point of view, both methods are comparable.[19] An interesting meta-analysis of 16 papers showed no difference in lymph node yield between LRC and ORC groups. Furthermore, there were fewer positive lymph node invasions and a significantly lower PSM rate in LRC patients ($p = 0.05$ and $p = 0.006$, respectively).[20] In our study, PSM

Table 3. Positive surgical margins in the ORC group vs the LRC group

Group	Ureteral PSM	Urethral PSM	Uterus or prostate involvement	Perivesical fat	Rectum involvement
LRC	4	14	11	52	2
ORC	10	10	24	57	1

LRC – laparoscopic radical cystectomy; ORC – open radical cystectomy; PSM – positive surgical margins.

were found in both groups, but the difference was statistically significant only in the case of ureteral PSM.

Oncological safety, defined by the presence of PSM and the number of lymph nodes removed, was questioned in a few older series.[21] Herr et al. proposed that if 10–14 lymph nodes were removed and the PSM were less than 10%, the oncologic results of LRC could be considered equivalent to those of ORC.[22] In our study, the mean number of lymph nodes removed was 10 in the LRC group and 12 in the ORC group, which was sufficient for oncologic staging. Surgical margins were positive in 18 of 129 (13.95%) patients in the ORC group and 15 of 131 (11.4%) patients in the LRC group. This is slightly higher than the accepted cumulative PSM ratio, but it is generally positive in high-volume disease (pT4).[22] Among the patients with lower-risk disease (pT2 and lower), none had PSM. The higher percentage of PSM in the ORC group than in the LRC group can suggest, that the PSM percentage does not depend on the surgical approach but more on the local advancement of the disease. Therefore, both methods can be considered similar regarding oncologic safety.

Laparoscopic radical cystectomy is a demanding procedure, but our data suggests that it can also be used to treat localized MIBC. In our study, the learning curve was not that long. After 50 cases, it was possible to achieve operating times, intraoperative blood loss, and the length of hospital stays comparable, or better, than those reported in the largest published ORC series.[23] It seems that in experienced hands, the operating time can be shorter than for ORC. Such short operating times can be achieved only by an experienced laparoscopic team. At the beginning of the LRC program, our team was well-experienced in laparoscopic prostatectomy and nephrectomy, so the learning curve was much shorter than expected. During LRC, there is a better visualization of all the anatomical structures (particularly in the pelvis minor) than in the classical open surgery. In experienced hands, LRC is easier and more manageable than ORC. It seems that with improved skills and experience on the part of surgeons, LRC could replace ORC.

With the emergence of robotics surgery, RARC is rapidly replacing laparoscopy.[24] The learning curve is shorter and postoperative results are comparable. In a comparative analysis between LRC and RARC, Abraham et al. concluded that both can be performed safely with good oncological results.[24] However, RARC is more expensive and cannot be used in secondary or tertiary urological departments. Blood loss was significantly lower in the LRC group. Only 18 out of 131 patients needed transfusions, and only 34 blood units were required in total. In comparison, 80 patients out of 129 needed transfusions in the ORC group, and 198 blood units were used. The same significant results were noted when recorded in subgroups, adjusted for type of urine derivation. This could be expected since the highest blood loss is noted usually during bladder resection, which is a part of radical cystectomy, rather than during urine derivation. The same trend was observed in a large meta-analysis by Tang et al., where the blood loss and transfusion rate from 16 studies were significantly lower in the LRC group than in the ORC group ($p = 0.001$ and $p = 0.002$, respectively).[20] It seems that better visualization and greater comfort during surgery improves hemostasis. Hospital stay is one of the most noticeable differences for patients. Recent, unpublished data from our team shows that the advantages of LRC together with the Early Recovery After Surgery (ERAS) program, enable safe hospital discharge 5–7 days after surgery. In this study with older data, the hospital stay was 8.18 days in the LRC group and 11.63 days in the ORC group. The simpler urine derivation generally used in the LRC group could partially explain such a difference. However, when the length of the hospital stay was calculated for the LRC subgroup of patients with ileal conduit, the difference was almost the same. A shorter hospital stay has also been noticed in large series.[25,26] It significantly reduced the cost of hospitalization but was possible only with the aid of outpatient department control.

A lower need for analgesia was also observed in the LRC group. In the previously mentioned meta-analysis, the need for analgesia in the LRC group was also significantly lower compared to the ORC group ($p = 0.001$). With the implementation of the ERAS program, most patients can get out of bed on the 2nd or 3rd day after surgery and are encouraged to do so.

The rate of perioperative complications is one of the most important markers of surgical safety. In this study, complications were predominantly level 2 in the ORC group and level 1 and 2 in the LRC group. Conversion from LRC to ORC was not common. It happened only in one case, where the disease load was very high and made LRC too risky. The same significant results were noted when recorded in 2 subgroups adjusted for type of urine derivation. It is interesting that even when LAP subgroup with ileal conduit was compared to the subgroup with open simple ureterocutaneostomy, the number of complications showed the superiority of LAP technique ($p = 0.0004$), even when complications usually appeared with more complicated urine derivation. Most life-threatening complications are associated with urine leaks and with later intestinal complications due to urine contact with bowel anastomosis.[27] For this reason, uretero-cutaneostomy was considered in the cases of very high disease load or poor general status. In the LRC group, the general condition of patients was more serious than in the ORC group, and the mean ASA score was also significantly higher ($p = 0.001$). Older patients with ASA scores of III and IV were subjected to minimally invasive surgery with great success, with the same level of perioperative safety as in the ORC group. From our unpublished data, it seems that even older patients (above 80 years) with serious comorbidities can be operated safely using the laparoscopic approach.

Laparoscopic radical cystectomy is safe to treat advanced urothelial disease, with similar oncological results and

faster recovery compared to ORC. After 50 cases in experienced hands, LRC was at least as easy to perform as ORC. The level of perioperative complications, lower blood loss and faster hospital discharge time seen in the LRC group, lead to the conclusion that this technique can be developed in secondary and tertiary urological centers as the method of choice for patients with advanced urothelial disease.

In general, during the learning curve of LRC, this approach seems very difficult to manage and is applicable only in selected cases. However, after 50 operations, LRC was easier to perform than ORC, and difficult cases could be managed just as easily.

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Antibiotic-resistant bacterial colonization increases the number of hospitalizations in patients after solid organ transplantation or with non-communicable diseases

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Abstract

Background. Healthcare-associated infections could affect the rate of morbidity, mortality and post-discharge hospitalization among patients. They are also dangerous to healthcare professionals and generate significant cost to the healthcare system.

Objectives. The aim of this study was to evaluate the occurrence rate of colonization with various antibiotic-resistant (AR) bacteria among patients admitted to the Department of Immunology, Transplantology and Internal Diseases.

Material and methods. The study used retrospective analysis of patients (n = 280) with no clinical signs of infection admitted into the department between November 2015 and May 2017. The observational period lasted until January 2019. Collected data included sex, age at admission, location directly prior to current hospitalization, and medical history. Nasal and rectal swabs were collected, and stool and urine samples were obtained on the day of admission. Specimens were cultured according to standard microbiological procedures. In all cases, the appropriate bioMerieux (Marcy-l'Étoile, France) media were used. Isolates were identified using mass spectrometer (Vitek MS; bioMerieux).

Results. One-hundred ninety-one (68.2%) of patients were colonized with AR bacteria. The incidence of colonization was not influenced by age or sex. The risk of colonization was associated with admission from another hospital and history of kidney transplantation ($p = 0.0136$ and $p < 0.001$, respectively). The number of hospitalizations during the whole observational period was higher in the group of colonized patients compared to non-colonized (2.76 ± 2.4 vs 2.07 ± 1.68, $p = 0.0099$). The number of hospitalizations correlated positively with the number of positive cultures obtained from the same patients (rho = 0.18, $p = 0.0274$).

Conclusions. The rate of colonization at admission to the ward could be high, depending on previous hospitalization and medical history. Colonization significantly increased post-discharge hospitalization rate.

Key words: hospitalization rate, healthcare-associated infections, bacterial colonization

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Introduction

Healthcare-associated infections (HAI), including those originating in hospitals, are currently one of the most important challenges for modern medicine. They are a common cause of morbidity and mortality, and result in adverse effects. Healthcare-associated infections are responsible mainly for pneumonia, as well as bloodstream, urinary tract and surgical site infections. The estimated number of HAI in American hospitals in 2002 was approx. 1.7 million.[1] Especially dangerous are infections with antibiotic-resistant (AR) bacteria. It has been proven that such infections affect disability-adjusted life-years (DALYs).[1–3] They are also associated with prolonged hospital stays and lengthy treatment with antibiotics or steroids.[4]

Besides the serious consequences to patients, HAI are also dangerous to healthcare professionals and generate significant costs to the healthcare system. It was estimated that in 2007 (after adjusting from previous data), the cost resulting from HAI treatment in American hospitals exceeded 33 billion dollars, and the benefits from prevention exceeded 6 billion dollars.[5] Therefore, recently, great emphasis is being placed on HAI and special preventive procedures such as enhanced active surveillance, patient isolation, hand hygiene, surface disinfection, and enhanced education of patients and healthcare professionals are being implemented in healthcare institutions. Despite these procedures, more invasive medical technologies and the demographics of society will increase the chances of HAI.

The risk factors for HAI are predominantly previous hospitalization, urinary tract catheterization, terminal illness, and broad-spectrum antibiotic therapy.[6,7] The challenge for medical personnel is to prevent and minimize the occurrence of healthcare infections, which are partly unavoidable. Healthcare-associated infections has become a patient safety problem and a public health problem.[1] Patients with infectious diseases display specific clinical symptoms, while carriers usually are admitted to hospitals due to other clinical conditions. Therefore it is of great importance to detect carriers of bacteria at admission and isolate them in single-patient rooms to prevent further spread of infection.[4,8] International recommendations prefer active screening rather than passive; however, these strategies depend mainly on respective hospital recommendations.[9]

Properly diagnosed carriers could be decolonized. It has been proven that decolonization reduces the risk of surgical site infection and the rate of infections in intensive care units. In the case of methicillin-resistance *Staphylococcus aureus* (MRSA), decolonization with chlorhexidine and mupirocin lowers the risk of infection by 30% compared to education regarding decolonization alone.

The aim of this study was to evaluate the occurrence rate of colonization with various bacteria among patients admitted to the Department of Immunology, Transplantology and Internal Diseases (ITID). We focused on the quantitative rather than qualitative aspect of the problem. In each case, a detailed microbiological diagnosis was performed, including identification of antibiotic resistance, and appropriate medical therapy was implemented; however, this data was not considered in this study.

Material and methods

The study protocol was approved by the Bioethics Committee of the Medical University of Warsaw (approval No. AKKB/123/2019). The study used retrospective analysis of data regarding patients admitted into the ITID, Infant Jesus Teaching Hospital, Medical University of Warsaw, Poland, between November 2015 and May 2017. Data regarding the number of hospitalizations since enrollment into the study was collected in January 2019.

Patients enrolled in the study (n = 293) had been admitted into the ITID for the first time or with no history of hospitalization during the previous year other than hospitalization immediately prior to the admission. Patients had been admitted to the ITID from another hospital, another ward of the same hospital or from home. None of them had clinical signs of infection. Patients for one-day hospitalization were excluded from the study. Because of missing data, the investigated group consisted of 280 patients. For each participant, data collected included sex, age at admission, data regarding previous location, and medical history of transplantation or other non-communicable diseases. The characteristics of the investigated group are included in Table 1. In all patients, nasal and rectal swabs were collected, and stool and urine samples were obtained on the day of admission. They were examined for carriage of the following pathogens: vancomycin-resistant *Enterococci* (VRE), extended spectrum beta-lactamases (ESBL) producing *Enterobacteriaceae* as well as carbapenemase producing *Enterobacteriaceae* (CPE). Moreover, patients were examined for the presence of *Clostridium difficile* (stool samples only). A patient was recognized as colonized if any of the above bacteria were found. In connection with usual multiresistance, ESBL- or/and carbapenemase-producing *Escherichia coli* in this work were called antibiotic-resistant *E. coli* (AR *E. coli*), and antibiotic-resistant *Klebsiella pneumoniae* (AR *K. pneumoniae*) was named similarly.

All microbiological procedures were performed at the Chair and Department of Medical Microbiology of the Infant Jesus Teaching Hospital. The various specimens from the patients were cultured according to standard microbiological procedures, depending on the kind of specimen and the aim of examination (infection/colonization). In all cases, the appropriate bioMerieux (Marcy-l'Étoile, France) media were used. Isolates were identified using a mass spectrometer (Vitek MS; bioMerieux). Susceptibility testing was performed using the Vitek 2 or disc diffusion method and the results were interpreted

Table 1. Demographic and clinical details of the investigated group

Factor	Description	n	% of n = 280
Sex	male	142	50.7%
	female	137	49.3%
Admission group	home	173	61.8%
	other hospital	79	28.2%
	another ward in the same hospital	28	10.0%
Medical history	kidney transplantation (KTx)	145	51.8%
	liver transplantation (LTx)	64	22.9%
	heart transplantation (Htx)	2	0.7%
	other non-communicable disease (INT)	71	25.4%
Factor	**Mean (SD)**	**Min–Max**	**Median**
Age	50.19 ±15.02	37–61	51

according to European Committee on Antimicrobial Susceptibility Testing (EUCAST) recommendations.[11] For detection of carbapenemase production, the combination disk test was performed and results were confirmed using biochemical CarbaNP test.[12,13] Production of extended spectrum beta-lactamases (ESBLs) was examined with modified double disc synergy test (DDST).[14] All tests were recommended by EUCAST.[15]

Statistical analysis

Statistical analysis used R software v. 3.5.0 (www.r-project.org). Descriptive statistics of the data were generated using standard statistical parameters: percentage, mean and standard deviation (SD), median, and minimum and maximum (min and max). Median values in the 2 independent groups were compared using the nonparametric Mann–Whitney U test. The correlations between pairs of numerical parameters were studied using χ^2 test or Fisher's test. Multivariate analysis was conducted using a logistic regression model based on Wald's statistics. In all analyses, results were considered significant if the p-value was <0.05.

Results

From the investigated group, 191 of patients were colonized with AR bacteria (men, n = 93, women, n = 98), which comprised 68.2% (accordingly, 48.7% of men and 51.3% of women) of all study population. The incidences of colonization were not influenced by age or sex. From the colonized group, 58.6% were admitted from home (n = 112), 32.5% were admitted from another hospital (n = 62) and the rest was admitted from another ward of the hospital (8.9%, n = 17). Among the colonized patients, 57.6% (n = 110) had a history of kidney transplantation, 19.4% (n = 37) had a history of liver transplantation, 1% (n = 2) were after heart transplantation, and 23% (n = 44) were admitted to the ward due to other non-communicable diseases. The χ^2 test revealed a significant correlation between kidney transplantation and colonization, regardless of the localization of colonization (p = 0.0065).

According to the results of the logistic regression analysis, the risk of colonization was associated with admission from another hospital and a history of kidney transplantation (p = 0.0136 and p < 0.001, respectively). The risk of colonization among patients admitted from another hospital was almost 2.2 times higher compared to the group of patients who were admitted from home (odds ratio (OR) = 2.151, 95% confidence interval (95% CI) = 1.139–5.062), and were almost 2.4 times higher among patients after kidney transplantation compared to other patients (OR = 2.353, 95% CI = 1.383–4.003).

The most common type of pathogen among the investigated group was vancomycin-resistant *Enterococci* spp. (VRE) (24.9%). Less common were AR *E. coli* (21.5%) and AR *K. pneumoniae* (15.4%). *Clostridium difficile* was diagnosed in 14.3% of patients. A similar panel of pathogens was observed in the group of patients with different medical history. None of the investigated parameters (age, sex, medical history, and the place of previous location) influenced the incidence of particular pathogens colonization, excluding AR *E. coli* and VRE. Women were infected by AR *E. coli* more often than men (p = 0.022). Infection with VRE was less common among patients with non-communicable diseases compared to the rest of the investigated group (p = 0.047). The Fisher's test revealed that more patients free of VRE colonization were admitted from home than from another hospital (p = 0.0013). The particular pathogen colonization in the group of patients after heart transplantation were not considered due to the small number of such participants.

Forty patients were colonized with *C. difficile*, which comprised 14.3% of the whole investigated group. From this group, 23 patients (57.5%) were admitted to the ITID from home, 13 patients were admitted from another hospital (32.5%) and 4 patients were admitted from another ward of the hospital (10%).

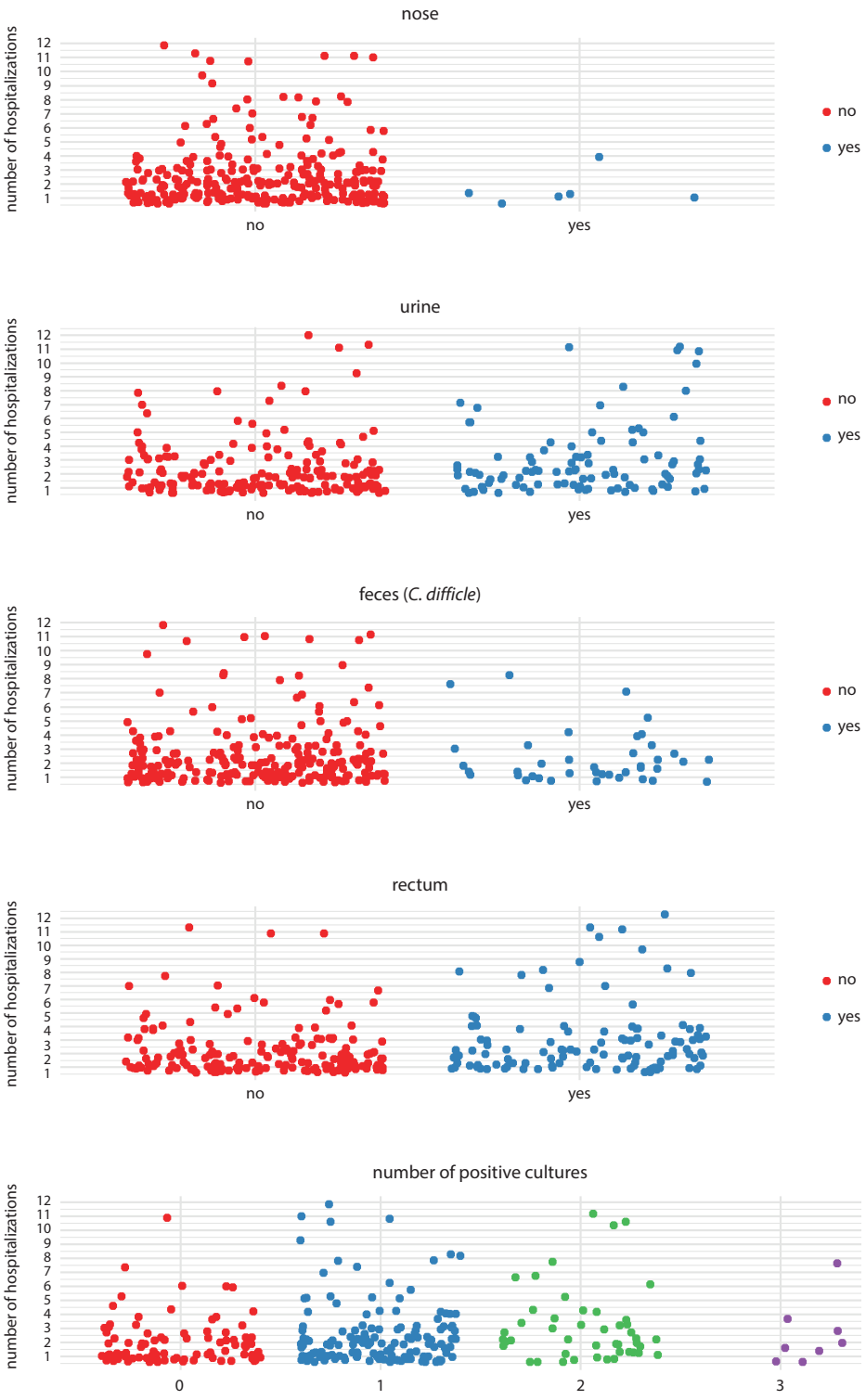

Fig. 1. The number of hospitalizations during the whole observational period of patients belonging to the whole investigated group, divided into subgroups: those who had a positive culture of rectum swabs (n = 110), feces samples (*C. difficile*, n = 40), urine samples (n = 97) and nasal swabs (n = 6), and according to the number of positive cultures obtained in each patient (1, 2 or 3)

The number of hospitalizations during the whole observational period was higher in the group of colonized patients compared to non-colonized in the whole investigated group (2.76 ±2.4 vs 2.07 ±1.68, p = 0.0099). The same was observed in the group of patients with positive urine culture in comparison to those with negative urine culture (n = 97, 2.95 ±2.5 vs n = 183, 2.33 ±2.02, p = 0.0012), and in the group of patients with positive rectal swab in comparison to those with negative rectal swab (n = 110, 3.0 ±2.57 vs n = 170, 2.25 ±1.9, p = 0.0044). *Clostridium difficile* colonization did not influence the number of hospitalizations. The influence of positive nasal swabs on the number of hospitalizations was not considered due to the small number of such patients. The number of hospitalizations correlated positively with the number of positive cultures obtained from the same patients; however, the correlation was weak (Spearman's rho = 0.18, p = 0.0274). The results described above shown in Fig. 1.

Discussion

Our results showed that almost 70% of patients free of any clinical symptoms of infection at admission to the hospital were colonized by various AR bacteria. This is very high rate but similar results had already been reported.[6,7] However, other studies reported much lower rates.[4] The high rate of colonization in our patients could result from their medical history. The majority of them had undergone organ transplantation and were under chronic immunosuppression treatment. We showed that renal transplantation increased the risk for colonization almost 2.5 times. Colonized patients could be a potential source of infection; however, the transmission rate depends on the epidemiology of the pathogens.[8,16] The most frequently diagnosed pathogen in our study was VRE. According to literature data, the frequency of particular pathogens' colonization and infection rates differ depending on the specificity of the healthcare facility.[17] In hematopoietic stem cell transplant recipients, *Staphylococci* spp. and *Enterobacteriaceae* were the most frequent pathogens causing infections, and in the oncologic ward, *Enterococci* spp. were detected in more than 40% of patients.[4,18] The epidemiology of pathogens do not vary significantly with regard to age, sex or medical history. Similar results were obtained by Chmielarczyk et al.[19] Special attention should be placed on *C. difficile* colonization. *Clostridium difficile* is a Gram-positive, spore-forming anaerobic bacteria, and it is recognized as one of the most important pathogens in healthcare settings as it is the major cause of antibiotic therapy associated infections infections and could contribute to increased mortality.[19,20] It is distinct from other microorganisms due to the fact that its spores can persist in the environment for a long time. Asymptomatic colonization of *C. difficile* is not clearly defined; however, it is recognized in the absence of infection symptoms.[21] The number of colonized patients is higher than symptomatic,[22] but it varies between different patient groups. In a healthy population, colonization with these bacteria varied between 0% and 15%, while in hospitalized patients, it reaches 30%, which is more or less in line with our findings, because in our group more than 14% of patients were colonized at admission to the hospital.[21] In the case of *C. difficile*, transmission from person to person usually takes place, but colonized asymptomatic patients have the potential to contaminate the environment and subsequently other patients.[23]

In this study we focused on quantitative analysis of the AR bacteria colonization rate among patients of ITID. We found that colonized patients are most frequently admitted from other hospitals. This is in line with previously recognized risk factors for colonization, e.g., hospitalization within 2 months prior to current hospitalization[22]; however, it is worth noting that the majority of colonized patients in our study were admitted to the ward from their homes. Therefore, we recommend also treating such patients as potentially colonized and a potential source of infection. However, we should note that patients after organ transplantation usually had a history of many previous hospitalizations despite the fact that in this study they were not hospitalized in 1 year prior to the study. Another important finding is that colonization is a risk factor for an increase in the number of hospitalizations over the 2.5-year observational period, especially in the case of urinary tract and gastrointestinal tract colonization. Surprisingly, colonization with *C. difficile* did not increase the number of hospitalizations. Similar results were obtained by Huang et al. Patients who underwent decolonization of MRSA infection had lower risk of hospitalization over 1 year after discharge.[10]

Interventions to reduce transmission depend on the epidemiology of the bacteria and the main route of acquisition. It was shown that identification of carriers and implementation of preemptive isolation, and contact precautions had little effectiveness on transmission of, for instance, MRSA, VRE or HRE (highly resistant *Enterobacteriaceae*). Hand hygiene is effective in protection from MRSA and VRE transmission, while the data regarding the effectiveness of chlorhexidine bathing is conflicting. It should be noted that MRSA and VRE can also colonize the skin and environment.[8] Therefore, other methods should be considered to protect everyone from bacterial transmission, such as decolonization.[8] Also, current guidelines do not recommend active screening for *C. difficile* colonized patients because its impact on infection transmission prevention is low.[21] The reason is that there is limited information on the duration of the latent period in the case of contact with *C. difficile*; therefore, patients exposed but not yet colonized could not be detected.[24] Another reason is that the treatment for colonized asymptomatic patients is not recommended. Nevertheless, intensive infection control practices like the use of gloves and enhanced environmental cleaning should be implemented with regard to the high number of patients potentially colonized with *C. difficile*.[21,23]

Conclusions

The rate of colonization at admission to the ward could be high, depending on previous hospitalization and medical history. The rate of colonization in patients admitted to the ward from home is also high enough to treat them as potentially colonized. The epidemiology of the pathogens do not vary significantly with regard to age, sex, medical history, or location directly prior to current hospitalization, excluding AR *E. coli*, which was more often in urine samples from women, and VRE, which were less common among patients with non-communicable diseases and admitted from home. Colonization significantly increased the post-discharge hospitalization rate.

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Role of chromatin remodeling complex SWI/SNF and VDR in chronic rhinosinusitis

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Abstract

Background. The SWI/SNF (SWItch/sucrose non-fermentable) chromatin remodeling complex enables glucocorticoid receptor (GR) and vitamin D receptor (VDR) to function correctly and is engaged in inflammation response. The SWI/SNF may play an important role in chronic rhinosinusitis (CRS).

Objectives. The aim of this study was to assess the following: 1) the gene and protein expression of the SWI/SNF complex subunits in sinonasal mucosa; 2) relation of SWI/SNF complex and VDR expression; and 3) correlation with clinical data.

Material and methods. The study population consisted of 52 subjects with CRS without nasal polyps, 55 with CRS with nasal polyps and 59 controls. The SWI/SNF protein expression level was analyzed in immunohistochemical (IHC) staining. Human nasal epithelial cells (HNECs) was stimulated using lipopolysaccharide (LPS), Staphylococcal enterotoxin B (SEB) and vitamin D3 (vitD3) in vitro. The transcript level of the SWI/SNF subunits was measured with polymerase chain reaction (PCR).

Results. In the control group, the intensity of the IHC staining for SWI/SNF subunits was significantly higher than in both groups of patients with CRS ($p < 0.05$). A positive correlation of the SWI/SNF protein expression was noticed with VDR expression level ($p < 0.043$). Association between SWI/SNF protein expression level and allergy, neutrophils and body mass index (BMI) has been observed ($p < 0.05$). The decreased transcript level of the SWI/SNF subunits genes in HNECs was observed after LPS stimulation and increased after vitD3 stimulation.

Conclusions. The SWI/SNF complex may influence CRS through steroid hormone signaling and VDR. Thus, modification in therapy may be mandatory in patients with CRS and altered SWI/SNF signaling, reflecting resistance to steroids treatment.

Key words: chronic sinusitis, nasal polyps, vitamin D, steroids

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Background

Chronic rhinosinusitis (CRS) is a heterogeneous disease characterized by symptomatic inflammation of paranasal sinuses and nasal cavity. Its prevalence is estimated at 14% in the American and 10.9% in the European population, and it is one of the most common human chronic diseases.[1,2] The pathophysiology of CRS is multifactorial. Various theories on the etiology, such as allergy, bacterial and fungal infections, as well as structural abnormalities, have been proposed; however, the pathogenesis remains largely unknown.[3]

The CRS is typically classified in 2 phenotypes including CRS with nasal polyps (CRSwNP) and CRS without nasal polyps (CRSsNP). Symptoms in CRSwNP are associated more closely with clinical complaints of nasal obstruction and olfactory loss. The CRSwNP is more often combined with comorbidities such as asthma and aspirin hypersensitivity (this phenomenon is named Samter's triad). Moreover, CRSwNP tissues are characterized by more intense eosinophilic infiltration and a Th2-based cytokine profile. The CRSsNP tissues have been infiltrated mostly by neutrophils and Th1 cytokines. However, the distinct role of Th1/Th2 profiles in the subtypes of CRS gives rise to some controversy.[4]

Current medical knowledge does not explain in a detailed way the pathomechanism of CRS. However, over the past 20 years, the development of science in the field of immunology and molecular biology has allowed, at least in part, to understand the various pathophysiological processes taking place at the cellular level and involved in CRS.[1,5]

Regardless of the pathomechanism leading to CRS formation, the characteristic features of this disease are inflammation and tissue remodeling. One of the regulators of the inflammatory response is the SWI/SNF (SWItch/sucrose non-fermentable) complex. Studies show that after lipopolysaccharide (LPS)-induced inflammation in macrophages, the SWI/SNF complex is necessary for the anti-inflammatory response.[6] The SWI/SNF is a multi-protein complex (15–20 subunits) that has the ability to provide a DNA sequence to the transcriptional apparatus. The SWI/SNF consists of the following: the core part, formed by 1 of the 2 ATP-ases BRG1 and BRM, which hydrolyze ATP, bind acetylated histones, regulate transcription and act as tumor suppressors; BAF155, BAF170, and INI-1 subunits that stabilize the core of the complex[7,8]; and a range of external proteins responsible for joining the complex in a tissue-specific manner.

For the first time, the SWI/SNF type chromatin remodeling complex was identified in the yeast *Saccharomyces cerevisiae*.[9] Among the many processes regulated by the SWI/SNF complex, it has been shown that it is involved in the regulation of gene expression encoding the regulators of many different processes in the cell, such as adhesion, differentiation, hormonal response, and cell cycle.[8,10] Further studies on the function of this complex will help us to better understand the mechanisms of tissue remodeling resulting from chronic inflammation.

The treatment of CRS should be started with pharmacological treatment and then – if there is no improvement in the clinical condition of the patient – surgery should be considered. The pharmacotherapy of CRS is primarily aimed at limiting inflammation. The drugs of first choice, in this case, are glucocorticosteroids (GS).[2] They work by reducing neutrophil accumulation in the inflammatory foci; in addition, they reduce the production of inflammatory mediators by inhibiting the release of arachidonic acid from cell membranes, decreasing the permeability of blood vessels and limiting the production of secretions through the mucous glands.[11]

The SWI/SNF complex may play a significant role in the development and treatment of CRS. The subunits of the chromatin SWI/SNF remodeling complex interact directly with the glucocorticoid receptor (GR) and additionally regulate the expression of the GS response genes through binding to repeating DNA sequences called hormone response elements (HREs).[12,13] Therefore, proper function of GR depends on the SWI/SNF complex, which determines the regulation of GS-dependent genes expression. Additionally, the latest findings indicate that the SWI/SNF complex is able to promote anti-inflammation processes in combination with activation of vitamin D receptor (VDR).[14] The role of vitamin D and its receptors has been vastly addressed in recent studies on CRS pathophysiology. Authors present their crucial role in regulation of the immune function in paranasal sinuses.[15–19]

Objectives

In the present study, we investigated: 1) the protein expression of the main SWI/SNF subunits (BAF155, BRM and BRG1) in sinonasal mucosa of patients with CRSwNP and CRSsNP, and in control group (CG); 2) gene expression of the SWI/SNF subunits in human nasal epithelial cells (HNECs) after treating with LPS, SEB and vitamin D3 (vitD3) in vitro; 3) correlation of obtained results with clinical data, e.g., allergy and steroids therapy data, and with VDR expression in sinonasal mucosa.

Material and methods

The study was conducted in accordance with the ethical standards of the Local Ethics Committees of Warsaw Medical University, Poland, approval No. KB/209/2016, and with the Helsinki Declaration. All participants signed informed consent.

Material

The study population consisted of 166 patients (63 females and 103 males) operated in the Ear, Nose and Throat (ENT) Department at the Faculty of Medicine and

Dentistry of the Medical University of Warsaw. Patients were divided into 3 groups (Table 1). Study groups with CRSsNP and CRSwNP were distinguished on the basis of the interview, physical examination including endoscopic examination of nasal cavities, and paranasal sinuses computed tomography (CT) scan according to the clinical criteria of EPOS 2012.[2] A CG included patients with anatomical abnormalities of nasal structures without additional inflammation in nasal cavity and paranasal sinuses.

All patients with CRS underwent a 3-month conservative therapy (adequate medical therapy (AMT)).[2] Then, after ineffective AMT, the patients were qualified for surgical treatment.

The study material in all groups was collected from the ostiomeatal complex region during endoscopic nasal/sinus surgery, fixed in formalin and embedded in paraffin blocks:

– group 0 – CG (59 patients) fragments of healthy nasal mucous membrane (the ostiomeatal complex region) taken from patients undergoing endoscopic nasal surgery due to the non-inflammatory nasal diseases (septal deviation, anatomy variations of the lateral nasal wall – concha bullosa);

– group 1 – CRSsNP group (52 patients) fragments of the mucous membrane (the ostiomeatal complex region) taken from patients undergoing an endoscopic sinus surgery due to CRSsNP;

– group 2 – CRSwNP group (55 patients) fragments of the mucous membrane (the ostiomeatal complex region) taken from patients undergoing an endoscopic sinus surgery due to CRSwNP.

Exclusion criteria were the following: sinonasal tumor, external sinus surgery in the past, systemic disease (cystic fibrosis, sarcoidosis, autoimmune disease), fungal rhinosinusitis, and possibility of pregnancy.

Clinical data

All patients underwent CT of the paranasal sinuses, assessed according to the CT Lund–Mackay scale (L–M), and completed a questionnaire for the evaluation of sinus complaints SNOT-22 (Sino-Nasal Outcome Test).[2] Data on allergy status were collected based on a medical interview, a skin prick test and total immunoglobulin E (IgE) levels in the blood. Bronchial asthma was determined according to Global Initiative for Asthma (GINA) 2015 criteria.[20] Moreover, during ENT examination, CRS patients were assessed according to the Lund–Kennedy scale (L–K).[21] Complete blood count was analyzed in order to calculate the blood cells, especial eosinophils, and neutrophils in all examined groups (ALAB laboratory, Warszawa, Poland).

Immunohistochemistry

Immunohistochemical staining was performed on 3.5-micrometer sections of tissue sheared from paraffin blocks. The assay was performed in all examined patients. The study was carried out using the EnVision FLEX + detection system, Mouse, High pH Detection System (Dako, Glostrup, Denmark). After deparaffinization in xylene, the slides were rehydrated and incubation was carried out with optimal dilutions of antibodies: anti-SMARCC1/BAF-155 (1:200, per 1 h in 25°C) monoclonal antibodies (D7F8S) (Cell Signaling Technology, Danvers, USA), BRG1 (1:100, per 12 h in 5°C) (G-7) (Santa Cruz Biotechnology, Santa Cruz, USA) and BRM (1:200, per 1 h in 25°C) (D9E8B) XP (Santa Cruz Biotechnology) – forming part of the chromatin remodeling complex SWI/SNF type. The colored

Table 1. Patients' characteristics

Characteristic variable	CG (n = 59)	CRSsNP (n = 52)	CRSwNP (n = 55)	Statistical analysis	p-value
Age range average	 19–70 36.3	 18–77 42.5	 22–83 50.1	K–W, $\chi^2 = 20.66$	<0.001
Sex female male	 24 35	 20 32	 19 36	$\chi^2 = 0.46$	0.79
Average Lund–Kennedy scores	–	4.8	8.9	K–W, $\chi^2 = 55.5$	<0.001
Average CT Lund–Mackay scores	0.7	6.9	16.5	K–W, $\chi^2 = 140.28$	<0.001
Average SNOT-22	1.23	1.61	1.59	K–W, $\chi^2 = 14.21$	<0.001
Asthma	1	1	18	$\chi^2 = 13.58$	<0.008
Allergy	12	18	23	$\chi^2 = 14.76$	<0.006
Eosinophil count range average	 0.02–0.47 0.14	 0.02–0.80 0.21	 0.01–1.59 0.41	K–W, $\chi^2 = 34.36$	<0.001
Neutrophil count range average	 2.04–9.80 4.56	 1.01–9.24 4.03	 1.08–11.35 4.73	K–W, $\chi^2 = 6.59$	<0.037

CG – control group; CRSsNP – patients with chronic rhinosinusitis without nasal polyps; CRSwNP – patients with chronic rhinosinusitis with nasal polyps. The data was assessed according to the age, endoscopic examination – Lund–Kennedy scoring system, CT Lund–Mackay scoring system, SNOT-22, asthma, and allergy status. AERD – aspirin-exacerbated respiratory disease. Statistical tests: Kruskal–Wallis (K–W) and χ^2 test.

reaction product was obtained using 3,3'-diaminobenzidine tetrahydrochloride (Dako). Then, hematoxylin staining was performed in 1 min. In the final stage, the sections were embedded in balsam and examined with light microscopy.

In order to obtain the most accurate results, the cells were counted by 2 independent specialists to avoid bias, and both scores were averaged and recorded. The color intensity was evaluated using the H-score method. First, the results were recorded and assessed by a four-grade scale of staining intensity: 0 – none, 1 – weak, 2 – moderate, and 3 – strong for BRG1, BRM and BAF155 staining for each cell in a fixed field (100 cells). The H-score was based on predominant staining intensity. Using this method, the percentage of cells at each staining intensity level was calculated and, finally, a H-score was assigned using the following formula:

H-score = [1 × (% cells 1) + 2 × (% cells 2) + 3 × (% cells 3)]

Cell culture

For the experiments, HNECs were grown in the following conditions: 37°C, 5% CO^2 and 90% humidity. When cells reached 80–90% confluence, media was removed and the cells were washed with phosphate-buffered saline (PBS; 37°C, pH 7.4) and fresh media containing LPS (Sigma-Aldrich Germany, Darmstadt, Germany; 5 µL/mL), SEB (Staphylococcal Enterotoxin B; Sigma-Aldrich, St. Louis, USA; 1 µL/mL), vitD3 (25-hydroxyvitamin D3 solution, Sigma-Aldrich Germany; 3 µL/mL), or nothing (CG) were added to the cells and incubated for 24 h. Moreover, the HNECs were stimulated by both vitD3 and LPS or SEB. After incubation, cells were collected and stored in –80°C for further analysis.

Quantitative reverse-transcription polymerase chain reaction

Total RNA was isolated from the HNEC human nasal epithelial cells (PromoCell GmbH, Heidelberg, Germany) using RNA Isolation Kit (ReliaPrep RNA Cell Miniprep System; Promega, Madison, USA) according to the protocol. All procedures were conducted according to the manufacturers' instructions. The reverse transcriptase reaction was performed using Transcriptor First Strand cDNA Synthesis Kit (Roche, Basel, Switzerland). Expression of *BRG1*, *BRM*, *BAF 155*, *BAF 170*, and *INI1* genes was measured with SybrGreen (BioRad, Hercules, USA) with *UBIQUITIN* as reference gene using following primers: (UBC-Fq ATTTGGGTCGCGGTTCTTG, UBC-Rq TGCCTTGACATTCTCGATGGT) for *BRG1*: reverse BRG1qR GCAACAGTACTGCCAGCAAC, forward BRG1qF GACATTCCAGTCTCGACCCC, for *BRM*: forward hBRMqF CGGTTTGATTGTGCCTGGTT, reverse hBRMqR GCTTTTGTTCAGATCATAGAGCAT and for *BAF 155*: forward BAF155Fq GCCTGGCTTTCTCACTTCAC, reverse BAF155Rq CTGAGGGTTTGAAAGGCAAA, for *BAF170*: forward BAF170Fq ACAGCAGAATGAACTCCGCT, reverse BAF170Rq GTCTGAGTGCTGCAGGTAGG, for *INI1*: forward INI1Fq GACCAGGACAGGAACACGAG, reverse INI1Rq CAAATGGAATGTGTGCCGG. Gene transcript levels of *BRM*, *BRG-1*, *BAF 155*, *BAF 170*, and *INI1* were quantified using the ddCt method.

Statistical analysis

The data collected was saved in Microsoft Excel 2010 (Microsoft Corp., Redmond, USA) spreadsheet and analyzed using SAS v. 9.2 (SAS Intitute, Cary, USA). This allowed for a descriptive analysis, including averages, standard deviations (SD), medians, and lower and upper quartiles. In the first step, correlations between variables were calculated using Spearman's correlation coefficients. Several non-parametric tests were used in the analysis, such as Mann–Whitney U test, Kruskal–Wallis test (for many comparisons) and Wilcoxon test (comparison of 2 dependent data samples). We also used the χ^2 test to compare discrete data. Several multiple regression models were used. Multiple regression presents in the tables the relations of the clinical and laboratory variables. In all statistical analyses, the level of significance was determined at the level of $p < 0.05$.[22]

Results

The SWI/SNF protein expression in a tissue section

Evaluation of the presence of SWI/SNF complex subunits showed that all proteins (BRG1, BRM, and BAF155) are expressed in the specimens. The stoichiometry in all groups was preserved. BRG1, BRM, and BAF155 were detected in all groups: CRSsNP group, CRSwNP group and CG (Fig. 1). Proteins were localized in the nuclei. The higher intensity of this staining was marked in CG and less intensive in patients with CRSsNP and CRSwNP.

The SWI/SNF protein expression in CRSsNP group and CRSwNP group vs CG

All the examined protein expression levels in the CG were significantly higher in comparison to patients with CRSwNP and CRSsNP (Fig. 2). We found no statistically significant difference in both CRS groups in regard to all subunits (BRG1, BRM and BAF155).

Moreover, we analyzed the SWI/SNF protein expression in patients treated with oral steroids during AMT and compared the results with those of patients not subject to this therapy in the CRSwNP group. We noticed differences for all SWI/SNF subunits in both parameters but without statistical significance (Fig. 3,4).

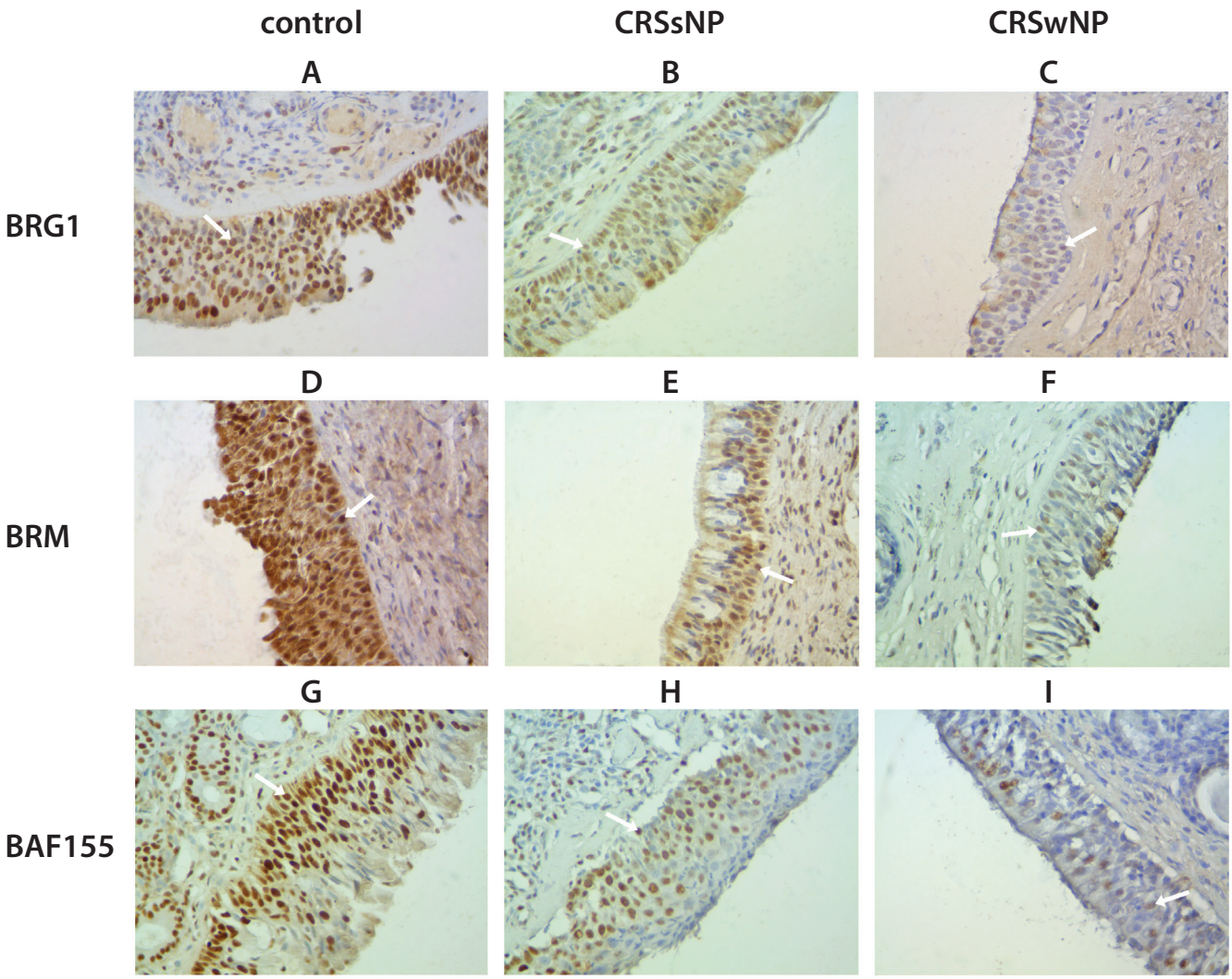

Fig. 1. Immunostaining of cells with BRG1 (A,B,C), BRM (D,E,F) and BAF155 (G,H,I) – antibodies in sinonasal epithelial cells. Dilution of the antibody: 1:100 – BRG1; 1:200 BRM; 1:200 BAF155

CRSsNP – chronic rhinosinusitis without nasal polyps; CRSwNP – chronic rhinosinusitis with nasal polyps. Magnification ×400. The arrows show the nuclei after IHC staining.

H-score for the SWI/SNF correlations in patients with CRSwNP

Furthermore, according to Spearman's test, we found a correlation between protein expression levels of BRG1, BRM and BAF155 in the CRSwNP group and other clinical parameters as follows. In the CRSwNP group, no correlations were observed.

Protein expression level of BRG1 in CRSwNP correlates inversely with serum level of eosinophils ($R = -0.3$; $p < 0.03$), neutrophils ($R = -0.3$; $p < 0.03$) (Fig. 5,6) and allergy ($R = -0.3$; $p < 0.03$).

Analyzes of association of BRM subunit with other clinical data in CRSwNP group displayed an inverse correlation of BRM protein level with allergy ($R = -0.3$; $p < 0.05$).

The protein level of BAF155 correlates positively with VDR protein expression in CRSwNP group ($R = 0.3$; $p < 0.043$) (Fig. 7).

Results of multiple regression for the SWI/SNF subunits and other parameters

The multiple regression analysis was performed in Stata v. 11.0 (StataCorp LLC, College Station, USA). The following clinical data was used in statistical models: allergy, asthma, white blood cell count, eosinophils blood count, neutrophils blood count, usage of oral steroids and nasal steroids, previous sinus surgery, SNOT-22 scale, L–M score, smoking, and H-score VDR.

In the CRSwNP group, allergy was the only factor significantly influencing BRM expression level ($F = 6.28$ and $p = 0.015$). The patients with allergy had significantly lower BRM expression than patients in the CRSwNP group without allergy (Table 2).

Additionally, in the CRSwNP group, 2 other factors significantly influenced BRG1 expression: neutrophils and body

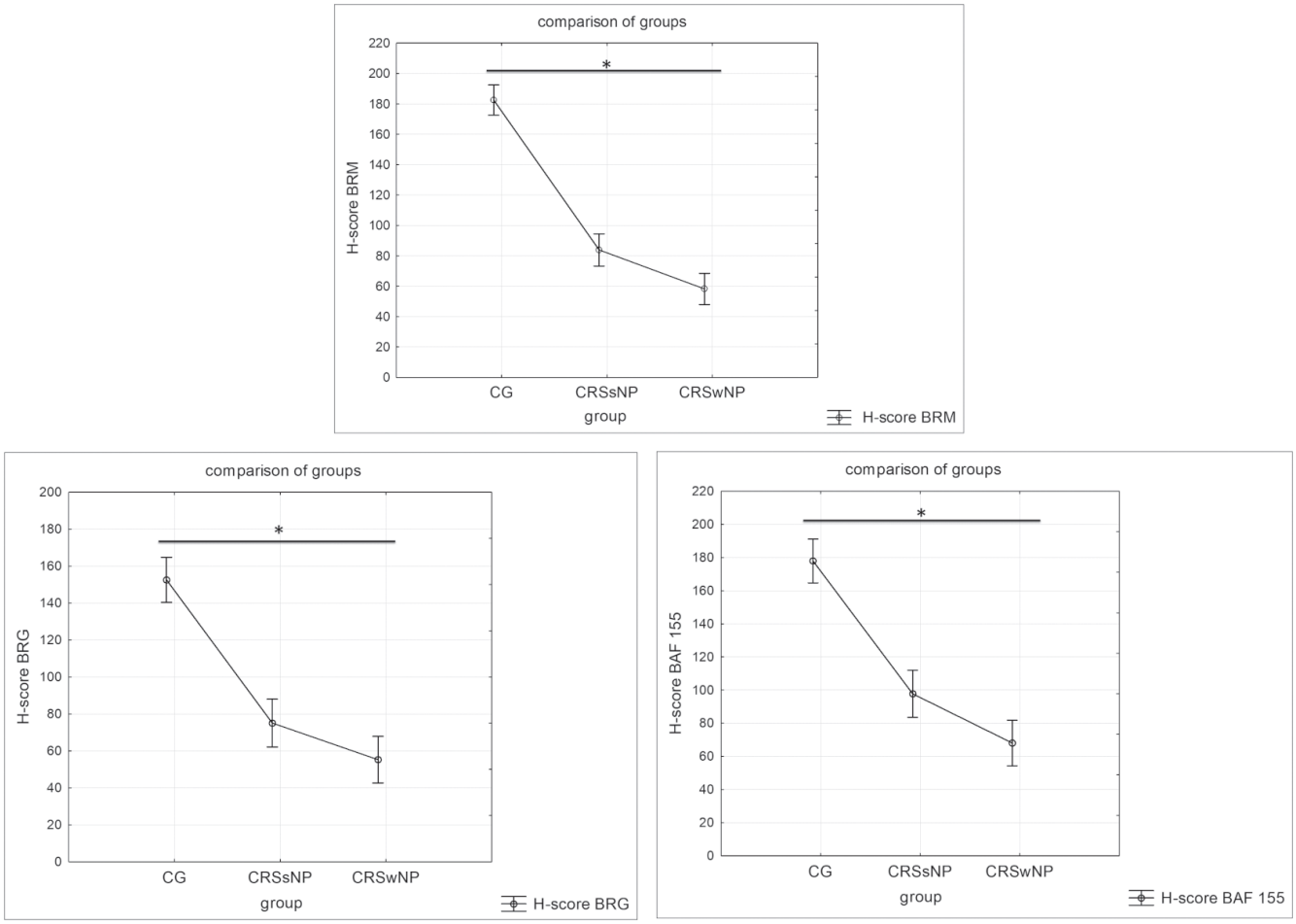

Fig. 2. Higher H-score for BRG1, BRM and BAF 155 staining intensity in control group (CG) in comparison to the CRSsNP group and the CRSwNP group (*p < 0.05)

CRSsNP – chronic rhinosinusitis without nasal polyps; CRSwNP – chronic rhinosinusitis with nasal polyps.

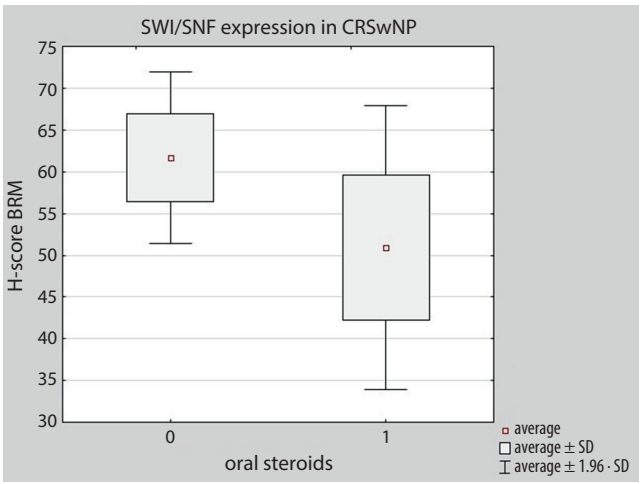

Fig. 3. Higher H-score for BRM staining intensity in CRSwNP – group of CRS with nasal polyps without oral steroids therapy (0) in comparison to patients with systemic treatment (1) (p > 0.05)

CRSwNP – chronic rhinosinusitis with nasal polyps; CRS – chronic rhinosinusitis.

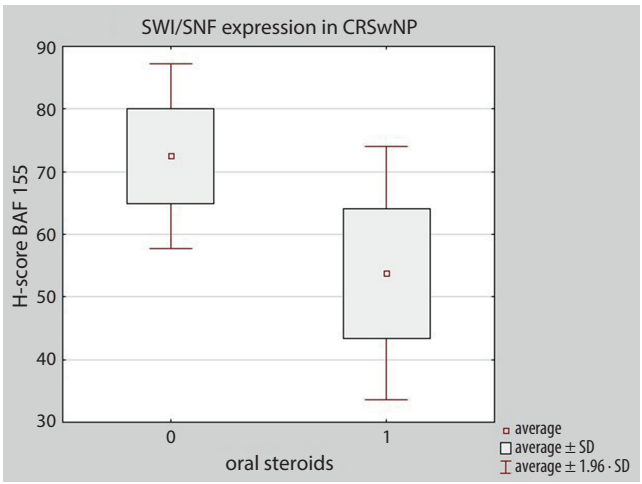

Fig. 4. Higher H-score for BAF 155 staining intensity in CRSwNP – group of CRS with nasal polyps without oral steroids therapy (0) in comparison to patients with systemic treatment (1) (p > 0.05)

CRSwNP – chronic rhinosinusitis with nasal polyps; CRS – chronic rhinosinusitis.

mass index (BMI). Patients with higher neutrophils blood count (F = 9.23 and p = 0.004) had a significantly lower H-score BRG1 (Table 2). Moreover, in the CRSwNP group, allergy coexistence (F = 4.12 and p = 0.048) and VDR expression level (F = 4.19 and p = 0.048) significantly influenced BAF155 protein expression level (F = 4.3 and p = 0.02) (Table 2).

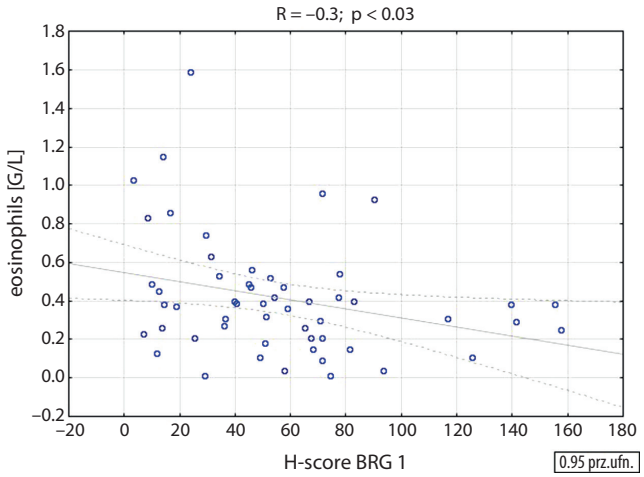

Fig. 5. Correlation of protein expression level of BRG1 with serum level of eosinophils (R = –0,3; p < 0.03) in CRSwNP group

CRSwNP – chronic rhinosinusitis with nasal polyps.

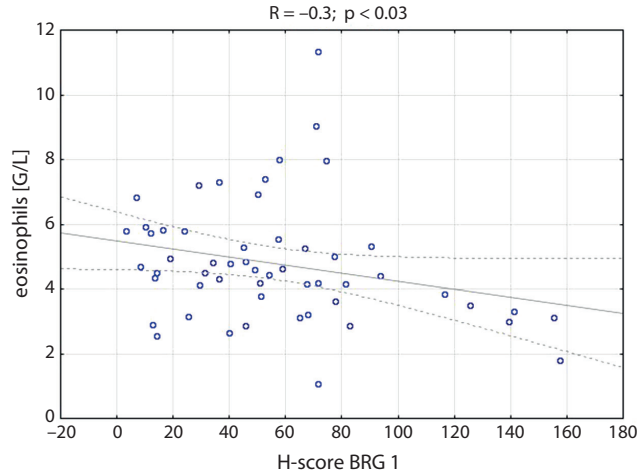

Fig. 6. Correlation of protein expression level of BRG1 with serum level of neutrophils (R = –0,3; p < 0.03) in CRSwNP group

CRSwNP – chronic rhinosinusitis with nasal polyps.

In the CRSsNP group, L–M score was the only factor significantly influencing BRG 1 expression level (F = 6.19 and p = 0.02). The patients with higher L–M scores had a significantly lower BRG 1 expression than patients with lower L–M scores (Table 3).

Table 2. The multiple regression analysis in chronic rhinosinusitis group with nasal polyps (CRSwNP) shows correlations for BRM expression and allergy, BRG 1 with neutrophils and BMI and BAF 155 with allergy and expression of VDR (p < 0.05)

Variable		Parameter estimate	F	p-value	Model	
					F	p-value
BRM	intercept	68.6	145.86	<0.001	6.28	0.015
	allergy	–21.8	6.28	0.015		
BRG 1	intercept	5.5	0.04	0.8	7.54	0.001
	neutrophils	–8.14	9.23	0.004		
	BMI	3.34	10.78	0.002		
BAF 155	intercept	63.82	34.53	<0.0001	4.3	0.02
	allergy	–24.52	4.12	0.048		
	H-score VDR	0.33	4.19	0.048		

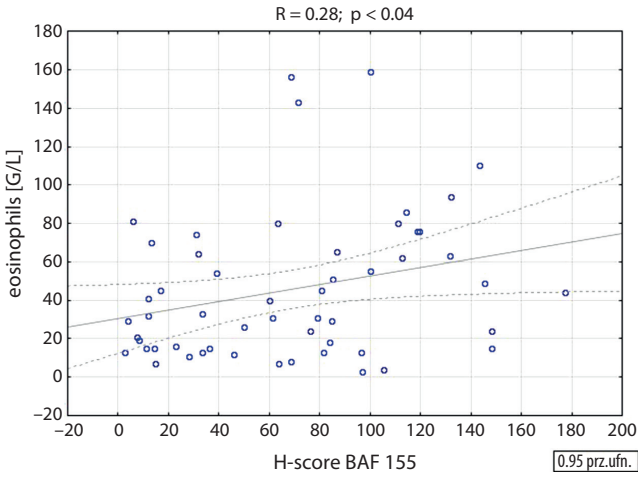

Fig. 7. The Spearman correlation for BAF155 protein expression level and VDR expression in CRSwNP group. BAF155 protein expression presented a positive correlation with VDR protein expression level (p < 0.05)

CRSwNP – chronic rhinosinusitis with nasal polyps; VDR – vitamin D receptor.

The SWI/SNF subunits expression in HNECs after treating with LPS, SEB and vitamin D3

To examine if an expression of the SWI/SNF subunits encoding genes can be modulated by infection or vitD3, the HNEC cell line was stimulated with LPS, SEB and vitD3 for 24 h. Moreover, HNECs were incubated with both vitD3 and LPS or SEB. The expression level of main SWI/SNF subunits was measured using quantitative reverse-transcription polymerase chain reaction (qRT-PCR) method with transcript-specific primers. Stimulation with LPS decreased significantly the transcript level for BRG1, BAF155 and INI1. The level of BAF 170 and INI1 increased after SEB stimulation. Moreover, the vitD3 and SEB stimulation of HNECs increased significantly the transcript level of BAF155 and BAF170, but decreased it for INI1. Increase of INI1 transcript level after vitD3 stimulation was higher for HNECs with LPS in comparison to HNECs without LPS. In the other cases, the transcript level for measured genes did not change (Fig. 8).

Discussion

In this study, we showed the SWI/SNF chromatin remodeling complex as an important factor that may influence steroids and vitamin D signaling pathways in sinonasal mucosa, thus contributing to the pathogenesis and treatment of CRS. The main findings of our study are the following: 1) higher

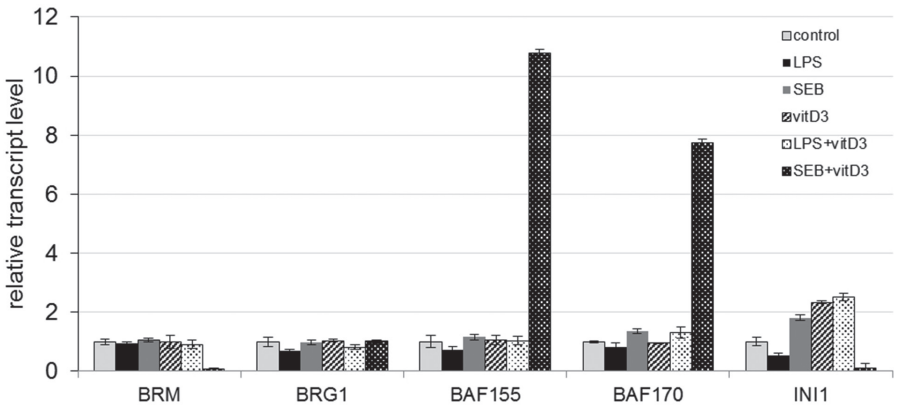

Fig. 8. Relative transcript level for the SWI/SNF main subunits BRG1, BRM, BAF155, BAF170 and INI1 after treating with LPS, SEB, vitD3, or both vitD3 and LPS or SEB compared with control untreated cells

LPS – lipopolysaccharide; SEB – staphylococcal enterotoxin B; vitD3 – vitamin D3.

Table 3. The multiple regression analysis in chronic rhinosinusitis group without nasal polyps (CRSsNP) shows inverse correlation for BRG1 expression and Lund–Mackay CT score (L–M) ($p < 0.05$).

Variable		Parameter estimate	F	p-value	Model	
					F	p-value
BRG 1	intercept	110.27	33.75	<0.001	4.09	0.049
	L–M	–5.14	4.09	0.049		

protein expression levels for BRG1, BRM and BAF155 in CG in comparison to CRSsNP group and CRSwNP group ($p < 0.05$); 2) statistically significant negative correlation of the BRG1 protein expression with eosinophils and neutrophils in the CRSwNP group ($p < 0.05$); 3) statistically significant positive correlation of the BAF155 protein expression with VDR expression level in the CRSwNP group ($p < 0.05$); 4) relation in the multiple regression between the SWI/SNF protein expression level with allergy, neutrophils and BMI value ($p < 0.05$) in CRS patients; 5) decreased transcript level of the SWI/SNF subunits in HNECs after LPS stimulation, but increased after SEB stimulation.

Many authors presented the important role of the SWI/SNF complex in the regulation of the inflammatory response and hormone metabolism.[8,12,13] Ramirez-Carrozzi et al. showed that the SWI/SNF complex is involved in the inflammatory response stimulated with LPS murine macrophages. The constitutive association of BRG1 with the promoters of early primary inflammatory response genes suggests that the SWI/SNF complex might contribute to the initial establishment of "open" chromatin structures during macrophage development.[6] Additionally, Hu et al. noticed that the SWI/SNF complex modulates the transactivation of the late-primary inflammatory response genes in macrophages in response to microbial challenges. The study showed the inhibition of the SWI/SNF complex recruitment to set gene promoter regions by lincRNA-Cox2 siRNA-A in LPS-treated cells.[23] According to the above data, we assumed that the SWI/SNF complex may play an important role in the inflammatory process contributing to CRS. Our results were based on the quantitative and qualitative analysis of the SWI/SNF complex core subunits expression in sinonasal mucosa. We observed a significantly higher protein expression level of the SWI/SNF subunits (BRG1, BRM and BAF155) in the CG compared to patients with CRS.

Therapy for CRS is currently based on both intranasal and oral GS. Oral steroids therapy is ordered in patients who manifest massive nasal polyps and worse prognosis in medical therapy. In this respect, it is important to analyze various aspects of GS activities and their interaction in relation to the SWI/SNF complex. In many cases, glucocorticoid therapy is ineffective; however, the reason remains unknown. It has been shown that proper functioning of GR is associated with the SWI/SNF complex. The SWI/SNF complex regulates genes expression following the response to GS. Hormones initiate a binding process of GR to glucocorticoid response element (GRE), in cooperation with the SWI/SNF complex, resulting in global changes in gene expression. Without the SWI/SNF complex, GR-dependent gene expression is blocked and GR function can be impaired.[12,13,24] The decreased level of the main SWI/SNF subunits in CRS patients can suggest that steroid therapy can be inefficient (Fig. 9). The results of our study showed the lower expression of all examined SWI/SNF complex subunits in the CRSwNP group in comparison to CG ($p < 0.05$). Moreover, we noticed higher SWI/SNF protein expression level in patients without oral steroids, though not statistically significant, in comparison to patients who underwent this treatment in the CRSwNP group ($p > 0.05$). Therefore, our results suggest that treatment with steroids in CRSwNP patients showing dysfunctional SWI/SNF signaling may not be effective. In consequence, the alternative medical treatment should be proposed.

To better identify recalcitrant patients who are unresponsive to steroids therapy, we analyzed the SWI/SNF subunits and other clinical data. We found that the BRG1 and ATPase SWI/SNF subunit abundance presented

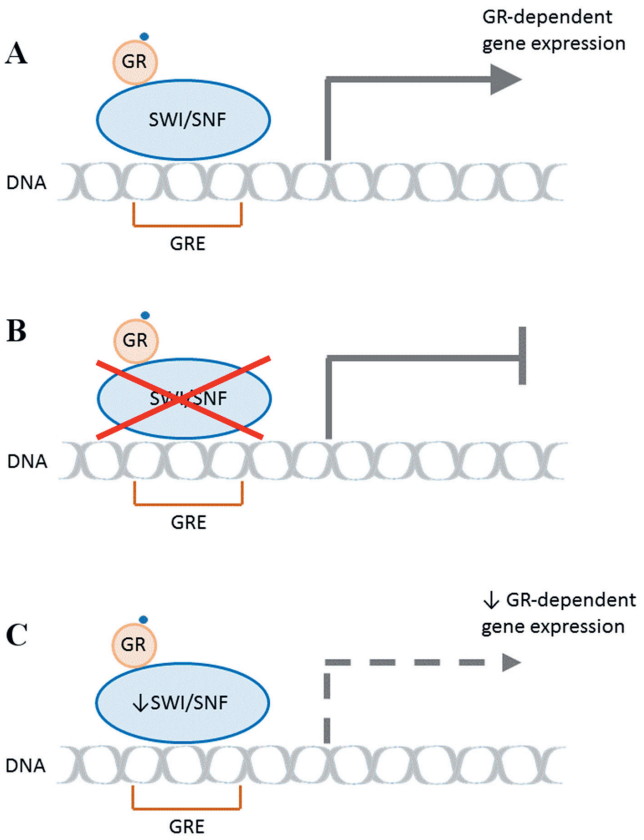

Fig. 9. A model describing GR and the SWI/SNF complex cooperation in response to steroids treatment. Hormone (small blue dot) is shown binding the GR. It enters the nucleus and binds target sequences, such as the GRE to the DNA. Next, the complex SWI/SNF cooperates with the promoter through interaction with GR, and then the nucleosomes are repositioned. A. The SWI/SNF complex is correct, proper GR response gene expression. B. Loss of the SWI/SNF complex, blocked GR-dependent gene expression. C. The SWI/SNF complex deficiency, reduced GR-dependent gene expression

GR – glucocorticoid receptor; GRE – glucocorticoid response element.

an inverse correlation with the number of eosinophils and neutrophils in the blood of patients with CRSwNP. For a better understanding of the disease course and prediction of treatment outcomes, some authors categorized CRSwNP into various subtypes, such as eosinophilic CRSwNP, neutrophilic CRSwNP and noneosinophilic nonneutrophilic CRSwNP.[25,26] Other data indicated that blood eosinophilia is related to the extent of sinonasal mucosal involvement, the severity of nasal disease, size of nasal polyps, and a higher risk of disease recurrence.[27,28] Moreover, Fokkens et al. proposed another form of management for patients with eosinophilic CRS.[29] Our study showed lower expression of BRG1 in the CRSwNP group with a higher level of blood eosinophils and neutrophils in comparison to CRSwNP individuals without eosinophilia and neutrophilia. Therefore, this negative correlation of eosinophils and neutrophils with the SWI/SNF complex may expound the worse prognosis in this group of patients. Thus, blood analysis of the amount/number of eosinophils and neutrophils could be a prognostic factor for GR effectiveness through the SWI/SNF complex abundance. In the group of patients with CRSwNP and eosinophilia/neutrophilia nasal steroids, there will probably be poor effectiveness and some treatment modification will be needed. These findings, i.e., high number of eosinophils and neutrophils, might be an efficient and simple clinical marker to identify patients with CRSwNP who present impaired SWI/SNF expression, meaning that they either respond worse or not at all to medical treatment with oral steroids. To our knowledge, this is the first report on the SWI/SNF complex subunits expression in CRS in relation to the treatment.

Further, we analyzed the correlations of our results with VDR expression. Our previous study on CRS pathophysiology presented VDR and 1α-hydroxylase expression in sinonasal mucosa. This study showed a statistically significant decrease of VDR expression in CRSsNP and CRSwNP patients in comparison to the CG.[30] Additionally, Wei et al. analyzed the role of the SWI/SNF complex (BAF and PBAF) subunits in relation to VDR and vitamin D in pancreatic β-cells protection. Ligand binding promotes VDR association with the SWI/SNF subunit, resulting in an anti-inflammatory response in the murine type 2 diabetes model.[14] Likewise, VDR signaling plays an important role in the regulation of the immune processes in paranasal sinuses.[15–19] In our study, the protein expression level of BAF155, a core SWI/SNF chromatin remodeling complex subunit, correlates positively with VDR expression level in the CRSwNP group. Further, after administration of vitD3 to the HSNEC, we observed a significant increase in the BAF170 and INI1 transcript level. Therefore, we assume that VDR, upon ligand- (vitD3) binding, may reduce the pro-inflammatory response in sinonasal mucosa via the SWI/SNF-induced transcriptional changes, thus contributing to the treatment of CRS.

Furthermore, in multiple regression models, we found the relation of the SWI/SNF subunits with numerous clinical findings, i.e., L–M scores, allergy, BMI values, neutrophils, and VDR abundance. The BRG1 protein expression level was associated with L–M score. The L–M CT score is a useful tool to assess the radiological stage of rhinosinusitis.[31] Our results confirm lower BRG1 expression in CRSsNP patients with higher L–M scores. In the CRSwNP group, BRM expression level was associated with allergy. The expression of BRG1 protein is associated with the amount of neutrophils in blood and BMI value in the CRSwNP group. Additionally, interesting results were observed for BAF155 protein level, e.g., its relation with allergy and VDR abundance. Therefore, we showed that there might be a link between allergy, vitamin D and VDR in the CRS pathophysiology.[15–19] Our results suggest a new mechanism of VDR action in cooperation with the SWI/SNF chromatin remodeling complex.

Moreover, we analyzed the SWI/SNF genes expression after HNECs line has been treated with vitD3, LPS, SEB, and both vitD3 with LPS or SEB. The vitD3 treatment increased the INI1 encoding gene transcript level. The SEB had an increased BAF170 and INI1 genes expression.

The LPS treatment of HNECs decreased the expression of BRG1, BAF155 and INI1 genes; however, it did not affect BRM and BAF170 genes transcription. The vitamin D and SEB stimulation of HNECs significantly increased BAF155 and BAF170 transcript level. The role of vitamin D and its receptors has been vastly addressed in recent studies on CRS pathophysiology. Vitamin D is recognized as the factor influencing the CRS.[3] Research results from the last decade show the immunomodulatory effect of vitamin D on the mechanisms of CRS by reducing inflammation,[32,33] inducing cathelicidin (hCAP18), which is the only antimicrobial peptide produced by the human body,[34] and by stimulating neutrophils and macrophages in anti-inflammatory responses.[35] Other studies on SEB have demonstrated that SEB lead to T-cell proliferation (CD4+ and CD8+) and pro-inflammatory cytokine production, and can influence the activity of immunomodulatory and pro-inflammatory effector epithelial cells and, therefore, may have a potentially important role in the pathogenesis of CRS. The presence of nasal polyps in CRS seems to be associated with inflammatory mechanisms resulting from microbial products.[36–38] The LPS is a cell wall surface antigen of Gram-negative bacteria and a biologically active substance activating many transcription factors. It triggers an inflammatory signaling cascade.[39] Therefore, SEB and LPS may contribute to CRS pathogenesis through respectively increasing or decreasing gene expression encoding for the SWI/SNF complex subunits, subsequently compromising anti-inflammatory signaling pathways of steroids, thus contributing to recalcitrant course of the disease.

The SWI/SNF interacts with GR and regulates genes expression following the response to GS. Without the SWI/SNF complex, GR-dependent gene expression is blocked and GR function can be impaired.[12,13,24] Additionally, as a continuation of our previous studies,[30] we found a link (positive correlation) between VDR and the SWI/SNF complex in patients with CRSwNP. Moreover, GR and vitD3 presents antagonistic activity in human cells signaling.[40] Due to the fact that the SWI/SNF complex may be involved in both GR and VDR signaling pathways, it is very likely that decreased expression of this complex in the sinonasal mucosa of CRSwNP patients can disturb anti-inflammatory function of steroids because of competition with vitD3 in the sinonasal mucosa contributing to CRS. Results of our study are of clinical relevance due to the fact that based on the correlation of the SWI/SNF proteins expression and clinical data, it is possible to identify responders and non-responders to steroids treatment in the CRSwNP group.

Conclusions

The strength of this study is that it included an analysis of a large group of patients with CRS with or without polyps compared to CG. Furthermore, this is the first report on the expression of the SWI/SNF complex subunits in the sinonasal mucosa and its clinical associations. We proved the significant differences in the protein expression of the SWI/SNF subunits in the sinonasal mucosa between the groups. We found a positive correlation for BAF155 protein expression with VDR level and a negative correlation of BRG1 subunit with blood eosinophils and neutrophils. Moreover, we analyzed the expression of genes encoding for SWI/SNF subunits in HNECs after vitD3, LPS and SEB stimulation. Our results contributed to the knowledge of the molecular inflammatory process in sinonasal mucosa in the CRS and showed the need for alternative treatment options in recalcitrant CRS. Further analysis of the function of the SWI/SNF complex in response to steroids and vitamin D may be beneficial for an understanding of the pathophysiology of CRS, especially in patients with a recalcitrant course of the disease.

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The effect of middle ear effusion on the inner ear condition in children

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Abstract

Background. Otitis media with effusion (OME) is the most common cause of hearing impairment among children in developed nations. Middle ear (ME) fluid accumulation leads to progressive hearing impairment, usually of the conductive type. In some cases, mixed hearing loss associated with OME has been noted. It was reported that effusion in the ME has a negative impact on the vestibular system of the inner ear.

Objectives. The aim of this random-sample cohort study was to evaluate postural stability and the influence of ME drainage on vestibulospinal reflexes in children with OME, and to determine whether disturbances in the vestibular organ correlate with a sensorineural component in OME-related hearing loss.

Material and methods. The study group consisted of 53 children with bilateral OME who were treated with bilateral ME drainage. The study group was divided into subgroups according to hearing loss. The control group consisted of 29 healthy children. Vestibular function and hearing evaluation were performed before and 4 weeks after drainage.

Results. A comparison of the stabilograms of the study group and the control group revealed elevated parameters in most of the tests. In the subgroup with mixed hearing loss, either before or after ME drainage, elevated stabilogram parameters were found in all tests. Posturography revealed vestibular system disturbances before and after ME drainage in the subgroup with mixed hearing loss, especially before ME drainage. The stabilogram parameters in the subgroup with conductive hearing loss after ME drainage were better in most tests in comparison to those before the procedure.

Conclusions. The presence of effusion in the ME has a negative effect on the inner ear. We highlight the importance of monitoring the condition of the vestibular system in all children with OME, especially in cases with mixed hearing loss and more advanced clinical stages of the disease.

Key words: vertigo, balance disorders, otitis media with effusion

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Background

Otitis media with effusion (OME) is the most common cause of hearing impairment among children in developed nations.[1–3] Middle ear (ME) fluid accumulation[4–8] leads to progressive hearing impairment, usually of the conductive type. In some cases, mixed hearing loss associated with OME has been noted.[9,10] It was reported that effusion in the ME has a negative impact on the vestibular system of the inner ear.[7,11–14]

Objectives

The aim of this random-sample cohort study was to evaluate postural stability and the influence of ME drainage on vestibulospinal reflexes in children with OME and to determine whether disturbances present in the vestibular organ correlates with a sensorineural component in OME-related hearing loss.

Material and methods

Material

The study group consisted of 53 children (20 females and 33 males) aged 4–14 years (mean age: 8 years; SD: 2.5 years) diagnosed with bilateral OME. Those children were treated with bilateral ME drainage.

The study group included children with recurrent or persistent bilateral OME persisting 3 months or longer after conservative treatment had proven ineffective, with a hearing level in the better ear of 25–30 dBHL or worse, averaged at 0.5 kHz, 1 kHz, 2 kHz, and 4 kHz (or an equivalent dBA where dBHL was not available), and with type B tympanometry.

Children with a birth weight of less than 2,500 g; a history of neonatal asphyxia; congenital malformations of the external, middle or inner ear; temporal bone fracture; neurological diseases or any other serious illness (epilepsy); a history of meningitis; and a history of vestibulotoxic or ototoxic drugs were excluded from the study.

The study group was divided into the following subgroups according to hearing loss:

- conductive hearing loss (25 children: 14 boys and 11 girls) aged 4–11 years (average age: 7.76 years; SD: 1.8 years) and
- mixed hearing loss (28 children: 19 boys and 9 girls) aged 4–14 years (average age: 8.18 years; SD: 2.85 years).

The subgroup with mixed hearing loss included children with more severe pathological changes, including retraction pockets or local atrophy of the tympanic membrane, or those with a past history of ME drainage and a disease lasting longer than 2 years.

The control group consisted of 29 healthy children (13 girls and 16 boys) aged 4–17 years (average age: 10 years; SD: 3.8 years), who had been scheduled for a tonsillectomy operation in the Ear, Nose and Throat (ENT) Department. The children's medical history was obtained from their parents.

Methods

All of the children underwent a complete otoneurological examination. A detailed case history was collected, with particular attention paid to the current disease, previous ENT diseases, and the presence of vertigo and/or disequilibrium. The children and parents were asked whether the children had suffered from frequent falls; had difficulty riding a bicycle, or climbing or descending stairs; whether they disliked swings; had a tendency to bump into objects or to misjudge distances; experienced vertigo, dizziness, tinnitus, or disequilibrium; or whether they exhibited unexplained clumsiness, delayed gross motor development or recurrent headache.

Each child underwent a full physical otolaryngological examination. An assessment of ME status and the function of the ventilation tubes was performed using pneumatic otoscopy, performed by a certified otoscopist.

Tympanometry was performed using a Madsen Zodiac 801 tympanometer and done by a qualified audiologist prior vestibular testing. An audiometric evaluation using a Madsen OB 822 (Otometrics, Denmark) audiometer was performed by means of conventional audiometric methods in a soundproof room. The collected data included air conduction and bone conduction thresholds in each ear at frequencies of 0.5 kHz, 1 kHz, 2 kHz, and 4 kHz. The pure-tone average for bone conduction in each ear was calculated for each patient as the average of bone conduction thresholds at 0.5 kHz, 1 kHz, 2 kHz, and 4 kHz. The air–bone gap in the affected ear was calculated as the difference between air conduction and bone conduction thresholds at 0.5 kHz, 1 kHz, 2 kHz, and 4 kHz. Pure-tone audiometry and tympanometry was performed before vestibular testing. Audiometry was carried out before drainage and 4 weeks postoperatively. Tympanometry was only performed prior to ME drainage.

Vestibulospinal reflexes were evaluated through stato-posturography using a Posturographer PE 62 Model 04 (Neurocom, Luxembourg). The diagnostic system consisted of an an IBM microcomputer (IBM Corp., Armonk, USA) with additional converters, a static posturographic 40 × 40 cm platform with pressure sensors recording deflections in the range of ±10 cm (0.05 mm accuracy), and a visual stimulator connected to a TV screen. In each case, a set of 3 tests was performed in order to evaluate static balance. Each child was tested under different conditions: test 1 was performed standing with eyes open (E-O); test 2 was performed standing on the platform with eyes closed (E-C); and test 3 was performed with eyes open and feet rested on the platform, the child observing a moving point of light, which reflects the current

position of the center of gravity, and making slight movements of the body in order to self-correct their current body position (feedback test (F-T)). Three trials, lasting 30 s each, were performed for each condition. The field of developed area (FDA; the area described by the center of mass [mm^2]) and the average sway velocity (ASV) [mm/s] were analyzed.

Vestibular testing was performed by a technician who had no knowledge of each child's ear status. Vision tests were not carried out.

Children with bilateral OME were examined while hospitalized, before the bilateral insertion of ventilation tubes and 4 weeks after the procedure during their postoperative check-up.

Informed consent was obtained from all parents/legal guardians of individual participants included in the study. The study was conducted in accordance with the Declaration of Helsinki after obtaining approval from the local Bioethics Committee (approval No. KB-29/2005). One or both parents accompanied each child during all testing procedures.

Statistical analysis was carried out using Student's t-test to compare the average values of parameters before and after drainage. A p-value <0.05 was considered to be statistically significant for all tests. The statistical analysis was performed using STATISTICA v. 13.0 (StatSoft, Inc., Tulsa, USA).

Results

Otoscopic testing showed OME symptoms in all of the selected patients. Before the ventilation tubes were inserted, none of the patients complained of vertigo or balance disorders. In the study group, impedance audiometry showed bilateral type B tympanometry before ME drainage in all 53 children (100%). Before drainage, pure-tone audiometry showed bilateral conductive hearing loss in the range of 40–50 dB in 25 children (47%) and bilateral mixed hearing loss with an average air conduction threshold of 35–45 dB at high frequencies in 28 children (53%).

Control pure-tone audiometry performed after ME drainage revealed:

- in the subgroup with conductive hearing loss – normal hearing in 22 children (88%) and bilateral conductive hearing loss with cochlear reserve approx. 15 dB in 3 children (12%), and
- in the subgroup with mixed hearing loss – a decrease or lack of cochlear reserve and bilateral high-frequency hearing loss in the range of 35–45 dB.

Posturography

A comparison of the stabilograms of the study group and the control group revealed elevated parameters for FDA and ASV in most of the tests. The findings showed statistically significant values ($p < 0.001$) before ME drainage for FDA in all tests and for ASV with E-O and E-C in the study group. After the ME drainage procedure, there were statistically significant values for FDA with E-O and E-C (Fig. 1–4).

The stabilogram parameters were compared between subgroups, divided according to the group with hearing loss and the control group.

In the subgroup with mixed hearing loss, either before or after ME drainage, elevated stabilogram parameters were found for FDA and ASV in all tests in comparison with those of the subgroup with conductive hearing loss. Before drainage, there were statistically significant values ($p < 0.05$) for FDA with E-O and E-C and for ASV with E-O. After ME drainage, statistically significant values ($p < 0.05$) were found for FDA with the F-T (Fig. 5,6).

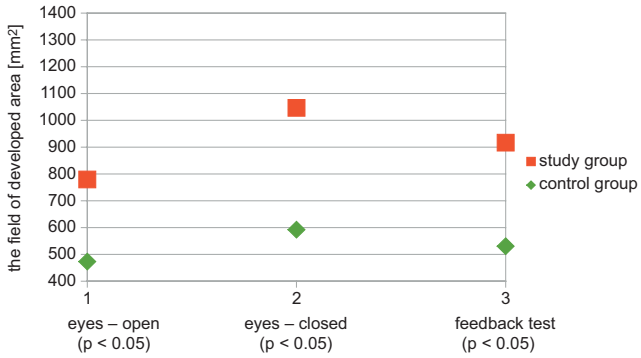

Fig. 1. The FDA in the study and control groups before ME drainage

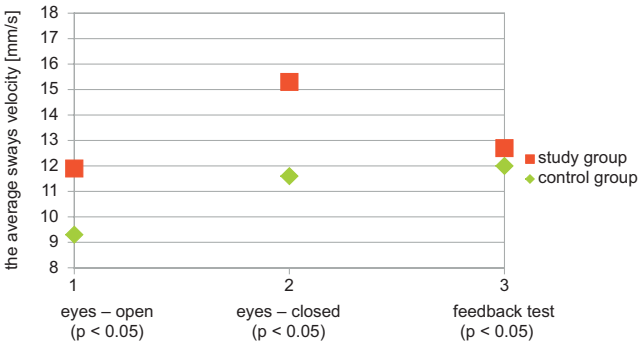

Fig. 2. The ASV in the study and control groups before ME drainage

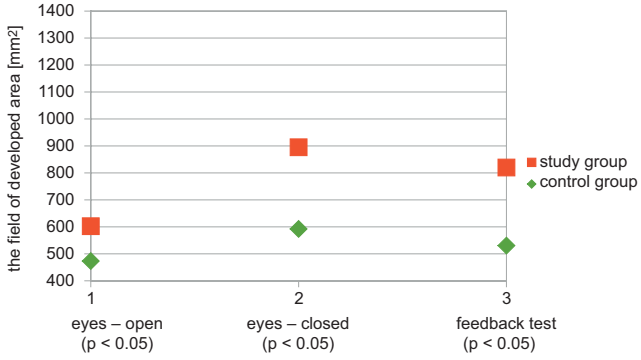

Fig. 3. The FDA in the study and control groups after ME drainage

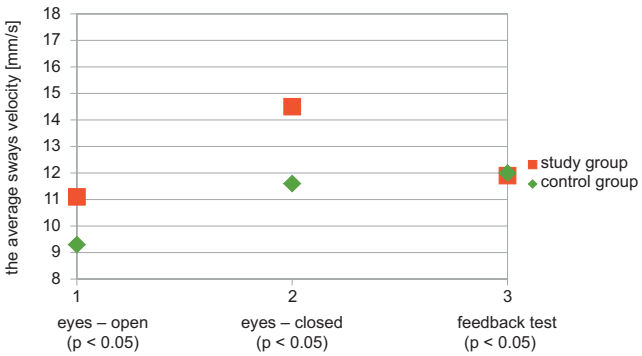

Fig. 4. The ASV in the study and control groups after ME drainage

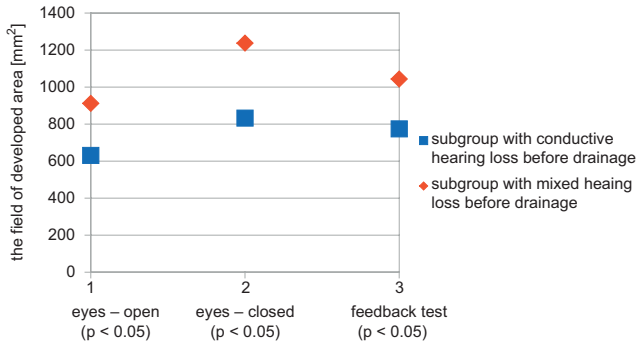

Fig. 5. The FDA in the subgroups with conductive hearing loss and with mixed hearing loss before ME drainage

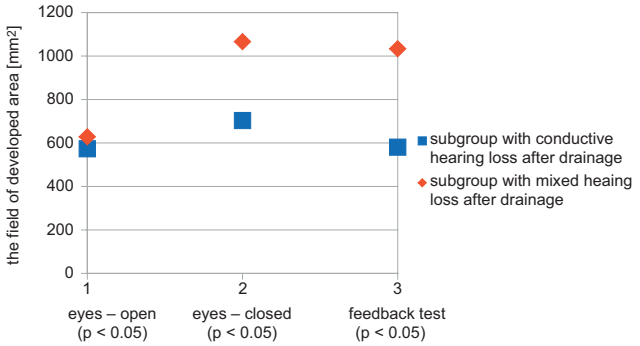

Fig. 6. The FDA in the subgroups with conductive hearing loss and with mixed hearing loss after ME drainage

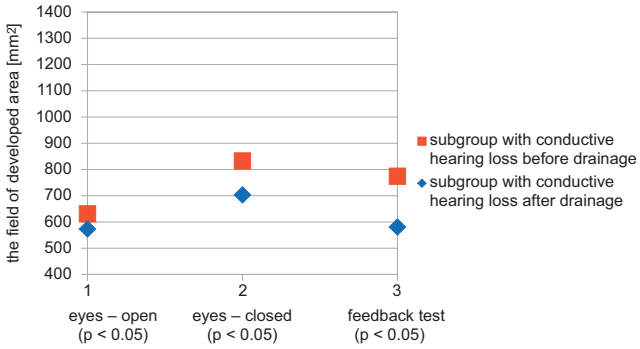

Fig. 7. The FDA in the subgroup with conductive hearing loss before and after ME drainage

Posturography revealed more significant vestibular system disturbances before and after ME drainage in the subgroup with mixed hearing loss compared to the subgroup with conductive hearing loss, especially before the tympanostomy procedure.

The stabilogram FDA and ASV parameters in the subgroup with conductive hearing loss after ME drainage were better in most tests in comparison to those before ME. Statistically significant values ($p < 0.05$) for FDA were found with E-C and F-T (Fig. 7).

In the subgroup with mixed hearing loss, the stabilogram parameters after ME drainage were lower in all tests than those before drainage. Statistically significant values were revealed ($p < 0.001$) for FDA and ASV with E-O.

Discussion

The precise pathomechanism of balance disorders in the course of OME is not fully understood. The study conducted at the beginning of the 20th century by Merica et al. (According to: Grace A, Pfeiderer A. Dysequilibrium and otitis media with effusion: What is the association? *J Laryngol Otol*. 1990;104(9):682–684) demonstrated that the functional insufficiency of the Eustachian tube causes dizziness in children. Golz et al. reported that the presence of effusion in the ME is the main cause of vertigo in children.[12] One of the theories states that changes in hydrostatic pressure in the ME are transmitted through the round window and subsequently lead to secondary changes within the inner ear fluids. Due to numerous reports detecting bacteria cultures in the ME effusions, it is believed that bacterial toxins may penetrate into the labyrinth resulting in dizziness.[15] The balance disorders in children with OME may also be a result of recurrent and frequent episodes of acute otitis media. Some authors report that vestibular system disturbances, but also the development of sensorineural component in OME-related hearing loss, are the result of changes within the kinocilia and stereocilia ionic channels.[16]

The results of our study confirm the negative effect of ME effusion on the vestibular system in children with OME. Elevated stabilogram parameters before and after ME drainage were found in the study group in comparison with the control group. Similar results were reported by other authors studying the nature of postural stability and the effect of ME drainage on vestibulospinal reflexes in children with OME.[13,14,17,18] After 4 weeks, there was an improvement in stabilometry parameters, though they did not completely normalize in the study period and were elevated in comparison to healthy children. Some authors emphasize the possibility of the chronic nature of vestibular system deficits, which may have a negative impact on a child's proper motor development.[12,17] Casselbrant et al. evaluated the vestibular system in children with OME and studied visual dependency for balance by assessing

the influence of optical flow on postural stability. Their study showed that maintaining postural stability in children with OME requires the excessive involvement of other, non-vestibular sensory information; this is not the case in healthy children.[13]

The presence of an effusion in the ME may lead to difficulties in the sensory integration, whose proper functioning is needed to develop language, and social integration. Children with vestibular dysfunction sometimes show delayed development of gross motor skill milestones compared with children without vestibular dysfunction. This emphasizes the importance of assessing balance function in children.

It was revealed that a higher number of myringotomies and ventilation tube insertions and a longer duration of OME are risk factors for developing permanent hearing loss, both conductive and sensorineural.[19] Sorri et al. stated that worse thresholds, especially at high frequencies, in children with recurrent acute otitis media and OME indicate inner ear involvement.[20] Mixed hearing loss associated with OME has been reported in various types of otitis media, including OME.[9,10] Its precise pathogenesis is unclear. One of the theories is that inflammatory agents present in ME effusion pass through the round window membrane into the inner ear and cause temporary threshold shifts or permanent threshold shifts limited to the cochlear basal turn.[21] Mutlu et al. stated that changes in the ME lead to a temporary inhibition of the vibratory movement of the oval and/or round window membrane or to an alteration of the ionic composition of the inner ear fluids. This subsequently leads to a reversible cochlear dysfunction, which usually appears as a depressed threshold region around 2 kHz.[22] Another theory claims that mixed hearing loss is a consequence of a true disturbance of inner ear function, which does not resolve after an episode of OME.[22,23] It has been revealed that elevated levels of hypoxia-inducible factor 1-α in ME effusion may play an important role in the pathogenesis of the bone conduction impairment associated with OME. In children with mixed hearing loss, a more significant elevation of stabilogram parameters was found compared to children with conductive hearing loss. Before ventilation tube placement, disturbances in posturographic tests were greater in children with mixed hearing than in children with conductive hearing loss. After ME drainage, the stabilogram parameters improved, though the improvement was more significant in children with conductive hearing loss than in children with mixed hearing loss, which included children with more advanced OME.

None of the parents or children had any complaints connected to vestibular organ pathology. According to the parents, their children's motor function development was normal, and no impairments in the child's psychomotor milestone achievement were reported. Interestingly, some of the parents noticed an improvement of their child's motor skills after ME drainage. The children were more willing to partake in physical activities requiring greater coordination and balance, such as climbing a ladder on the playground or riding a bicycle. This trend was also reported in the literature.[14,15,18,24]

Conclusions

The presence of effusion in the ME has a negative effect on the inner ear. Our findings highlight the importance of monitoring the condition of the vestibular system in all children with OME, especially in cases with mixed hearing loss and more advanced clinical stages of disease. The more pronounced improvement of vestibular function in children with conductive hearing loss after ME drainage in comparison to children with mixed hearing loss suggests that impairment of inner ear function affects its vestibular and cochlear parts.

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Estimating brain volume loss after radiation therapy in children treated for posterior fossa tumors (Corpus callosum and whole brain volume changes following radiotherapy in children)

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Abstract

Background. More than half of pediatric tumors of central nervous system (CNS) primarily originate in the posterior fossa and are conventionally treated with radiation therapy (RT).

Objectives. The objective of this study was to establish whether corpus callosum volumes (CCV) and whole brain volumes (WBV) are correlated and to determine the impact of whole-brain low- vs high-dose RT on brain parenchymal volume loss as assessed using each technique.

Material and methods. Of the 30 identified children (6–12 years) with newly diagnosed posterior fossa tumors treated with cranial RT, including focal and whole-brain RT, suitable imaging was obtained for 23. Radiotherapy regimens were the following: no whole-brain RT (Group 1, n = 7), low-dose whole-brain RT (<30 Gy, Group 2, n = 9) and high-dose whole-brain RT (>30 Gy, Group 3, n = 7) in addition to focal boost. Magnetic resonance images (MRIs) were analyzed at baseline and follow-up (median 14 months). The CCVs were manually segmented on midline sagittal slice (n = 23), while WBVs were segmented semi-automatically using Freesurfer (n = 15). This was done twice (6-month interval) for all baseline CCV measurements and 5 randomly selected WBV measurements to establish measurement reproducibility. Correlations between CCV and WBV were investigated and percentage of children demonstrating reduction in CCV or WBV noted.

Results. Correlation between baseline CCV and WBV was not significant (p = 0.37). Measurement reproducibility was from 6% to –9% for CCV and from 4.8% to –1.2% for WBV. Among the children studied, 30.4% (7/23) had >9% reduction in CCV at follow-up, while 33.3% (5/15) had >1.2% reduction in WBV. Five of 7 patients with CCV loss were not picked up by WBV measurements. Similarly, 3 of 5 patients with WBV loss were not picked up by CCV measurements.

Conclusions. The CCV and the WBV are unrelated and may indicate different brain parenchymal losses following RT. Up to a third of posterior fossa tumors treated with RT have measurable CCV or WBV loss; incidence was equivalent in low- vs high-dose whole-brain RT.

Key words: posterior fossa tumor, radiotherapy, brain volume

Introduction

More than half of pediatric tumors of the central nervous system (CNS) primarily originate in the posterior fossa and are conventionally treated with radiation therapy (RT).[1] Such therapy is extremely effective and has led to an increase in long-term survivorship. However, this treatment induces neurotoxicity, manifesting as various forms of motor and cognitive long-term impairment, and is problematic in individuals in whom there is a long life expectancy: quality of life in children surviving treatment of CNS tumors is recognized as being of vital importance.[2,3] Neurobehavioral morbidity is a late outcome measure, with limited management options, which is why early objective indicators of likely motor or cognitive deficit are increasingly sought in order to implement appropriate management strategies early.[3]

Magnetic resonance imaging (MRI) has revolutionized anatomic assessments of the normal and diseased brain and has enabled characterization and monitoring of structural changes within the brain resulting from RT. Various degrees of parenchymal volume loss and generalized white matter signal changes have been reported in children treated for medulloblastoma (MDL).[4–7] Most studies used a fully automated hybrid neural network segmentation as well as a classification method to quantitatively derive volumes of brain parenchyma from these images.[4,7,8] Volumes of the corpus callosum (CC) have also been advocated as a surrogate to quantify volumes of neuroparenchyma, because the nearly 180 million myelinated axons within this white matter commissure make it susceptible to radiation-induced damage.[5,9] Nevertheless, a correlation between CC volume (CCV) and whole brain volume (WBV) has not been demonstrated. The purpose of this study, therefore, was to establish whether CCVs and WBVs are correlated and to determine the impact of low- vs high-dose RT on brain parenchymal volume loss as assessed using each technique.

Material and methods

Patients

Approval for this study was obtained from the Institutional Review Board. Informed consent from the parents was waived.

We searched the pediatric oncology database of the Royal Marsden Hospital (Sutton, London, UK) regarding a period from 2000 to 2013 and identified 30 children between the age of 6 and 12 with newly diagnosed posterior fossa tumors, who were treated with cranial RT and in whom baseline (pre-RT) and follow-up (at least 6 months after treatment) MRI scans were available. Radiotherapy regimens were either focal to the posterior fossa only or included whole-brain RT at low (<30 Gy) or high (>30 Gy) dose.

Children below the age of 6 and those above the age of 12 were excluded from the study to ensure the similar age of our cohort. Other exclusion criteria were the following: 1) radiological evidence of surgery-related intracranial bleeding (excluding asymptomatic, resolving hemorrhagic changes associated with recent surgery and the presence of punctate hemorrhage in the tumor), 2) any disease or condition that disabled compliance according to the appropriate radiation regimen, 3) prior diagnosis of malignancy and disease during the last imaging follow-up, 4) previous cranial irradiation, 5) prior systematic anticancer therapy, and 6) images with artefact that precluded brain parenchymal volume measurements.

MRI evaluation

Selection of the MRI scans for the 2nd timepoint was based on the completeness of image data available. Selected images where anonymized and placed in a Cancer Research UK (CRUK) Cancer Imaging Centre repository.

All images were visually inspected for quality. T2-weighted images, pre- and post-contrast T1-weighted images and fluid attenuated inversion recovery and diffusion weighted imaging/apparent diffusion coefficient maps were reviewed for each patient. The MRI scans were retrospectively and quantitatively analyzed by a specialist pediatric neuroradiologist and a biomedical scientist. Both readers were blind to group membership and the timepoint when the scans were taken.

Corpus callosum volume measurement

The anonymized images were opened in OsiriX imaging Software (Pixmeo, Geneva, Switzerland) and a region of interest (ROI) was manually drawn on the mid-sagittal slice of T1-W images. Manual segmentations of CC were retrospectively performed by a pediatric radiologist with 5 years of experience in pediatric brain MRI. The CCV was calculated using the FMRIB Software Library's (FSL) stats command (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSL). To account for differences in acquisition, the volume of ROI was normalized by slice thickness.[10] Six months after the initial ROIs were drawn, a 2nd set of CC ROIs were manually drawn by the original observer on each mid-sagittal images to establish repeatability of the method.

Brain volume measurement

A mask of each patient's brain was generated using the autorecon1 command in FreeSurfer v. 5.3 (https://surfer.nmr.mgh.harvard.edu).[11] Each brain mask was then manually corrected by a trained biomedical scientist with 5 years of experience in pediatric neuroimaging. The brain masks were transformed into halfway space and further masked to ensure that only regions within the field of view at both timepoints were included in the volume analysis. Whole

brain volumes were calculated by summing the voxels within the brain mask using FMRIB Software Library's (FSL) stats command (https://fsl.fmrib.ox.ac.uk/fsl/fslwiki/FSL). The MRI examinations of 5 patients were randomly selected from the dataset and measurements repeated 6 months later to establish reproducibility of the method.

Statistical analysis

Statistical analysis was performed using Prism v. 7.0 software (GraphPad Software, San Diego, USA). Descriptive statistics were used to summarize the measurements. As the data were non-parametric, a Spearman's rank correlation tested the association between CCV and WBV.

The intra-observer repeatability for each of the measurement techniques – absolute CCV or WBV – were assessed using a Bland–Altman method to calculate 95% confidence intervals (95% CIs) of the measurement. A p-value of less than 0.05 was considered significant. The percentage reduction in CCV and WBV beyond these 95% CIs was assumed to represent real reductions beyond measurement variability. The number of patients in each group that exceeded the measurement reproducibility limits was noted, but small numbers precluded meaningful statistical comparisons.

Results

Patients' characteristics

Images of 23 children with complete data were included in final analyses. The cohort consisted of 17 males and 6 females with a median age of 10 years (range: 6–12 years) at the time of diagnosis. Out of 23 children, 7 were diagnosed with high-risk MDL (MDL HR), 9 with standard-risk MDL (MDL SR), 3 with ependymoma, 3 with low-grade glioma (LGG), and 1 with atypical teratoid rhabdoid tumor (ATRT). Children diagnosed with MDL and ependymoma had received adjuvant treatment. Those with MDL had also received craniospinal irradiation with boost to the posterior fossa and chemotherapy, whereas patients diagnosed with gliomas had not (Table 1).

Of 23 children, 7 received focal RT to the posterior fossa only (group 1), 9 were treated additionally with whole-brain RT of 23.4 Gy in 13 fractions of 1.8 Gy (classified here as low-dose RT of <30 Gy – group 2), while the other 7 received >30 Gy to the whole brain and were considered high-dose patients (5 received whole-brain RT of 36 Gy in 20 daily fractions of 1.8 Gy, while 2 received 39 Gy in 30 fractions of 1.3 Gy to the posterior fossa – group 3).

Intra-observer measurement variability

Reproducibility estimates for the CCV ranged from 6% to –9% (Fig. 1A). The 95% CI for absolute CCV ranged from +0.48 cm^3 to –0.77 cm^3. The WBV values had a smaller variability ranging from 4.8% to –1.2% (Fig. 1B); absolute values ranged from +5.5 cm^3 to –1.5 cm^3.

Table 1. Patient characteristics: age, tumor type, radiation dose, time interval between the end of RT and the follow-up MRI. All patients underwent surgical resection prior to RT

ID	Age [years]	DGN	WB RT dose [Gy]	Time interval [months]
1	10	MDL HR	>30	6
2	10	MDL SR	<30	14
3	9	MDL SR	<30	73
4	9	LGG	0	21
5	11	MDL HR	>30	9
6	12	EPND	0	27
7	9	MDL SR	<30	48
8	9	MDL SR	<30	11
9	10	MDL SR	<30	60
10	11	MDL SR	<30	9
11	7	MDL HR	>30	12
12	8	ATRT	0	10
13	10	MDL HR	>30	13
14	10	MDL SR	<30	60
15	8	LGG	0	6
16	6	MDL HR	>30	38
17	10	MDL SR	<30	83
18	8	LGG	0	96
19	10	EPND	0	80
20	11	MDL SR	<30	74
21	11	MDL HR	>30	12
22	11	MDL HR	>30	10
23	6	EPND	0	6

RT – radiotherapy; MRI – magnetic resonance imaging; ID – patients anonymized number; DGN – diagnosis/tumor type; WB RT dose – RT dose to whole brain: 0 (only focal RT), <30 Gy or >30 Gy; time interval – time in months between the end of RT and the follow-up MRI; MDL HR – high-risk medulloblastoma; MDL SR – standard risk medulloblastoma; ATRT – atypical teratoid rhabdoid tumor; EPND – ependymoma; LGG – low-grade glioma.

Comparison of corpus callosum and brain volume measurements as indicators of brain parenchymal loss

Measurements at follow-up were performed in a range from 6 to 96 months after RT (median: 14 months, lower quartile (LQ): 10 months, upper quartile (UQ): 60 months).

Poor image quality made the derivation of the automated WBV segmentation error-prone, so that WBV measurements were obtainable in 15 out of 23 patients. Absolute CCV values for 23 children at both timepoints (baseline and follow-up), and WBV values in 15 children at those timepoints are summarized in Table 2. At baseline, CCV values ranged from +4.4 cm^3 to –11.0 cm^3 (median: 8.1 cm^3),

Table 2. Corpus callosum volume (CCV) and whole brain volume (WBV) measurements performed by the same observer twice at baseline and twice at follow-up (FU)

ID	Baseline CC vol 1 [cm^3]	FU CC vol 1 [cm^3]	Baseline CC vol 2 [cm^3]	FU CC vol 2 [cm^3]	Baseline WBV 1 [cm^3]	FU WBV 1 [cm^3]	Baseline WBV 2 [cm^3]	FU WBV 2 [mm^3]	Percentage change CCV	Percentage change WBV
1	8.2	8.1	8.4	8.1					–1.6	
2	8.1	8.4	8.4	8.2	947.7	990.2	934.5	996.5	3.4	4.5
3	9.0	7.9	9.3	7.5	1,119.4	1,083.5	1,087.7	1,071.4	–13.6	–3.2
4	8.8	7.2	8.8	7.4					–21.2	
5	8.4	7.7	8.5	7.6					–9.2	
6	6.8	6.2	6.8	6.3					–8.8	
7	9.5	9.6	9.5	9.5	1,166.8	1,186.0	1,129.1	1,160.1	1.0	1.6
8	10.0	6.8	11.0	6.9	1,242.3	1,256.4			–46.9	1.1
9	10.9	8.5	11.2	8.5	1,308.1	1,311.0	1,283.5	1,277.1	–29.2	0.2
10	6.8	6.8	6.9	6.8					0.0	
11	7.3	7.1	7.4	7.2					–2.1	
12	6.4	6.7	6.3	6.6					3.3	
13	8.1	7.5	8.1	7.6	695.1	718.0			–7.4	3.3
14	7.4	8.3	8.6	8.3	1,304.9	1,096.8			10.8	–15.9
15	6.7	6.5	6.8	6.6	1,157.7	1,160.6	1,164.78	1,159.4	–1.8	0.3
16	6.2	5.3	6.2	5.2					–17.0	
17	7.0	7.7	7.1	7.7	1,319.2	1,331.0			9.5	0.9
18	4.4	4	4.4	4.2	1,054.1	977.3			–9.7	–7.3
19	5.8	5.9	5.7	5.8	1,049.0	1,059.7			0.7	1.0
20	8.0	8.3	8.0	8.2	1,338.7	1,400.7			3.0	4.6
21	6.9	7	6.8	7.1	1,144.4	1,122.2			1.1	–1.9
22	8.7	7.9	8.6	8.1	1,364.3	1,330.2			–9.2	–2.5
23	8.91	9.1	8.98	9.05	1,146.2	1,143.9			2.1	–0.2

Baseline CC vol 1 – first measurement of CCV at diagnosis; FU CC vol 1 – first measurement of CCV at follow-up; Baseline CC vol 2 – repeated measurement of baseline CCV at diagnosis; FU CC vol 2 – repeated measurement of CCV at follow-up; Baseline WBV 1 – first measurement of the WBV at diagnosis; FU WBV 1 – first measurement of the WBV at follow-up; Baseline WBV 2 – repeated measurement of the WBV at diagnosis; FU WBV 2 – repeated measurement of the WBV at follow-up.

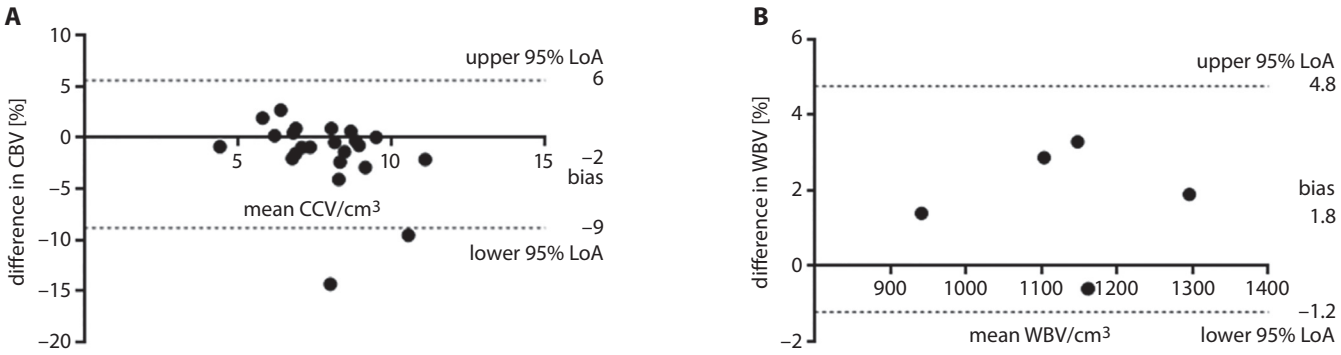

Fig. 1. A. Bland–Altman plots showing intra-observer reproducibility and upper and lower 95% CIs (dotted lines) of manually delineated corpus callosum volumes (CCV). B. Bland–Altman plots showing intra-observer reproducibility and upper and lower 95% CIs (dotted lines) of automated whole brain segmentations (whole brain volume (WBV)); LoA – limits of agreement.

while WBV values ranged from 695.1 cm^3 to 1,364.3 cm^3 (median: 1,157.8 cm^3). Correlation between baseline CCV and WBV was not significant (r = 0.25, p = 0.37; Fig. 2).

At follow-up, CCV values ranged from 4.2 cm^3 to 9.5 cm^3 (median: 7.5 cm^3). Seven out of 23 patients (30.4%) had a greater than 9% reduction in CCV at the 2nd timepoint, meaning a real reduction beyond measurement variability (Fig. 3). At follow-up, WBV ranged from 718.0 cm^3 to 1,400.7 cm^3 (median: 1,144.0 cm^3). Five out of 15 patients (33.3%) had a greater than 1.2% reduction in WBV at the 2nd timepoint, indicating a real reduction beyond measurement variability.

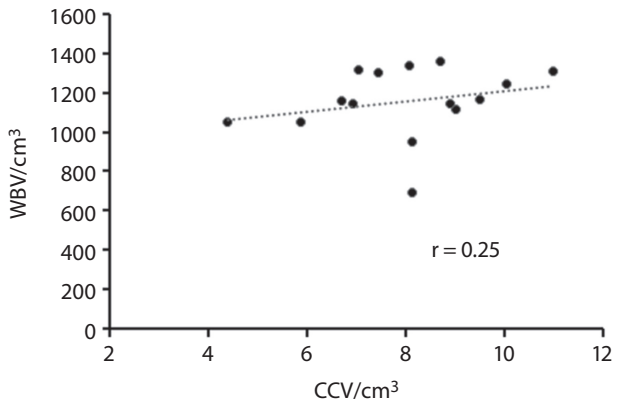

Fig. 2. Scatter plot showing the relationship between the measurements of corpus callosum volume (CCV) and whole brain volumes (WBV)

Five out of 7 patients who were recognized as having CCV loss at follow-up were not picked up by the WBV measurements. Similarly, 3 out of 5 patients identified as having WBV loss at follow-up were not picked up by the CCV measurements, confirming the poor correlation between the 2 assessments of brain parenchymal loss.

Corpus callosum and WBV loss in low- vs high-dose radiation regimens

Of the 7 patients with CCV loss greater than measurement variability, 2 were in group 1, 3 in group 2 and 2 in group 3. Of the 5 patients with WBV loss greater than measurement variability, 1 was in group 1, 2 in group 2 and 2 in group 3. The mean CCV loss was –5.1 in group 1, –6.9 in group 2 and –5.5 in group 3, while for WBV the values were –1.6, –0.8 and –0.3, respectively.

Discussion

To our knowledge, this study is the first to evaluate the impact of RT on brain parenchyma in children using 2 different techniques for quantifying brain volume. Our study showed that both CCV and WBV decrease after RT in up to a third of the children. Importantly, however, this study has shown that CCV does not correlate with WBV, so that significant reductions in these measurements were seen in different individuals. They may represent different ways of estimating brain parenchymal volume loss and should not be assumed to be interchangeable.

In this small cohort of patients, it was not possible to demonstrate the dose distribution of RT in relation to the CC to establish whether a greater dose was received by this structure in those patients demonstrating the profound effects of white matter loss within this structure.

The CCV has been used to quantify white matter loss in a variety of oncologic and non-oncologic applications. A reduced CC area was noted among children with adrenoleukodystrophy, an effect of demyelination.[12] Similar results have been observed in patients with attention deficit hyperactivity disorder, and among those with Williams syndrome.[13–15] In oncology, white matter damage was observed primarily in children with MDL (as it is the most common malignant solid tumor in children) and CCV was used to detect white matter loss.[4,9] Palmer et al. described a decrease in the CCV in 35 MDL patients following craniospinal irradiation.[9] Other reports on white matter volume also show that the volume of normal-appearing white matter decreases in children treated for MDL with craniospinal irradiation.[4] Data from the present study

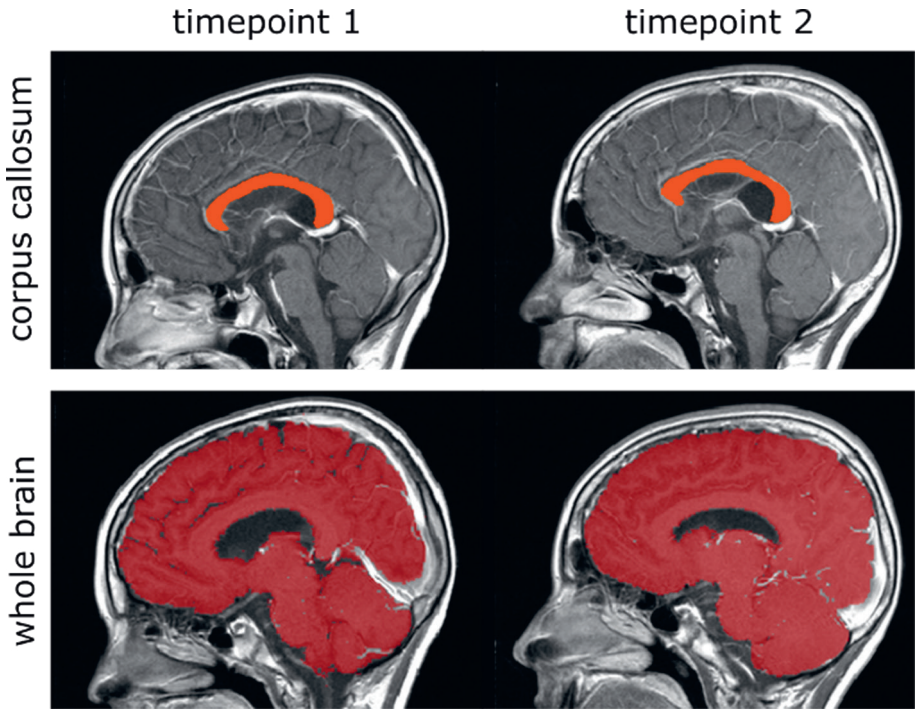

Fig. 3. Nine-year old patient with standard-risk medulloblastoma (SR MDL). Figures showing region of interest delineating the corpus callosum (CC) on a mid-sagittal T1W image (top row, A) and a mask segmenting the whole brain to derive a whole brain volume (WBV; bottom row, B) The cerebrospinal fluid (CSF) spaces are excluded in B

is consistent with these previous reports. The CCV measurements have also been used in children with primitive neuroectodermal tumors treated with high-dose thiotepa after hyperfractionated accelerated craniospinal radiotherapy (HART), revealing mild to severe neuroparenchymal volume loss following intensive sequential high-dose therapy with thiotepa given after HART regimen.[5] This confirmed previous results from a similar study, but where a different method of estimating brain volume was used.[6]

Linear increases in white matter volumes, occurring during normal maturation, have been documented in a large-scale study of 145 healthy individuals aged between 4 and 20 years.[13] The size of the CC also increases with age into early adulthood: this has been demonstrated in a study of 109 healthy subjects aged 7–32 years.[16,17] The growth of CC is regarded as the direct result of myelination of callosal axons present at birth. Therefore, a decline in CCV and WBV is opposite to what would be expected with normal maturation and is likely to be directly related to the effects of RT. In the timeframe of this study, where follow-up times were lengthy and patient growth and maturation would have occurred, it may well be that increases in CCV (and even in WBV) as a result of maturation would have masked any treatment-related reduction in CCV and WBV. Nevertheless, the 7 patients who showed CCV loss were followed up between 9 and 73 months (median: 21 months), while the 5 patients with WBV loss underwent follow-up imaging between 10 and 96 months (median: 60 months), indicating that follow-up time is not a primary confounding factor here.

Measurement error was greater for CCV than for WBV, but this is unsurprising as it depended on manual delineation rather than a computer-based automated process, albeit one that required manual correction. Manual segmentation is highly intensive and time-consuming, can be prone to errors, and may suffer from both inter- and intra-rater variability.[18] In several studies, automation has improved the reliability of ROI delineation and hence of the derived measurements.[19,20] Nevertheless, manual measurement of CCV enabled us to exclude artifacts from areas of surgical resection, which can potentially affect the assessments. However, an accurate outlining of the CC remains a challenge. One of the factors that may affect the actual accuracy of measurement is the impact of RT on CC irregularity of size and shape in relation to other brain structures.[21–23] Another challenge for precisely outlining the CC may result from its decreased signal intensity as maturation of the axonal cytoskeleton occurs. Signal intensity of the CC decreases during childhood and adolescence, thereby reducing image contrast between the CC and surrounding brain parenchyma, which may have affected outlining in our cohort who were mainly above the age of 8.[16]

The Freesurfer-based approach employed for WBV measurements was more robust than the approach for segmenting the CC, as it reduced the potential for rater bias. The resulting whole-brain segmentations also included more tissue, minimizing the effect of any variability. However, performing the measurements was laborious and time-consuming, as extensive manual correction of the masks was required. Even with semi-automated segmentation, the time required for each mask was around 3 h. This meant that it was only feasible to perform the repeatability study in 5 cases, although ideally all patients should have been included.

There are several limitations of this study. Firstly, its retrospective nature meant that time after RT was variable (6–96 months), although we included only those patients in whom a reasonable time following treatment had elapsed (6 months). Nevertheless, this meant that we could not control neither for ongoing effects of RT nor for brain maturation that may have confounded the measurements. Secondly, the imaging was performed using a variety of protocols, which meant that differences in T1- and T2-weighting would have affected image contrast and the conspicuity of the structures being outlined, leading to measurement variability. To avoid these types of errors, we outlined sequences on the sagittal T1-W and repeated the measurement after 6 months to verify measurement repeatability. Because of the retrospective nature of this study, variations in protocols meant that imaging parameters such as in-plane resolution also varied between patients and timepoints. This variation in protocols, combined with a restricted field of view and variable image quality, resulted in the misclassification of tissue in the automated brain segmentation. As a result, extensive manual correction of the segmentations was required. In addition to this, the field of view used for the images resulted in the lateral portions of the brains being clipped. We compensated for the clipping by only considering brain regions that were included at both timepoints. Finally, because we only selected patients in whom paired, reasonably good quality imaging studies were available, our sample size was small and resulted in a low statistical power.

In summary, our study has confirmed a decline in both CCV and WBV values in around 1/3 of the cases following cranial RT in children, but this decline was not related to radiation dose. However, this also suggests that both these measurements may not be used interchangeably, and may actually be sensitive to different factors. Both measurements, however, were relatively robust and even manually delineated CCV measurements would be expected to detect volume decreases of more than 9%. The appropriate measurement method should be selected, and its variability established when using CCV or WBV assessments in clinical trials.

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Veno-occlusive disease in children and adolescents after hematopoietic stem cell transplantation: Did the Modified Seattle Criteria fit the characteristics of pediatric population?

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Abstract

Background. Hepatic veno-occlusive disease (VOD) is a life-threatening complication following hematopoietic stem cell transplantation (HSCT) and associated with a high mortality rate. Therefore, accurate and immediate diagnosis is crucial for implementing appropriate treatment.

Objectives. In our single-center retrospective study, we assessed the accuracy of the Modified Seattle Criteria in children and adolescents undergoing HSCT, and compared them to the diagnostic criteria recently established by the European Society for Blood and Marrow Transplantation (EBMT).

Material and methods. Retrospective analysis of medical records of 951 HSCT procedures performed in 850 children and young adults in the years 2001–2015 in the Department of Pediatric Hematology, Oncology and Bone Marrow Transplantation of Wroclaw Medical University Supraregional Center of Pediatric Oncology "Cape of Hope" in Wrocław, Poland.

Results. Among the 850 children, 48 were diagnosed with VOD according to the Modified Seattle Criteria (5.05%). Thirteen patients (27%) developed VOD later than within 20 days after transplantation, as required in the diagnostic criteria. Five of the 6 patients who died from VOD were diagnosed with late-onset VOD. Using the categories of symptoms described in the Modified Seattle Criteria, hepatomegaly and weight gain were the most common symptoms in the analyzed cohort (81.25% and 68.75%). Fourteen patients (29%) never demonstrated elevated plasma bilirubin level (>2 mg/dL), as suggested in the Modified Seattle Criteria. Twenty-nine patients (64%) had increased platelet consumption requiring daily transfusions. Only 5 patients with decreased plasma antithrombin III (ATIII) activity level (<80%) on the day of HSCT developed VOD despite supplementation of ATIII.

Conclusions. The Modified Seattle Criteria seemed to not meet the special needs of the pediatric population. The new diagnostic criteria proposed by the EBMT appear to be more adequately tailored to the pediatric population and may significantly change the conception of VOD in the future. The surprisingly low incidence of VOD in our cohort may suggest a beneficial role of monitoring and early supplementation of ATIII.

Key words: hematopoietic stem cell transplantation, pediatric, veno-occlusive disease

Introduction

Hematopoietic stem cell transplantation (HSCT) has been used as a curative therapy for various kinds of disorders, both malignant and nonmalignant. Despite the increasing rate of successful transplantation procedures, the widespread use of the treatment is limited by concerns of life-threatening complications.

Hepatic veno-occlusive disease (VOD), also known as sinusoidal obstructive syndrome (SOS), is frequent and may be one of the most severe complications in the early post-HSCT period. This syndrome is characterized by clinical features like rapid weight gain, ascites, painful hepatomegaly, and jaundice.[1] Clinical suspicion of VOD may be supplemented by noninvasive imaging, such as ultrasonography (USG), particularly to identify attenuated or reversed hepatic venous flow, a typical USG finding in VOD.[2,3] However, none of the diagnostic criteria, laboratory tests or USG findings are specific to VOD. Its incidence in the adult population historically has been reported in up to 60%[1,4–6] of patients undergoing HSCT, but nowadays it appears to be 13.7%, with values ranging from 0% to 60.2% in different studies.[7] Such great range of results depends on the diagnostic criteria used – the Baltimore or the Seattle Criteria (Table 1).[6,8] In the face of the newly presented European Society for Blood and Marrow Transplantation (EBMT) diagnostic criteria for VOD in both the adult and pediatric population, the prevalence of this complication may change significantly in upcoming years (Table 2).[9,10]

Several risk factors for the development of VOD have been identified and inclusion of patients with each of these risk factors has differed between studies. The most widely accepted risk factors are allogeneic HSCT, previous liver disease, history of abdominal radiation, advanced disease (beyond the 2nd complete remission or relapse) and busulfan- and cyclophosphamide-based regimens.[4,7,11] In the pediatric population, low body weight, low albumin level, autologous HSCT for neuroblastoma, younger age, some underlying diseases (i.e., hemophagocytic lymphohistiocytosis (HLH) and osteopetrosis) and previous treatment (i.e., eculizumab, gemtuzumab ozogamicin) were identified as specific risk factors for VOD.[12–15] The severity of VOD could be retrospectively classified as mild, moderate or severe based on the dysfunction of the liver and associated organs.

Table 1. Comparison of Baltimore and Modified Seattle VOD Diagnostic Criteria

Modified Seattle Criteria	Baltimore Criteria
Two of following occurring within 21 days of transplantation:	Bilirubin serum level >2 mg/dL and at least 2 of following within 20 days of transplant:
hepatomegaly or right upper quadrant pain	hepatomegaly
bilirubin serum level >2 mg%	ascites
unexplained weight gain >2% from baseline	weight gain >5% from baseline

Table 2. European Society for Blood and Marrow Transplantation (EBMT) diagnostic criteria for hepatic VOD/SOS in children

No limitations for time of the onset of VOD/SOS
Presence of 2 or more of the following[a]: – unexplained, consumptive, transfusion-refractory thrombocytopenia[b]; – otherwise unexplained weight gain in 3 consecutive days despite use of diuretics or weight gain >5% baseline; – hepatomegaly (best confirmed by imagining) above baseline[c]; – ascites (best confirmed by imagining) above baseline[c]; – rising bilirubin from a baseline value on 3 consecutive days or bilirubin ≥2 mg/dL within 72 h.

[a] with the exclusion of other potential differential diagnosis;
[b] ≥ on weight-adjusted platelet substitution/day to maintain institutional transfusion guidelines;
[c] imaging immediately before HSCT to determine baseline value.
VOD – veno-occlusive disease; SOS – sinusoidal obstructive syndrome.

In addition to the recently proposed diagnostic criteria, the EBMT has established a new severity classification that would affect the structure of VOD incidence in transplant populations.[9] Therapy options for VOD are still very limited and defibrotide is the only drug approved by both the European Union and the USA for VOD treatment. Many previous and ongoing trials provide promising results about the safety and efficacy of defibrotide as a crucial agent in both prophylaxis and treatment regimens for VOD.[16–20] Additional treatment and prophylactic strategies using other agents such as heparin, antithrombin III, prednisone, or ursodiol have been studied, but none of them have been proven unequivocally effective.[21–25]

Given the limited number of large cohort studies on VOD in the pediatric population, in our single-center retrospective study, we have reported detailed VOD characteristics in children. In our research, we have evaluated the usefulness of the Modified Seattle Criteria, which have been widely used for over 25 years, particularly compared to the recently published EBMT VOD criteria in the pediatric population. We have also assessed the influence of antithrombin III (ATIII) supplementation on the prevalence of VOD in our center.

Material and methods

We retrospectively analyzed the medical records of 951 HSCT procedures performed on 850 children and young adults in the years 2001–2015 in the Department of Pediatric Hematology, Oncology and Bone Marrow Transplantation of Wroclaw Medical University Supraregional Center of Pediatric Oncology "Cape of Hope" in Wrocław, Poland. Seven hundred sixty-two patients underwent a single HSCT procedure, 76 patients required 2 HSCTs, 3 HSCTs were performed in 10 patients, and 4 HSCTs in 2 patients. Among the 951 transplantations performed, 246 were autologous and 705 were allogeneic. Among the allo-HSCTs, the stem cells were derived from bone marrow in 236 cases and from peripheral blood in 451 cases. Eighteen patients

Table 3. Characteristic of study cohort

Parameter	Value
HSCT analyzed autologous allogeneic	951 246 705
Patients after 1 transplantation after 2 transplantations after 3 transplantations after 4 transplantations	850 762 76 10 2
Male/female	534/316
Age	3 months–21 years (median: 8.87 years)
Stem cells source bone marrow peripheral blood cord blood unit	 237 696 18
Donor characteristic autologous MSD MUD MMUD	 246 184 420 101

MSD – matched sibling donor; MUD – matched unrelated donor; MMUD – mismatched unrelated donor.

Table 4. Indications for HSCT in study cohort

Diagnosis		Number of patients	
Hematological malignancies	ALL AML CML MDS LYM	227 125 36 65 53	total n = 506
Anemias	SAA BDA FA	62 10 13	total n = 85
Solid tumors	neuroblastoma Ewing sarcoma other	100 40 35	total n = 175
Immuno-deficiencies	SCID X-CGD Omenn syndrome other	26 11 8 21	total n = 66
Metabolic disorders	ALD MLD other	7 2 9	total n = 18

ALL – acute lymphoblastic leukemia; AML – acute myeloblastic leukemia; CML – chronic myeloid leukemia; MDS – myelodysplastic syndrome; LYM – lymphoma; SAA – severe anaplastic anemia; BDA – Blackfan–Diamond anemia; FA – Fanconi anemia; SCID – severe congenital immunodeficiency; X-CGD – chronic granulomatous disease; ALD – adrenoleukodystrophy; MLD – metachromatic leukodystrophy.

received stem cells from a cord blood unit. The complete clinical and demographic analysis of the patient cohort is presented in Tables 3 and 4.

Definitions

Each case of VOD was diagnosed using the Modified Seattle Criteria, which requires at least 2 of the following clinical findings within 20 days of transplantation: painful hepatomegaly, weight gain >2% above baseline and plasma bilirubin level >2 mg/dL (34 mcmol/L). The severity was evaluated on the basis of criteria proposed by McDonald et al.[1]: mild for cases that resolved spontaneously, moderate when treatment was required but the symptoms were resolved completely and severe when there was associated multiorgan failure (MOF) or the symptoms were not resolved by day 100 post-HSCT.

Prophylaxis and treatment

Every patient was scheduled for careful ATIII activity monitoring, starting from the first day of conditioning. Measurements of ATIII were repeated twice a week and continued till day 100 post-HSCT. If a patient developed low levels of ATIII, its activity was measured more often, i.e., every day.

Every patient who demonstrated decreased plasma ATIII activity in the period between the beginning of the conditioning up to 100 days post-HSCT received an additional supplementation of ATIII to maintain its activity over 80%. The treatment strategies for patients with confirmed VOD depended on the severity of the symptoms and any coexisting multiorgan failure. They varied from conservative measures such as fluid restriction and ursodiol in mild cases up to combined treatment with defibrotide (daily dose 25 mg/kg b.w.) and additional diuretics, anticoagulant agents and multiple platelets transfusions.

Results

Veno-occlusive disease was diagnosed in 48 patients – 20 females and 28 males. Forty-seven patients developed VOD after the 1st HSCT. There was only 1 case of VOD following consecutive transplantation. Patients were from 7 months to 19.5 years of age at the time of HSCT. Veno-occlusive disease developed in 26 of the 246 autologous HSCT recipients (10.6%) and in 22 of the 705 allogeneic HSCT recipients (3.1%). Mild VOD occurred in 7 patients (14.6% of VOD), and severe occurred in 39 patients (81.25% of VOD). Two patients developed moderate VOD. The overall incidence of VOD in the study cohort was 5.05%. There was a significantly higher difference in VOD incidence when the analyzed autologous HSCT was compared to allogeneic HSCT ($p < 0.05$). The indications for transplant in the VOD cohort was most commonly solid tumors (26 patients), mainly neuroblastoma. Conditioning regimens, as a well-known risk factor for VOD, were also assessed. All patients, except for one, who were diagnosed with VOD, received a myeloablative regimen prior to HSCT. The majority of patients (38) received busulfan/melphalan, while 9 received a TBI-based regimen (Table 5).

Time from HSCT to VOD onset varied between 3 days to 67 days post-transplantation (median: 14.5 days). Cases of VOD diagnosed after 20 days post-transplantation (13 patients, 27%) were confirmed by the presence of specific

Table 5. Detailed characteristic of VOD patients

Parameters		Values
Sex	male	28
	female	20
Type of transplant and donor	AUTO	26
	ALLO: MSD MUD MMUD	22 6 13 3
Stem cells source	bone marrow	7
	peripheral blood	41
Conditioning regimen	MAC: TBI BU-MEL MEC	47 9 36 2
	RIC	1
Diagnosis	AL	16
	CML	1
	IE	1
	ID	1
	LYM	3
	solid tumor: NBL Ewing Yolk sac Wilms	26 20 4 1 1
Staging at the moment of HSCT	advanced	40
	non-advanced	8
Age [years]	range: 0.6–19.5, median: 6.75	

AUTO – autologous transplantation; ALLO – allogeneic transplantation; MSD – matched sibling donor; MUD – matched unrelated donor; MMUD – mismatched unrelated donor; MAC – myeloablative conditioning; TBI – total body irradiation 12 Gy; BU-MEL – busulfan (16–19 mg/kg)+melphalan (140 mg/m^2)-based regimen; RIC – reduced intensity regimen; RTC – reduced toxicity regimen (Busulfan (9–12 mg/kg)+Fludarabine 150 mg/m^2); MEC – Melphalan (140 mg/m^2)+Etoposide (60 mg/kg) conditioning regimen; AL – acute leukemia; IE – metabolic disorders; ID – immunodeficiency; LYM – lymphoma; NBL – neuroblastoma. Non-advanced disease – 1CR (1st complete remission); CML – chronic phase, non-malignant disease; advanced – anything below non-advanced.

findings in an abdominal USG such as attenuated or even reversed blood flow in the portal vein. Nine cases of late-onset of VOD were confirmed with liver biopsy.

Among the diagnostic criteria used in the Modified Seattle Criteria, hepatomegaly was most commonly observed in our patients (n = 39; 81.25%). Ascites and weight gain occurred accordingly in 32 (66.7%) and 33 (68.75%) patients. Plasma bilirubin level range at diagnosis was 0.7–32.5 mg/dL (median 2.83 mg/dL). Fourteen patients (29%) never demonstrated a bilirubin level higher than 2 mg/dL, as indicated in the Modified Seattle Criteria, neither on the day of diagnosis nor during the course of treatment for VOD.

In the entire study cohort, 19% of patients (n = 181) demonstrated decreased ATIII activity level on day 0 and received immediate ATIII supplementation. Antithrombin III activity range on day 0 was 44–152% (median: 101%). Only 5 patients, among those who demonstrated decreased ATIII levels on the day of HSCT, developed VOD, despite being given prophylaxis. In the VOD cohort, 10 patients (21%) had normal ATIII activity levels since the beginning of pre-HSCT conditioning through the resolution of symptoms of VOD (9 patients) or death (1 patient). The remainder of patients that were diagnosed with VOD (n = 33, 68.8%) developed decreased ATIII plasma activity between 19 days prior, up to the day of diagnosis of VOD (median: 3 days before diagnosis of VOD).

Twenty-nine patients (64%) demonstrated increased platelet consumption followed by refractory thrombocytopenia and required daily platelet transfusions to maintain transfusion criteria (platelet count below 20,000/mcL).

Resolution of VOD symptoms was defined as platelet level sustainability and platelet transfusion independence, resolution of painful hepatomegaly, resolution of jaundice, and reduction of ascites (which was measured by a decrease of waist circumference to the patient's baseline as well as weight reduction). Using this definition, duration of VOD symptoms ranged from 3 to 47 days (median: 19 days). Eight patients (16.6%) died before the resolution of symptoms.

During therapy, 29 patients (60.4%) with VOD received defibrotide. The response rate for defibrotide was 82.76%. The time of treatment ranged from 4 days to 26 days (median: 12 days). Each patient was given a standard dose of 25 mg/kg b.w. daily.

Day 100 mortality rate in the VOD cohort was 21% (n = 10), compared to the 15.52% (n = 132) day 100 mortality rate in the entire cohort of patients. In the VOD cohort, 10 more patients died after day 100 but their causes of death were not directly related to the HSCT.

Day 100 mortality rate among patients treated with defibrotide was 17% (n = 5). Veno-occlusive disease was recognized as the cause of death in 6 patients (12.5%). Two more patients died from septic shock and gastrointestinal hemorrhage with coexisting active VOD. Importantly, only 1 patient among those who died of VOD was diagnosed within 20 days post-HSCT. Detailed cause-of-death information is presented in Table 6.

Table 6. Causes of day +100 transplant-related mortality in veno-occlusive disease (VOD) cohort

Cause of death	n = 10
VOD	6
Infection	3
Hemorrhage	1

Discussion

The incidence of VOD in our study was 5.05%. Other publications that have also focused strictly on VOD in children have reported incidence ranges from 13% to 28%.[12–15,26–30] This high variability in rates of VOD might be caused by the selected study cohort, different risk factors and, of course, ambiguous diagnostic criteria.

However, in light of the recently implemented diagnostic criteria, the incidence of VOD in the pediatric population may shift drastically. The most recent research conducted by Corbacioglu et al.[9] demonstrates major differences in VOD prevalence depending on the criteria used, suggesting that VOD may be more common than previously thought. Given the criteria proposed by EBMT, there is also a possibility that VOD was underdiagnosed in our center, even taking into account 13 late-onset cases which did not meet the Modified Seattle Criteria.

Our study revealed that patients undergoing autologous-HSCT more likely develop VOD than patients receiving allogeneic graft – 10.6% compared to 3.12%. This result is quite opposite to those presented in the literature concerning adult patients, as allogeneic stem cell transplantation is a well-known risk factor for VOD in the adult population. There are only a few studies performed on very limited study cohorts (86 and 116 patients) analyzing VOD after autotransplantations in children. Those studies report an extremely high incidence of VOD, reaching 39%.[31,32] The difference in frequency of VOD after autologous and allogeneic transplantations presented in our analysis is most likely caused by the fact that almost all of the patients treated with autotransplantation were suffering from neuroblastoma, a known risk factor for VOD.[32] The exact mechanism for this increased risk in neuroblastoma is not known, but it likely relates to the association with other known VOD risk factors such as previous abdominal radiation, specific chemotherapy agents including tandem transplantations and lower albumin level prior to HSCT.[32]

The majority of patients in our VOD cohort were recipients of a 1st HSCT. Only 1 out of 88 patients receiving multiple transplants developed VOD. This observation may indicate that it is not the cumulative doses of chemotherapeutic agents that are a risk factor for VOD, but rather the intensity of treatment in a limited period of time that has a significant impact, given that several other studies have found a correlation with increased risk following subsequent transplantation.[13]

The prevalence of late-onset VOD has never been properly assessed in the pediatric population.[33] Several studies report a minority of VOD occurrences diagnosed later than 20 days post-transplantation. In our cohort, almost 1/3 (27%) of patients did not meet the time criterion of VOD required in the Modified Seattle Criteria. Our results are in compliance with the absence of time limitation in the newly proposed diagnostic criteria, where VOD may be diagnosed regardless of the time when the first symptoms occurred. This may be particularly important given the high mortality rate associated with late-onset VOD in our cohort. Five of the 6 patients who died of VOD in our cohort were diagnosed with late-onset VOD. New EBMT VOD criteria for adults (Mohty et al.[10]) have highlighted for the first time the presence of anicteric VOD. This phenomenon seems to be even more frequent in children, which has been emphasized by Corbacioglu et al.[9]

Fourteen patients (27%) never demonstrated a bilirubin level over 2 mg/dL, as suggested in the Modified Seattle or Baltimore VOD diagnostic norms. A similar percentage of anicteric patients were reported in other studies[26,27]; nonetheless, bilirubin level, even if not mandatory, still remained a part of the VOD diagnostic criteria. The novel approach to VOD emphasizes the importance of bilirubin kinetics, instead of bilirubin plasma levels alone. Although elevated bilirubin level is a common VOD symptom, lack of hyperbilirubinemia should not lead to a delay in diagnosis and treatment. As reported by Corbacioglu,[16] the response to treatment with defibrotide is significantly lower when implemented more than 2 days from diagnosis. Therefore, a proper and immediate diagnosis, based on adequate criteria, is crucial for general patient outcome.

Bilirubin levels and its kinetics are suggested to be more a prognostic factor than a strict diagnostic criterion.[9]

Increased platelet consumption was a prominent feature of our VOD cohort. Sixty-four percent of patients required daily platelet transfusions to maintain adequate hematologic parameters. The median time of transfusion dependence related to VOD was 14 days. Even if mentioned in the very first studies about VOD, refractory thrombocytopenia (RT) has been overlooked as a VOD symptom for years. Despite not being a part of the diagnostic criteria, in our center, RT has remained an important symptom and diagnostic hint for VOD. Correlation of ATIII activity level and development of VOD is still unclear. Only half of patients demonstrated a sudden drop in ATIII activity, as indicated in previous studies. Even if such an evident drop was observed, it mostly happened only 3 days before a diagnosis was made. Haussmann et al. demonstrated an even shorter time lapse between ATIII activity decline and diagnosis of VOD.[24] This makes plasma ATIII activity drop a poor prognostic factor for VOD. Although both Haussman and Peres emphasize a beneficial effect of early intervention with ATIII,[24,25] it has never been highlighted in the major expert opinions. Looking at the relevantly lower incidence of VOD, this beneficial effect of ATIII supplementation seems to be confirmed by our study.

Conclusions

Veno-occlusive disease is a quite frequent, partially unexpected and potentially fatal complication following HSCT. New VOD diagnostic criteria provided by EBMT seem to be more adequate for the pediatric population than the recently used Modified Seattle Criteria. The newly proposed criteria correlate better with actual clinical findings in children diagnosed with VOD after HSCT. Removal of the time factor and hyperbilirubinemia, and incorporation of RT into the diagnostic criteria were crucial. The lower incidence of VOD in our center might suggest that early supplementation of ATIII to maintain its activity over 80% could be an efficacious prophylaxis against VOD. This novel finding requires further studies.

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Effect of glucocorticoids on the function of microvascular endothelial cells in the human femoral head bone

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Abstract

Background. The pathogenesis of glucocorticoid (GC)-induced osteonecrosis (ON) of the femoral head remains unclear. Recent research has suggested that it is closely associated with injured bone microvascular endothelial cells (BMECs). However, few studies have used BMECs to perform research pertaining ON of the femoral head.

Objectives. The objective of this study was to investigate the functional changes of BMECs treated with a GC and to detect the changes in related genes using microarrays.

Material and methods. Cells were isolated using an enzymatic method and identified with EC markers, such as von Willebrand factor (vWF), CD31 and vascular endothelial cadherin (VE-cadherin). Bone microvascular endothelial cells were treated with 0.1 mg/mL and 0.3 mg/mL of hydrocortisone to establish a GC-damaged model of BMECs. The mRNA microarrays were used to detect the differential expression profiles between BMECs with and without GC damage.

Results. Primary cells appeared as having a cobblestone-like morphology. Immunofluorescence staining revealed that the cells were 100% positive for vWF and CD31, and near 100% positive for VE-cadherin. It also confirmed that the cells were BMECs. Bone microvascular endothelial cells treated with 0.1 mg/mL of hydrocortisone showed shrinkage, and those treated with 0.3 mg/mL of hydrocortisone mostly showed apoptosis. The mRNA microarray showed that genes associated with endothelial cells, such as endothelin 1 (ET-1) receptor, angiotensin II (AII) receptor, intercellular adhesion molecule 1 (*ICAM-1*), and plasminogen activator inhibitor 1 (*PAI-1*), were upregulated, and genes associated with endothelial nitric oxide synthase (eNOS), endothelin 1 (*ET-1*), prostaglandin I_2 (PGI_2) synthase, PGI_2 receptor, vascular endothelial growth factor (VEGF), prostaglandin E (PGE) synthase, and PGE receptor were downregulated. The results of quantitative polymerase chain reaction (qPCR) validation were consistent with the findings of mRNA microarrays.

Conclusions. Glucocorticoids promoted BMECs to express vasoconstrictors and procoagulant factors and related receptors, and decreased the expression of vasodilators and their receptors.

Key words: glucocorticoids, microarray analysis, cell culture techniques, microvascular endothelial cells, real-time polymerase chain reaction

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Introduction

Non-traumatic avascular necrosis of the femoral head is a refractory hip joint lesion. The causes of the disease are the use of glucocorticoids,[1] alcoholism,[2] decompression sickness,[3] sickle cell anemia,[4] and idiopathic causes.[5] However, glucocorticoid (GC)-induced osteonecrosis (ON) of the femoral head (ONFH) accounts for a large proportion of this occurrence,[6–9] primarily occurs in young individuals, and shows characteristics of short course and early collapse. In addition, middle-aged and older people can have ONFH. However, the pathogenesis of steroid-induced ONFH is still not very clear. Presently, osteoblast apoptosis,[10,11] lipid metabolism abnormality,[12] intravascular coagulation,[13] and vascular endothelial dysfunction[14] are hypothesized as factors causing ONFH. However, a study indicated that the pathogenesis of steroid-induced ONFH was the result of a comprehensive effect of multiple factors.[15] Recently, more and more authors believe that steroid-induced ONFH is closely related to the damage to bone microvascular endothelial cells (BMECs) in the femoral head. Vogt and Schmid-Schönbein[16] found that a large dose of dexamethasone could induce apoptosis of mesenteric microvascular endothelial cells. Li et al.[17] pointed out that endothelial cell injury, an increase of local procoagulant factors and low fibrinolysis could be responsible for GC-induced ON. Yang et al.[18] found that reactive oxygen species (ROS) concentration was higher in the femoral head BMECs than in the control group; there was a positive correlation between ROS concentration and GC levels, suggesting that high GC levels lead to increased ROS concentration in endothelial cells and cause irreversible damage to cells, eventually leading to cell death. The above studies showed that glucocorticoids damaged the microvascular endothelial cells, leading to endothelial dysfunction, apoptosis and death. However, only a few studies have been reported on the kind of changes that occur after BMECs injury. Endothelial cells have endocrine functions[19]; they can synthesize and secrete various bioactive substances and play an important role in regulating local blood flow and body fluid balance. Their functions involve vasoconstriction, coagulation and fibrinolysis, angiogenesis and cell proliferation, oxidative stress, cell adhesion, and inflammatory mediation, which include endothelial nitric oxide synthase (eNOS), prostaglandin I_2 (PGI_2), endothelin 1 (*ET-1*), intercellular adhesion molecule 1 (*ICAM-1*), vascular cell adhesion molecule 1 (*VCAM-1*), tissue plasminogen activator (t-PA), plasminogen activator inhibitor 1 (*PAI-1*), vascular endothelial growth factor (*VEGF*), von Willebrand factor (vWF), fibroblast growth factor (FGF), interleukin 1 (IL-1), and IL-6. In addition, there are some vasoactive substances in the blood, such as angiotensin II (AII); although endothelial cells cannot be synthesized, they still play a role in regulating vasoconstriction, coagulation and fibrinolysis by binding with endothelial cell receptors.

Do BMECs in the femoral head have the same functions as other endothelial cells? It was considered that steroid-induced ONFH was characterized by systemic coagulation and fibrinolytic dysfunction. For example, van Veldhuizen et al.[20] and Glueck et al.[21] found that *PAI-1* expression increased and t-PA expression decreased in patients with ON. Asano et al.[22] found the polymorphism of *PAI-1* gene in patients with ONFH after renal transplantation. They showed that the vasoactive substances secreted by the endothelial cells of necrotic femoral head had changed. Therefore, it can be speculated that glucocorticoids may cause severe damage to BMECs. However, the abovementioned research was relatively rare, and the cells used were either from animal models or from other organ-derived endothelial cells. Our study was based on a few previous experiments[18,23,24] and involved hydrocortisone being used on in vitro-cultured human femoral head BMECs. Herein, we established a GC-damaged model of BMECs and detected differentially expressed genes using high-throughput expression chips and real-time quantitative polymerase chain reaction (RT-qPCR) to study function change of the cytokine and the related receptor of GC-induced human femoral head damage model of BMECs.

Material and methods

Ethics statement and patients

The protocol described herein was approved by the ethics committee of the China-Japan Friendship Hospital, Beijing, China, and is in compliance with the tenets

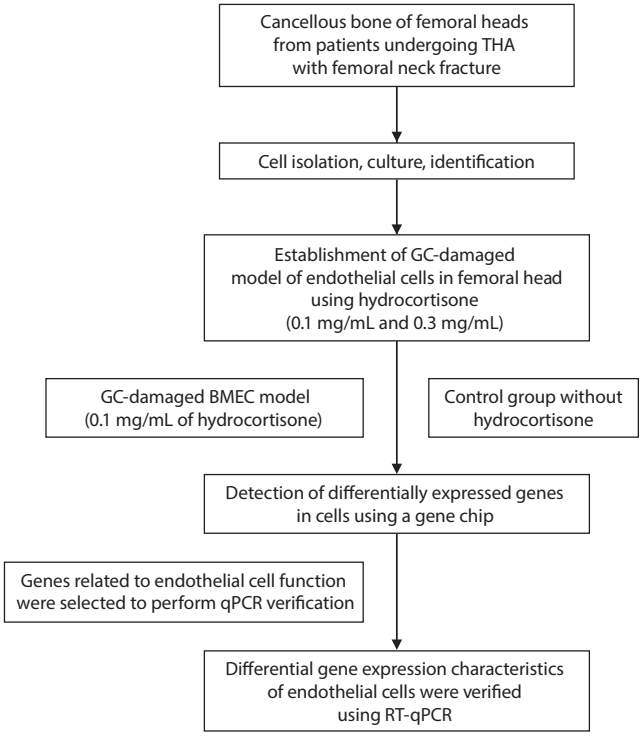

Fig. 1. Flow diagram illustrating cell culture and gene chip experiments

THA – total hip arthroplasty; PCR – polymerase chain reaction; RT-qPCR – real-time quantitative PCR; BMEC – bone microvascular endothelial cell; GC – glucocorticoid.

of the Declaration of Helsinki on ethical principles for medical research involving human subjects (Institutional Clinical Trials Register Number is 2014-34). All participants provided written informed consent. All patients were enrolled at the China-Japan Friendship Hospital between October 1, 2013 and February 28, 2014. Patients with femoral neck fracture requiring hip arthroplasty were included. Exclusion criteria were the following: patients with ONFH, ankylosing spondylitis, rheumatoid arthritis, other inflammatory joint diseases involving the hip joint, hemophilic arthropathy, hip tuberculosis, pyogenic infection, perihip tumors, and systemic diseases (e.g., diabetes). A total of 9 patients met the above criteria, including 1 man and 8 women (average age: 79.1 years, age range: 62–92 years). Our entire experimental process was shown in Fig. 1.

Isolation of human femoral head bone microvascular endothelial cells

The femoral heads were obtained from patients with femoral neck fractures who underwent total hip arthroplasty. The soft tissue and cartilage of the femoral heads were removed, and the cancellous bone was bitten into granule with a rongeur under aseptic conditions in the operating room (Fig. 2A). The cancellous bone was placed into a centrifugal tube containing 30 mL of heparinized, serum-free Dulbecco's modified Eagle's medium (DMEM; Hyclone Laboratories, South Logan, USA) and carefully transferred to the laboratory. Samples were shaken repeatedly for 3–5 min and the medium was removed. Then, the cells were transferred into a new centrifugal tube and washed several times with Hanks' balanced salt solution (HBSS; Gibco, Carlsbad, USA) until the rinse was clear. Then, the rinse fluid was removed from the bone, and DMEM containing 1.5–2% collagenase type I (Solarbio, Beijing, China) was added to the bone tissue, with the liquid level slightly higher than that of bone granule; then, the sample was incubated in a water bath for 30 min at 37°C. Thereafter, 0.25% trypsin and 0.53 mM ethylenediaminetetraacetic acid (EDTA; Solarbio) were added, and incubation was done in a water bath at 37°C for 5 min. Immediately, these digestive juices were transferred to a 70-micrometer cell strainer and then placed on a 50-milliliter Falcon tube. Then, the suspension was subjected to centrifugation at 200 × g for 10 min and the resulting pellet was collected. The pellet was re-suspended in M199 culture medium (Hyclone) supplemented with 20% fetal bovine serum (FBS; Gibco), 10 ng/mL of VEGF (Sino Biological Inc., Beijing, China), 100 ug/mL of streptomycin (Solarbio), 100 U/mL of penicillin (Solarbio), and 40 U/mL of heparin (Xinbai Pharmaceutical, Nanjing, China) in a Petri dish pre-coated with gelatin 2% (Sigma-Aldrich, St. Louis, USA). The cells were incubated at 37°C under 5% CO_2 conditions.

After 24 h of incubation, the cells started showing attachment to the dish. Unattached blood cells and debris were removed by changing the 20% FBS serum-supplemented medium. Medium change was performed every 3–5 days according to the color of the medium. Phase-contrast microscope (Olympus IX71; Olympus Corp., Tokyo, Japan) was used to monitor cell growth. When about 80% confluence was reached (about 7–10 days after primary culture), the cells were treated with 1 mL 0.25% trypsin and 0.53% EDTA for 3 min for harvesting. Trypsin was carefully aspirated; the cells were subjected to centrifugation at 200 × g for 10 min. Then, the pellet was resuspended with 10 mL of M199 culture medium (Hyclone). The suspended cells were split in 1:2 ratio and cultured using gelatin-coated plates; then, they were maintained in M199 culture medium as described above.

Characterization of endothelial cells

Cells from 2 or 3 generations of patients were characterized using the endothelial cell characterization kit (Bioss Antibodies, Woburn, USA) according to manufacturer instructions. The cells were grown on a glass coverslip, and the coverslip was washed with phosphate-buffered saline (PBS) 3 times for 5 min, and then the cells were fixed with 4% paraformaldehyde for 30 min at room temperature. Next, the cells were washed (3 × PBS) for 5 min and incubated at room temperature for 1 h with blocking buffer comprising 0.05% Triton X-100 (Solarbio) and 10% FBS in 3 × PBS. The primary antibody (rabbit anti-human IgG) (Bioss Antibodies) was diluted with a working concentration of the blocking buffer; next, the cells were incubated with the primary antibodies vWF, CD31 and vascular endothelial cadherin (VE-cadherin; Bioss Antibodies) overnight at 4°C. Then, secondary antibodies (goat anti-rabbit IgG; Bioss Antibodies) labeled with fluorescein isothiocyanate were added, along with Hoechst33342 stain solution (Sigma-Aldrich), and the cells were incubated for 90 min. Next, the cells were fixed on adhesive slides with an anti-fluorescence attenuating agent. Homologous antibodies were used as negative controls. Finally, the cells were washed with 1 × PBS and analyzed using a fluorescent microscope (Olympus Corp.).

Establishment of glucocorticoid-damaged BMEC model

The 2nd or 3rd generation cells of 8 patients (the cells from a 92-year-old woman had poor cell growth and were excluded) were selected to establish GC-damaged BMEC model. When the cells reached about 80% confluence, hydrocortisone (Tianjin Kingyork, Tianjin, China) was introduced to the culture medium of the experimental groups, and the final concentration was 0.1 mg/mL and 0.3 mg/mL. Hydrocortisone was not added to the control group. The cells were monitored under an inverted microscope (Olympus Corp.) after being cultured for 6 h, 12 h, 18 h, and 24 h after drug administration. After 24 h, the total RNAs of cell samples were extracted with TRIzol reagent (Invitrogen, Germany).

Detection and data analysis of gene chips

Total RNA was isolated using the TRIzol reagent according to the manufacturer's protocol. After the qualitative and quantitative detection of mRNA, qualified crystal core® mRNA expression profiling chip (CapitalBio, Beijing, China) was used for detection. Specific experimental steps refer to the biological experimental scheme of CapitalBio Corporation (http://cn.capitalbio.com). The hybridization pictures were acquired and analyzed using Agilent Feature Extraction software v. 10.7 (Agilent Technologies, Santa Clara, USA). The data were normalized and the differences between the groups were analyzed using Agilent GeneSpring software (Agilent Technologies).

Quantitative real-time PCR assays

Differentially expressed genes associated with endothelial cell function were selected for quantitative polymerase chain reaction (qPCR) verification. After the cells from the experimental group with 0.1 mg/mL of hydrocortisone and those from the control group were cultured for 24 h, TRIzol reagent was used to extract total RNA from the cells. Thereafter, reverse transcription into cDNA was performed according to the manufacturer's protocol. The PCR amplification was done in 96-well plates. The conditions of the PCR reaction were the following: 95°C for 10 min, and amplification for 40 cycles at 95°C for 15 s and 60°C for 60 s. In addition, a melting curve step was performed at 95°C for 15 s and at 60°C for 1 min, and then the temperature was increased at a rate of 0.11°C/s until reaching 95°C to measure fluorescence signals. The experiment was repeated 3 times to get the average value.

Statistics

The data was represented as mean ± standard deviation (SD) for n = 8. The expression level of each gene was calculated using the ΔCt method. To find significant differences, the values were assessed with Student's t-test using SPSS v. 18.0 software (SPSS Inc., Chicago, USA). A p-value <0.05 was considered to indicate statistically significant differences between means.

Results

Observation of cell morphology

After 24 h in primary culture, spindle-shaped or polygonal cells were scattered and occasionally reached confluence as observed using phase-contrast microscopy. Further, 7–10 days later, the cells showed a cobblestone-like appearance reaching confluence, basically covering the dish bottom (Fig. 2B) The cells grew densely, and the fused cells were in a swirl shape. The morphology of passaged cells was slightly different from that of primary cells as the former were mostly spindle-shaped, polygonal or branched (Fig. 2C). After 6 h of treatment with 0.1 mg/mL of hydrocortisone, cells in the experimental group showed shrinkage and were thus smaller than those in the control group. After 12–24 h, the cells showed growth inhibition, poor state and decreased cell density (Fig. 2D). However, most cells showed apoptosis after 0.3 mg/mL of hydrocortisone treatment (Fig. 2E). The cells in the control group grew well and gradually fused (Fig. 2F). Therefore, we chose 0.1 mg/mL hydrocortisone-treated cells for mRNA chip detection.

Endothelial cell characterization

Primarily, endothelial cells are observed and characterized based on the morphology observed under a phase-contrast microscope, wherein endothelial cells were confirmed by identifying the cobblestone morphology. The expression of several endothelial cell-specific markers, such as vWF (Fig. 2G), CD31 (Fig. 2H) and VE-cadherin (Fig. 2I), was confirmed using immunocytochemistry analysis. Corresponding to Hoechst 33342 cell nuclear staining, immunofluorescence staining revealed that the cells were 100% positive for vWF and CD31, and near 100% positive for VE-cadherin. The cells in the negative control group were not stained, showing that the cells cultured were BMECs.

mRNA microarray gene screening results

Compared with the control group, 519 genes were differentially expressed in BMECs of the experimental group, of which 337 genes were upregulated and 182 genes downregulated. These genes include vasoactive substances synthesized by BMECs, coagulation and fibrinolytic cytokines, and their related receptors. In addition, these are genes that affect cell apoptosis, angiogenesis, cell signal transduction, protein processing, and cell cycle regulation. Among these, 12 genes were associated with endothelial cell function (Table 1).

qPCR results

According to the results of mRNA chip detection, we selected the genes of *ICAM-1*, eNOS, *ET-1*, ET-1 receptor, PGI_2 synthase, *PAI-1*, *VEGF*, AII receptor, prostaglandin E (PGE) synthase, and PGE receptor for qPCR verification (primer sequence: see Table 2). The relative mRNA expression of *ICAM-1*, ET-1 receptor, *PAI-1*, and AII receptor in BMECs of the experimental group was significantly increased compared with the control group, whereas eNOS, *ET-1*, PGI_2 synthase, *VEGF*, PGE synthase, and PGE receptor expression were significantly decreased (Fig. 3A–J). The results of qPCR were consistent with those of mRNA chip analysis.

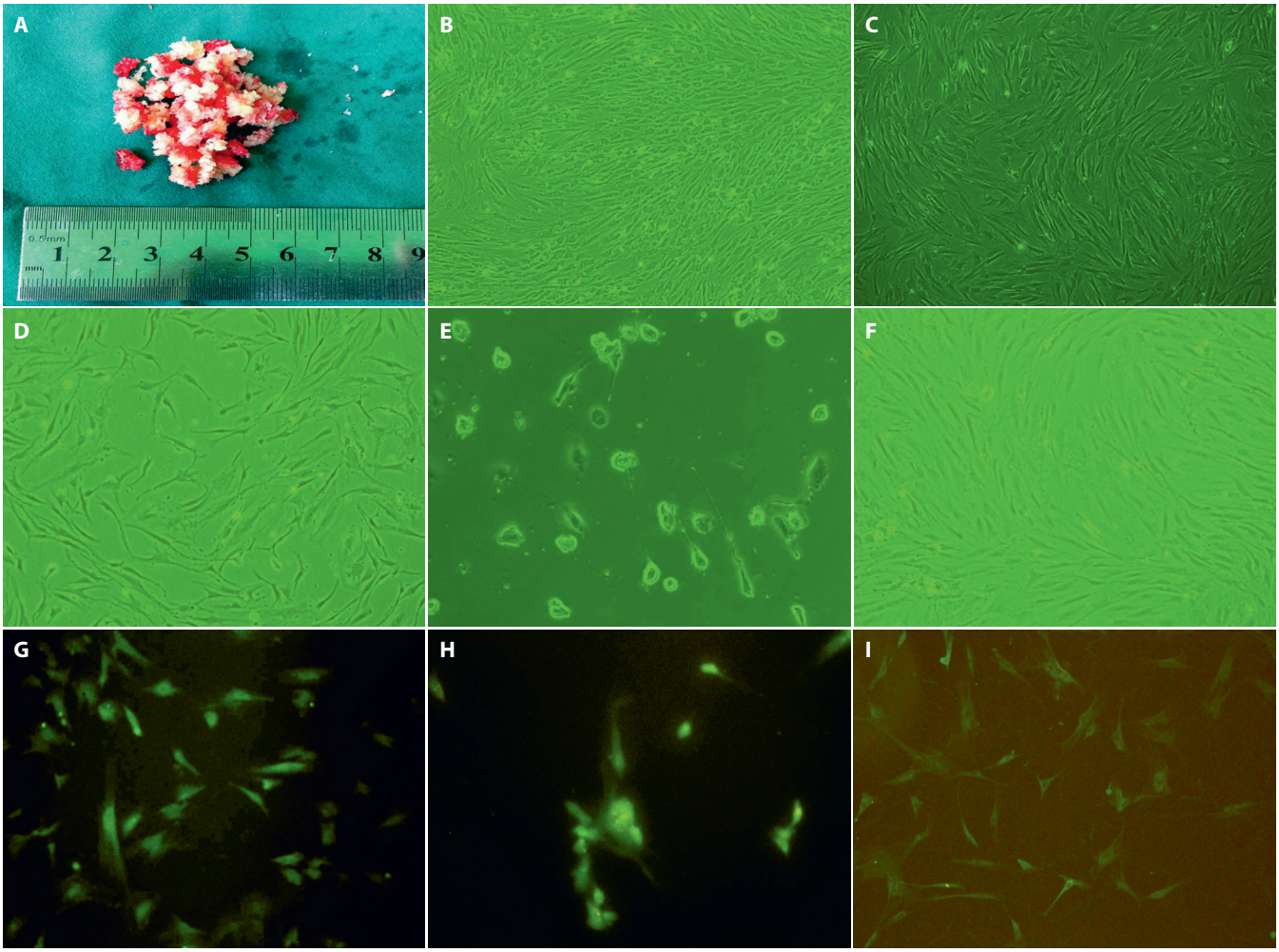

Fig. 2. Isolation and characterization of bone microvascular endothelial cells (BMECs) from human femoral head at ×100 magnification. A. Cancellous granules of the femoral head from patients undergoing total hip arthroplasty (THA) with femoral neck fracture. B. BMECs at P0 form confluent monolayers showing cobblestone morphology. C. BMECs at P3 with mostly fusiform, polygonal and branched cell morphology. D. After 24 h of 0.1 mg/mL hydrocortisone treatment, the cell growth condition was poor and the density decreased. E. After 24 h of 0.3 mg/mL hydrocortisone treatment, most cells showed apoptosis. F. Cells in the control group developed properly. G. Positively immunostained BMECs using rabbit anti-human von Willebrand factor (vWF). H. Positively immunostained BMECs using rabbit anti-human CD31. I. Positively immunostained BMECs using rabbit anti-human VE-cadherin

Table 1. Differentially expressed genes in femoral head BMECs experimental group and control group

Genbank accession	Ratio	Gene name	Regulation	p-value
NM_001955	−1.24 ±0.55	*ET-1*	down	0.002
NM_001256283	2.34 ±1.17	ET-1 receptor	up	0.009
NM_000961	−0.55 ±0.47	PGI_2 synthase	down	0.012
NM_000960	−0.40 ±0.32	PGI_2 receptor	down	0.044
NM_001287044	−0.75 ±0.26	VEGF A	down	0.003
NM_005429	−0.30 ±0.42	VEGF C	down	0.007
NM_000686	0.44 ±0.23	AII receptor	up	0.011
NM_004878	−0.84 ±0.45	PGE synthase	down	0.012
NM_000956	1.37 ±0.71	PGE receptor	down	0.010
NM_001160111	−0.24 ±0.19	eNOS	down	0.042
NM_000201	0.52 ±0.39	*ICAM-1*	up	0.037
NM_000602	0.13 ±0.03	*PAI-1*	up	0.003

eNOS – endothelial nitric oxide synthase; PGI_2 – prostaglandin I_2; ET – endothelin; *ICAM-1* – intercellular adhesion molecule 1; *VEGF* – vascular endothelial growth factor; AII – angiotensin II; *PAI-1* – plasminogen activator inhibitor 1.

Discussion

Non-traumatic femoral head necrosis is a common disease. There were 8.12 million patients with this disease in China in 2013.[25] Steroid-induced ONFH accounts for 30–50% of all non-traumatic ONFH cases,[26] and thus far, its pathogenesis is not clear. However, there has been increasing evidence confirming intraosseous microvascular endothelial cell injury and microvascular thrombosis as the pathogenesis of ONFH.[18,27,28] The microcirculatory vessels in the deep femoral head are mainly irregular sinusoidal capillaries, and the endothelial cells in these structures are characterized by fenestration, large gaps and no septa. The basement membrane of the bone microvessels was incomplete, absent or intermittent, thus facilitating the passage of mature red blood cells.[29,30] Yang et al.[18] reported that hydrocortisone at a concentration >0.1 mg/mL could cause different degrees of damage and even

apoptosis to BMECs of the femoral head cultured in vitro. The degree of cell damage was directly proportional to the concentration of hydrocortisone, and the cells died at the concentration of 1 mg/mL. Reportedly, hydrocortisone concentration of 0.1–0.3 mg/mL is the most suitable concentration of corticosteroids for establishing a GC-damaged BMEC model. On the basis of this study, we also established a model of GC-induced BMEC damage in vitro and screened differentially expressed genes using microarray analysis to detect the mRNA of the cell model. We found that some genes related to endothelial cell function, such as *ET-1*, ET-1 receptor, *ICAM-1*, PGI_2 synthase,

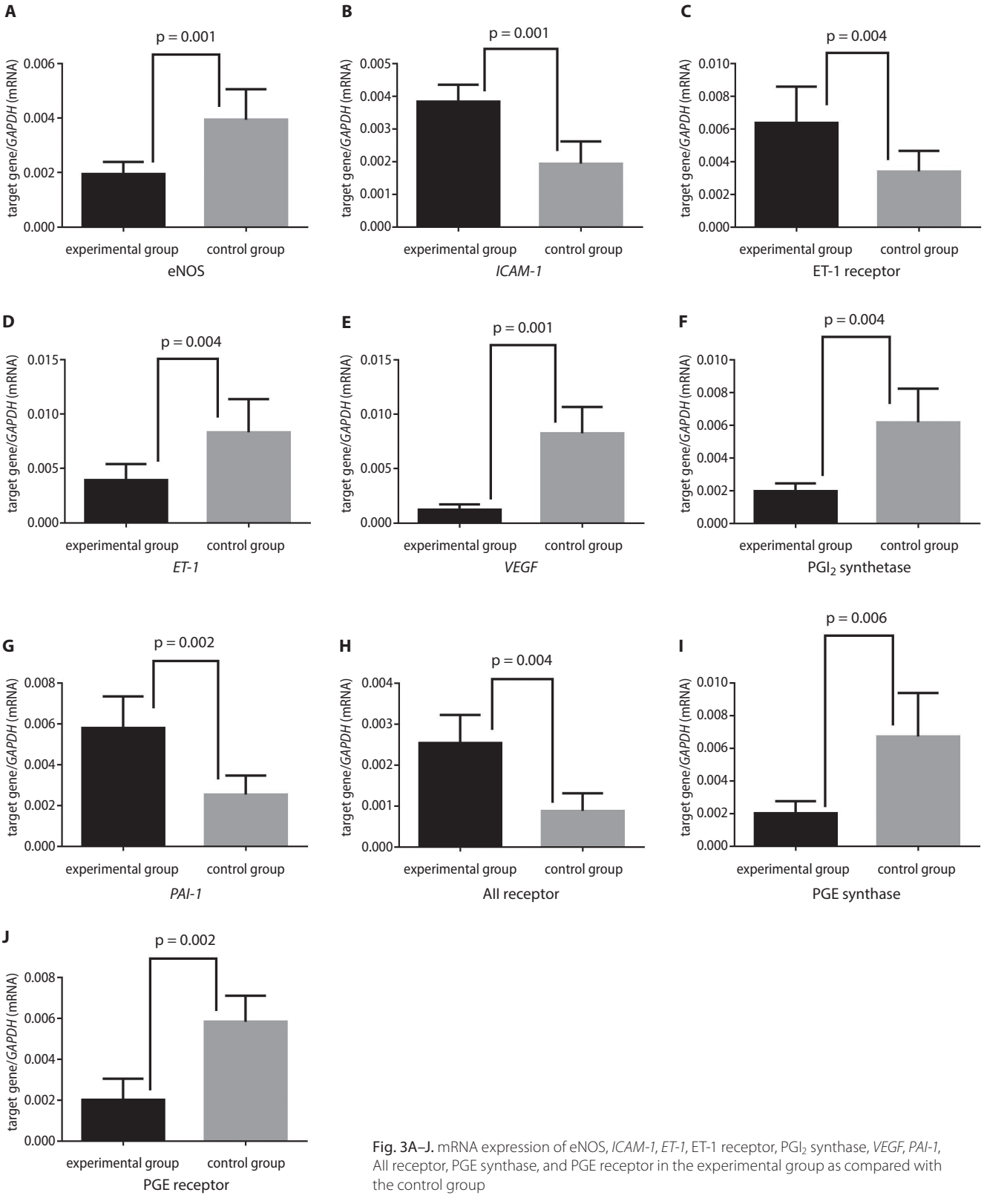

Fig. 3A–J. mRNA expression of eNOS, *ICAM-1*, *ET-1*, ET-1 receptor, PGI_2 synthase, *VEGF*, *PAI-1*, All receptor, PGE synthase, and PGE receptor in the experimental group as compared with the control group

Table 2. Primer sequences of target genes and housekeeping genes

Gene	Primer	
	forward	reverse
ICAM-1	5′-AGCTTCGTGTCCTGTATGGC-3′	5′-GACACTTGAGCTCGGGCAAT-3′
eNOS	5′-ACCGGCATCACCAGGAAGA-3′	5′-TTGTCGCCTTCACTCGCTTC-3′
ET-1	5′-GAGCTCCAGAAACAGCAGTCTTA-3′	5′-CTTTATCCATCAGGGACGAGCA-3′
ET-1 receptor	5′-TCACTCCCACACCCAAGAAG-3′	5′-AGTGCTGAATACAACACGCAG-3′
PGI_2 synthase	5′-CTGCTCCCAATTCACCTCGT-3′	5′-CGGGCCATGCTAGCTCATAA-3′
PAI-1	5′-TTGCAGGATGGAACTACGGG-3′	5′-GTGGCAGGCAGTACAAGAGT-3′
VEGF	5′-GGGAGCTTCAGGACATTGCT-3′	5′-GGCAACTCAGAAGCAGGTGA-3′
AII receptor	5′-GCCTGTTTGTCCTCATTGCC-3′	5′-CCAGCTGACCATTGGGCATA-3′
PGE synthase	5′-CCTCCCAAGGTTTGAGTCCC-3′	5′-AGGGGACATTTGCAGTTTCCA-3′
PGE receptor	5′-CCTTGGGTCTTTGCCATCCT-3′	5′-GACCTCAAAGGTCAGCCTGT-3′
GAPDH	5′-TGTTGCCATCAATGACCCCTT-3′	5′-CTCCACGACGTACTCAGCG-3′

eNOS – endothelial nitric oxide synthase; PGI_2 – prostaglandin I_2; PGE – prostaglandin E; ET – endothelin; *ICAM-1* – intercellular adhesion molecule 1; *VEGF* – vascular endothelial growth factor; AII – angiotensin II; *PAI-1* – plasminogen activator inhibitor 1.

PGI_2 receptor, eNOS, *VEGF*, AII receptor, *PAI-1*, PGE synthase, and PGE receptor, were significantly changed. The results of qPCR verification of changed gene expressions were consistent with microarray results, indicating a certain relationship between GC-induced ONFH and expression changes of these genes.

Endothelial nitric oxide synthase and ET-1 are 2 vasoactive factors secreted by vascular endothelial cells and have opposite effects. An imbalance between them leads to damaged endothelial cell function. Endothelial cells synthesize and release ET-1, which is the strongest known vasoconstrictor factor. Endothelin 1 binds to the receptors on its endothelial cells and vascular smooth muscles in the bone, and exerts a strong vasoconstrictive effect, thus reducing intraosseous blood flow. A study by Drescher et al.[31] showed that ET-1 enhanced the contractile effect of the intraosseous artery in the femoral head in vitro in pigs treated with glucocorticoids; however, other vasoactive substances, such as norepinephrine, substance P and bradykinin, exerted no such effect. Endothelial nitric oxide synthase is an endothelial-derived vasodilator primarily synthesized by eNOS in endothelial cells. Endothelial nitric oxide synthase can inhibit the secretion of endothelin and antagonize its effect on vasoconstriction. In addition, eNOS has several anticoagulant actions, such as blood vessel dilatation, platelet aggregation prevention and monocyte–endothelium adherence inhibition. Therefore, the GC-induced decrease in the bioavailability of eNOS elicits vascular endothelial dysfunction, thus causing insufficient peripheral circulation, which is a potential mechanism for GC-induced ON. Angiotensin I (AI) conversion to AII occurs through the removal of 2 C-terminal residues by angiotensin-converting enzyme. Angiotensin II can damage endothelial cells by binding to its receptors on endothelial cells. In addition, it can inhibit eNOS expression and stimulate ROS production by NADP/NADPH oxidase in smooth muscle cells.[32] Furthermore, research has shown that vasoconstrictor enhancement (e.g., AII) and eNOS synthesis reduction damaged endothelial cells and further reduced blood supply to tissues.[33] Both PGI_2 and TXA_2 are important members of the prostaglandin family. Prostaglandin I_2 is produced by PGI_2 synthase in endothelial cells; it strongly dilates blood vessels and inhibits platelet aggregation by stimulating IP receptors in vascular endothelial cells and platelets. TXA_2 is produced by platelets and causes vasoconstriction and platelet aggregation by activating thromboxane (TP) receptors in vascular endothelial cells and platelets. Both PGI_2 and TXA_2 are dynamically balanced under physiological conditions. This imbalance plays an important role in the occurrence of vascular diseases. 6-ketone prostaglandin Fα (6-keto-$PGF_{1\alpha}$), a PGI_2 metabolite, is considered an endothelial cell injury marker. He et al.[34] reported that when compared to controls, the level of 6-keto-PGF1α significantly decreased in a rabbit ON model induced by endotoxin and GC. This suggests that ON presents with another endothelial cell impairment, which is likely to be GC-mediated. The PGE has the function of dilating blood vessels, increasing the blood flow to organs and reducing the resistance of peripheral blood vessels. A research study[35] has found that PGE protects endothelial cells and upregulates eNOS mRNA and protein expression. The results of our study suggested that the expressions of ET-1 receptor and AII receptor were significantly upregulated, and the expressions of eNOS, PGI_2 synthase, PGE synthase, and PGE receptor were significantly downregulated after 24-hour GC treatment. The results showed that the vasoconstriction effect is enhanced, whereas the vasodilator effect is decreased after the GC-induced damage to BMECs. In addition, the effect on endothelial cell injury and the factors promoting thrombosis was strengthened, whereas the protective

function of endothelial cells was decreased. Therefore, a vicious circle has been formed regarding the damage of endothelial cells. Interestingly, our study showed that *ET-1* expression was significantly reduced, suggesting that the effects of GC on BMECs damage and function were exerted via a complex process. *PAI-1* is a major inhibitor of plasma t-PA. Abnormal expression of *PAI-1* is instrumental in the formation of atherosclerosis. PAI-1 is mainly produced by vascular endothelial cells. Increased expression of *PAI-1* promotes the formation of not only intravascular thrombosis but also atherosclerotic plaques. Zeng et al.[36] performed a meta-analysis, identified 5 articles that met the criteria for review and concluded that there was an association between *PAI-1* gene 4 G/5 G polymorphism and increased susceptibility to ONFH. Yamamoto et al.[37] showed that dexamethasone could upregulate the expression of *PAI-1* gene in human umbilical vein endothelial cells, and they also pointed out that *PAI-1* could promote coagulation in both dexamethasone exposure and tumor necrosis factor α (TNF-α) stimulation. A study by Kim et al.[38] suggested that ON was associated with a decrease in fibrinolytic activity induced by increased *PAI-1* expression. This is in agreement with our current results. Intercellular adhesion molecule 1, a protein also known as cluster of differentiation 54, is encoded in humans by the *ICAM-1* gene. This gene codes for a cell surface glycoprotein that is typically expressed on endothelial cells. Intercellular adhesion molecule 1, an important adhesion molecule, mediates the adhesion reaction. It is expressed at low levels in resting vascular endothelial cells, and the intercellular adhesion mediated by it plays a role in many aspects of the immune response. It enhances the adhesion between leukocytes, inflammatory cells and endothelial cells, promotes the activation of endothelial cells and makes it easier for them to penetrate the endothelium. A study by Lawson and Wolf[39] showed that with the increase in *ICAM-1* expression of endothelial cells, atherosclerosis became more severe. A high dose of GCs can damage BMECs, increase the expression of *ICAM-1* and promote the formation of microcirculatory thrombosis, which may be an important factor in the pathogenesis of steroid-induced femoral head necrosis. Vascular endothelial growth factor acts directly on vascular endothelial cells and induces angiogenesis. A research study by Li et al.[40] showed that high-dose GCs can reduce VEGF synthesis in bone marrow progenitor cells. Vascular endothelial cells can also produce VEGF. Our study showed that a high dose of GCs decreased the expression of *VEGF* in BMECs, but did not affect the expression of the VEGF receptor.

There were several limitations in our study. Firstly, the BMECs examined in this study were from the elderly (>60 years), while most patients with GC-induced ONFH are young. With the increase of age, the function of the BMECs may weaken and the results might differ as well; therefore, selection bias existed in the study. Secondly, our sample size was small. Only 8 patients were selected as the study population. It is necessary to select more patients in a multicenter study to increase the accuracy of the study. Thirdly, this is an in vitro study. The effect of GCs on bones in vivo is much more complex than in vitro, and the disease-producing dose of GCs is also different.

In conclusion, our study found that GCs promoted the expression of vasoconstrictors, procoagulant factors and the related receptors secreted by BMECs, and decreased the expression of vasodilator and corresponding receptors. Therefore, it can be deduced that complex functional changes happen after GC damage to BMECs, resulting in intraosseous microvascular thrombosis, microcirculation failure, and ultimately bone tissue ischemia and necrosis.

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A prospective observational study on perioperative use of antibacterial agents in implant surgery

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Conflict of interest
Marzena Dominiak reports having received consulting fees from Pierre Fabre Pharmaceutical Laboratories.
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Abstract

Background. Dental implant surgery has become routine practice for replacing missing teeth. Little is known about the use of local antisepsis to control the development of bacterial plaque and to facilitate healing, as current practice guidelines do not address this issue.

Objectives. The objectives of this study were to describe antiseptic practices for implant surgery and to assess plaque control at the operative site as well as the investigator's satisfaction.

Material and methods. This prospective, observational study conducted in 4 European countries enrolled 911 adult patients receiving a single or multiple implant on the day of inclusion. Any medication prescribed during the pre- or postoperative periods was documented, particularly antibiotics, antiseptic mouthwashes and topical antiseptic gels. At a follow-up visit, the presence of plaque was documented on teeth adjacent to the implant and its extent determined using the Silness–Löe index.

Results. Oral antibiotics were prescribed prior to surgery in 53.8% of the patients. Antiseptic mouthwashes were prescribed to patients (49.6–65.7%) according to country. Following dental implant placement, 84.1–94.7% of patients were prescribed oral antibiotics, 45.6–86.5% of patients were prescribed antiseptic mouthwash and 72.8–100% of patients were prescribed an antiseptic gel. At the follow-up visit, plaque was observed in 45.4% of the patients. The mean Silness–Löe plaque index was 0.7 or 0.8, indicating a low level of plaque accumulation. The Löe and Silness gingival index was 0.6 or 0.7, which is consistent with a low level of gingival inflammation.

Conclusions. Use of antibiotics pre- and post-surgery is frequent in implant surgery, despite it being discouraged in practice guidelines. Use of antiseptic mouthwashes and topical antiseptic gels is widespread, although treatment paradigms vary widely. Practice guidelines covering antisepsis provision would be useful, since those products could be used as an alternative to antibiotics to facilitate wound healing.

Key words: dental implants, chlorhexidine, antibacterial agents, antiseptic gel, mouthwash

Introduction

Dental implant surgery has become routine practice for replacing missing teeth.[1,2] In 1988, it was estimated that 100,000–300,000 dental implants were placed every year worldwide,[3] whereas more recent data indicates that 3,000,000 people in the USA alone now have dental implants and that this number is growing by 500,000 per year.[4]

Over the last 30 years, considerable evidence has accumulated demonstrating the long-term stability of dental implants, with long-term success rates >95% after 5 years[5,6] and >90% after 15 years.[7] However, long-term survival of the implants may be compromised by inflammatory damage of the surrounding peri-implant tissues, leading to bone loss and subsequent implant loss.[8] This is a consequence of peri-implant mucositis, characterized by inflammation of the mucosa surrounding the implant, or of peri-implantitis, where both the mucosa and the underlying bone are affected.[8] Peri-implant mucositis may affect 80% of the subjects with dental implants and 50% of the implants, and peri-implantitis up to 56% of subjects and up to 40% of the implants.[8,9]

Both abovementioned peri-implant diseases are caused by accumulation of bacteria from dental biofilms. As with natural teeth, there is a direct relationship between plaque accumulation and peri-implant disease. For this reason, good oral hygiene and maintenance strategies are critical for the long-term success of dental implant placement. Although international[10–13] and national[7,14–16] guidelines on different aspects of dental implant surgery have been published, none of these have specifically focused on peri- or postoperative local antiseptic management aimed at preventing plaque accumulation. In particular, there is no guidance for practitioners on the optimal timing and type of local antiseptic therapy to provide for their patients.

Local antiseptic therapy, using a mouthwash or a gel form containing an active antiseptic agent such as chlorhexidine, is widely accepted in oral surgery to prevent plaque formation and consequently to facilitate wound healing. The purpose of this local antisepsis is firstly to control the development of bacterial plaque in the direct environment of the operative wound, such as on the sutures in soft tissues, on healing screws (used in one-stage treatment procedures) and on adjacent teeth, and secondly to facilitate the physiological healing process by preventing potential delays due to localized inflammatory phenomena. Antiseptic gels may be of particular interest as they are applied directly in situ to the site of the operative wound, where they may provide a barrier effect, and present a longer-lasting antiseptic action compared to a mouthwash. This may provide a perceived benefit to patients who may be reluctant to interfere with the wound site during the postoperative period due to pain and fear of "weakening" the implant. In preclinical models, it has been demonstrated that application of a chlorhexidine-based gel may speed up the rate of healing of an intrabuccal surgical wound in experimental animals.[17]

The lack of explicit consensus practice guidelines for perioperative (especially postoperative but also preoperative) antiseptic management in dental implant surgery has resulted in a range of individualized practices, whose use and outcomes are very insufficiently documented. In addition, disparities in national and even regional practice may exist, especially due to the workings of various healthcare systems. It is thus important to collect information on the use of local antiseptic product in routine clinical practice.

The objectives of this study, conducted in 4 European countries, were to describe antiseptic practices for implant surgery and to assess plaque control at the operative site as well as the investigator's satisfaction. Recommendations for dental care provided by the practitioner after surgery were also evaluated.

Material and methods

This prospective, longitudinal, observational study was conducted in 4 European countries; Poland, Bulgaria, Portugal, and Greece. The investigator sites were dental surgeons' offices specialized or with a special interest in implantology and with regular implant activity of at least 1 implant per week. The study was performed between October 2015 and January 2016.

Participating centers

Dental surgeons were selected from the national register and stratified by region. Potential sites were randomly selected from each region based on the total number of surgeons in the region. To minimize selection bias, each dental practice was contacted sequentially in the order of the list and invited to participate in the study. Any reasons for refusal were recorded. This process continued until the required number of sites was obtained.

Patients

Adult patients (aged 18 years or older) for whom implant surgery had been indicated by the investigator and who received on the day of inclusion a single or multiple implant for which the surgical site was bordered by at least 1 tooth were eligible to be enrolled in the study. Patients were enrolled on the day they presented for the procedure. The dentist was free to decide if a one- or two-stage procedure and either a single or multiple implant therapy was appropriate and all surgical conditions were accepted. Participants were required to have healthy periodontal tissue or stabilized periodontal disease and patient management was expected to include at least 1 intra-oral antiseptic in the postoperative phase. Participants were excluded

if they were already enrolled in a clinical trial or were totally edentulous. All patients were required to provide informed consent.

Procedures and follow-up

In line with the observational nature of the study, no study-specific interventions were required. Patients were included in the study on the day on which dental implant placement was performed. Each investigator was encouraged to follow their normal routine. Participants received no additional incentives to adhere to the recommendations other than those which the investigator employed in their normal routine. The participant returned after a period of 7–21 days for a follow-up visit to assess the sutures and wound healing, according to routine practice. The time until the follow-up visit was decided by the investigator on a case-by-case basis. Participating sites were monitored throughout the study in order to optimize the quality of the data collected.

Data collection

Data was collected at the inclusion visit on the day of the procedure and on the day of the follow-up visit. At the inclusion visit, the dentist documented the patient's general health and periodontal disease, as well as the type of surgery performed. Any medication prescribed during the preoperative or postoperative periods was documented, and in particular, systemic and local antibacterial treatment – antibiotics and antiseptics (mouthwashes, gels). Other prescribed medications (non-steroidal anti-inflammatory drugs (NSAIDs), oral corticosteroids, oral analgesics, topical analgesics, and scar-healing products) have been also documented (data not shown). Any specific postoperative recommendations or counseling provided were also recorded.

At the follow-up visit, the presence of plaque was documented on teeth adjacent to the implant and its extent determined by the Silness–Loë plaque index.[18] This index measures the accumulation of plaque on 4 surfaces of individual teeth on a four-point Likert scale ranging from 0 (no plaque) to 3 (abundance of soft matter within the gingival pocket and/or on the tooth and gingival margin). The tooth score is calculated as the mean of the individual scores for each of the 4 surfaces. A global score can be calculated as the mean values for several individual teeth. The state of the gums at the operative site (measured on the teeth adjacent to the implant site or on the provisional prosthesis) was scored using the Löe and Silness gingival index.[19] This index measures gingival inflammation in proximity to individual teeth on a five-point Likert scale ranging from 0 (absence of inflammation) to 4 (severe inflammation defined by the presence of erythema, edema, marginal gingival hypertrophy of the unit or spontaneous bleeding, papillary, congestion, or ulceration). Individual tooth scores or global scores are calculated as for the plaque index. The dentist's satisfaction with the oral antiseptic gel used during the postoperative period was evaluated with respect to 5 items, each rated on a ten-point numerical rating scale ranging from 1 (very unsatisfied) to 10 (very satisfied). Any adverse events reported during the study were to be documented on the case report form.

Sample size determination

The targeted sample size for the study was determined a priori in order to estimate the key variables at an assumed frequency of 50% with an accuracy of 5%. To achieve this, a total of 1,064 (266 per country) patients would be needed to be included. To achieve this, the target number of participating centers was 60 (15 per country), assuming that 70% of the centers would be active at the end of the inclusion period.

Statistical analysis

Analysis of the data was principally descriptive and no hypothesis was tested in the study. Categorical variables are reported as frequency counts and percentages, and continuous variables as mean values with standard deviation (SD) or median values with range. Missing data were not replaced. Data from each country were analyzed separately. The statistical analysis was carried out using SAS® v. 9.4 software (SAS Institute, Cary, USA).

Ethics

The study was conducted in accordance with the Guidelines of Good Practice in Epidemiology and pertinent international and national legislation. Approval was received from the national ethics committees in Greece, Poland and Portugal. In Bulgaria, ethics committee approval was waived since the study did not influence patient care. Written informed consent was obtained from all patients and no nominative information was recorded in the study database.

Results

Overall, 911 subjects receiving a dental implant were recruited into the study: 257 in Poland, 275 in Bulgaria, 207 in Greece, and 172 in Portugal. Two subjects in Portugal who were under 18 years of age were excluded from the analysis. The remaining 909 subjects were eligible for analysis. The demographic characteristics of these subjects are presented in Table 1. These were essentially similar between countries, with a mean age of around 50 years and a slight preponderance of women.

Clinical characteristics are presented in Table 2. Patients were comparable between countries, although Greek par-

Table 1. Socio-demographic characteristics of the subjects enrolled

Variable	Poland n = 257	Bulgaria n = 275	Greece n = 207	Portugal n = 170
Gender men women	n = 247 109 (44.1%) 138 (55.9%)	n = 271 134 (49.4%) 137 (50.6%)	n = 202 85 (42.1%) 117 (57.9%)	n = 166 75 (45.2%) 91 (54.8%)
Age, mean ±SD [years]	n = 251 48.0 ±12.2	n = 274 47.0 ±13.2	n = 204 52.6 ±13.2	n = 166 51.2 ±14.1
BMI, mean ±SD [kg/m^2]	n = 253 24.3 ±3.7	n = 274 24.9 ±4.0	n = 203 25.6 ±4.3	n = 156 24.4 ±3.3

SD – standard deviation; BMI – body mass index.

Table 2. Clinical characteristics of the subjects enrolled

Variable	Poland n = 257	Bulgaria n = 275	Greece n = 207	Portugal n = 170
Periodontal disease history	n = 251 46 (18.3%)	n = 261 71 (27.2%)	n = 193 84 (43.5%)	n = 166 22 (13.3%)
Type of periodontal disease chronic periodontitis aggressive periodontitis other type of periodontitis	n = 42 34 (81.0%) 1 (2.4%) 7 (16.7%)	n = 70 65 (92.9%) 4 (5.7%) 1 (1.4%)	n = 78 65 (83.3%) 9 (11.5%) 4 (5.1%)	n = 21 16 (76.2%) 1 (4.8%) 4 (19.0%)
Oral hygiene: presence of plaque very good (no plaque) average (<30% of teeth with plaque) poor (>30% of teeth with plaque)	n = 249 128 (51.4%) 119 (47.8%) 2 (0.8%)	n = 269 152 (56.5%) 113 (42.0%) 4 (1.5%)	n = 206 67 (32.5%) 125 (60.7%) 14 (6.8%)	n = 163 65 (39.9%) 92 (56.4%) 6 (3.7%)
Comorbidities diabetes mellitus heart disease other comorbidity	n = 257 8 (3.1%) 10 (3.9%) 25 (9.7%)	n = 275 4 (1.5%) 9 (3.3%) 30 (10.9%)	n = 207 8 (3.9%) 9 (4.3%) 3 (1.4%)	n = 170 4 (2.4%) 3 (1.8%) 3 (1.8%)
Tobacco use current smoker	n = 252 36 (14.3%)	n = 271 65 (24.0%)	n = 206 54 (26.2%)	n = 162 19 (11.7%)

Table 3. Characteristics of implant surgery performed

Variable	Poland n = 257	Bulgaria n = 275	Greece n = 207	Portugal n = 170
Procedures one-stage surgery two-stage surgery one-stage surgery and two-stage surgery	n = 257 58 (22.8%) 195 (76.8%) 1 (0.4%)	n = 275 104 (38.2%) 168 (61.8%) –	n = 207 49 (23.8%) 155 (75.2%) 2 (1.0%)	n = 167 102 (61.1%) 63 (37.7%) 2 (1.2%)
Immediate temporization (one-stage surgery)	n = 59 3 (5.1%)	n = 104 33 (31.7%)	n = 51 4 (7.8%)	n = 104 20 (19.2%)
Implant placement single multiple	n = 254 152 (59.8%) 102 (40.2%)	n = 275 156 (56.7%) 119 (43.3%)	n = 207 99 (47.8%) 108 (52.2%)	n = 170 111 (65.3%) 59 (34.7%)

ticipants tended to have a higher frequency of periodontal disease and poorer dental hygiene. Comorbidities were documented in less than 10% of subjects and smoking rates were lower than national averages.

Implant surgery was performed in 2 stages for 64.1% of patients, with the exception of patients in Portugal, where one-stage surgery was more frequently performed (Table 3). In the case of one-stage surgery, immediate temporization was only performed in a minority of patients (<10% in Poland and Greece). Multiple sites were implanted in the same procedure for 42.8% of patients.

Preoperative prescription

Around 3/4 of patients (2/3 in Bulgaria) were prescribed an antibiotic or an antiseptic mouthwash, and most frequently both. Oral antibiotics were prescribed prior to surgery in 53.8% of patients, ranging from 48.0% in Poland to 58.6% in Bulgaria (Fig. 1). The most frequently prescribed class of antibiotics were beta-lactams (Table 4). Combinations of more than 1 antibiotic were prescribed to 38.8% of patients in Bulgaria, whereas this practice was uncommon (<5% of patients) in other countries (Table 4).

Table 4. Treatments used prior to dental implant placement

Variable	Poland n = 257	Bulgaria n = 275	Greece n = 207	Portugal n = 170
Treatment	n = 252	n = 274	n = 205	n = 164
neither antibiotic nor antiseptic mouthwash	69 (27.4%)	94 (34.3%)	51 (24.9%)	43 (25.9%)
antibiotic only	22 (8.7%)	46 (16.8%)	46 (22.4%)	42 (25.3%)
antiseptic mouthwash only	65 (25.8%)	20 (7.3%)	43 (21.0%)	34 (20.5%)
both antibiotic and antiseptic mouthwash	96 (38.1%)	114 (41.6%)	65 (31.7%)	47 (28.3%)
Oral antibiotic	n = 118	n = 160	n = 111	n = 89
beta-lactam	60 (50.8%)	57 (35.6%)	94 (84.7%)	76 (85.4%)
macrolide	11 (9.3%)	11 (6.9%)	2 (1.8%)	6 (6.7%)
combination therapy	3 (2.5%)	62 (38.8%)	5 (4.5%)	2 (2.2%)
Protocol	n = 97	n = 153	n = 104	n = 81
once only prior to procedure	23 (23.7%)	72 (47.1%)	79 (76.0%)	23 (28.3%)
other	74 (76.3%)	81 (52.9%)	25 (24.0%)	58 (71.7%)
Antiseptic mouthwash: treatment duration	n = 157	n = 132	n = 106	n = 79
starting 7 days prior to surgery	29 (18.5%)	103 (78.0%)	21 (19.8%)	4 (5.1%)
starting 24 h prior to surgery	12 (7.6%)	13 (9.9%)	50 (47.2%)	17 (21.5%)
starting on the day of the procedure	112 (71.3%)	14 (10.6%)	35 (33.0%)	55 (69.6%)
other treatment duration	4 (2.6%)	2 (1.5%)	0	3 (3.8%)
Antiseptic mouthwash: treatment frequency	n = 32	n = 107	n = 36	n = 19
number of times per day (mean ±SD)	2.6 ±0.5	2.2 ±0.8	2.6 ±0.5	2.5 ±0.5

SD – standard deviation.

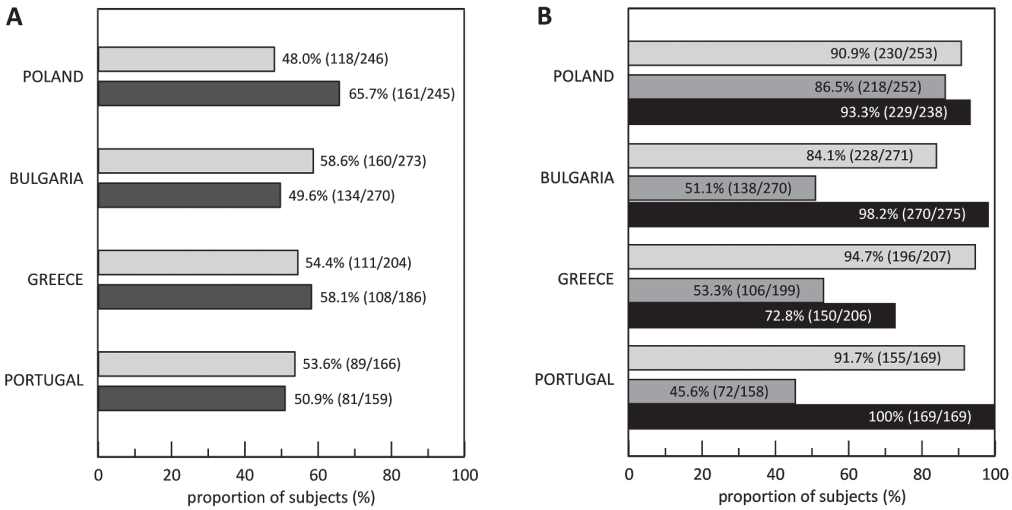

Fig. 1. Antibacterial agents in implant surgery

Greece was the only country where were the majority of patients were instructed to take the antibiotic only once prior to the procedure (Table 4). Antiseptic mouthwashes were prescribed in all countries, the frequency of use ranging from 49.6% of patients in Bulgaria to 65.7% in Poland (Fig. 1). In Poland and Portugal, the mouthwash was most frequently started on the day of the procedure, whereas in Bulgaria the majority of patients were prescribed an antiseptic mouthwash to be used for 7 days prior to the procedure (Table 4).

Postoperative prescription

Over 90% of patients received either a systemic antibiotic or an antiseptic mouthwash or both (Table 5). Oral antibiotics were prescribed following dental implant placement to the majority of patients in all countries, ranging from 84.1% in Bulgaria to 94.7% in Greece (Fig. 1). Again, beta-lactams were the most frequently prescribed class of antibiotic (Table 5). A relatively high proportion of patients in Bulgaria (30.7%) and, to a lesser extent, in Greece (20.4%) were prescribed more than 1 antibiotic (Table 5). An antiseptic mouthwash was prescribed to around half of the patients, except in Poland, where 86.5% received such a product (Fig. 1). For all but 18 patients who were prescribed a mouthwash, the active antiseptic ingredient was chlorhexidine. A topical antiseptic gel containing chlorhexidine was prescribed to nearly all patients, except in Greece, where this was the case for 72.8% of patients (Fig. 1). The gel was generally to be applied 2 or 3 times a day, and using a finger. In up to 40.4% of patients (in Portugal), a brush was used to apply the gel (Table 5). The duration of treatment was generally around 10 days. Use of an antiseptic gel did not differ according

Table 5. Treatments prescribed for the postoperative period

Variable	Poland n = 257	Bulgaria n = 275	Greece n = 207	Portugal n = 170
Treatment neither antibiotic nor antiseptic mouthwash antibiotic only antiseptic mouthwash only both antibiotic and antiseptic mouthwash	n = 255 15 (5.8%) 22 (8.6%) 10 (3.9%) 208 (81.6%)	n = 275 25 (9.1%) 112 (40.7%) 22 (8.0%) 116 (42.2%)	n = 207 4 (1.9%) 97 (46.9%) 7 (3.4%) 99 (47.8%)	n = 170 7 (4.1%) 91 (53.5%) 8 (4.7%) 64 (37.7%)
Oral antibiotic beta-lactam macrolide combination therapy other	n = 230 120 (52.2%) 43 (18.7%) 16 (7.0%) 48 (20.9%)	n = 228 72 (31.6%) 18 (7.9%) 70 (30.7%) 51 (22.4%)	n = 196 144 (73.5%) 10 (5.1%) 40 (20.4%) 5 (2.6%)	n = 155 121 (78.1%) 17 (11.0%) 6 (3.9%) 12 (7.7%)
Chlorhexidine-based antiseptic gel	n = 257 238 (92.6%)	n = 275 270 (98.2%)	n = 207 150 (72.5%)	n = 170 169 (99.4%)
Time after procedure initiated, mean ±SD [h]	n = 229 11.4 ±13.2	n = 253 8.8 ±10.5	n = 137 12.1 ±7.3	n = 156 4.6 ±6.0
Number of applications per day 1 2 3 4 or more	n = 233 13 (5.6%) 106 (45.5%) 105 (45.1%) 9 (3.9%)	n = 266 4 (1.5%) 88 (33.1%) 56 (21.1%) 118 (44.4%)	n = 143 0 69 (48.3%) 54 (37.8%) 20 (14.0%)	n = 164 1 (0.6%) 56 (34.2%) 51 (31.1%) 56 (34.2%)
Duration of treatment, mean ±SD [days]	n = 200 10.1 ±4.0	n = 234 10.5 ±4.5	n = 100 11.5 ±4.6	n = 141 12.3 ±5.7
Method of application brush finger tongue brush and finger	n = 199 36 (18.1%) 160 (80.4%) 0 3 (1.5%)	n = 237 1 (0.4%) 232 (97.9%) 4 (1.7%) 0	n = 121 30 (24.8%) 89 (73.6%) 0 2 (1.7%)	n = 141 31 (22.0%) 84 (59.6%) 0 26 (18.4%)

SD – standard deviation.

to the characteristics of the patient or of the surgery undergone (data not shown).

In addition, recommendations on appropriate hygiene techniques were given to 80.2% of the patients and recommendations on cleaning the remaining teeth given to 80.5%. Overall, 88.5% of patients were advised not to eat on the side of the operation and 87.8% to apply ice to relieve swelling. The pattern of these recommendations was similar between countries (Table 6), with the exception of Bulgaria, where patients were more frequently advised to refrain from smoking (76.7% of patients) and less frequently advised to apply ice (63.1%) than in the other 3 countries (Table 6).

Clinical follow-up

The clinical follow-up visit was made on average 9.7 ±6.5 days after surgery, although this interval was highly variable (range: 0–97 days). The median interval was 8 days in all countries except Greece, where it was 7 days. At the follow-up visit, plaque was observed in 45.4% of patients, with

Table 6. Recommendations for postoperative care

Variable	Poland n = 257	Bulgaria n = 275	Greece n = 207	Portugal n = 170
Avoiding eating on the side of the wound	n = 254 223 (87.8%)	n = 275 245 (89.1%)	n = 207 174 (84.1%)	n = 169 159 (94.1%)
Avoiding sucking on the side of the wound	n = 254 151 (59.5%)	n = 275 219 (79.6%)	n = 207 108 (52.2%)	n = 169 108 (63.9%)
Recommendations on appropriate hygiene techniques	n = 254 196 (77.2%)	n = 275 238 (86.6%)	n = 206 151 (73.3%)	n = 168 139 (82.7%)
Brushing the operated area with an appropriate material	n = 255 71 (27.8%)	n = 275 164 (59.6%)	n = 206 67 (32.5%)	n = 168 112 (66.7%)
Recommendations for brushing the remaining teeth	n = 253 209 (82.6%)	n = 274 205 (74.8%)	n = 207 178 (86.0%)	n = 170 136 (80.0%)
Not smoking	n = 249 87 (34.9%)	n = 275 211 (76.7%)	n = 205 52 (25.4%)	n = 162 89 (54.9%)
Applying ice	n = 254 245 (96.5%)	n = 255 161 (63.1%)	n = 207 202 (97.6%)	n = 170 170 (100%)

Table 7. Clinical follow-up

Variable	Poland n = 257	Bulgaria n = 275	Greece n = 207	Portugal n = 170
Presence of visible plaque	n = 234 101 (43.2%)	n = 253 97 (38.3%)	n = 182 101 (55.5%)	n = 151 73 (48.3%)
Mean plaque score on tooth/teeth adjacent to operating site	n = 231 0.7 ±0.7	n = 251 0.8 ±0.7	n = 192 0.8 ±0.7	n = 136 0.8 ±0.7
Löe and Silness gingival index				
Mean score on tooth/teeth adjacent to operating site	n = 228 0.7 ±0.6	n = 250 0.6 ±0.6	n = 196 0.7 ±0.7	n = 138 0.7 ±0.6
Mean score on healing screws (for one-stage operations)	n = 82 0.6 ±0.6	n = 90 0.7 ±0.7	n = 54 0.8 ±0.6	n = 85 0.4 ±0.6

no major differences being observed between countries (Table 7). The mean Silness–Löe plaque index on teeth adjacent to the operating site was 0.7 or 0.8 in all 4 countries, indicating a low level of plaque accumulation (Table 7). The Löe and Silness gingival index was 0.6 or 0.7 according to country, both for teeth adjacent to the operating site and on the healing screws in the operating site (Table 7). This is consistent with a low level of gingival inflammation.

Physician satisfaction

In all countries, physicians expressed high levels of satisfaction with oral antiseptic gels for use in the postoperative period (Table 8). On all 5 items, mean satisfaction scores were ≥7.8 on a scale ranging from 1 to 10. Satisfaction levels were generally lowest in Portugal (range: 7.8–8.5 according to item) and highest in Bulgaria (8.5–9.5). With regard to individual items, the lowest scores were observed for plaque control (range: 7.8–8.5 according to country) and highest for suitability for postoperative management after implant surgery (8.5–9.5).

Discussion

This observational study performed in 4 European countries indicates that antiseptic therapy using mouthwashes and, in the postoperative period, topical antiseptic gels is a well-established part of the implant surgery protocol. Some differences were observed between countries in these practices, for example in the duration of treatment before surgery or in the extent of use in the postoperative period. Prior to surgery, around half of patients received oral antibiotics (53.8%) and a similar proportion received an antiseptic mouthwash (56.3%), although 28.7% received neither. These proportions were similar between countries. These 2 types of treatment were administered in the postoperative period. Around 90% of patients were prescribed an oral antibiotic in all countries, around 50% were prescribed an antiseptic mouthwash, except in Poland, where this was the case for 86.5% of patients. Topical antiseptic gels were prescribed to >90% of patients in all countries, except in Greece (72.8%). A likely explanation of this practice in Greece is that clinicians prescribe antibiotic treatment post-surgically to ~95% of patients (the highest percentage among the 4 countries) and, for this reason, local antiseptic gel is only prescribed adjunctively to the antibiotic. In Poland, due to the emphasis on reducing antibiotic therapy in generally healthy dental patients, local antisepsis with a mouthwash or gel is generally encouraged in pre-procedural and post-surgical protocols. In Portugal, clinicians are used to prescribe antibiotics in conjunction with implant surgery. Since bone grafts are very commonly used together with implant surgery, this could justify the need for antibiotics. The use of oral antiseptic rinses has been considered for many years to be

Table 8. Physician satisfaction with oral antiseptic gels for use in the postoperative period

Variable	Poland n = 257	Bulgaria n = 275	Greece n = 207	Portugal n = 170
Plaque control at the operating site(s)	n = 256 8.2 ±1.7	n = 268 8.5 ±1.4	n = 202 8.1 ±1.5	n = 164 7.8 ±1.9
Inflammatory status at the operative site(s)	n = 255 8.1 ±1.6	n = 268 8.9 ±1.5	n = 202 8.2 ±1.5	n = 164 8.2 ±2.8
Quality of wound healing of surgical site	n = 255 8.6 ±5.2	n = 268 9.1 ±1.5	n = 202 8.4 ±1.4	n = 163 8.3 ±1.8
Effectiveness for postoperative management	n = 236 8.4 ±1.5	n = 265 9.3 ±1.4	n = 147 8.7 ±1.1	n = 161 8.4 ±1.5
Suitability for postoperative management	n = 235 8.6 ±1.5	n = 263 9.5 ±1.3	n = 144 8.7 ±1.1	n = 161 8.5 ±1.5

good practice in most of the postoperative care protocols. In spite of certain practice differences between countries, due to diversity in education, healthcare system organization and economic level, practice was in general similar and the outcome in terms of absence of plaque around the implantation site was also similar.

No apparent differences were observed in the extent of antiseptic use according to the type of surgery performed. Practitioners in all countries encouraged appropriate hygiene techniques to ensure that plaque did not build up at the operative area. Ice was recommended to reduce pain and inflammation and refraining from smoking was advised to encourage healing.

Scientific evidence for the benefits of antibiotic prophylaxis during implant placement is limited.[20] In the early era of dental implant placement, implant surgery was seen as posing a high risk of infection and, for this reason, prophylactic antibiotic treatment before surgery was proffered systematically as a precautionary measure.[21] However, the evidence accumulated from randomized clinical trials of perioperative antibiotic use in dental implant surgery suggests that the benefits are marginal.[21–24] Current evidence is insufficient to recommend or discourage the use of prophylactic systemic antibiotics to prevent complications and failures of dental implants.[20] Nonetheless, given the problem of antibiotic resistance, it is now recommended that use of antibiotics prior to uncomplicated dental implant placement surgery be limited to patients who are at specific risk for infections, such as immunosuppressed patients, or possibly in patients at high risk of failure, such as those undergoing immediate post-extractive implant placement.[21,23,25,26] The potential benefit needs to be assessed at the individual patient level and carefully weighed against the risk of adverse reactions, side effects and the emerging problems with antibiotic resistance.[20,21] In the present study, around 50% of the patients received antibiotics prior to surgery and around 90% received them for the postoperative period. These proportions suggest that prescription of antibiotics in routine practice in all participating countries extended well beyond the population of patients expected to be at specific risk.

With respect to antiseptic use during the postoperative period, a topical gel was prescribed to virtually all patients and around half received an antiseptic mouthwash as well. All the gel products and most of the mouthwashes contained chlorhexidine. Considerable variation was observed within countries, and to a lesser extent between countries, in the recommendations given to patients on when to start using the antiseptic gel after surgery, on how many times a day the gel was to be applied and for how many days the treatment should be continued following surgery. It may be helpful for practitioners and for planning of health service provision to develop standard protocols for the use of topical antiseptic gels after dental implant surgery.

Whatever the antibacterial protocol used for this study, the outcome at the follow-up visit was satisfactory. Both the Silness–Loë plaque index and the Löe and Silness gingival index on the teeth adjacent to the implantation site were <1, indicating good gingival status. Since the follow-up visit occurred at most 3 weeks after surgery, this is a good result. In addition, participating physicians reported being very satisfied with the suitability and effectiveness of topical antiseptic gels for postoperative management following dental implant surgery.

This study has several strengths and limitations. The strengths include the large number of patients included and the use of an identical protocol in 4 countries with very different healthcare systems. The limitations include the fact that, since dentist participation in the study was voluntary, it was not possible to ensure that their practice is representative of all implant surgery in the country. Given the design of the study, caution should therefore be exercised in interpreting the results. In addition, no long-term follow-up data was collected which could provide information on the benefits of antibiotic or antiseptic treatment in the postoperative period on periodontal health or on the need for supportive periodontal therapy a year following surgery.

Conclusions

The use of antiseptic mouthwashes prior to dental implant surgery and use of topical antiseptic gels after surgery is widespread in the countries participating in the study. However, treatment paradigms vary widely and it would be helpful to develop practice guidelines covering antisepsis provision in this field. Use of antibiotics is still widespread, in spite of this being discouraged in current practice guidelines, and may not have been justified in certain patients. Where antibiotics are not justified, antiseptics could be used as an alternative to ensure satisfactory wound healing. Nevertheless, interventional studies should be conducted to support this hypothesis and to identify the most appropriate protocol of administration. Education programs for dental surgeons on the issue of antibiotic use in implant surgery would be useful.

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Pain assessment and management in children in the postoperative period: A review of the most commonly used postoperative pain assessment tools, new diagnostic methods and the latest guidelines for postoperative pain therapy in children

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Abstract

Pain is one of the most common complaints expressed by hospital patients and is the main reason they seek medical help. Pain is always subjective, so its severity should be assessed individually for each patient. The main issue with pain management in children is the difficulty involved in evaluating it. Numerous studies have developed tools that would allow for an accurate assessment of the intensity of pain in children in the postoperative period. Adequate postoperative pain assessment in pediatric patients may significantly improve their comfort and quality of life. Postoperative pain prolongs recovery and hospitalization; therefore, the severity of the pain should be part of a routine assessment. Whichever tool is applied to measure pain, it should take into account the child's age, language, ethnicity, and cognitive ability. There is no one universal method for pain assessment which is appropriate for every pediatric patient. This article provides a review of the available subjective methods of postoperative pain assessment, including new objective diagnostic methods and the latest guidelines for postoperative pain therapy in a group of pediatric patients.

Key words: pain, postoperative pain, pain treatment, pediatric

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Introduction

The main issue with pain management in children – especially young children – is the difficulty involved in evaluating it. When a patient's level of pain cannot be accurately assessed, effective analgesia cannot be prescribed. When children are not sufficiently treated for pain, stress hormones are released into their systems, resulting in increased catabolism, immunosuppression and hemodynamic instability.

Pain is defined by the International Association for the Study of Pain as an unpleasant sensory and emotional experience associated with actual or potential tissue damage, or described in terms of such damage, and is caused by pain stimuli (also known as nociceptive stimuli). Pain is induced in the appropriate receptors, the nociceptors, and then transmitted through nociceptive pathways to the central nervous system, by signals that trigger a cascade of changes in the somatosensory system. These changes increase the response to further stimuli, thus increasing the pain.[1]

Pain is one of the most common complaints experienced by hospital patients[2] and is the main reason they seek medical help. Pain is always subjective, so its severity should be assessed individually for each patient. Pain consists of everything the patient describes as pain, regardless of the objective symptoms associated with it. A lack of verbal communication does not equate to a lack of pain sensation, so appropriate analgesic treatment may still be required. In 1992, the American Academy of Pediatrics and the American Pain Society issued a statement aimed at providing comprehensive treatment for pain and suffering in all children and adolescents, suggesting that attention should be focused on interdisciplinary treatment, including pharmacological, cognitive behavioral, psychological, and physical treatment.[3] Compared to adult patients, it is more difficult to assess and treat pain in children, a fact which often results in insufficient analgesics being administered. The available literature documents the harmful physiological effects of pain on young patients[4,5] as well as the beneficial results of effective analgesia in children.[6] The commonly available methods of assessing pain intensity are based on the patient's own account or depend on a clinical evaluation performed by medical personnel. It is necessary to increase the use of the available pain assessment tools and scales that allow for the most objective assessment of pain severity and the most effective analgesia possible.

Description of current knowledge

Numerous studies have developed tools that would allow for an accurate assessment of the intensity of pain in children in the postoperative period. In this review, the PubMed database was queried with the keywords 'postoperative', 'pain assessment' and 'pain scale', searching for articles published between 1950 and 2019. In total, 8,769 articles were found. After filtering for age from infancy to 18 years, 1,944 articles were left. Articles in any language other than English were not considered. An evaluation of the titles and abstracts excluded a further 1,666 studies which – despite the age range filter – also referred to adult patients and to scales used in a period other than the postoperative one, leaving 278 articles eligible for review. A total of 10 distinct common pain scales were identified. All scales were used in the postoperative period in children. A summary of the scales is provided in Table 1.

Table 1. Most often used pain scales from the literature which meet the search criteria

Acronym	Age range	First reference (author)	Number of citations
CHEOPS	1–7 years	McGrath PJ et al.[8]	33
FLACC	2 months–7 years	Merkel S et al.[11]	26
CHIPPS	0–5 years	Büttner W et al.[14]	9
OPS and MOPS	8 months–13 years	Broadman LM et al.[22] Wilson GAM et al.[24]	23
Poker Chip Tool	from 3 years	Hester N et al.[27]	4
Oucher Scale	3–12 years	Beyer JE et al.[35]	10
Wong-Baker FACES® Pain Rating Scale	from 3 years	Whaley L et al.[30] Wong DL et al.[31]	34
FPS-R	from 4 years	Hicks CL et al.[39]	31
VAS	from 5 years	Hayes MH et al.[45]	92
NRS	from 8 years	Jensen MP et al.[49]	16

CHEOPS – Children's Hospital of Eastern Ontario Pain Scale; FLACC – Face, Legs, Activity, Cry and Consolability; CHIPPS – Children and Infants Postoperative Pain Scale; OPS/MOPS – Objective Pain Scale/Modified Objective Pain Scale; FPS-R – Faces Pain Scale – Revised; VAS – Visual Analogue Scale; NRS – Numeric Rating Scale.

Pain assessment tools: Evaluation of pain according to the patient's age

As a result of growth differences, the expression of pain is different in each age group.

Infants and toddlers

Self-assessment scales do not apply to the youngest group of patients due to their inability to communicate verbally. According to Pawar and Garten,[6] the symptoms of pain in this age group include body rigidity, facial overexpression (furrowed eyebrows and exaggerated eye closure), loud crying, sleep disorders, resistance, and shifting the painful part of the body away from touch. Toddlers may do any of the following: exhibit verbal aggression, cry because of the pain, show regressive behavior, repel the harmful stimuli, or defend the part of the body exposed to pain.

Behavioral parameters – even non-specific ones – can be used in conjunction with physiological parameters, such as heart rate, blood pressure or palm sweating. A number of behavioral scales have been developed which include these symptoms.[7] The most commonly used ones are the Children's Hospital of Eastern Ontario Pain Scale (CHEOPS),[8–10] Face, Legs, Activity, Cry and Consolability (FLAAC)[9,11–13] and the Children and Infants' Postoperative Pain Scale (CHIPPS).[14–17]

Children's Hospital of Eastern Ontario Pain Scale

The CHEOPS is a behavioral scale used to assess postoperative pain in young children aged 1–7 years. According to this scale, pain assessment should be performed every 3 h, 15–20 min after intravenous analgesics and 30–45 min after oral or rectal analgesics. The child's behavior is assessed according to the criteria shown below (Table 2);

Table 2. The Children's Hospital of Eastern Ontario Pain Scale (CHEOPS)

Parameter	Criteria	Score	Definition
Cry	no cry	+1	Child is not crying.
	moaning	+2	Child is moaning or quietly vocalizing silent cry.
	crying	+2	Child is crying, but the cry is gentle or whimpering.
	scream	+3	Child is in a full-lunged cry; sobbing; may be scored with complaint or without complaint.
Facial	smiling	0	Score only if definite positive facial expression.
	composed	+1	Neutral facial expression.
	grimace	+2	Score only if definite negative facial expression.
Child verbal	positive	0	Child makes any positive statements or talks about other things without complaint.
	none	+1	Child not talking.
	other complaints	+1	Child complains, but not about pain, e.g., 'I want to see mommy' or 'I am thirsty'.
	pain complaints	+2	Child complains about pain
	both complaints	+2	Child complains about pain and about other things, e.g., 'It hurts' and 'I want my mommy'.
Torso	neutral	+1	Body (not limbs) is at rest; torso is inactive.
	shifting	+2	Body is in motion in a shifting or serpentine fashion.
	tense	+2	Body is arched or rigid.
	shivering	+2	Body is shuddering or shaking involuntarily.
	upright	+2	Child is in a vertical or upright position.
	restrained	+2	Body is restrained.
Touch	not touching	+1	Child is not touching or grabbing at wound.
	reach	+2	Child is reaching for but not touching wound.
	touch	+2	Child is gently touching wound or wound area.
	grab	+2	Child is gently touching wound or wound area.
	restrained	+2	Child is grabbing vigorously at wound area.
Legs	neutral	+1	Legs may be in any position but are relaxed; includes gentle swimming or separate-like movements.
	squirm/kicking	+2	Definitive uneasy or restless movements in the legs and/or striking out with foot or feet.
	drawn up/tensed	+2	Legs tensed and/or pulled up tightly to body and kept there.
	standing	+2	Standing, crouching or kneeling.
	restrained	+2	Child's legs are being held down.

Adapted from: McGrath PJ, Johnson G, Goodman JT, et al. CHEOPS: A behavioral scale for rating postoperative pain in children. *Adv Pain Res Ther.* 1985;9:395–402. Accessed February 8, 2019 with permission from Patrick McGrath.

Table 3. Face, Legs, Activity, Cry and Consolability (FLAAC) scale

Categories	Scoring		
	0	1	2
Face	no particular expression or smile	occasional grimace or frown, withdrawn, disinterested	frequent to constant frown, clenched jaw, quivering chin
Legs	normal position or relaxed	uneasy, restless, tense	kicking, or legs drawn up
Activity	lying quietly, normal position, moves easily	squirming, shifting back and forth, tense	arched, rigid or jerking
Cry	no cry (awake or asleep)	moans or whimpers, occasional complaint	crying steadily, screams or sobs, frequent complaints
Consolability	content, relaxed	reassured by occasional touching, hugging or being talked to, distractible	difficult to console or comfort

Merkel S, Voepel-Lewis T, Shayevitz S, Malviya S. The FLACC: A behavioral scale for scoring postoperative pain in young children. *Pediatr Nurs*. 1997;23(3): 293–297. Copyright© 2002, The Regents of the University of Michigan. All rights reserved.
Each of the 5 categories (F) Face; (L) Legs; (A) Activity; (C) Cry; (C) Consolability is scored 0–2, which results in a total score between 0 and 10.

the minimum score is 4 points and the maximum score is 13 points. A score ≥5 should be considered for the administration of an analgesic, whilst a score ≥8 should be interpreted as requiring the administration of an analgesic.

Face, Legs, Activity, Cry and Consolability

The FLAAC scale (Table 3) is a tool for assessing the intensity of postoperative pain in young children and infants or in children without contact, who are asleep, aged from 2 months to 7 years, with exposed body and limbs; the observation should last 2–5 min. Each parameter is evaluated on a scale from 0 to 2; the total score is interpreted as follows: 0 = relaxed and comfortable, 1–3 = mild discomfort, 4–6 = moderate pain, 7–10 = severe discomfort/pain. A score of more than 3 points suggests the need for analgesics.

Children and Infants' Postoperative Pain Scale

The CHIPPS (Table 4) is intended to assess the intensity of postoperative pain in infants and children under the age of 5 years. The pain evaluation should be carried out within 15 s. In the postoperative period, values from 0 to 3 points indicate that there is no pain, whilst a score ≥4 points indicates the need for analgesics.

COMFORT scale

It is also necessary to emphasize the COMFORT scale, which is less frequently used in surgical wards due to the complexity of measuring blood pressure and heart rate. This scale is used primarily for patients in a critical care setting. The COMFORT[18] scale is used to assess both behavioral and physiological elements in children. This scale has 8 indicators: alertness, calmness/agitation, respiratory response, physical movement, blood pressure, heart rate, muscle tone, and facial tension.

Each parameter is scored from 1 to 5. The overall score is between 8 and 40 points. A score between 17 and 26 indicates adequate sedation and pain control. Values above 26 indicate that the patient is experiencing pain. Initially,

Table 4. Children and Infants Postoperative Pain Scale (CHIPPS)

Item	Structure	Points
Crying	none moaning screaming	0 1 2
Facial expression	relaxed/smiling wry mouth grimace (mouth and eyes)	0 1 2
Posture of the trunk	neutral variable rear up	0 1 2
Posture of the legs	neutral, released kicking about tightened legs	0 1 2
Motor restlessness	none moderate restless	0 1 2

Büttner W, Finke W. Analysis of behavioural and physiological parameters for the assessment of postoperative analgesic demand in newborns, infants and young children: A comprehensive report on 7 consecutive studies. *Paediatr Anaesth*. 2000;10(3):303–318.

the COMFORT scale was used to evaluate the level of sedation or distress and procedural pain.[19,20] Currently it is also used in the postoperative period.[21]

Preschoolers

Children aged 3 to 7 years are able to describe the severity of their pain on an individual basis and grow increasingly expressive with age in describing the severity, location and value of pain. They can understand pain as punishment, they complain and refuse to cooperate with parents, a nurse, or a doctor, they try to push away harmful stimuli, they demand emotional support, and – as with younger patients – they may suffer from sleep disorders.[6] At this age, both scales based on observation of the child and those that require the patient's self-assessment are used. The most commonly used scales are the Objective Pain Score (OPS)[22,23] and the modified version of it, the Modified Objective Pain Scale (MOPS),[24–26] the Poker Chip Tool (Pieces of Hurt Tool),[27–29] the Wong–Baker FACES® Pain

Rating Scale,[30–34] the Oucher Scale,[35–38] and the Faces Pain Scale – Revised (FPS-R).[39–42]

Objective Pain Scale and Modified Objective Pain Scale

The Objective Pain Scale (OPS) and the Modified Objective Pain Scale (MOPS) (Table 5) are used to evaluate both the physiological parameters of pain and the behavioral changes in children which accompany pain or discomfort after surgical procedures. Wilson and Doyle[24] modified the OPS by substituting posture assessment for blood pressure in order to assess pain in children ranging in age from 8 months to 13 years. The MOPS can be used by a patient's parents, and the criterion 'holds injury site' could be substituted for the type of surgery performed. The minimum score is 0 and the maximum is 10; the higher the score, the greater the pain experience for the child.

Table 5. Modified Objective Pain Scale (MOPS)

Criteria	Finding	Points
Crying	none consolable not consolable	0 1 2
Movement	none restless thrashing	0 1 2
Agitation	asleep/calm mild hysterical	0 1 2
Posture	normal flexed holds injury site	0 1 2
Verbal	asleep/no complaint complains/cannot localize complains/can localize	0 1 2

Wilson GAM, Doyle E. Validation of three pediatric pain scores for use by parents. *Anaesthesia*. 1996;51(11):1005–1007.

Poker Chip Tool

The Poker Chip Tool (Fig. 1) is based on using 4 red poker chips. In the beginning, the child is asked whether he/she has any pain right now. If the child replies 'no', 0 is recorded. If the child says 'yes', he/she is given 4 chips. The child selects the number of chips that reflects the intensity of his/her pain, where 0 chips indicate little pain and 4 chips indicate the worst pain. It is used to assess the severity of pain in children aged from 3 to 18 years.[43]

The Wong–Baker FACES® Pain Rating Scale

The Wong–Baker FACES® Pain Rating Scale (Fig. 2) represents a series of faces from a 0-value happy face (which represents a lack of pain) to a 10-value crying face (which suggests the worst possible pain). On this basis, the patient chooses the face that best describes his/her level of pain.

Oucher Scale

The Oucher Scale (Fig. 3–5) is a combination of 2 separate scales: a photographic facial scale and a numerical scale from 0 to 10. The photographic scale contains 6 images of the same child, whose expressions suggest different levels of pain. The advantage of this scale is that there are different ethnic versions, e.g., presenting examples for white, black and Hispanic children. A vertical numerical scale from 0 to 10 is adjacent to these photographs. A numerical scale can be used by children who can count up to at least 100 and who understand, e.g., that 77 is more than 43. Children who do not understand the digits should use only the photographic facial scale.

Faces Pain Scale – Revised

The revised Faces Pain Scale (FPS-R) (Fig. 6) has been adapted to the commonly used metrics from 0 to 10 on the basis of the Faces Pain Scale (FPS).[44] It presents faces in a horizontal row, where the one on the left side indicates no pain and the one on the right side indicates the greatest possible pain. The researcher should explain to the child, 'These faces show how much something can hurt. This face [pointing to the face on the far left] shows no pain. The faces show more and more pain [pointing to each one from left to right] up to this one [pointing to the face on the far right], which shows very much pain.

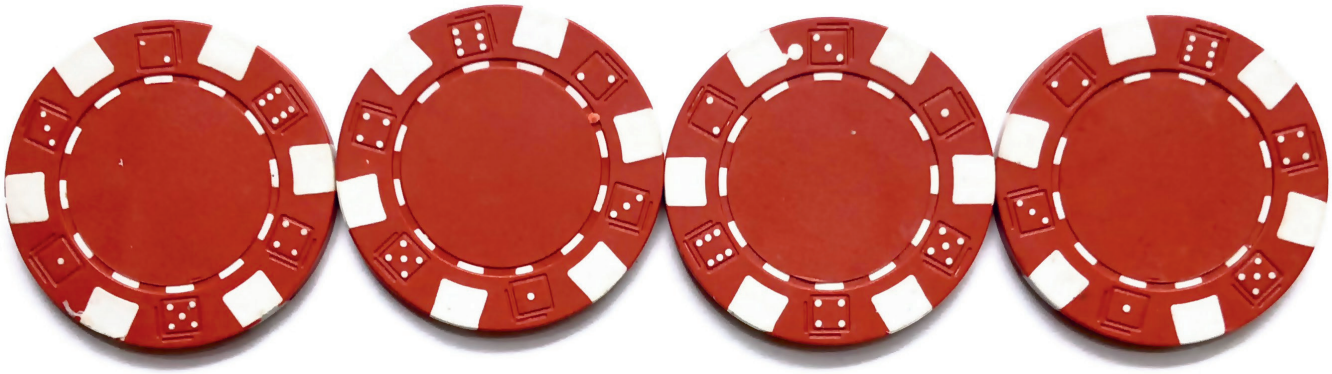

Fig. 1. The Poker Chip Tool (Pieces of Hurt Tool)

Fig. 2. Wong–Baker FACES® Pain Rating Scale

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Fig. 3. Caucasian Oucher scale

Fig. 4. African-American Oucher scale

Fig. 5. Hispanic Oucher scale

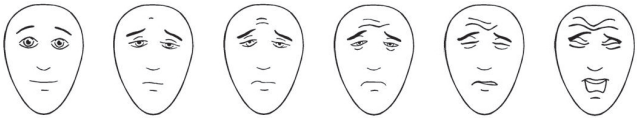

Fig. 6. Faces Pain Scale – Revised (FPS-R)

Point to the face that shows how much you hurt [right now].' Additionally, under each facial image are the numbers 0, 2, 4, 6, 8, and 10, ranging from the lowest to the highest intensity of pain, which can be seen on the back of the sheet and are not visible to the patient. The FPS-R is used to assess the intensity of the child's pain, which indicates how he or she feels; the researcher does not analyze the appearance of the patient's face or correlate it with the images.

School-age children

The verbalization of pain is common in this age group and is a great diagnostic facilitator. In addition, school-age children may experience nightmares associated with pain, increased muscular tension or body rigidity, e.g., clenching their fists and teeth or wrinkling their forehead. Adolescents may deny pain in the presence of their peers, experience appetite disorders or show regressive behavior in the presence of family members.[6]

The most commonly used scales at this age include the Visual Analogue Scale (VAS),[45–48] the Faces Pain Scale – Revised (FPS-R)[39] and the Numeric Rating Scale (NRS).[33,49–52] These are the gold standard for pain assessment.

Visual Analogue Scale

The VAS (Fig. 7) is a line that is typically 10 cm long, with markings '0' and '10' on opposite sides. Zero stands for no pain and 10 indicates very strong pain. The patient is asked to mark a line or to select a point on the scale to indicate the intensity of their pain. There are many versions of VAS in the literature, and the differences between them include units of measurement, e.g., centimeters or millimeters, length – 10 or 15 cm – and whether the scale is shown as a vertical or horizontal line.

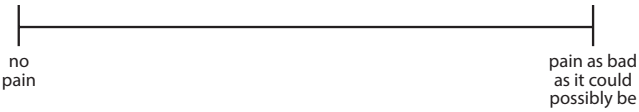

Fig. 7. Visual Analogue Scale (VAS)

Numeric Rating Scale

The Numeric Rating Scale (Fig. 8) is a segmented numerical version of the VAS scale in which patients chooses the integer from 0 to 10 which best reflects the severity of their pain. The assessment of pain intensity is as follows: no pain = 0, mild pain = 1–3, moderate pain = 4–6, and severe pain ≥7.

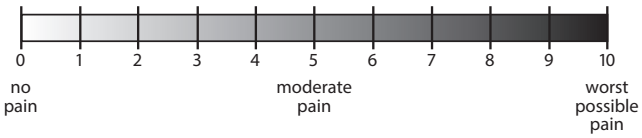

Fig. 8. Numeric Rating Scale (NRS)

New diagnostic methods – towards an objective assessment of pain

Skin conductance

Stimulation of the autonomic nervous system by nociceptive stimuli leads to skin conduction changes caused by the action of acetylcholine released in pain response to muscarinic receptors, with the subsequent release of sweat.[53] This reduces the electrical resistance of the skin and increases its conductivity. Fluctuations in skin amplitude and conductivity frequencies can then be used to assess pain.[54] This is a nociceptive phenomenon which is not affected by the changes in heart rate, blood pressure or body temperature that might occur in response to a child's anxiety, e.g., hunger, fear of separation from parents or a foreign environment. A skin conductance algesimeter is used to measure the severity of pain in children and adults, unconscious patients, patients under general anesthesia or with verbal communication limitations. This type of pain assessment is therefore considered to be the most objective,[55–57] despite the fact that the literature describes technical issues which may cause artifacts, such as electrode dislocation, wire stretching or excessive sweating by the patient.[58] Measuring skin conduction changes is an excellent pain detector, requiring further validation and clinical trials.

Analgesia Nociception Index

Analgesia Nociception Index (ANI)[59] is a non-invasive tool based on an analysis of fluctuations in heart rate, which combines electrocardiography and respiratory rate with high-frequency heart rate variability (HRV), in a frequency domain analysis. Heart rate variability is mediated primarily by changing the levels of parasympathetic and sympathetic outflow from the central nervous system to the sinoatrial node of the heart. The ANI monitor records the ECG signal continuously, enabling quantitative evaluation of respiratory variations in heart rate, which decreases during nociceptive stimulation. The ANI monitor was developed for patients over 2 years of age. Most of the ANI evaluation studies were performed in adult patients under general anesthesia or in the immediate postoperative period and showed that the ANI measurement was significantly correlated with the severity of pain.[60] To date, very little data is available on the usefulness of ANI in children.

Newborn Infant Parasympathetic Evaluation Index

Newborn Infant Parasympathetic Evaluation Index (NIPE)[61] provides an analysis of the parasympathetic response to a nociceptive stimulus. This indicator of nociception and analgesia effectiveness is also based on an algorithm for evaluating heart rate variability (HRV). As mentioned above, ANI was developed for adults and children over 2 years of age. Newborns and infants up to 2 years of age, due to the immaturity of the autonomic nervous system and the higher initial HR level, require a modified HRV analysis. The NIPE index is a modified version of ANI, and can reach values from 0 to 100. A score close to 100 indicates a higher level of patient comfort. Values below 50 indicate discomfort, stress or pain, which suggests a modification of the analgesic therapy. Few pediatric studies have yet validated this tool, although the NIPE index seems to be related to EDIN (Échelle Douleur Inconfort Nouveau-Né, neonatal pain and discomfort scale), for postoperative neonatal pain.[62]

Principles of pain management in children

It is important that children, regardless of their age, receive effective postoperative analgesia. The type and dosage of analgesics should be selected on the basis of scientific evidence, as well as standards and guidelines developed by local, national and international organizations. In this context, it is important to establish the standards of postoperative pain management in children that they expect from surgical procedures. The administration of basic analgesics (nonsteroidal anti-inflammatory drugs, paracetamol, etc.) intravenously, orally and rectally is crucial for pain management in children, and these drugs are to be found in most medical centers and hospitals around the world, even in resource-limited facilities. In addition, the effective use of basic analgesics has a significant impact on reducing the use of opioids,[63,64] which are reserved for the intraoperative and early postoperative period, in conditions where adequate monitoring is provided and a continuous opioid infusion requires the availability of specially trained personnel 24 h a day.

Table 6. Dosage suggestions for systemic analgesia in children in the postoperative period

Basic level	Intermediate level	Advanced level	Dosage suggestions
Rectal NSAIDs			
ibuprofen	ibuprofen	ibuprofen	10 mg kg^{-1} every 8 h
diclofenac	diclofenac	diclofenac	1 mg kg^{-1} every 8 h
naproxen	naproxen	naproxen	5–7.5 mg kg^{-1} every 12 h
Oral NSAIDs			
ibuprofen	ibuprofen	ibuprofen	10 mg kg^{-1} every 8 h
diclofenac	diclofenac	diclofenac	1 mg kg^{-1} every 8 h
Intravenous NSAIDs			
–		ketorolac	0.5–1 mg kg^{-1} up to 30 mg for a single intraoperative dose of 0.15–0.2 mg kg^{-1} (max 10 mg) every 6 h (short-term therapy, max 48 h)
–		ketoprofen	1 mg kg^{-1} every 8 h
Rectal paracetamol (if rectal NSAID in not available)			
paracetamol	paracetamol	paracetamol	20–40 mg kg^{-1} (15 mg kg^{-1} if <10 kg). Single loading dose in association with anesthesia; the higher dose is due to poor bioavailability from rectal route of administration.
Oral paracetamol			
paracetamol	paracetamol	paracetamol	10–15 mg kg^{-1} every 6 h
Intravenous paracetamol			
–		paracetamol	<10 kg: 7.5 mg kg^{-1} >10 kg: 15 mg kg^{-1} Intravenous preparation: 10 mg mL^{-1}
Intraoperative/postoperative intravenous metamizole			
–		metamizole	10–15 mg kg^{-1} every 8 h. 2.5 mg kg^{-1} h^{-1} (continuous infusion following an intraoperative loading dose). Due to the risk of agranulocytosis after long-term use metamizole is recommended for short term postoperative use in a hospital setting only.

NSAIDs – nonsteroidal anti-inflammatory drugs.
Adapted with a permission from: Vittinghoff M, Lönnqvist PA, Mossetti V, et al. Postoperative pain management in children: Guidance from the pain committee of the European Society for Paediatric Anaesthesiology (ESPA Pain Management Ladder Initiative). *Paediatr Anaesth*. 2018;28(6):493–506.

In 2018, the European Society for Paediatric Anaesthesiology (ESPA) Pain Committee published guidelines[65] to improve postoperative pain management in children. Although these guidelines are primarily aimed at Europe, the authors of the guidelines hope that they can also be used in other countries around the world, and that postoperative pain therapy can be adapted based on the availability of medicines, national recommendations and drug registration rules in different countries. The ESPA consensus on postoperative pain management in children is presented in Table 6. Special precautions should be taken when prescribing opioids in patients with obstructive sleep apnea, due to the increased risk of ventilation disorders in the postoperative period.[66]

Conclusions

Adequate postoperative pain assessment in pediatric patients may significantly improve their comfort and quality of life. Postoperative pain prolongs recovery and hospitalization,[67] so the severity of pain should be assessed routinely, using tools appropriate for the patient's age and disease. The research has been reviewed by selecting the scales most commonly used and validated in the postoperative period (Table 1). In order to establish simple criteria for scale selection, it seems most appropriate to categorize the patient's age into ≤5 years and >5 years of age. For patients up to 5 years of age, the CHEOPS and FLACC scales should be used; they are behavioral scales and do not require self-assessment by the patient. For children over 5 years of age, who are able to describe the severity and intensity of their pain, it is recommended to use mainly pictorial scales – such as the ethnically differentiated Oucher scale, the Wong–Baker FACES® Pain Rating Scale, or the FPS-R – or the most commonly used VAS. Whichever tool is applied to measure pain, it should take into account the child's age, language, ethnicity, and cognitive ability. Without a doubt, more than one tool is required, because no individual scoring system will be appropriate for assessing pain in all children and in all contexts. Only by considering all of these parameters can an objective evaluation of the complex nature of pain be conducted.[68]

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The ways of using machine learning in dentistry

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Abstract

Innovative computer techniques are starting to be employed not only in academic research, but also in commercial production, finding use in many areas of dentistry. This is conducive to the digitalization of dentistry and its increasing treatment and diagnostic demands. In many areas of dentistry, such as orthodontics and maxillofacial surgery, but also periodontics or prosthetics, only a correct diagnosis ensures the correct treatment plan, which is the only way to restore the patient's health. The diagnosis and treatment plan is based on the specialist's knowledge, but is subject to a large, multi-factorial risk of error. Therefore, the introduction of multiparametric pattern recognition methods (statistics, machine learning and artificial intelligence (AI)) is a great hope for both the physicians and the patients. However, the general use of clinical decision support systems (CDSS) in a dental clinic is not yet realistic and requires work in many aspects – methodical, technological and business. The article presents a review of the latest attempts to apply AI, such as CDSS or genetic algorithms (GAs) in research and clinical dentistry, taking under consideration all of the main dental specialties. Work on the introduction of public CDSS has been continued for years. The article presents the latest achievements in this field, analyzing their real-life application and credibility.

Key words: dentistry, clinical decision support systems, machine learning, artificial intelligence, CDSS

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Background

Digitalization in dentistry has increased significantly over the last 10–20 years. In most developing countries, the shortage of medical and dental professionals stimulates the need for technology, especially artificial intelligence (AI) software. This can reduce costs, time, the need for human knowledge, and the number of medical errors.

Applications in the field of dental science vary according to needs – from dental emergencies, through differential diagnosis of pain in the mouth, interpretation of radiographic images, analysis of facial growth in orthodontics, to planning the optimal prosthetics for a particular patient. Despite the recognized demand for clinical decision support systems (CDSS), the production of these systems has been limited and slow to date. This can be due to the lack of formal evaluation of the systems, challenges in programming development, cost, and skepticism about the value and feasibility of CDSS. The use of digitalization technology in dental practice has grown rapidly in recent years. It is hoped that the incidence of dental and periodontal diseases as well as deaths related to oral and maxillofacial diseases will be reduced, making it easier to take care of the patient.

According to Mendonça,[1] CDSS can be divided into several basic groups depending on the applied data analysis technique. The types of these systems are listed in Table 1.

This paper presents the general assumptions of the expert systems in dentistry and the latest 10-year results of research published in all fields of dentistry, based on the medical databases of MEDLINE (PubMed) and Dentistry & Oral Sciences Source (EBSCO).

Applications of expert systems in different dental specializations

The topics in which CDSS have been used are systematized in Table 2. Every topic has been extended in the text below.

Orthodontics

The decision to remove teeth for orthodontic treatment is important and difficult, because it tends to rely on the physician's experience. An AI expert system has been developed to diagnose extraction using the neuronal network (NN; type of machine learning), and to evaluate the effectiveness of this model. Using the back-propagation (BP) algorithm, 4 models of NN learning were constructed and evaluated for the diagnosis of extraction. Success and classification accuracy on the studied models were 93% for extraction diagnostics compared to non-extraction diagnostics, and 84% for detailed diagnosis of extraction patterns. Despite many limitations of this study (only a small, exact group of cases could be examined), the results suggests that orthodontic systems may be useful in the case of expert systems with machine learning in NN which would be a great help for the less practiced clinicians.

Table 1. Types of clinical decision support systems (CDSSs) in medicine

Type of clinical decision support systems	Mode of action	Examples of first applications
Algorithmic systems	Used in logical classification methods, represented as DTs and flowcharts that lead the user to a desired endpoint. This approach does not depend on large sample sizes of data and can be applied across patient populations.	– recommendation of chemotherapy drugs for breast cancer[2] – a diagnostic aid for oral pathology[3,4]
Neural networks	Algorithms that require training to create a set of solutions to a problem.	– first implemented in the 1940s[5] as a biological model of the brain – particularly successful at narrow and well-defined clinical problems such as classifying textual output of images diagnosis support[6–9] and prognosis evaluation[10,11] – commercialized for image recognition and used in uterus cervix cytology labs – applied in dentistry to identify people at risk of oral cancer and pre-cancer[12] – also tested in lower third molar treatment planning decisions[13]
Probabilistic systems	Incorporate rates of diseases or problems in a population and the likelihood of various clinical findings in order to calculate the most likely explanation for a particular clinical case.	– Oral Radiographic Differential Diagnosis (ORAD), a program to assist in oral radiographic diagnosis[14] and a system that assists pulpal diagnosis[15]
Logical/deductive systems	Branching logic – a collection of if-then rules – to make decisions.	– RHINOS, a consultation system for diagnosis of headache and orofacial pain[16]
Critiquing model	Program that reacts to proposed diagnosis or treatment with agreement or alternatives.	– ATTENDING, HyperCritic and RaPiD. Both HT-ATTENDING[17] and HyperCritic[18] are systems designed to critique the management of hypertensive patients. RaPiD uses both an automated and critiquing model for removable partial denture design[19]
Hybrid systems	Combine both deductive rules and probabilistic reasoning in the same CDSS.	– HEME, a system used to diagnose blood diseases in 1950s

Table 2. Application of CDSS in various dental fields

Dental specialization	Application of CDSS
Orthodontics	– the diagnosis of extractions with NN machine learning[20] – computational formulation of orthodontic tooth-extraction decisions[21] – design and implementation of a hybrid GA and ANN system for predicting the sizes of unerupted canines and premolars[22] – ANN modeling for deciding if extractions are necessary prior to orthodontic treatment[23] – factors affecting the clinical approach to impacted maxillary canines[24] – paraconsistent ANN as auxiliary in cephalometric diagnosis[25] – use of automated learning techniques for predicting mandibular morphology in skeletal class I, II and III[26] – an automatic method for skeletal patterns classification using craniomaxillary variables on a Colombian population[27]
Conservative dentistry and prosthodontics	– modeling the longevity of dental restorations by means of a CBR system[28] – the prediction in computer color matching of dentistry based on GA+BP NN[29] – an ontology-driven, case-based clinical decision support model for removable partial denture design[30] – decision support system for predicting color change after tooth whitening[31]
Periodontology	– diagnosis of periodontal diseases using different classification algorithms (a preliminary study)[32] – ANNs for the diagnosis of aggressive periodontitis trained with immunologic parameters[33] – supervised machine learning-based classification of oral malodor based on the microbiota in saliva samples[34] – predicting recurrent aphthous ulceration using GA-optimized NNs[35]
Temporomandibular joint disorders	– BBN analysis applied to determine the progression of TMD using MRI[36] – outcome of 3 screening questions for TMD (3Q/TMD) on clinical decision-making[37] – use of ANN in differentiation of subgroups of temporomandibular internal derangements (a preliminary study)[38]
Endodontics	– the reliability of ANN in locating minor AF (a cadaver study)[39] – a new approach for locating the minor AF using ANN[40]
Dental surgery	– performance of ANN for vertical root fracture detection (an ex vivo study)[41] – CDSS for dental treatment[42] – CDSS in dental implantology[43]
Maxillofacial surgery	– oral cancer prognosis based on clinicopathologic and genomic markers using a hybrid of feature selection and machine learning methods[44] – ANN analysis to assess hypernasality in patients treated for oral or oropharyngeal cancer[45] – application of FL in oral cancer risk assessment[46]

Improved performance has been achieved with elements such as proper selection of input data, appropriate organization of modeling and preferred generalization.[20]

The topic of decision support for orthodontic extraction was also raised several years earlier. The authors developed a mathematical model that simulates whether or not to extract teeth in optimizing orthodontic treatment outcome and formulates the morphologic traits sensitive to optimizing the tooth-extraction/non-extraction decisions. Orthodontic records of patients with good treatment outcomes were collected, and dentofacial morphologic traits, along with their degrees of influence in the optimized model, were determined. The rate of coincidence between the recommendations given by the optimized model and the actual treatments performed was found to be 90.4%.[21]

Also, other studies have been published in the field of orthodontics. They aimed at developing a hybrid genetic algorithm (GA) and artificial NN (GA-ANN) to evaluate the size of canine teeth and premolars that did not emerge during tooth replacement. The data was derived from measurements of tooth models. The GA-ANN hybrid algorithm was used to find the best reference teeth and the most accurate mapping function. During each iteration, the GA introduced the reference teeth into the ANN. The ANN tried to find the best mapping function for relating the reference inputs to the targets. Next, the algorithm checked the stopping criteria. If satisfying, the results were reported; if not, the GA moves to the next generation, searching for better possible candidates among the reference teeth. This process was repeated until the algorithm found a result that satisfied the stopping criteria or until the number of generations exceeded the predefined value.[22] The percentage of predictive errors and the ratios of over- or underestimation when using the GA-ANN hybrid algorithm were lower than in the case of linear regression analysis. The method is very promising, but was only tested in 1 ethnic group. More generalized studies in different ethnic groups are needed to validate the feasibility of the proposed method. There are still no updates of the method.

The experiment in orthodontics was aimed at building a decision-making expert system for the treatment of orthodontic patients aged 11–15 years in order to determine using ANN whether extraction is necessary. In particular, factors affecting the decision-making process were discovered. The ANN designed in this study was 80% effective.[23] This BP ANN employs the error BP learning algorithm. The basic principle of the BP algorithm is the propagation of errors from the output layer backward to the input layer by each layer that shares the error with neurons of each layer. The 20 test samples proved successful in evaluating factors that affect the decision-making process.

A very interesting and complicated topic about impacted canines from the border of orthodontics and dental surgery is discussed in the article by Nieri et al.[24] The aim of the study was to apply Bayesian networks (statistics)

to evaluate the relative role and possible causal relationships among various factors affecting the diagnosis and final treatment outcome of impacted maxillary canines. The demographic, orthodontic and periodontal variables were recorded and analyzed by means of NN. The network identified possible relationships among the variables considered for diagnosis and treatment of impacted canines. It confirmed the results of previous investigations on the same population in which the final periodontal outcomes after the surgical-orthodontics repositioning of maxillary impacted canines were unrelated to pretreatment diagnostic variables on the panoramic radiographs.[25]

Another work dealing with the subject of diagnosis in orthodontics presents the application of the paraconsistent artificial neural network (PANN) in the analysis of cephalometric variables and provides an orthodontic diagnosis. Patient input cephalometric values were compared with means drawn from individuals considered normal from the cephalometric point of view by the PANN. The analysis was targeted to measure skeletal and dental discrepancies and establish a cephalometric diagnosis. The analysis results were expressed in degrees of skeletal, anteroposterior, and dental discrepancy, pertinent to upper and lower incisors. A sample of 120 orthodontic patients was processed by the proposed model and 3 orthodontic experts. Comparisons between the model and the human experts' performance provided kappa indexes that varied from moderate to almost perfect agreement. The agreement between the model and specialist's performance was equivalent. In addition, the model pointed out contradictions presented in the data that were not noticed by the orthodontists, which highlights the contribution that this kind of system could make in orthodontics decision support.[26]

Niño-Sandoval et al.[27] used an already existing machine learning software (as the only one of the articles described). RapidMiner predicts the mandibular morphology through craniomaxillary variables on lateral radiographs in patients with skeletal class I, II and III. The researchers use 2 machine learning techniques – ANN and support vector regression (SVR). Standardized lateral radiographs were used to create mandibular measurements. They were evaluated through a correlation coefficient using a ridge regression between the real value and the predicted value. The authors came to a conclusion that the used craniomaxillary variables showed a high predictability ability of the selected mandibular variables, which may be the key to facial reconstruction from specific craniomaxillary measures in the 3 skeletal classifications. The ANN compared with the SVR had a better performance and classification accuracy reflected in its higher coefficients.[27]

Despite the fact that the abovementioned algorithms were very promising in the orthodontic diagnosis, none of them were introduced into general orthodontic practice.

Conservative dentistry and prosthodontics

The lifespan of dental restorations is limited. Longevity depends on the material used and different dental characteristics. One recent study by Aliaga et al.,[28] based on a set of data from graphs, notes and radiological information from information analysis performed using AI, attempted to determine the most suitable material for the restoration of cavities and long-term monitoring of the reconstruction process. In order to classify the cases, a multilayer perceptron and a mixture of experts evaluated the data. The multilayer perceptron uses a NN which facilitates the combination of outputs obtained by both methods and reduces error. As shown, the output of the classifiers corresponds to the input of NN. The 2 classifiers were combined by means of NN. The conclusion of the presented study suggests that the system enables us to determine the type of reconstruction best suited to the patient, predicting the longevity of each procedure. The system adapts to new cases that are acquired by incorporating new data into the system database and updating information which is then used for new prognoses. This is an important issue which can lead to improved results in new cases introduced into the system. The technology has not been transferred to the clinical usage, though.[28]

The material needed to fill or replace the defect is just as important to the patient as the color of the selected filling or prosthetic restoration. Although the use of computer-based color matching can reduce the impact of subjective factors, matching the color of the natural tooth to ceramic one is still one of the most demanding topics in esthetic dentistry. Back propagation of neural networks (BPNN) has already been introduced to computer color matching in dentistry, but it has disadvantages such as instability and low accuracy. The study used GA to optimize the initial mass and threshold values in BPNN in order to improve the precision of matching. In accordance with expert knowledge, BPNN was combined with GA as a novel method for computer color matching in dentistry. Experiments have shown that the proposed method improves the precision and predictability of color matching in complementary dentistry. The existing improved BPNN has a low convergence rate and it is difficult to devise a suitable network structure. However, it has high practical application value and, with the development of computer science, dental practices will have more ability to provide better services for patients in the future.[29]

Also, in the field of prosthodontics, an initial study by Chen et al. was published[30] that presented a CDSS model for specific design of removable partial dentures (RPDs). The authors developed an ontological paradigm to represent knowledge of patient's oral conditions and denture component parts as well as a cosine similarity algorithm to calculate similarity values between input patients and standard ontology cases. All the similarity metrics described in the article demonstrated the efficiency

of the model. In conclusion, the authors admitted that the methodology merits further research development to match clinical applications.

The use of CDSS was also introduced in predicting the color of the teeth after the bleaching procedure. The authors implemented the CDSS using the regression model as an intelligent part of the system. The system consisted of 3 parts: patient individual data, pre-bleaching color input, and prediction of post-treatment color (output). The results obtained have demonstrated that the CDSS can predict the color change obtained with an in-office whitening system using colorimetric values.[31]

Periodontology

An attempt to apply AI was also made in the field of periodontal diseases – in periodontology. In recent studies, an identification unit has been developed for the classification of periodontal diseases using SVM, decision trees (DT) and NN. Patients were divided into 2 groups. The codes created for risk factors, data related to the periodontium and, radiographically, to bone loss were created as a matrix and treated as input data for the classification unit. In total, 6 conditions of the periodontium were the results of the classification unit. The accuracy of the proposed methods was compared according to their resolution and operation time. The DTs and SVM achieved the best accuracy rate at classifying periodontal diseases with high accuracy depending on clinical trials. The SVM and DT results were 98% with a total calculation time of 19.91 s and 7.00 s, respectively. The worst correlation between input and output variables was for ANN, and its efficiency was estimated at 46%. The SVM and DT proved to be complex enough to reflect all factors associated with the periodontal condition, and simple enough to be understandable and useful as a decision-making aid for predicting periodontal diseases. The program offered a supportive diagnostic tool for periodontal diseases with high accuracy and opened a new area for identifying periodontal diseases. However, further research with a wider population is recommended, including research on advanced algorithmic models that use clinical and imaging data.[32]

Tests were also carried out in the field of periodontology using ANNs to diagnose and treat aggressive periodontitis (AgP) resulting from autoimmune diseases. To date, no clinical, microbiological or histopathological markers or their combination have been developed to identify AgP in patients with chronic periodontitis (CP). The aim was to determine the probability density function of clinical and immunological data sets from patients with periodontal diseases and develop NNs to correctly classify patients into either AgP or CP class. Matching probability distributions to data sets was examined by means of the Akaike information criterion (AIC). The ANNs were trained by cross-entropy (CE) values estimated on the basis of the probability of demonstrating certain levels of immune parameters and the probability of the reference mode proposed by the kernel density estimation (KDE). In the case of ANNs, the weight distribution regularization parameter of method was determined using tenfold cross-validation. Possible evidence for 2 clusters of patients in cross sections and longitudinal weight loss measurements was revealed using KDE. Data sets with CD4/CD8 ratio showed from 2 to 7 clusters, CD3, monocytes, eosinophils, neutrophils, and lymphocytes, levels of interleukin (IL)-1, IL-2, IL-4, interferon gamma (INF-γ) and tumor necrosis factor α (TNF-α) from monocytes, as well as antibody levels against *Actinobacillus actinomycetemcomitans* (Aa) and *Porphyromonas gingivalis* (Pg). The ANNs gave 90–98% accuracy in classifying patients into both groups. The best overall predictor was ANN from CE monocytes, eosinophils, neutrophils, and CD4/CD8 as inputs. The ANNs may be invaluable in classifying periodontal patients into AgP or CP when they are supplied with CE values based on KDE. Therefore, ANNs can be used to accurately diagnose AgP or CP with relatively simple and conveniently obtained parameters, such as the peripheral blood leukocyte count. This will enable clinicians to better tailor specific therapeutic protocols for patients with AgP and CP. The algorithm can effectively classify periodontitis patients. Future work should be anticipated on bigger samples, extending the results of the present study and employing a wider array of parameters that can turn personalized treatment of periodontitis from concept to reality.[33]

In the field of periodontology and mucosal diseases, studies have been conducted on the composition of saliva and microorganisms in the mouth. Recent studies have shown an effective method for classifying yeasts from salivary microorganisms using SVM, ANNs and DTs. This approach uses the concentration of methyl mercaptan in the oral air as an indicator of oral odor and the peak areas of restriction fragment length polymorphisms (T-RF) of the *16S rRNA* gene as data for supervised machine learning methods without identifying specific species producing inflammatory oral compounds. Using T-RF proportions and frequencies, models have been developed to classify the presence of methyl mercaptan, volatile sulphur-containing compounds that cause yeasts in the oral cavity. The SVM classifiers successfully classify the presence of highly specific methyl mercaptan, and it is expected that such classification will be useful for saliva screening in respiratory tract allergies prior to visits to specialist clinics. Classification using SVM and ANN does not require the identification of oral species of microorganisms responsible for halitosis, and ANNs do not require T-RF proportions.[34] Interesting findings from ANN studies, also in the field of mucosal diseases, presented in publication by Dar-Odeh et al.,[35] provide an opportunity to develop and optimize NN that can predict the occurrence of recurrent rheumatoid arthritis ulcers (RAU) based on a set of relevant input data. Artificial

neural networks that take advantage of GA to optimize NNs architecture were used. Input and output data from 86 subjects (predisposing factors and participants' status for recurrent aphthous ulceration) were used to construct and train NNs. Optimized NNs were tested using unqualified data from 10 subsequent subjects. Based on data analysis, ANN found significant correlations between specific environmental, individual and behavioral factors and the occurrence of recurrent aphthosis. This is of great importance for screening patients and for the proper education of the exposed group.

Temporomandibular joint disorders

The latest AI technology has also been used to help in extremely complicated joint diseases in terms of analysis, diagnosis and treatment. This study investigated the analysis of magnetic resonance imaging (MRI) results to diagnose temporomandibular joint disorders (TMD). The purpose of the study was to determine the progression of TMD, focusing on how each discovery affects the others.

Briefly, 1.5-T MRI (33 variables) and diagnosis (bone changes and disk displacement) of patients with TMD in the years 2007–2008 were selected. The data was modified according to the TMD diagnostic criteria. Bayesian belief network (BBN) accuracy was compared using 11 algorithms (necessary path condition, path condition, greedy search-and-score with Bayesian information criterion, Chow–Liu tree, Rebane–Pearl polytree, tree-augmented naïve Bayes model, maximum log likelihood, Akaike information criterion, minimum description length, K2, and C4.5), multiple regression analysis, and an ANN with the use of usefulness validation and tenfold cross-validation. The BBN path condition algorithm was >99% accurate when using the regression validation and tenfold cross-validation. The BBN may represent cause-effect relationships between different results and attribute conditional probabilities that can then be used to interpret TMD progression. The research was made on the great dataset and the results are accurate. The program results would be helpful in clinical practice, especially when dealing with such a difficult topic. Effort should be made to implement the program in dental practice.[36]

The solution published by Lövgren et al.[37] also helps in the field of TMD. The aim of the study was to determine the use of ANN to predict 2 subgroups of internal changes in temporomandibular joints (TMJ) and normal joints using characteristic clinical signs and symptoms. Clinical symptoms and diagnosis of 161 patients with temporomandibular joint internal derangement (TMJID) were considered a golden standard and were used to train NNs. After training, symptoms and the diagnosis of 58 new patients were used to test the ability of the network to diagnose. The diagnosis from ANN was compared with the diagnosis of a surgeon experienced in TMD. The sensitivity and specificity of ANN in predicting TMJ ID subtypes were assessed using clinical diagnosis as a golden standard. Eight cases assessed as bilaterally correct in a clinical trial were found to be normal by ANN. In the detection of unilateral anterior disc displacement with reduction (ADDwR), the sensitivity and specificity of ANN were 80% and 95%, respectively. In detecting unilateral anterior disc displacement without reduction, sensitivity and specificity of ANN were 69% and 91%, respectively. In bilateral ADDwR detection, sensitivity and specificity of ANN were 37% and 100%, respectively. In the case of bilateral ADDwRn, sensitivity and specificity of ANN were 100% and 89%, respectively. In detection of ADDwR on one side and ADDwR on the other side, sensitivity and specificity of ANN were 44% and 93%, respectively.

Great progress in the use of CDSS has been made by Bas et al.,[38] who used it practically in a clinical screening study of patients with TMD. Considering factors such as gender, age, dental pain, 300 TMD-positives and 500 TMD-negatives were randomly selected from the study population (SPSS v. 22 for Macintosh; IBM Corp., Armonk, USA; random numbers) who needed or did not need TMD treatment. On the basis of the CDSS analysis, conclusions have been drawn which factor the influence on the higher risk of developing TMD (there was no difference in the occurrence of dental care or in the rate of subscription of dental care). Among TMD-positives, there was no significant difference in the proportion of women and men receiving treatment related to TMD. No significant association was found between which fee systems the individual belonged to and any specific type of treatment, and no significant association with TMD treatment for the independent variables of sex, age, profession of the examiner, or dental care subscription. The research was one of the first real clinical and practical uses of the CDSS in dentistry.

Endodontics

Artificial intelligence has also been introduced into the field of endodontics. The accuracy of ANN was evaluated in an attempt to simulate the clinical evaluation of the working length of the root canals of human teeth. It was measured on 50 extracted teeth by an endodontist. The working length was confirmed using radiography. The position of the endodontic file relative to the minor apical foramen (AF) was classified as distant, close and accurate by ANN, by endodontist prior to extraction and using stereomicroscope after extraction. There were significant differences between the data obtained by endodontists and ANN and the data obtained by endodontists and actual measurements with a stereomicroscope after extraction. The evaluation of endodontists was accurate in 76% of the teeth. The ANN found correct anatomic position in 96% of the teeth and was more accurate than endodontic markings compared to actual working length measurements using a stereomicroscope as a golden standard after tooth extraction. An ANN can be used

to exactly determine the working length, which will be of great importance in clinical practice and treatment success as well as in the long-term maintenance of root canal-treated teeth.[39]

A very similar study, conducted in the same year and in the same research center, presented a new approach to finding minor AF.[40] The new approach was developed to locate AF using feature extraction from radiography, followed by data processing using ANN as CDSS. Fifty straight single-rooted teeth placed in the alveolar process of the jaw of a dissected skull were selected. Access to the chamber and canal of the tooth was established and the file was placed in the canals in order to determine the working length. A radiograph was performed to evaluate the location of the file relative to AF and the location was re-evaluated after tooth removal. The file end location was split identically to the previously described experiment. Each radiograph was used to extract relevant functions using unsupervised machine learning method K-means for clustering, Otsu's method and the Wavelet protocol. Thirty-six functions were used for training, and the others were used to evaluate the multilayer perceptron ANN model. Analysis of radiographic images (test samples) using ANN showed that in 93% of the samples, AF location was correctly determined by false rejection and acceptance of errors. It has been shown that ANNs may be the second opinion in locating AF on radiographs to increase the accuracy of radiography in determining the working length. In addition, ANN can function as CDSS in various similar clinical situations.

Dental surgery

A study presenting NN developed for the detection of vertical root fractures may be also placed in the field of endodontics or at the border of dental surgery. This is an important and difficult issue because vertical fractures are often difficult to diagnose by a clinician due to cracks that overlap anatomical structures in 2D dental X-rays.

A probabilistic NN design was used to assess whether the root of the tooth was healthy or had a vertical fracture. Two hundred photos from digital radiography – used to train and test NNs – were divided into 3 groups according to the number of training and test sets. Both the training data and the test data were evaluated using gray data on the line passing through the root. The function deviation in the reconnaissance data was estimated between 0 and 1 to select the best NN performance. Neural network results were evaluated using a diagnostic test. The NN designed in this study had sufficient sensitivity, specificity and accuracy to be a model for detecting vertical root fractures. However, before extending to a clinical application, further research is needed in order to work around several clinical problems, i.e., the grey-scale data in the experimental root fracture without any radio density material certainly differ from the variety of root canals in clinical cases.[41]

Another article connected to dental surgery has been published by Mago et al.[42] The authors proposed an expert system based on fuzzy logic (FL) that was supposed to help to decide which treatment should be introduced in the case of the broken tooth.

Fuzzy logic is a form of AI that uses a collection of membership functions and fuzzy rules, instead of traditional bivalued logic. Fuzzy logic systems are capable of dealing with imprecise values or vague concepts and are able to imitate human reasoning capabilities, which must deal with imprecise or not-well-defined terms. The authors concluded that the system is identical to dentists' predictions with respect to treatments. The system has been rigorously tested and the predictions are consistent with those supplied by the tested dentists, so it is concluded that the system is operating similarly to the intelligent behavior of the dentists. This system can be treated as an expert "second opinion" to help dentists during the decision-making process of choosing treatment for a cracked or broken tooth. However, the software still remains only in the academic usage.

The field of dental surgery includes implantology, which merges prosthetics, orthodontics and periodontology. Therefore, the clinician needs to analyze huge amount of data before the decision about implantation. The article by Polášková et al.[43] presents a web application that provides recommendations for application of the implant based on anamnesis and medical examination such as 3D measurements, diagnostic information for treatment planning and objective measurement of implant placement. The core of the program concerned expert knowledge programming, such as DT. The structure model had 4 basic components: inference engine, knowledge base, working memory, and explanation. This CDSS is based on the comprehensive appraisal of the morphologic features of the proposed implantation site and can practically help to formulate an objective treatment plan in general practice, one that would include implant objectification. The system would be greatly helpful for dentists as long as it would be widely available and widespread.

Maxillofacial surgery

The field of dentistry that is particularly important in everyday practice is maxillofacial surgery, which involves, i.a., the diagnosis and treatment of oral cancer. A potential diagnostic error may cost the life of patients. Therefore, it would be advisable to introduce objective solutions, allowing for 100% correct diagnosis. Chang et al.[44] focused on this problem. The main aim of this research was to apply a hybrid of feature selection and machine learning methods in oral cancer prognosis based on the parameters of the correlation of clinicopathologic and genomic markers. In the 1st stage of this study, 5 methods of feature

selection were proposed and a set of data on oral cancer prognosis was experimented with. In the 2nd stage, the model with the functions selected from each feature selection method was tested on the proposed classifiers. Four types of classifiers were selected, namely adaptive neuro-fuzzy inference system (ANFIS), NN, SVR, and logistic regression. Results have shown that prognosis is superior to the presence of clinical and genomic markers. The research was conducted on a small sample size mostly because of the medical confidentiality problems. Some patients, especially with such a difficult health problem as cancer, do not wish to reveal any information about their diseases to others, and are unwilling to donate their tissues for research/educational purposes. As for clinicians, some may not want to share patients' data with others, especially those from the non-medical fields, while some do not keep their medical records in the correct medical form. Among the available cases, some patients' clinicopathologic data were incomplete, some tissues were missing due to improper management and some were duplicated cases. Due to that, the number of cases that could actually be used for this research was very limited. In order to overcome the problem, the authors employed the feature selection methods on their dataset to choose the most optimal feature subsets based on the correlations of the input and output variables. The features selected were fed into the proposed classifier. The method is very promising and should be extended to as many clinical centers as possible.[44]

Neural network analysis has been also used to assess hypernasality in patients treated for oral or oropharyngeal cancer. The researchers investigated the applicability of NN feature analysis of nasalance in speech to assess hypernasality in the speech of patients treated for the abovementioned cancers. The speech recordings were evaluated regarding hypernasality, articulation, intelligibility, and patient-reported speech outcome. Objective measurement with ANN was not able to differentiate between patient for tumor stage and tumor location, while trained listeners are able to differentiate between patients for tumor stage, but not for tumor location. In conclusion, ANN is not yet able to substitute trained raters.[45]

The topic of the risk and causes of the malignization is presented in a recently published article by Scrobotă et al.,[46] in which the authors aimed to estimate the oxidative stress related-cancerization risk of the potentially malignant processes. They used FL to interpret the values in the input data and to assign values to the output in order to implement the multi-criteria CDSS. The risk was estimated as a concrete numerical value on a scale from 1 to 10 depending on the input numerical/linguistic value. The system can be a great achievement in oral cancer screening. However, it needs to be tested on a greater sample (in the research, only 16 samples were tested) before it could be admitted as a reliable instrument to infer the screening in this very challenging field of medicine.

Conclusions and comments

1. As in another disciplines, SVM brings the best accuracy and performance because of method construction. However, the articles present the contemporary achievements in CDSS without their critical assessment, largely displaying a very optimistic approach to the subject.
2. The validity of CDSSs is mostly established in narrow domains under varying conditions and technologies. Most of the systems have not been formally evaluated, and their value for clinical practice could not be established. Currently, CDSSs, in general, are developing as fragments and isolated systems with a few clinic- or hospital-wide exceptions in academic centers.
3. All of the algorithms and projects presented in the review have been validated only internally. The algorithms use small, inner databases. None of them have been shown to affect real clinical decision-making.
4. A small sample size is a common problem associated with medical datasets. The costs that have to be incurred and the usually inconsistent, incomplete or noisy samples make it almost impossible to gather adequate samples. The small sample size problem is mostly visible in the oral cancer research.
5. The validation of the datasets is not performed truly independently. The cross-validation method is used, which might have too many variables that can improperly modify the developing and training of the classifier, which is why it is necessary to assess its performance on completely independent datasets, preferably from different institutions.
6. The presented ideas have a great potential to be a very helpful key for the clinicians to shorten the diagnostics and make it simpler and more reliable.
7. To successfully implement CDSSs in dental practice, there is a need to overcome many practical difficulties:

a) A dentist's work is rarely concentrated around a university clinic, where there are a few dentists for 1 program; dentists have their own private practice, often one-person. This means that the CDSSs would have to be financially available for the average dentist.

b) Only a few dentists specialize in one area, such as periodontics, surgery, orthodontics, or endodontics. Most of them perform treatments in many fields of dentistry, which means that a very extensive program should be created, making decisions in a very wide range of areas.

c) Ideally, the program should be immediately linked to an electronic patient database, so that the physician only writes data from the interview and clinical trial – otherwise the program would take too much time in the chair and it would not be cost-effective for the dentist. The "currency of the chair-time" refers to the time that a physician must devote to performing a particular procedure.

8. The focus should be on creating a precise and practical program in the most specialized and the most difficult areas of dentistry. This should occur primarily in academic hospitals, such as facial and maxillofacial surgery

departments, especially for the assessment of the advancement and treatment of orthostatic neoplasms and orthognathic surgery, which are fields that link orthodontics and facial-jaw surgery, treating dentomandibular, mostly severe genetic malocclusions.

9. The impact of the abovementioned techniques in clinical dentistry is inconsiderable so far, but they are very promising in the future.

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Evaluation of the potential of nanoparticles containing active substances in selected chronic diseases

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Abstract

Currently, over 80% of all deaths result from the incidence of chronic diseases. The challenge of modern medicine is to develop innovative and effective methods of diagnosis and therapy of these disorders. Different types of particles can be obtained with the use of nanotechnology, including nanoliposomes, solid lipid nanoparticles (SLN), nanospheres, dendrimers, as well as carbon nanotubes (CNT) or fullerenes. All of these nanoparticles (NPs) are suggested to have potential, both in medicine and in diagnosis of many diseases, giving a chance for recovery or longer life for the patients. The studies concerning the usage of NPs show their effective role in most cases. However, there are also concerns about their toxicity or long-term adverse effects. The aim of this literature review was to discuss the results of the latest available studies concerning the efficacy of selected drug-loaded nanocarriers in several chronic diseases, i.e., cardiac disorders, cancer, Alzheimer's disease (AD), Parkinson's disease (PD), and wound healing. We also focused our attention on the methodology of NPs preparation, materials used for their preparation as well as on positive and negative aspects of these nanocarriers.

Key words: cancer, cardiac diseases, nanoparticles, neurodegenerative diseases, wound healing

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Introduction

In recent decades, the average life expectancy has significantly increased. Advances in medicine and the improvement of living conditions have significantly reduced mortality caused by infectious diseases and inflammatory conditions of various etiologies. Currently, over 80% of all deaths are a result of chronic diseases.[1] The challenge of modern medicine is to develop innovative and effective methods of diagnosis and therapy for chronic conditions. Currently, modern controlled drug release systems are being extensively studied.[2] However, one of the important issues in the process of developing new drugs is the bioavailability of active pharmaceutical ingredient (API). To overcome this potential problem, several methods can be used, including micro- and nanonization, which can help reduce a substance to a micro- or nanosize without changing its chemical properties. They also increase the surface area of drug particles, especially in the case of nanoparticles (NPs), leading to faster dissolution rates and increased bioavailability in the body.[3]

The use of NPs containing various active substances is widely analyzed in the areas of diagnostics, medicine and pharmacy. Nanoparticles included in the diagnostic tests can help in quick determining the patient's disease state as well as precise indicating the location of the pathological cells in the human body.[4] Nanoscale methods used in pharmacy make it possible to manufacture spherical- or fibrous-shaped, and single- or multilayered NPs. The new drug delivery systems (DDS) enable API to reach the target site. In addition, the structure of API can be modified. Due to the size of NPs (ranging from 1 nm to 100–200 nm) it is possible to suspend them in a gas and thereby to obtain a therapeutic aerosol.[5] The NPs can also be suspended in a liquid or embedded into a solid matrix.[6] The specificity of the nanopreparation is associated with a reduced dose or concentration of API. In addition, reduced adverse effects of API can also be observed. Currently, intensive work is being conducted on biodegradable polymers and natural biopolymers, used in the production of NPs, which will be compatible with the cells of the human body.[7] It is important to improve technology for the manufacture of NPs; obtained NPs should be characterized by high homogeneity, monodispersity and, thus, optimal efficiency. Nanoscale techniques currently in use have many disadvantages, among which are difficulties in obtaining a product with high stability and low toxicity. During the manufacturing process, some polymorphic transformations can possibly appear, as well as a tendency towards aggregation. In addition, these techniques are time-consuming and require high financial outlays. The results of experimental studies suggest, however, that the use of NPs in medicine may revolutionize the therapeutic success of treating many diseases, i.e., neoplastic diseases, heart diseases, but also neurodegenerative diseases or wounds difficult to heal.[7–11] On the other hand, controversies about their long-term toxicity, which is not yet fully known, have also been raised.[12] These concerns are the reason why work is being done to develop the legal regulations regarding nanotechnology methods.[13] At present, the so-called soft international law regulates the safety of using innovative applications of nanotechnology, which includes recommendations with regard to current standards.[14]

The aim of this literature review was to discuss the results of the latest available studies concerning the efficacy of certain drug-loaded nanocarriers in selected chronic diseases, with special emphasis on methodology as well as materials used during their preparation.

Characteristics of selected drug nanocarriers

The following types of NPs can be distinguished in relation to the material used: lipid-based (e.g., nanoliposomes, lipospheres, including solid lipid NPs (SLN) and nanostructured lipid carriers), polymeric-based (e.g., nanospheres, nanocapsules) or carbon-based (fullerenes, carbon dots, carbon nanotubes (CNT)).[6]

Nanoliposomes are spherical structures up to 100–200 nm in size, with the walls formed by a lipid bilayer with an aqueous phase as a core of a vesicle. The structure of the liposomes/nanoliposomes, as well as methods of their preparation and division, are well-understood and have been described in previous studies.[6] Nanoliposomes show a number of advantages, e.g., lack of toxicity and immunogenicity, full biodegradability and structural versatility as carriers. However, a number of disadvantages of liposomal carriers can be noted. Studies demonstrated, among others, low stability, especially in the case of aquatic environment, high accumulation in the liver and spleen, difficulty in overcoming the blood–tissue barrier, oxidation of the lipid, hydrolysis of the ester moiety, or API release to the external phase.[6,15] Many studies have, therefore, been performed to modify the structure of liposomes sufficient to obtain the efficient transport of active substances.[15,16] Nanoliposomes can be often coated with polyethylene glycol (PEG), which may, among other things, affect the half-life of the drug-molecule-transporter conjugate in the blood.[16]

Another spherical lipid-based particles are lipospheres with a size of up to 500 nm, in which 2 types can be distinguished: SLN and nanostructured lipid carriers (NLC).[6] The structure of SLN resembles the oil/water (o/w) emulsion, in which the liquid lipid has been replaced by a lipid solid at room temperature.[17] The biologically active compounds are arranged in an aqueous phase containing surfactants between the chains of the hydrophobic compounds. The lipid content in the lipospheres is approx. 0.1–30% and leads to good in vivo tolerability and reduced toxicity.[18] Solid lipid nanoparticles provide better stability for encapsulated proteins, preventing their proteolytic

degradation, in addition to their prolonged release. In contrast with SLN, the matrix in NLC can form both solid and liquid lipids (e.g., Miglyol, Capmul, MCM).[19] Nanostructured lipid carriers can be manufactured in high-pressure hot homogenization in the form of multiple systems such as oil/solid lipid/water suspension. A dispersing method using a high-speed mixer and a method for emulsion formation and emulsification with evaporation or diffusion of an organic solvent can also be used for the preparation of lipospheres.[20,21] Both SLN and NLC may provide better stability of the drug, and a reduction of the first-pass-effect after oral administration, as well as mask its bitter taste.[21]

Among polymer-based NPs, nanospheres, nanocapsules and dendrimers are the most studied in the context of drug delivery.[6,22–24] The polymers used to manufacture these particles should be biodegradable and biocompatible. Simultaneously, the drug should be released at the target site and at a concentration within the therapeutic range. In nanospheres, the active ingredient is incorporated (suspended) in a biodegradable polymer matrix. The mechanism of API degradation is chemical (polymer hydrolysis) and physical as well (matrix erosion). The size of nanospheres (10–200 nm) allows for their free penetration into tissues and cells and prevents them from being absorbed by the cells of the immune system of the body.[6] Nanocapsules are in the form of colloidal nanovesicles sized 100–500 nm. The core of this structure (o/w) is surrounded by a polymer membrane.[22] Synthetic (e.g., polycaprolactone, polyacrylamide, methyl polymethacrylate) and natural (e.g., gelatine, chitosan, albumins) polymers are most commonly used to obtain nanocapsules.

In turn, dendrimers are organic polymer compounds with a highly branched structure in the shape of a sphere. These kinds of NPs consist of the core and radially branching dendrons. Their unique trait is that they can transport the drug in 2 ways: via covalently linking the drug molecules to surface groups of the NPs and by encapsulating the drug in the spaces between the branches of the polymer. In addition, in anticancer therapy these NPs may carry many other substances apart from the anticancer drugs, including fluorescent markers, photosensitizers or monoclonal antibodies.[23,24]

In the field of carbon-based NPs, the best-known and most analyzed are fullerenes, CNT, dots, and nanodiamonds. Fullerenes are an allotropic form of pure carbon with a diameter of approx. 1 nm. The fullerene C60 achieves the fullest chemical stability and high symmetry. These particles are obtained using an electric or laser arc technique. To use fullerenes for therapeutic purposes, their interiors, as well as their surface, can be modified (e.g., through coating with polyvinylpyrrolidone (PVP), by PEG addition or by complexes with cyclodextrins). The following types of fullerenes may be distinguished: endohedral, heterofullerenes and exohedral. Other atoms or molecules can be placed in the middle of the endohedral fullerenes (e.g., metals, lanthanides carbides). In turn, in heterofullerenes, the carbon atoms may be partially or completely replaced by other atoms while exohedral ones can be obtained by surface modifications. Fullerenes show strong antioxidant and protective properties.[25] This is primarily due to the presence of conjugated double bonds and an unfilled molecular orbital. They are characterized by a high ability to attach free oxygen radicals, so-called radical sponges.[26]

Carbon nanotubes are cylindrical graphene films which are open or closed on one or two sides, with carbon atoms arranged in hexagonal structures. These NPs exhibit many excellent properties, including electronic, thermal and mechanical features. The following types of CNT, due to the number of layers they form, may be distinguished: single-walled CNT (SWCNT), double-walled CNT (DWCNT) and multi-walled CNT (MWCNT). To obtain SWCNT and MWCNT, electric arc discharge methods, laser graphite evaporation and chemical vapor deposition are used. In turn, double-walled nanotubes may be obtained with the encapsulation of C60 fullerenes inside the single-walled carbon nanotubes.[27] As with fullerenes, the surface of CNT may be modified. Previous studies have shown that CNT can penetrate cell membranes and are effective carriers of various molecules, including peptides, proteins, plasmids, nucleic acids, and chemotherapeutics.[28,29] Simultaneously, low toxicity of CNT as well as structures displaying a lack of immunogenicity were also observed.[28,29] On the other hand, with regard to the safety of using CNT, some data revealed that their toxicity was comparable with asbestos fibers, which induced some pro-inflammatory mechanisms, among others, the secretion of interleukin 1β (IL-1β) from lipopolysaccharide (LPS)-primed macrophages.[30] It was also suggested that, much like asbestos, CNT may cause genotoxic effects.[31]

Table 1 summarizes the characteristics of selected types of NPs (description of their structure and diameter, as well as exemplary material which may be used to prepare particular NP) depending on the nature of the raw material from which these structures can be obtained.

Figure 1 shows a schematic structure of the described NPs. Available data from animal models demonstrated that, regardless of the route of administration of a drug, they tend to penetrate into the bloodstream quickly and are then distributed in organs and tissues. Their elimination from the body is slow. Most tissues and organs are able to remove NPs after cessation of exposure (with the exception of the reproductive organs and brain, which have been found to have increased retention). Nanoparticles can be eliminated from the body by the mononuclear phagocyte system (MPS) and by organs with the extensive reticuloendothelial system (RES) such as the liver and spleen.[32] In turn, nanocarriers based on biodegradable polymers (e.g., lactic acid polymers (PLA), glycol acid polymers (PGA) or a mixture of them (PLGA)) are completely degraded in the body, and their degradation products are excreted in the urine after reaching the renal threshold.[33]

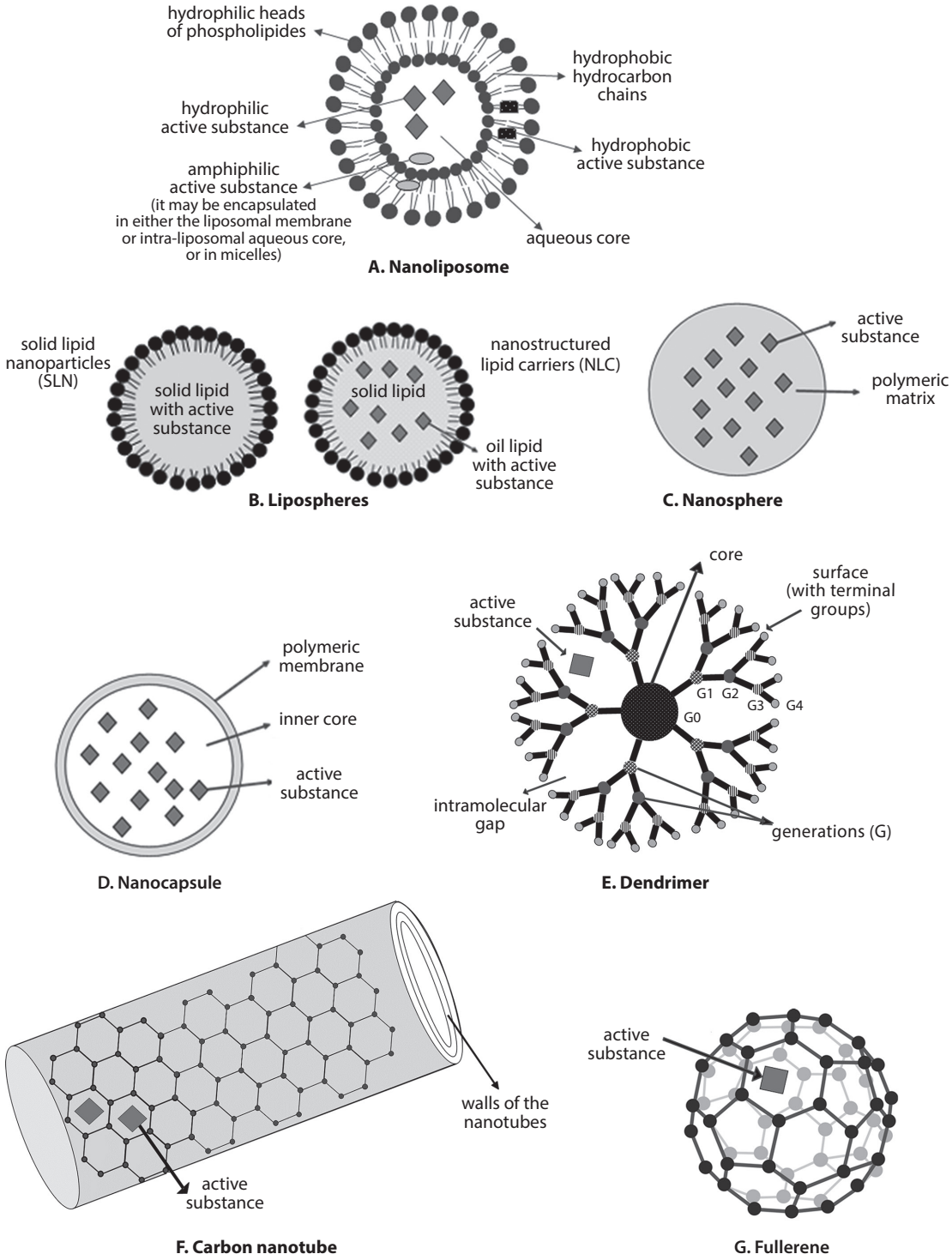

Fig. 1. Schematic structure of selected nanocarriers of active substances: (A) nanoliposome; (B) lipospheres; (C) nanosphere; (D) nanocapsule; (E) dendrimer; (F) carbon nanotube; (G) fullerene (original drawings)

The toxicity of NPs correlates with their physicochemical properties, i.e., size, shape, chemical composition, surface structure, surface charge, degree of aggregation, solubility, degradation time, presence or absence of functional groups of other chemical compounds, as well as reactivity with the surrounding tissue. Nanoparticles may affect the phagocyte system by initiating stress reactions, and consequently inflammation. Slowly decomposing and/or non-biodegradable NPs may accumulate in internal organs. They are able to pass through cell membranes, while inhaled NPs may reach the liver and heart via the bloodstream. Nanoparticles may affect the proper course of biochemical processes in the human body, including regulatory mechanisms of enzymes and proteins. Table 2 summarizes the possible adverse effects, which apply to polymer and carbon NPs.

Table 1. Characteristics of selected NPs

NPs depending on the nature of the raw material	Types of NPs		Short description of the particular NP	Diameter of NPs	Exemplary material used for NPs manufacture	Reference
Lipid-based NPs	nanoliposomes		spherical structures with the walls formed by a lipid bilayer and an aqueous phase inside	usually below 100–200 nm	phospholipid, cholesterol and phosphatidic acid which contains specified charge, soybean lecithin	6, 11, 38
	lipospheres	SLN	structure of SLN resembles the o/w emulsion; liquid lipid is replaced by a lipid solid in room temperature	lipospheres show an average size below 500 nm, whereby SLN reach larger sizes than NLC	glyceryl monostearate soybean lecithin	6, 11, 21
		NLC	NLC consist of oil droplets containing the drug, placed in the solid lipid matrix		solid and liquid lipids (e.g., Miglyol, Capmul, MCM)	6, 11, 21
Polymer-based NPs	nanospheres		API is incorporated (suspended) in a biodegradable polymer matrix	usually 10–200 nm	PLA, PGA or a mixture of them (PLGA), PMMA	6–11, 35
	nanocapsules		the form of colloidal nanovesicles in which the core of this structure (o/w) is surrounded by a polymer membrane	100–500 nm	synthetic (e.g., polycaprolactone, polyacrylamide, methyl polymethacrylate) and natural (e.g., gelatine, chitosan, albumins) polymers	6, 22
	dendrimers		tree-like structure consisting of a core (or focal group), the interior of the polymer and a surface	approx. 20 nm	polylysine and polyamidoamine, poly(aryl ether)	6, 8, 9, 23, 24
Carbon-based NPs	nanotubes		cylindrical graphene films which are open or closed on one or two sides, with carbon atoms arranged in hexagonal structures	below 25 nm	their surface can be modified	6, 8, 11, 26–28
	fullerenes		an allotropic form of pure carbon	approx. 1 nm	their structure can be modified by coating with PVP or PEG	6, 8, 9, 11, 25, 26

NPs – nanoparticles; SLN – solid lipid NPs; NLC – nanostructured lipid carriers; API – active pharmaceutical ingredient; PLA – lactic acid polymers; PGA – glycol acid polymers; PMMA – poly(methyl methacrylate); PVP – polyvinylpyrrolidone; PEG – polyethylene glycol.

Table 2. Possible adverse effects of selected NPs

NPs depending on the nature of the raw material	Types of NPs	Possible adverse effects
Polymer-based NPs	nanospheres	Cytotoxicity depends on size, shape and surface charge. The small size of the nanospheres and the aggregation process are the main causes for their cytotoxicity.
	dendrimers	Dendrimer cytotoxicity depends on the generation, the number of surface groups, and the nature of terminal moieties (anionic, neutral or cationic). Higher-generation dendrimers as well as dendrimers with positive charges on the surface present higher cytotoxicity.
Carbon-based NPs	nanotubes	Similarly to asbestos fibers, carbon nanofibers are likely to induce some pro-inflammatory mechanisms, i.e., the secretion of IL-1β from LPS-primed macrophages. In addition, CNT may cause genotoxic effects.
	fullerenes	Fullerene colloid prepared with tetrahydrofuran demonstrates toxic effects in different mammalian cells, including mitochondrial depolarization, and lipid peroxidation resulting in necrotic cell death.

NPs – nanoparticles; LPS – lipopolysaccharides; IL-1β – interleukin 1β; CNT – carbon nanotubes.

Application of drug nanocarriers in selected diseases

The use of NPs in medicine and pharmacy was mentioned earlier as being intensively studied in the diagnosis and treatment of a rising number of diseases. In this section, we discussed the results of studies analyzing the use of API nanocarriers in selected chronic diseases, i.e., cardiovascular disease, cancer and neurodegenerative diseases, as well as in wound healing. Since this topic is so broad, we have limited our discussion to results which we believed are the most interesting, relevant or innovative.

The potential use of nanoparticles in cardiovascular disorders

For several years, research on the possibilities of using nanotherapy to treat embolism and thrombosis has been conducted.[34] Nanoparticles loaded with fibrinolytic drugs

have been analyzed. They work to dissolve blood clots, a cause of myocardial infarctions, strokes and pulmonary embolisms. Korin et al. developed a targeted DDS to eliminate blood clots.[35] Nanoparticles were obtained using spray-drying solutions containing poly(lactic-co-glycolic acid) (PLGA 50:50, MW 17 kD). The surface of the NPs was coated with a tissue plasminogen activator (t-PA). Tissue plasminogen activator is bound by surface conjugation with the use of streptavidin/biotin. The authors tested the nanodrug activated by shear stress in a model of pulmonary embolism and mesenteric artery embolism in mice. From a carrier sized 180 ±70 nm they obtained microaggregates of a size similar to platelets, which were stable under physiological blood flow conditions. Within the artery narrowing, where the blood flow increases rapidly (shear stress of 1,000 dyn/cm^2), there was a spontaneous activation of the nanodrug, i.e., the breakdown of the microaggregate into NPs with t-PA. Korin et al. found that the released NPs accumulated within the thrombus and exhibited pharmacological activity. Dissolution of clots was obtained with a therapeutic dose of t-PA of 50 mg, which was 50-fold lower than a standard dose of t-PA administered intravenously, i.e., 2 mg/kg.[35] However, it is suggested that the NP–t-PA conjugation (biotin and streptavidin) may elicit an immune response.[36]

Lipid-based NPs, i.e., nanoliposomes as well as SLN are among the essential NPs in the targeted therapy for heart diseases. Dvir et al. investigated the efficacy of API encapsulation in nanoliposomes in the targeted therapy for myocardial infarctions. Nanoliposomes with a diameter of 142 nm were conjugated with a ligand specific for the angiotensin II type 1 (AT1) receptor. The obtained lipid-based NPs were administered by intravenous injection to an isolated rat heart and demonstrated high therapeutic efficacy.[37] Shao et al. investigated the therapeutic effect of Schisandrin B (Sch B) in the form of SLN in the model of the rat myocardial infarction.[38] Schisandrin B is isolated from the fruit of *Schisandra chinensis* and can be clinically applied in myocardial ischemia. Polyethylene glycol (PEG) and matrix metalloproteinase-targeting peptide (MMP-TP) conjugate was synthesized in the study. The MMP-Sch B liposomes, 130 nm in size, were prepared using the solvent displacement technique. The aqueous phase was prepared by dissolving the PEG-peptide, DOTAP (liposomal transfection reagent) and poloxamer in water. Glyceryl monostearate (GMS), COMPRITOL® 888 ATO (Gattefosse, Paramus, USA), which can serve as a lipid carrier, and soybean lecithin (SL), were sonicated in acetone to form an organic phase. Both phases were combined and mixed until the organic solvent was removed. Efficacy and biodistribution of the obtained nanodrug were tested both in vitro and in vivo. The authors demonstrated that the developed formulation is the optimal carrier of API for the myocardium tissues and effectively protects the heart against acute myocardial infarction.[38]

The possibility of using porous silicon nanoparticles (PSi) in cardiovascular disease therapy was also assessed. The Finnish team attempted to develop PSi-based nanosystems for targeted therapy and diagnosis of myocardial infarction. Peptide-modified nanovectors PSi NPs sized 10.7 nm, with a surface area of 282 m^2/g and a total pore volume of 0.75 cm^3/g, were manufactured in the course of the study and analyzed in terms of their distribution in the myocardium tissue after intravenous administration in the rat model.[39] The results obtained by the cited authors are promising and indicate the potential application of this formulation in the future. Similarly, positive results were demonstrated for thermally oxidized porous silicon (TOPSi) NPs in the isolated rat heart model.[40] The NPs presented biocompatibility and did not significantly affect cardiac function both before and after the myocardial infarction. In turn, Chan et al. conducted research on the safety of using silicon nanoparticles (SiNPs).[41] The toxicity of SiNPs, which were 150 nm in size, was measured in vivo in a mouse model. The mice were administered NPs intravenously and underwent observation for 14 days. After this time, the authors performed histopathological examinations and blood analysis to assess internal organ damage. In this study, the occurrence of adverse effects after the administration of SiNPs was not confirmed.[41] However, some studies have suggested that intravenous administration of SiNPs may lead to their distribution/accumulation in the liver, spleen and lungs, with subsequent damage of these organs.[42]

Myocardial infarction can lead to organ failure, thereby necessitating transplantation. Unfortunately, the number of people waiting for a heart transplant exceeds the number of available grafts; therefore, stem cell therapy seems prospective.[43] However, their survival is limited due to oxidative stress and ischemia-induced inflammation. Ma et al. suggested the usage of melatonin, which is a powerful endogenous antioxidant, for protecting cells against oxidative stress.[44] In order to increase the effectiveness of this antioxidant, the authors developed a technique for melatonin encapsulation in PLGA-mPEG nanoparticles (Mel-NPs). They observed that Mel-NPs achieved a higher melatonin bioactivity index over time and a better therapeutic effect than a conventional melatonin preparation in the model of rat myocardial infarction.[44] Myocardial infarction therapy using PLGA NPs was also proposed by Nakano et al.[45] who developed bioabsorbable irbesartan-loaded PLGA NPs. The efficacy of irbesartan-NPs was studied in the model of ischemia-reperfusion injury in the mouse heart, showing that such NPs may affect the limitation of infarct size.[45]

Promising results were also shown in the case of the therapeutic use of proteins in myocardium regeneration after myocardial infarction. A major barrier to the success of this therapy is the short half-life of proteins in vivo. It has been found that liraglutide loaded in PLGA-PEG

NPs (liraglutide-NPs) provides sustained release of API and retains its biological activity in vitro.[46] In addition, in the model of rat myocardial infarction, liraglutide-NPs improved heart function, reduced the extent of myocardial necrosis, promoted angiogenesis, and also prevented the apoptosis of cardiomyocytes.[46] Zhang et al. have shown that the cytotoxicity of PLGA-PEG NPs depends on their shape.[47] Needle-shaped NPs induced cytotoxicity in the tested cell lines. Lysosomal membrane damage, lysosomal enlargement, caspase-3 activation, and DNA damage were observed, which in turn induced cell apoptosis. The authors did not observe this process in the case of spherical PLGA-PEG NPs.[47]

Currently, attempts are being made to develop inhalation therapy, which would use biocompatible and biodegradable peptide NPs to treat heart failure.[48] The therapeutic efficacy of peptides delivered in a calcium phosphate nanoparticles (CaP-NP) with a size <50 nm in the rat and mouse models of diabetic cardiomyopathy was confirmed.[48] The proposed carrier allows for rapid distribution of the peptide from the lungs to the bloodstream, and subsequently to myocardium and cardiomyocytes. This study revealed that heart function was regenerated and improved.

The potential use of nanoparticles in the treatment of cancer

Cancer is a complex disease and still very difficult to treat. Classic radiotherapy, as well as chemotherapy, is not very effective, mainly due to the lack of specificity, low bioavailability and drug resistance.[49] Nanoscale technology may be a promising tool to overcome the limitations of conventional anticancer therapy. Because the NPs are coated with specific ligands or antibodies, an increased concentration of the drug in the tumor was found, along with reduced cytotoxicity in relation to healthy cells. The reason for this is that the ligand binds specifically to receptors on the surface of tumor cells, often those that overexpress the disease-related ones. In breast cancer, overexpression of the human epidermal growth factor 2 (HER2) is often observed.[50] The recombinant, humanized monoclonal antibody – trastuzumab – is used against the HER2 protein. It has been proven that cancer cells with an increased expression of the HER2 receptor show a more frequent and more intense drug resistance.[51] However, despite the potential for greater malignancy, their response to the combined treatment is very effective. Nonetheless, cytotoxicity of healthy cells remains an important problem.

In the case of polymeric-based NPs, many reports considered the use of dendrimers, especially polyamidoamine (PAMAM) dendrimers,[52] which come from polyamidoamine. Their core may be composed of a hydrophilic ethylenediamine[24] or, for example, of a hydrophobic diaminododecane. Kulhari et al. synthesized dendrimers with trastuzumab to facilitate the delivery of docetaxel to breast cancer cells.[53] The use of such a conjugate resulted in positive effects related to the possibility of reducing the dose of cytostatics, thus reducing the cytotoxicity in relation to healthy cells, but also reducing drug resistance.[53] The same type of dendrimer, but doxorubicin-loaded, was tested in mice by Zhong et al.[54] to analyze the possibility of reducing the lung metastases. Decreased cardiotoxicity was found in addition to increased accumulation of the drug in the tumor.

Unfortunately, apart from such promising reports related to the use of dendrimer conjugates with anticancer drugs, some rather more negative aspects of such therapies should be mentioned. All types of dendrimers are characterized by cytotoxic and hemolytic properties.[24] To eliminate or reduce these disadvantages, the surface of dendrimers can be modified, e.g., by binding to PEG (PEGylation). PEGylation, in addition to reducing cytotoxicity, carries another positive feature: it reduces the release of the drug from the formulation.[55] Furthermore, it was found that PEGylation did not affect the load capacity of the drug.[55]

Recently, the use of Au-Ag NPs in bladder cancer has also been analyzed.[56] Polydopamine (PDA)-coated Au-Ag molecules (Au-Ag@PDA NPs) were administered to T24 cells at different doses. These cancer cells were then irradiated with a laser wavelength of 808 nm. In addition to the in vitro study, the authors also verified the effects of therapy with the use of Au-Ag@PDA NPs in mice. Good structural stability, biocompatibility, photothermal effects, and potential anticancer efficacy were found.[56]

Similarly to dendrimers, carbon NPs can be conjugated to a ligand specific for a particular receptor and overexpressed in a given cancer. Among carbon-based NPs which can be used in cancer therapy, special attention has been paid to CNT and carbon dots (C-dots). These NPs were suggested as carriers of doxorubicin and gemcitabine. An interesting perspective is the coupling of CNT with cadmium telluride quantum dots (CdTe-QDs). This allows for optical imaging and drug administration using an external magnetic field. The CNT were filled with Fe_3O_4. On their surface, quantum dots coated with silica (SiO_2) were added. Magnetite crystals improved chemical stability, increased the load capacity of the drug and protected against agglomeration. Silica hybrid coatings reduced the toxicity of the entire system. The excellent load capacity of this nanosystem was discovered together with the possibility of delivering directional doxorubicin hydrochloride to Hela cells.[57]

On the other hand, in the study by Shi et al.,[58] the outer part of MWCNT was conjugated with PAMAM dendrimers. Such a nanosystem was observed to have high stability and biocompatibility. It was also effective in reaching cancer cells overexpressing folic acid receptors with high affinity, which can undoubtedly be used both in cancer diagnostics and therapy.

In the case of fullerenes, the data indicated that anticancer drugs conjugated with fullerenes may decrease their cytotoxicity on account of their role as radical sponges.[38,39] In the study of Chaudhuri et al.,[59] conjugate of fullerenol-doxorubicin (DOX) inhibited the proliferation of cancer cells in vitro, blocked the G2-M cell cycle and induced the process of apoptosis. The authors did not observe any toxicity of the obtained nanosystem, which even demonstrated its protective effect on cardiac muscle, kidney, lung, as well as venereal cells. Similar cardioprotective results were demonstrated in the study performed on Wistar rats by Torres et al.[60]

Forms and structures of different types of NPs allow for their usage in thermal and photodynamic therapy.[61] Recently, it has been found that phototherapy, induced utilizing a near-infrared laser with the use of modern photosensitizers, brings better treatment effects than previously used therapies. In their study, Sheng et al. used copper oxide sulfide NPs with polyethylene glycol diacrylate modified with copper sulfide (CuS) NPs (PEG-DA-CuS NPs).[61] Due to PEGylation, the solubility of CuS was increased, with a subsequent increase of its bioavailability. When these NPs reached the tumor, they were irradiated with a laser, which increased their temperature above 90°C.

For lipid-based NPs in the treatment of cancer, Yang et al. used lipospheres SLN, obtained using high-pressure homogenization, as carriers of the anticancer drug – camptothecin.[62] The authors have shown that SLN may prolong the release of camptothecin and other lipophilic drugs for even 1 week. In other research, linalool, which is a monoterpene found in essential oils of plants and herbs showing an antiproliferative activity in cancer cells, was encapsulated in SLN lipospheres with a yield of over 80%.[63] Controlled in vitro release profiles over a period of at least 72 h were obtained for this nanosystem. The authors also demonstrated the antiproliferative effects of SLN-loaded linalool on hepatocarcinoma (HepG2) and lung adenocarcinoma (A549) cells as well as higher inhibitory effects compared to free linalool.

Zhou et al.[64] encapsulated quercetin (QCT) into nanoliposomes and evaluated their morphology, particle size distribution, drug loading, encapsulation ratio, and the in vitro release of the drug. In addition, the authors carried out pharmacokinetics and anticancer activity examination on the mice model. Nanoliposomes were prepared using the thin-film hydration method with a modification using a short peptide containing arginine-glycine-aspartic acid (RGD). It was demonstrated that water-insoluble reagents, such as QCT, are readily dispersed in lipid solution and entrapped by the thin-film hydration. In addition, it was found that the size of the obtained nanoliposomes is suitable for the fenestrated blood vessels of the cancer tissues through the enhanced permeability retention effect. The biodistribution was evaluated using in vivo fluorescence imaging giving strong signals around the carcinoma cells. The release tests showed that the proposed QCT delivery system is useful because of its accumulation in cancer tissues and the reduced exposure of healthy tissues. The use of the RGD peptide to target integrin in the endothelium of the blood vessels of the tumor has confirmed the feasibility of a previously known method to inhibit angiogenesis and metastasis. The results indicate that the proposed RGD complex with nanoliposomes significantly increases the ability to inhibit tumor growth compared to normal liposomes.[64]

In the study conducted by Hasan et al.,[65] nanoliposomes containing curcumin, which is characterized by a broad spectrum of positive activities, e.g., antioxidant, antitumor, anti-inflammatory, as well as antimicrobial, were prepared using lecithin from salmon, soya and rapeseed. The average size of the obtained nanoliposomes was the smallest for those made from soya (110.3 ±0.8 nm) when compared with free liposomes and rapeseed and salmon nanoliposomes. The authors observed the highest entrapment efficiency of the curcumin in the case of salmon liposomes (67.3% ±1.1% vs 63.2% ±0.7% for rapeseed nanoliposomes and 65.0% ±1.1% for soya nanoliposomes). The in vitro antitumor activity of liposomal curcumin was analyzed on MCF7 cancer cells. The study demonstrated that curcumin-loaded nanoliposomes significantly increased the cancer cell cytotoxicity, while lower impact of free curcumin on cancer cells was found.

In turn, Chen and Liu[66] received nanoliposomes with bufalin (BF) using the high-pressure homogenization method. Empty liposomes (LP), liposomes modified with folic acid (FA-BF-LP), liposomes modified with transferrin (Tf-BF-LP), and complex liposomes modified with both FA and Tf (FA+Tf) BF-LP were administered into the mice with lung cancer. Bufalin has antiproliferative properties and induces apoptosis of tumor cells. However, it is toxic, insoluble in water and has a short half-life. Due to the abovementioned features, the authors focused on the possibility of closing BF into modern DDSs. They determined the morphology of the NPs as being spherical and of appropriate size. Particle size is an important parameter. Because NPs below 400 nm can emerge in the microspace of the tumor, smaller sizes can internalize into the cell through endocytic vesicles in a more efficient way. The anticancer efficacy was assessed after a period of 2 weeks after injection into A549 cells. The greatest effect of tumor suppression was observed after the (FA+Tf) BF-LP administration.[66]

In a study by Zabielska-Koczywąs et al.,[67] gold NPs (Au NPs) loaded with doxorubicin (DOX) and glutathione-stabilized (GSH) were analyzed in nude mice in feline injection-site sarcomas, which are malignant skin tumors. The obtained Au-GSH NPs had a size of approx. 5.5 nm. The Au-GSH NPs were observed to co-internalize with tumor-associated macrophages (TAM) close to the area of necrosis and in the tumor periphery. Further, the authors found no negative effect of AU NPs on liver and kidney parameters in nude mice.[67]

The potential use of nanoparticles in delivering API to the brain

The most important problems in the development of new drugs for these diseases are ineffective crossing of blood–brain barrier (BBB), the poor solubility of the drug as well as its low bioavailability. The size of active substance particles and endothelial permeability are the main factors that affect the crossing of the BBB, which separates the central nervous system (CNS) from the systemic circulation. Among other barriers limiting or preventing drug delivery to the brain, a barrier separating the blood from the cerebrospinal fluid (blood–cerebrospinal fluid barrier (BCSFB)) and some functional barriers in the forming transporter mechanisms (influx and efflux) of the CNS may be distinguished.[68]

A number of studies have been performed to analyze whether nanotechnology methods allow the delivery of active substances to the brain.[69–73] Experimental research on animal models demonstrated the effective delivery of nanoemulsions containing diazepam and risperidone into a rat brain.[69,70] Nanoemulsion with diazepam was prepared with the use of cold high-pressure homogenization and the following ingredients: triglycerides and soybean oil (oil phase), 0.1 M phosphate-buffered saline (PBS; pH 8, aqueous phase), and lecithin and polysorbate 80 (emulsifiers). Fast and intense initial distribution of diazepam into rat brain was demonstrated when nanoemulsions with 20% and 30% (w/w) oil content were used.[69] Another study of the same research team compared the effectiveness of different emulsifiers used during the preparation of nanoemulsions and showed that risperidone brain availability was increased in the case of poloxamer 80 in comparison with that of poloxamer 188 or Solutol® HS15 (Sigma-Aldrich, St. Louis, USA).[70]

Sadegh Malvajerd et al.[71] demonstrated that after the intravenous administration of a 4 mg/kg dose of curcumin in a rat, its amount available in the brain was significantly higher in the case of curcumin-loaded nanostructured lipid carriers than in the case of free curcumin and curcumin-loaded SLN. Curcumin was dispersed as amorphous in the analyzed nanocarriers.

Recently, some interesting studies showing the possible delivery of active substances to the brain via the nasal route have been published.[72,73] Liu and Ho[72] manufactured chitosan NPs using an ionic cross-linking method, loaded them with scutellarin (SCU), which is a traditional Chinese medicine used for the treatment of ischemic cerebrovascular disease, and aimed to deliver SCU to the brain through the nasal route. Increased accumulation of SCU delivered as encapsulated in NPs in the brain was observed when compared to SCU in solution. In a study by Sharma et al.,[73] PLGA midazolam-loaded NPs delivered intranasally demonstrated a drug release at 83% within 4 h and can, therefore, be proposed as a non-invasive DDS which improves drug entrapment and stability.

Neurodegenerative diseases

Since the number of people suffering from neurodegenerative diseases is increasing each year, they are a serious health problem all over the world.[74] The course of dementia, especially Alzheimer's disease (AD), has most often a slow beginning and may remain unnoticed for a long time. However, their impact on patients as well as the families' lives is enormous, both during the development of the disease and after the diagnosis.[74] Unfortunately, so far no drug has been developed to completely counteract the effects of neurodegeneration. Thus, the only way to reduce its progression is through early diagnosis and subsequent successful treatment.

Alzheimer's disease is caused by toxic effects of β-amyloid peptides that aggregate and are deposited as amyloid plaques in neural and vascular tissues. It all begins with the proteolytic cleavage of the amyloid precursor protein (APP).[75] It has also been shown in animal models that dysfunction of the cholinergic system can produce a memory deficit similar to AD.[76] Current treatment of AD is based on acetylcholinesterase (AChE) inhibitors or influencing inhibition of glutamate excitotoxicity.[77] However, tacrine, galantamine, rivastigmine, and memantine have been linked to some adverse effects including hepatotoxicity, nausea, vomiting, dizziness, confusion, or constipation, which may lead to the discontinuation of the treatment.[77] In the brains of rats, Wilson et al.[78] observed that the level of tacrine, which was encapsulated in NPS coated with 1% nonionic surfactant polysorbate 80, was elevated compared with the uncoated NPs as well as with the free drug. According to the authors, these coated NPs may be delivered to the brain through the interaction between polysorbate 80 coating and the endothelial cells of the brain microvessels.[78] What is more important, the toxicity of tacrine may be reduced with this drug form, as coating tacrine-loaded NPs with polysorbate 80 decreased its accumulation in the liver and spleen. Other studies also demonstrated the role of polysorbate 80 in the effective delivery of different active substances into the brain.[79,80]

Because of their many advantageous effects, nanoliposomes have been suggested to be a promising system for API delivery to the brain in AD, since they are non-toxic, biodegradable and non-immunogenic. The study conducted by Truran et al.[81] demonstrated that nanoliposomes composed of phosphatidylcholine, cholesterol and phosphatidic acid with a 70:25:5 molar ratios (20 mg lipid/mL) prevent β-amyloid peptide 1-42 (Aβ42) fibril formation. Nanoliposomes were prepared through the dissolution of the lipid mixture in chloroform, which was subsequently removed using a rotary evaporator, and then the dry lipid film was hydrated using a HEPES buffer and the mixture was sonicated.[81] A curcumin-decorated nanoliposomes was demonstrated to have a very high affinity for Aβ1-42 fibrils with their potential in the targeted delivery of new

diagnostic and therapeutic molecules for AD.[82] Very promising results have been linked to the studies concerning dendrimers as carriers of anti-amyloidogenic and anti-prionic substances.[83,84] The study conducted by Klementieva et al.[83] demonstrated that in vitro poly(propylene imine) (PPI) glycodendrimers of the 4th and 5th generation modified with maltose can reduce the toxicity of Aβ(1-42). Furthermore, Klajnert et al.[84] found a reduction of β-amyloid fibril formation when using gallic acid-triethylene glycol (GATG) dendrimer decorated with 27 terminal morpholine groups ([G3]-Mor).

In the case of using carbon-based NPs in the AD model, the study of Li et al.[85] demonstrated that SWCNT had the potential to inhibit Aβ(16-22) and full-length Aβ fibrillation. The study conducted by Yang et al.[86] demonstrated the successful delivery of acetylcholine to mice brains with experimentally induced AD using SWCNT with a diameter of 0.8–1.2 nm and a length of several microns. The authors precisely controlled the doses of obtained acetylcholine-loaded SWCNT, ensuring that the SWCNT preferentially penetrates the lysosomes instead of the mitochondria, which can happen when using high doses. In turn, Lohan et al.[87] indicated the potential role of MWCNT, coated with polysorbate/phospholipid and berberine-loaded, in reducing β-amyloid-induced AD. The analyzed formulation had a size of 186 nm and manifested 96% release of berberine over 16 h.

In Parkinson's disease (PD), overexpression of α-synuclein leads to the death of dopaminergic neurons. Therapy is limited to relieving the symptoms of the disease, both by using therapeutics or surgery.[88] At present, there is no treatment which could lead to regeneration of the brain tissue. However, many different neuroprotective and neuroregenerative molecules, including neurotrophic factors, antioxidants and RNA-based drugs, have been studied for their therapeutic capabilities in PD.[89] Unfortunately, their delivery to the brain is still a big challenge. Recently, a therapeutic device, which has the potential to deliver cerebral dopamine neurotrophic factor directly to the brain of PD patients, has also been designed.[90] There are also many reports indicating the advantages of using NPs in PD therapy.[10]

Hu et al.[91] analyzed Au NPs loaded with plasmid DNA and obtained through the combination of electrostatic adsorption and photochemical immobilization methods with regard to their therapeutic effects in PD models.[91] The authors observed that these systems could successfully cross the BBB and inhibit the apoptosis of PC12 cells and dopaminergic neurons in PD mice brain. In a study conducted by Cao et al.,[92] rat models of PD were separately given chitosan-coated levodopa liposomes/benserazide and levodopa/benserazide. The authors found that levodopa-loaded liposomes, coated with chitosan, may reduce dyskinesias inducing PD. In another study concerning the usage of nanoliposomes in the PD model, neutral (zwitterionic) nanoliposomes supplemented with cholesterol (NLP-Chol), both PEGylated or not, were demonstrated to reduce the neurotoxicity of α-synuclein, which aggregates to form fibrils in neuronal cells of PD patients.[93]

Interesting results were demonstrated by Al-Dhubiab et al.[94] who prepared selegiline-loaded poly(lactide-co-glycolide) nanospheres, which then served to impregnate a buccal film made with hydroxypropylmethylcellulose and eudragit. The physical properties, mucoadhesive strength and hydration of the film were found to be adequate. In vivo analysis of the buccal film revealed improved bioavailability in comparison with the oral solution, making it a possible alternative approach for the treatment of PD.[94]

Inhibiting the α-synuclein fibril formation is a potential therapeutic strategy in PD. Studies analyzing the possible role of carbosilane and viologen-phosphorus dendrimers in this process have previously been performed.[95,96] The authors also demonstrated carbosilane dendrimers as a partially protective factor against the toxic effect of rotenone, which is a compound of pesticides.[96] Rotenon is suggested to be a risk factor for PD, since it induces oxidative stress, aggregation of α-synuclein and dysfunction of the ubiquitin-proteasome system.

The potential use of nanoparticles in the treatment of wounds

Chronic wounds have a significant impact not only on patients' life, resulting in impaired mobility, limb amputation and even death, but also on the healthcare system. Wound healing is a complicated process that needs immediate management after the onset of injury. Ideal wound healing should be rapid, leaving minimal scarring with no negative esthetic effects. Many stages are involved in the whole process of healing, including coagulation, inflammation at the sites, proliferation, angiogenesis, remodeling, and restructuring of the tissues. The exact mechanisms of wound healing are not fully understood; however, it has been suggested that the use of silver NPs (Ag NPs) on wounds may cause a reduction in the activity of local matrix metalloproteinases and increase the destruction of neutrophils in the wound cells.[97] Silver NPs, which may be an alternative to antibiotics in the treatment of severe open wounds due to the increasing resistance of pathogenic bacteria to antibiotics, are intensively studied.[98–100] Silver-loaded NPs may reduce the levels of pro-inflammatory cytokines and, subsequently, accelerate wound healing or inhibit the action of tumor necrosis factor α (TNF-α) and interferon gamma (IFNγ) factors, which are important in the inflammation process.[99] However, the issue of silver accumulation and toxicity to the skin should also be considered. Experimental data indicates that absorption of Ag NPs through intact and damaged skin is detectable, while in the case of continuous skin continuity absorption is less pronounced.[100] The authors also

observed that in the case of damaged skin, the permeation of silver is higher than in healthy skin.

Ziv-Polat et al. stabilized the thrombin through binding it to maghemite (gamma-Fe(2)O(3)) NPs and compared its wound healing efficiency to free thrombin.[101] The authors treated incisional wounds on rat skin with the following: a mixture of fibrinogen, CaCl(2) solution and free or bound thrombin. The highest skin tensile strength after 28 days of therapy was demonstrated for thrombin bound to maghemite NPs and the finding was also confirmed during histological examination.[101]

In turn, Au NPs with epigallocatechin gallate and α-lipoic acid significantly accelerated wound healing through their anti-inflammatory and antioxidation effects both in cell culture and a murine model.[102] In the process of wound healing, Au NPs may also be used to deliver antioxidants as well as nucleic acids. Previous studies have shown the ability of Au NPs, based on antimicrobial peptide combined with pro-angiogenic (vascular endothelial growth factor (VEGF)) plasmids, to promote angiogenesis and inhibit bacterial infection in diabetic wounds, resulting in faster re-epithelization, improved granulation tissue formation and high VEGF expression.[103] Curcumin NPs were found to have strong antibacterial activity to suppress the growth of *Pseudomonas aeruginosa* and *Staphylococcus aureus*.[104] It was also demonstrated that curcumin NPs could reduce inflammation and induce cell expansion, which is useful in the reconstruction of damaged tissue.[105]

Nanoliposomes containing propylene glycol were previously observed to have elevated flexibility, as well as good stability and biocompatibility, and, therefore, may be a novel topical carrier of active substances.[106] Kianvash et al. obtained curcumin-propylene glycol liposomes approx. 145 nm in size to heal second-degree burns in the animal model.[107] The prepared nanoliposomes, containing 0.3% curcumin, demonstrated effectiveness with no adverse effects on intact skin. No detectable cytotoxicity on human dermal fibroblast was also found for 0.3% curcumin-loaded nanoliposomes. In turn, ethosomal 0.2% curcumin formulation applied once daily on rat dorsal improved the following aspects of wound repair: re-epithelization, neovascularization and collagen synthesis, among others. In addition, this formulation significantly inhibited the growth of the burn bacterial flora, including *Pseudomonas aeruginosa*.[108]

In turn, Li et al.[109] manufactured flexible nanoliposomes with daptomycin (DAP-FL) for topical delivery and bacteriostatic activity towards skin infections. The authors used lecithin and sodium cholate in a ratio of 17:1 (w/w) while lipid to drug ratio was 14:1 (w/w). The mean size of the obtained DAP-FL was 55.4 nm and the mean entrapment efficiency was 87.85% ±2.15%. The study demonstrated rapid and efficient antibacterial activity against *Staphylococcus aureus* and effective therapeutic levels of DAP were maintained for several hours.[109] Xu et al. prepared novel liposomes containing a hydrogel core of silk fibroin (SF) loaded with basic fibroblast growth factor (bFGF).[110] The obtained liposomes showed high encapsulation efficiency of bFGF and their size was about 100 nm. The authors observed that this specific liposomal system significantly improved the stability of bFGF in wound fluids, while maintaining cell proliferation activity with respect to conventional liposomes containing bFGF. Moreover, the bFGF-loaded liposomes with SF core accelerated wound healing very efficiently and induced regeneration of vascular vessels to an extent beyond that seen in free bFGF or conventional liposomes with bFGF.[110]

Since zinc oxide is a compound composed of many enzymes, it has a great impact on biological functions. However, various factors affect the extent of this impact, including microcrystals morphology, particle size, exposure time, concentration, pH, and biocompatibility. In a study by Kim et al.,[111] zinc oxide NPs were demonstrated to reduce mRNA expression of inflammatory cytokines by inhibiting the activation of nuclear factor kappa B cells. Ali et al.,[112] by using the co-precipitation method, were able to manufacture zinc peroxide NPs (ZnO_2-NPs) that were 15–25 nm in size and with a transition temperature of 211°C. The obtained NPs presented antimicrobial activity against *Pseudomonas aeruginosa* and *Aspergillus niger* strains isolated from burn wound infections. Also, the ZnO_2-NPs were found to have anti-elastase, anti-keratinase and anti-inflammatory properties, which were promising in wound healing in vivo.[112]

On the other hand, studies concerning PLGA NPs in wound healing have also been performed, since PLGA supplies lactate, which accelerates neovascularization. Chereddy et al.[113] prepared PLGA NPs with a peptide LL37, which modulates wound healing and angiogenesis, and fights infection. The treatment of wounds using PLGA-LL37 NPs significantly accelerated healing compared to those treated with PLGA or LL37 alone. The authors observed that PLGA-LL37 NPs improved angiogenesis, upregulated interleukin (IL-6) and VEGF expression, and modulated the inflammatory wound response. During the in vitro experiment, it was also demonstrated that these novel PLGA NPs had no effect on the metabolism and proliferation of keratinocytes.[113]

Conclusions

One of the main benefits of pharmacotherapy using NPs is the reduction of adverse effects. This is due to the significantly reduced dosage of the active substance compared with the currently available drug forms. In addition, the size of NPs and the possibility of their modification make it possible to design innovative solutions in the field of imaging, diagnostics and therapy.

However, the real threat is the free penetration of NPs into any place inside the body with subsequent accumulation

in internal organs such as the liver, bone marrow or heart. Opponents of the use of nanotechnology in medicine draw our attention to the dangers that this branch of science may bring, including the risk of cancer.[114,115] Nanoparticles can be embedded into the DNA, which can result in the degradation of nucleic acid and the formation of free radicals. Some data indicated comparable toxicity between CNT and asbestos fibers.[30]

On the other hand, promising results showing better efficacy of anticancer treatment using NPs may change the long-standing conviction that a diagnosis of cancer is a death sentence. Therefore, it seems that further studies will not be discontinued, since there is an urgent need to reduce the toxicity of anticancer drugs, while simultaneously enhancing their effectiveness. However, decision-makers should, undoubtedly, consider developing standards that would regulate the use of nanomaterials in medicine, with special attention given to their impact on the state of the natural environment.

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Geriatric assessment among elderly patients undergoing urological surgery: A systematic literature review

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

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Abstract

The elderly constitute the group of patients who most often undergo elective urological procedures, and they are at the highest risk of poor surgical outcomes because of comorbidity and frailty. The current model of qualification for surgery is often subjective and based on tools which do not address the characteristics of the elderly. The Comprehensive Geriatric Assessment (CGA) and screening tools can help in the evaluation of older, particularly frail patients. The aim of the study was to review the literature on the usefulness of preoperative geriatric evaluation in patients undergoing urological treatment. The review was based on MEDLINE/PubMed, Embase and Cochrane Library bibliographic databases from 2000–2017 for full-text, English-language publications meeting pre-defined criteria. Six prospective and 3 retrospective studies were selected for further analysis. The patient populations, methods of geriatric assessment, interventions, and outcome measures varied between the studies. None of the studies were randomized controlled trials. In 2 studies, the CGA was used; in other studies, rather basic screening tests were used. In only 2 studies, an intervention was performed after the CGA. In general, the variables of the CGA were both prospectively and retrospectively significant predictors of complications of urological surgery. Although the use of CGA is not a standard practice in everyday urological clinical practice, components of the CGA appear to be predictive of postoperative complications. Therefore, inclusion of geriatric assessment as part of routine preoperative care in geriatric urology patients should be considered. Because of the lack of randomized controlled trials on preoperative CGAs in urology patients, further studies are needed.

Key words: elderly, preoperative assessment, frailty, geriatric assessment, urological surgery

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Introduction

Older patients constitute a growing, very heterogeneous group with a variety of comorbidities and biological reserves.[1] Some of them present with frailty syndrome, which is by definition a state of increased vulnerability and a loss of resistance to external stressors, resulting in an increased risk of adverse outcomes. Frailty syndrome predisposes a patient to poor surgical outcomes,[2–6] including urological procedures.[7,8] A routine preoperative assessment of urology patients (based on medical history, physical examination, laboratory tests, as well as the American Society of Anesthesiologists (ASA), and the Eastern Cooperative Oncology Group (ECOG) scales) does not provide enough data to treat older patients with full regard for their specific health needs.[9,10] Aronson et al.[11] showed high inter-observer variability between staff members assigning ASA scores and a tendency to overestimate preoperative risk. Comorbidity – even when described using the Charlson Comorbidity Index (CCI) or the Cumulative Illness Rating Scale (CIRS), or with risk calculators – is still only based on previously diagnosed conditions and does not include an evaluation of subclinical physiological, nutritional or cognitive deficits.[12–14] Thus, there is a gap between the growing need for adequate, optimal preoperative assessment of older patients and the utility of commonly used preoperative assessment tools which were not developed specifically for elderly patients. Frailty seems to be a strong and important risk factor of poor surgical outcomes. The Comprehensive Geriatric Assessment (CGA) seems to be an efficient assessment tool that can identify frail older patients.[1]

Objectives

The aim of the study was to review the literature on the usefulness of preoperative geriatric evaluation in older patients undergoing urological treatment.

Material and methods

We searched the MEDLINE/PubMed Embase and Cochrane Library databases for publications from 2000 to 2017 (week 48). Two independent researchers (CM and KJ) screened all resulting abstracts according to the inclusion and exclusion criteria and any discrepancies were resolved through a third reviewer (PM). The databases were searched for the terms "geriatric assessment", "frailty" and "urology". Relevant papers were also identified through a manual search of the reference list of potentially relevant articles, and papers on screening for frailty were also considered.

Studies included in this review met the following criteria: full-text papers published in English between January 1, 2000 and November 30, 2017, prospective or retrospective study designs, and populations which included geriatric patients undergoing elective surgical procedures, preoperative assessments using the CGA domains or frailty screening tests as predictors of the patients' main surgical outcomes, which were: complications, 30-day mortality, discharge to an institution or other (length of stay, delay of operation or readmission). Studies in which only one specific outcome was measured (but not complications within 30 days) were excluded.

Results

The electronic and manual searches identified 191 potentially relevant publications for further evaluation. After duplicate removal and initial screening, 11 full-text articles were screened. Finally, 9 full-text English language articles met the inclusion criteria: 6 studies were prospective[7,15–18,19] and 3 were retrospective.[20–22] Figure 1 presents the flowchart of the search strategy based on PRISMA guidelines.

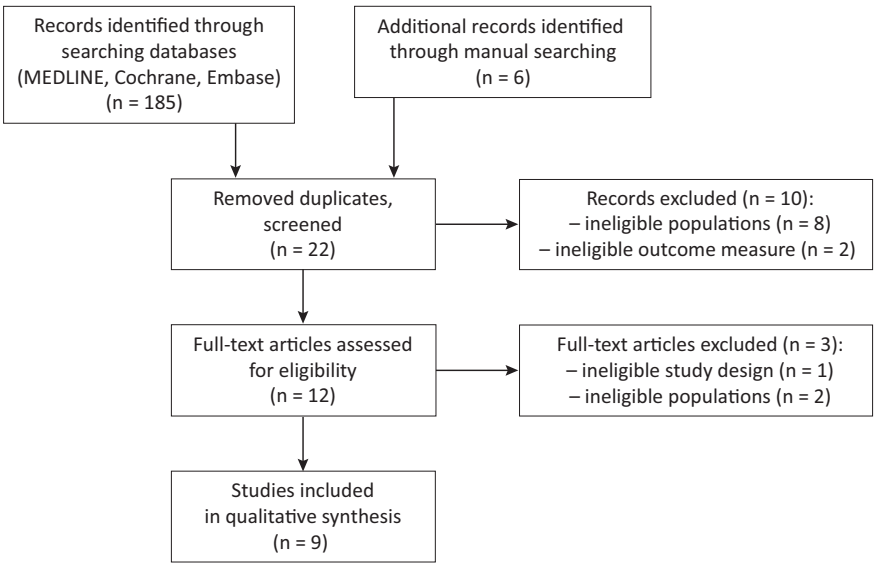

Fig. 1. The PRISMA flowchart

Prospective studies

None of the prospective studies were randomized trials. All studies were heterogeneous in population, study design and outcome measures, so meta-analysis was precluded.

In their prospective observational study, Dal Moro et al.[19] recruited 78 urology patients (86% men and 14% women) aged ≥70 years who had qualified for endoscopic transurethral resection of prostate (TURP), transurethral resection of bladder tumor (TURBT) with a tumor size of >4 cm or "open" procedures (radical cystectomy, radical prostatectomy or radical nephrectomy) in order to verify the predictive value of frailty for postoperative complications. Patients were evaluated for frailty with the Edmonton Frail Scale (EFS), which screens for cognitive impairment, dependence in instrumental activities of daily living (iADL), recent burden of illness, self-perceived health, depression, weight loss, medication issues, incontinence, inadequate social support, and mobility problems. Standard medical and urological histories were taken. Patients were evaluated with use of the Pre-operative Assessment of Cancer in the Elderly (PACE) components: the ASA classification, the Mini-Mental State Examination, activities of daily living (ADL), iADL, the Geriatric Depression Scale, the ECOG scale, and the Satarian Index of Comorbidities.

Postoperative outcomes were complications, both medical and surgical, mortality and rehospitalization within 3 months. The overall prevalence of frailty was 21.8% and male patients were frailer than female patients ($p = 0.003$). In both the open and endoscopic surgery groups, patients with complications were significantly frailer than those without complications in univariate analysis, but in multivariate analysis there was no significant correlation between frailty indices and the risk of major complications. The authors assumed this was probably due to the small number of cases and the low rate of complications. Despite these ambiguous findings, the authors stated that the EFS is a simple, quick and easy-to-administer test which assesses patients' physical and psychosocial characteristics. In consequence, the authors see a need for further well-designed studies focusing on urology to develop risk-reduction strategies for frail elderly patients.

Ellis et al.[15] conducted an evaluation of a nurse-led preoperative assessment service for elderly patients who had qualified for orthopedic, urological and general surgical procedures. The assessment consisted of basic investigation and diagnostic tools, such as the Mini-Mental State Examination for cognitive problems and the Barthel Index for the assessment of ADL. In the first 5 months, 141 eligible patients qualified for the control group (over 65 years of age with one or more of the following found in the preoperative assessment: cognitive or mobility problems or concerns about daily activities, falls or home circumstances). The need for additional intervention was noted, but no intervention was undertaken. In the next 6 months, 172 patients were evaluated and, if necessary, referred for appropriate intervention (physiotherapy, occupational therapy, dietician, social work, falls teams, family doctor's care, or other). In both groups, the mean age was similar (73.3 vs 72.7 years). Urological procedures (TURBT, TURP and "other renal" procedures) were performed in 32.6% of the control group and in 35.5% of the intervention group. Unfortunately, outcomes for urology groups as separate cohorts are unavailable. During the intervention phase, fewer operations were cancelled (5.2% vs 17.7%; $p < 0.001$), the mean length of stay was shorter (4.9 days vs 8.9 days; $p < 0.01$) and the rate of postoperative complications was lower (2.3% vs 8.5%; $p = 0.01$).

Revening et al.[16] recruited 80 patients over 18 years of age who had qualified for minimally invasive surgery. Most were urological procedures: 49 renal/urethral surgeries, 12 robot-assisted prostatectomies, 2 robot-assisted radical cystectomies, and 17 general-surgery operations. Standard preoperative assessments were performed. Additionally, patients were evaluated for frailty using the Fried criteria (shrinking, weakness, exhaustion, low activity, and slower walking speed). The primary outcome was the incidence of postoperative complications within 30 days of surgery, as assessed using the Clavien–Dindo scale. The secondary outcomes were mortality, length of stay and discharge to a skilled nursing facility. Only 2 patients were frail and 11 were intermediately frail; therefore, both groups were analyzed as a single group and compared with the non-frail group. Outcomes for the urology group as a separate cohort are unavailable. The mean age was 60 years (range: 19–87). The mean CCI was 3.99 ±1.85. Many of the patients (62.5%) had an ASA score ≥3 and 86.25% of the patients had an ECOG performance status of 0. The intermediately frail or frail patients comprised 16.25% of the study population. The 30-day postoperative rate of complications was 16.25%. Of these complications, according to the Clavien–Dindo classification, 15.4% were IIIa, 7.7% were IIIb and 7.7% were IV. Patients in the intermediately frail or frail group were 6 times more likely to experience postoperative complications (OR = 5.91; 95% CI = 1.25–27.96; $p = 0.025$). The authors were aware of the limitations of the research, but suggested the potential utility of preoperative frailty assessment in patients undergoing minimally invasive procedures.

Revening et al.[17] enrolled 189 patients over the age of 18 years in further research on preoperative assessment: 117 from urology clinics, 52 from surgical oncology clinics and 20 from general surgery clinics. In addition to standard preoperative evaluation, frailty was assessed with the Fried criteria. The primary outcome was postoperative complications within 30 days of surgery of any grade on the Clavien–Dindo classification. The mean age was 62 years. Patients who were intermediately frail or frail were more likely to experience postoperative complications (OR = 2.07, 95% CI = 1.05–4.08; $p = 0.036$). Of all other preoperative assessment tools, only hemoglobin levels had a significant correlation, and higher levels were

protective of complications within 30 days (p = 0.033). As with the other studies, outcomes for the urology group as a separate cohort were unavailable.

In another study by Revening et al.,[7] the researchers enrolled 351 patients who had qualified for major general and oncological urological surgeries – excluding endoscopic procedures such as TURBT. As before, a standard preoperative assessment was performed and the patients were evaluated for frailty using the Fried criteria. The primary outcomes were postoperative complications within 30 days of surgery – as assessed using the Clavien–Dindo scale – mortality and discharge to a skilled nursing facility. A predictive model for 30-day complications using frailty and other preoperative variables, such as the ASA score, the CCI, age, and serum hemoglobin and serum albumin levels, was constructed. In the end, 351 patients were analyzed. The mean age of the patients was 63 years (range: 19–87). The median age-adjusted CCI (ACCI) was 4. The ASA score was 1 or 2 in 24.8% of the patients and 3 or higher in 75.2%. An ECOG performance status of 0 or 1 was found in 96% of them and of 2 or higher in 4%. Urological procedures (e.g., radical or partial nephrectomy, radical cystectomy with urinary diversion, open or robotic-assisted radical prostatectomy, etc.) were performed in 205 patients (58.4%), and 146 patients (41.6%) had major general surgery. According to the Fried criteria, 255 patients (72.6%) were fit, 86 (24.5%) were intermediately frail and 10 (2.8%) were frail. Thirty-day major postoperative complications (of Clavien–Dindo grade III or higher) occurred in 50 patients (14.2%), and the 30-day mortality rate was 1.7%. Eight patients (2.3%) were discharged to a nursing care facility. Statistical analysis revealed a significant correlation between the Fried Frailty Criteria and the occurrence of 30-day complications (p = 0.002). Furthermore, shrinking and grip strength taken together performed as well as the full 5-component frailty criteria. The addition of ASA score and serum hemoglobin levels to the model of shrinking and grip strength resulted in the most sensitive and specific measure of 30-day complications (AUC = 0.632; p < 0.001, according to the authors).

Braude et al.[18] prospectively assessed the impact of introducing a geriatric service for urology patients, the Proactive Care of Older People Undergoing Surgery (POPS). They conducted the study in 2 phases. The aim of the 1st phase was to reduce postoperative length of stay, while the aim of the 2nd phase was to optimize the process: to improve the identification of geriatric syndromes, to facilitate proper intervention according to the CGA and to extend the application of the geriatric service to younger patients. In phase 1, patients aged ≥65 years who had qualified for elective or emergency urological surgery were enrolled into 2 groups: 112 patients into the control group (enrolled 1 year before the start of the intervention phase) and 130 patients into the intervention group. The intervention included a daily interdisciplinary round led by a POPS consultant or geriatric nurse, a weekly multidisciplinary team (MDT) meeting and a twice-weekly ward round, where patients whose cases were highlighted at the interdisciplinary round were discussed. The outcomes included cancellation of surgery, length of stay, postoperative complications, unplanned readmissions, and death within 30 days of discharge. After the intervention, the length of stay was shorter (4.0 vs 4.9 days) and the rate of postoperative complications was 4 times lower. The cancellation rate decreased from 10% to 5% and the readmission rate decreased from 8% to 3%, although the changes were not statistically significant (p = 0.12). Within the control group, 3 deaths occurred. In the 2nd phase (the quality improvement phase), several modifications were instituted: patients were included if they were ≥65 years old or were suspected for frailty, irrespective of age, the interdisciplinary round was replaced with a read-do Geriatric Surgery Checklist (GSCL) and 1 junior doctor from each of the 4 urology teams, an occupational therapist, a physiotherapist and a POPS social worker joined the group. The results of the follow-up survey completed by the staff confirmed that the POPS program had been successfully incorporated in the inpatient urology ward.

Retrospective studies

Lascano et al.[20] retrospectively compared a modified frailty index predicting poor surgical outcomes with other risk stratification tools among patients undergoing urological surgery due to malignancy. They searched the American College of Surgeons National Surgical Quality Improvement Program database (NSQIP) from 2005 to 2013 to identify patients undergoing major urological procedures. They modified the 11-variable Canadian Study of Health and Aging Frailty Index by adding 4 more variables relevant to oncology patients: weight loss, chemotherapy or radiation before surgery, history of metastasis and severe renal failure. The main outcome measures were mortality and Clavien–Dindo grade IV complications. A total of 41,681 patients were identified and included in the study. The elderly patients were concentrated in the groups of nephroureterectomy and radical cystectomy. The patients with a high frailty index score were at an almost fourfold higher risk of a Clavien–Dindo grade IV event (CI = 2.865–4.788; p < 0.0005) and an almost sixfold greater risk of 30-day mortality (CI = 3.72–9.51; p < 0.0005) than the non-frail patients, after adjusting for race, sex, age, smoking history, and type of surgery. Mortality after surgery was highest in the patients undergoing radical cystectomy (2.6%) and lowest in those undergoing radical prostatectomy (0.2%). The radical prostatectomy patients were a lower-risk group overall. The modified frailty index was comparable or superior to the CCI but inferior to the ASA classification in predicting postoperative complications. Compared to the ASA, the modified frailty index was superior to other tools in all aspects.

Suskind et al.[21] also used data from the NSQIP from 2007 to 2013, and they identified 95,108 patients aged ≥40 years who underwent common urological procedures appearing in the registry more than 1,000 times. Frailty was measured using the NSQIP frailty index. The main outcome was the rate of complications within 30 days of surgery. The majority of patients (67.8%) undergoing surgery were aged ≥61 years. The average frequency of complications was 11.7%, with the most common complications being readmission (6.2%), blood transfusion (4.6%) and urinary tract infection (3.1%). The rate of complications increased with increased frailty index (adjusted OR = 1.74; 95% CI = 1.64–1.85) regardless of the patient's age.

Moreover, Isharwal et al.[22] searched the NSQIP database from 2005 to 2011 to identify patients who had undergone urological procedures. They divided the patients into 2 groups: complex (inpatient) and simple (outpatient) procedures. Preoperative frailty was assessed using the Risk Analysis Index (RAI), a tool which uses preoperative history and physical examination without a detailed geriatric evaluation. The variables of the RAI were age, gender, admission to a nursing home in the last 3 months, weight loss, poor appetite, renal failure, chronic heart failure, shortness of breath, cancer, cognitive problems, and ADL. The primary outcomes were mortality and complications, whereas the secondary outcomes were length of stay, re-operation, 30-day readmission and discharge not to home (data only for 2011). A total of 42,715 patients were included: 25,693 in the complex procedure group and 17,022 in the simple procedure group. Complications, mortality rate and other measures of poor surgical outcomes increased with an increased RAI score. Interestingly, mortality in patients with a high RAI score were similar in the 2 groups, whereas the rate of complications was greater in the complex procedure group. The main characteristics of all studies included in this review are summarized in Tables 1 and 2.

Table 1. Characteristic of included studies

Study	Study time	Number of patients, gender	Inclusion criteria	Surgical procedures	Complications
Dal Moro et al.[19]	ND	78 (14% female)	Age ≥70, major urological procedure (endoscopic or open)	Radical cystectomy, prostatectomy, nephrectomy, TURP, TURBT (>4 cm of tumor size)	According to Clavien–Dindo scale within 3 months
Ellis et al.[15]	2009–2010	141 (62% female) in control group and 172 (55% female) in intervention group	Age ≥65, elective surgery	In urological group: TURBT, TURP, "other renal", general, surgery, orthopedic procedures	Wound problems, infections, alcohol withdrawal, other not specified
Revening et al.[16]	ND	80 (42.5% female)	Age ≥18, elective surgery	61.25% renal/ureteral surgeries, 15% robot-assisted prostatectomies, 8.75% hepatobiliary and pancreas surgeries, 6.25% gastric surgeries, 2.5% robot-assisted cystectomies	According to Clavien–Dindo scale within 30 days, mortality, discharge to a skilled nursing facility
Revening et al.[17]	ND	189 (40.2% female)	Age ≥18, elective surgery	Elective urological (62%) or general surgery, endoscopic procedures excluded	According to Clavien–Dindo scale within 30 days, mortality, discharge to a skilled nursing facility
Revening et al.[7]	ND	351 (39 % female)	Age ≥18, elective surgery	Elective urological (58.4%) or general surgery, endoscopic procedures excluded	According to Clavien–Dindo scale within 30 days, mortality, discharge to a skilled nursing facility
Braude et al.[18]	2007–2014	112 (13% female) in control group, 130 (18% female) in intervention group	Age ≥65, elective or emergency urological surgery	Elective or emergency urological surgery	Length of stay, surgery cancellation rate, unplanned readmission within 30 days, surgical/medical complications, death
Lascano et al.[20]	2005–2013	41,681 (16% females)	Elective urological surgery for malignancy	Elective major urological oncology procedures (cystectomy, prostatectomy, nephrectomy, nephroureterectomy)	Mortality, Clavien–Dindo grade IV
Suskind et al.[21]	2007–2013	95,108, no data for gender	Age ≥40, urological procedure that appears more than 1,000 times in the NSQIP database from 2007 to 2013	21 most common urological procedures	30-day complication rate
Isharwal et al.[22]	2005–2011	42,715, no data for gender	Patients undergoing urological in- or outpatient procedure	Common urological procedures both in- and outpatients	Mortality, Clavien–Dindo grade III, IV and V complications, length of stay, re-operation, readmission within 30 days

ND – no data; TURP – transurethral resection of the prostate; TURBT – transurethral resection of the bladder tumor.

Table 2. Characteristic of included studies

Study	Assessment tool	Usefulness of geriatric assessment	Comment
Dal Moro et al.[19]	CCI, ACCI, EFS, PACE	EFS – simple, easy and quick-to-administer PACE – complex and lengthy to administer	Prospective study, usefulness of PACE not clear. No significant relationship between frailty and complications
Ellis et al.[15]	MMSE, ADL, basic investigation	Preoperative assessment led by an intervention (if needed): significantly fewer cancellations, shorter stay, lower complications rate	Prospective study, nurse-led preoperative assessment, 2 groups: control group and intervention group, no data for urological patients separately
Revening et al.[16]	Frailty evaluation using Fried criteria, ASA, ECOG, CCI, standard preoperative assessment	Presence of frailty significantly increases risk of complications	Prospective study, mean age 60 years (range: 19–87 years) – age was not a predictor of complications. Low frailty rate – study population divided into 3 groups: not frail (83.75%), intermediately frail (13.75%) and frail (2.5%); no data for urological patients separately, but most procedures were urological
Revening et al.[17]	Frailty evaluation using Fried criteria, ASA, ECOG, CCI, CES-D (Center for Epidemiologic Studies Depression Scale), MNA (Mini Nutritional Assessment), ADL, standard preoperative assessment	Assessment of frailty is feasible in multidisciplinary patient population. Frailty is a predictor of postoperative complications	Prospective study, mean age 62 years (range: 19–82 years), no data for urological patients separately, but most were urological patients. Age was not a predictor of complications. Higher level of hemoglobin was protective for complications
Revening et al.[7]	Frailty evaluation using Fried criteria, ASA, ECOG, CCI, CES-D, MNA, ADL, standard preoperative assessment	Frailty is a predictor of postoperative complications. Shrinking and grip strength together performed equivalently to the full 5-component frailty criteria. Addition of ASA and serum hemoglobin level to the model of shrinking and grip strength demonstrated the most sensitive and specific predictor of complications	Prospective study, mean age 63 years (range: 19–87 years), no data for urological patients separately, but most were urological patients. Age was a predictor of complications
Braude et al.[18]	POPS CCI	After intervention followed the geriatric assessment: lower cancellation rate, shorter stay, lower complications rate, lower readmission rate	Prospective study. Two phases – the 2nd phase was the improvement phase
Lascano et al.[20]	MFI, CCI, ASA	High frailty index: 4-times higher risk of Clavien–Dindo IV grade complication and 6-times higher risk of 30-day mortality. MFI superior to CCI, but inferior to ASA. MFI associated with ASA was the best complications prediction tool	Retrospective study based on NSQIP database search. Mean age 61 years. Lack of detailed geriatric assessment
Suskind et al.[21]	NSQIP Frailty Index	Complications rate increased with the increase of frailty index regardless of patient's age	Retrospective study based on NSQIP database search. Lack of detailed geriatric assessment. Readmission and blood transfusion treated as complications; 67.8% procedures performed in patients ≥61
Isharwal et al.[22]	RAI using preoperative history, comorbidities, ADL	Complications rate increased with increasing RAI score, but prospective validation of RAI is needed	Retrospective study, no data for age. Lack of detailed geriatric assessment

CCI – Charlson Comorbidity Index; ACCI – Age-adjusted Charlson Comorbidity Index; EFS – Edmonton Frail Scale; PACE – Pre-operative Assessment of Cancer in the Elderly; MMSE – Mini-Mental State Examination; ADL – Activities of Daily Living; ASA – American Society of Anesthesiology; ECOG – Eastern Cooperative Oncology Group scale; CES-D – Center for Epidemiologic Studies Depression Scale; MNA – Mini Nutritional Assessment; POPS – Proactive care of Older People undergoing surgery; MFI – Modified Frailty Index; NSQIP – The American College of Surgeons National Surgical Quality Improvement Program; RAI – Risk Analysis Index.

Discussion

The World Health Organization (WHO) recognizes 60 years of age as the beginning of old age. However, the age of 65 years is very often encountered in the literature. The population of people aged 65 years or older is constantly growing. It currently represents about 14% of the Polish population, and in 2035 it will increase to 30–35%.[23] Half of all cancer cases and 2/3 of cancer deaths are among elderly patients.[24] Older people form the largest group requiring surgical treatment – almost 2/3 of urological surgeries are performed in elderly patients.[25] Therefore, urologists will be increasingly confronted with the difficulties of treating the elderly, especially due to the differences between them and younger patients.[26]

Currently, there is no widely accepted system developed specifically for the elderly that helps qualify them for specific oncological treatment. It is important to understand that one's biological age is often different from one's actual age. Unfortunately, the estimation of biological age by doctors is not entirely accurate. Several tools allowing the estimation of remaining life expectancy are available. Tables on life expectancy are available in most countries, but using observation or intuition is the most common method of estimating remaining life expectancy.[27] Older patients, including urology patients, are less likely than

younger patients to receive radical oncological treatment.[28] This may be due to the overestimation of their biological age as the sole risk factor for poor surgical outcomes.

In the treatment of muscle-invasive bladder cancer, radical cystectomy is the standard treatment, but among the patients between 70 and 80 years of age, only 40–50% undergo cystectomy; likewise, only 13–30% of 80-year-olds have such treatment.[29] In the management of organ-confined prostate cancer, the guidelines of urological societies suggest radical treatment in men whose estimated remaining life expectancy exceeds 10 years, though urologists and oncologists are typically not very accurate in estimating patients' remaining life expectancy. In 2005, Wilson et al.[30] showed that the estimation of patients' life expectancy by urologists and oncologists is very subjective; the same patient was often evaluated differently by the same physician, the accuracy of the assessment was based on the physician's own experience and the chances of 10-year survival were usually underestimated. All of this could lead to inadequate treatment: up to 34% of patients would not receive optimal treatment on the basis of an overly pessimistic estimation.

Schwartz et al.[31] also demonstrated the impact of age on decision-making in 2003. Suboptimal prostate cancer treatment was received by 14% of all subjects, but in the group of patients aged 70 years or older, the proportion was significantly greater: over 47% of those with a Gleason score of 5–7 and 73% of those with a Gleason score of 8–10. The risk factors for suboptimal treatment were age, comorbidities and Gleason score. The literature on elderly oncology patients with comorbidities currently suggests that chronological age should no longer be the basis for therapeutic decision-making, but that a broader geriatric assessment should be relied upon,[32] because a healthy and fit elderly person may be a better candidate for surgical treatment than a younger but burdened patient. This postulate was confirmed by the SIOG in 2010. They recommended classifying patients into 4 groups; "healthy," "vulnerable," "frail" and "terminal." Patients in the "healthy" and "vulnerable" groups should be offered the standard treatment, regardless of their age.[33]

Up to 75% of patients over the age of 85 are not frail, although frailty does tend to increase with age.[19] Frailty is a concept introduced by geriatricians that identifies elderly patients at an increased risk for falls, hospitalization and death. At present, this concept is more and more often adapted as a risk-stratification tool in surgically treated individuals. In numerous studies, screening for frailty was superior to traditional methods of evaluation; thus, frailty has become a broadly accepted risk factor of poor surgical outcomes in many surgical settings.[7,34] Frailty can be assessed using many screening tests, but the gold standard is a detailed geriatric assessment (GA). Moreover, it is worth noting that GA is not necessary in all patients, that it requires experience and it is time-consuming.[1] Therefore, a variety of screening tests may be useful (VES-13, GFI, G8, TRST, aCGA, Rockwood, Balducci, or Fried) in identifying patients requiring broader geriatric assessment.[35–42] The CGA as part of a preoperative assessment has been well-described in general surgery, thoracic surgery and orthopedics,[1,43–49] but not in urology. In most studies, screening for frailty and geriatric assessment were simple risk stratification tools for predicting poor surgical outcomes. In only a few studies was geriatric assessment followed by an intervention for which the concept of frailty and the CGA were constructed. Partridge et al.[50] performed a systematic literature review on the impact of geriatric assessment on postoperative outcomes in the elderly, including only prospective studies with preoperative evaluation, intervention and measurement of postoperative outcomes in phases, and excluding studies with frailty assessment and geriatric assessment as risk tools. Only 5 studies met the inclusion criteria, and 2 of those were randomized controlled trials. The results were encouraging and suggested that geriatric assessment is not only a risk-stratification tool, but is also beneficial in reducing poor postoperative outcomes in elderly patients if followed with a proper intervention.

Despite these findings, in only a few studies was the impact of frailty and GA on postoperative outcomes of urological surgery described. We searched MEDLINE/PubMed, Embase and Cochrane Library databases for publications from 2000–2017 (week 48), using the inclusion and exclusion criteria and the terms "geriatric assessment", "frailty" and "urology". The use of other terms did not yield more results. Nine studies were included in our review. None of these studies were randomized controlled trials. There were differences in the patient populations: in 2 studies with an intervention phase, only elderly patients were enrolled, while in others younger patients were also included.[15,18] We included these studies because the mean age of the participants and the type of surgery strongly suggested that most of the patients were elderly.

In all of the retrospective studies and in one of the prospective studies, only urology patients were evaluated; in the others, patients undergoing other types of surgery were included, and there was no data for urology patients as a separate cohort. The methods of geriatric assessment also differed. Braude et al.[18] and Ellis et al.[15] performed preoperative comprehensive geriatric assessment, while in the other studies only screening tests for frailty were used. The common outcome measures were complications within 30 days, mortality and – in several studies – length of stay, unplanned readmission or cancellation of operation. The high level of heterogeneity makes it impossible to compare these studies or to draw any meaningful conclusions. However, in all of the studies included in our review, either frailty screening or GA was confirmed as an important risk-stratification tool, and in 2 studies designed as prospective trials with an intervention phase, the effect of basing intervention on CGA encouraged further studies.

Practical aspects

In modern urology units, patients undergoing major surgery are admitted the day before surgery in most cases. This timeframe does not allow for a detailed geriatric assessment followed by any intervention. If it is known much earlier that a major oncological urological procedure is necessary, the optimal time for geriatric evaluation appears to be about 4 weeks before admission, which would allow for intervention or delayed surgery with a clear understanding of the planned procedure and associated risks.[51]

Conclusions

The current knowledge on preoperative geriatric assessment in elderly urology patients is sparse. Preoperative identification of frailty in such patients seems to be an important tool in daily urological practice. Moreover, proper stratification of preoperative frailty may lead to a decrease in postoperative complications. The traditional tools for preoperative evaluation seem to be inferior to frailty screening in predicting surgical risk. However, the latest literature does not provide strong data on the preoperative use of the CGA or its impact on surgical outcomes in elderly urology patients. Thus, further research in urological settings is needed, especially in multicenter randomized controlled trials.

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Does the status quo have to remain? The current legal issues of transsexualism in Poland

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Abstract

Transsexual persons often undergo the process of transition, which is a long, multi-stage procedure. One of the stages, often final, is the lawful reassignment of sex, which is often perceived by transsexual individuals as more meaningful than the medical interventions. The aim of the study was to analyze the current legal situation of transsexual individuals in Poland. An in-depth review of legal documents and their association with current medical knowledge on transsexualism together with a presentation of solutions established worldwide were performed. Analyzed aspects include surgical interventions, sex assignment and correction of birth certificate. The current legal situation of lawful sex reassignment in Poland is complex and far from friendly towards transsexual people. Recent attempts to improve the situation were unsuccessful and current strategies to help transsexual people seem to be ineffective. Apart from the medico-legal problems, a number of issues connected with transgenderism depend on the socio-political views. The most notable drawback of the currently binding judicial procedure of legal sex change is the requirement of suing parents, spouse and children. This could be avoided if the change was performed in a non-litigious mode of proceedings, in which the medical criteria of the World Health Organization (WHO) and an opinion of a strictly regulated team of experts were central factors.

Key words: patient advocacy, sex reassignment procedures, transsexualism

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Introduction

The commonly used umbrella term 'transgender' (as well as 'transsexual' or 'gender incongruent') addresses the situation in which the psychological gender identity of an individual differs from the gender established by the external sexual anatomy at birth. This article will focus strictly on the case of transsexualism, in which a person identifies with another gender and wants to undergo a transition, i.e., the process of aligning the desired gender. We do not analyze the issue of genderqueer persons, whose sexual identification is neither masculine nor feminine, or of those transgender individuals who neither seek medical help nor have a desire to undergo the transition process. The World Health Organization (WHO) ICD-10 *Classification of Mental and Behavioural Disorders* currently classifies this problem under the code F64.0.[1] The direct cause of transsexualism remains unknown; however, numerous genetic and perinatal hormonal factors are suspected to be involved.[2] The psychological experience of transsexualism, which is referred to as gender dysphoria, leads to chronic suffering. Transsexual persons are referred to according to their perceived/desired gender. Women in the bodies of men, i.e., persons who desire to live as women, are referred to as male-to-female (MTF) transsexuals. People in the opposite situation are called female-to-male (FTM).

The prevalence of gender incongruence varies, depending on the population and study method. The prevalence of clinically established transsexualism varies from 0.7/100,000 in Iran to 100/100,000 in Iceland, with a mean value of 9.2/100,000.[3–5] However, a meta-analysis of studies about transsexualism epidemiology, which were based on self-reported transgender identity, revealed a much higher transgender occurrence of 355/100,000.[4] Therefore, it may be suspected that gender incongruence is highly underdiagnosed. Most of the studies report a higher number of MTF transsexuals than of FTM.[4]

The process of diagnosing transsexuality is long and complicated. The gender identity has to exist for at least 2 years and no chromosomal anomalies should be present.[2] In addition, the identified problem should not be a result of any confounding psychiatric comorbidity (e.g., schizophrenia).[3] The full diagnostic process is most commonly performed in patients who want to undergo the gender transition process.

The diagnostic criteria of transgenderism are likely to be modified soon. On June 18, 2018, during the preparation of this article, the WHO announced the new ICD-11 classification on their website.[6] The new code is HA60 and the new name for the diagnosis is 'gender incongruence of adolescence or adulthood'. The new criteria read as follows: Gender incongruence of adolescence and adulthood is characterized by a marked and persistent incongruence between an individual's experienced gender and the assigned sex, as manifested by at least 2 of the following: 1) a strong dislike or discomfort with the one's primary or secondary sex characteristics (in adolescents, anticipated secondary sex characteristics) due to their incongruity with the experienced gender; 2) a strong desire to be rid of some or all of one's primary and/or secondary sex characteristics (in adolescents, anticipated secondary sex characteristics) due to their incongruity with the experienced gender; 3) a strong desire to have the primary and/or secondary sex characteristics of the experienced gender. The individual experiences a strong desire to be treated (to live and be accepted) as a person of the experienced gender. The experienced gender incongruence must have been continuously present for at least several months. The diagnosis cannot be assigned prior to the onset of puberty. Gender variant behavior and preferences alone are not a basis for assigning the diagnosis. As can be seen, much emphasis is put on the willingness to change the sex characteristics. This is similar in many aspects to the definition proposed by the DSM-V classification.[7] The official publication date of the new classification is scheduled for May 2019 and the implementation of the new classification by the associated countries is planned for 2022.

Transsexual individuals, in addition to the suffering caused by gender dysphoria, have to deal with social stigma.[8] The severity of social stigma differs depending on the tolerance level of the particular society, the legal status of transsexualism and the physical gender of the transgender individual.

The transition process is a complex procedure wherein the phenotype of an individual is transformed in order to match the gender identity. Currently, there is no method to change psychological identity and, therefore, transition remains the most effective method to reduce gender dysphoria in transsexual individuals.[9] In transgender men, this includes androgen hormonal therapy and an optional two-stage surgical therapy, including bilateral mastectomy and hysterectomy with male genital reconstruction.[9] The 2nd stage of the surgery is often not performed, as it is not desired by the patients because of unsatisfactory surgical results. In transsexual women, the transition includes hormonal therapy, which focuses on androgen suppression and estrogen supplementation, voice therapy and complex genital reconstruction surgery with optional additional plastic surgeries.[9]

The last aspect of the transition process, which creates plenty of problems for transgender patients, is the change of their legal status. The formal change of gender is often perceived by patients as the final goal of the transition process.

Status quo

The current legal procedure of the legal change of gender assignment in Poland is problematic in a multifaceted way. The general problem arises from the lack of a specific legal act which would thoroughly regulate this matter.

The present legal practice was created through case law, including mainly judicial activity of the Supreme Court of Poland. The judicial opinions on the legitimate procedure of the transition have been changing throughout the past years. The position expressed in various rulings of regional courts (sądy okręgowe, which have jurisdiction in such cases as the court of the first instance) on the subtleties of the proceedings on the change of legal sex differ significantly, which results from lack of specific legislation. These disparate opinions do not only create a lot of legal uncertainty, but are also inherently troublesome for transsexual individuals. It can be observed that many people seek legal advice in online communities for transsexuals. The question most commonly raised concerns before which particular court the legal sex change can be achieved most easily.[10] The 3 main legal issues in this field concern the following: the legality of the surgical removal of genitals from the viewpoint of criminal law (and discussing its potential necessity to the process of transition), the procedure of birth certificate correction, and finally, the action from Article 189 of the Civil Procedure Code,[11] which is now considered the legally appropriate way for transsexuals who want to change their legal gender.

Legality of surgical intervention

According to Article 156 of the Polish Penal Code,[12] whoever deprives another person of the ability to procreate, shall be subject to imprisonment for a period of 1–10 years. Apart from hormonal therapy, the surgical operation of sex correction includes the removal of one's genitals to create organs resembling genitals of the other sex. In most cases, this entails depriving the individual of the ability to procreate, prima facie fulfilling the premises of the offence described in the aforementioned Penal Code provision. The Polish criminal law does not provide an explicit exception for this kind of medical treatment, unlike, for example, the Austrian Criminal Code.[10] Despite the fact that not all transsexuals want to undergo this kind of treatment, this issue is still very important. The question of surgical treatment was discussed in Polish judicature as a potential prerequisite for a change of legal sex. It was perceived as a clear indicator that a person's feeling of being the opposite sex is permanent and irreversible, which was then considered as a requirement for a legal sex change.

Surgical treatment for MTF individuals consists primarily of genital surgery – vaginoplasty.[13] The techniques vary, depending on the performing center. The most commonly performed type of treatment is the penile inversion procedure, which includes orchiectomy, penile deconstruction with the creation of a sensitive neoclitoris and the creation of the vaginal cavity.[13] The procedure is irreversible, but only a small proportion of patients express regret or are not satisfied with their new organ's functionality.[14] Some transgender women perform additional surgeries, which include breast augmentation, thyroid gland reduction and facial feminization surgery. Those additional surgeries can reduce gender dysphoria to some extent.[15–17]

Surgical treatment in FTM may be considered as a two-step procedure. The first, most common procedure is breast reduction (bilateral mastectomy).[18] Naturally, it does not lead to the loss of reproductive capabilities of the individual. Most patients are satisfied with the procedure, as breasts are considered to be one of the most significant symbols of femininity. The next, more serious steps include an oophorectomy, hysterectomy and vaginectomy, followed by penile reconstruction surgery.[19] Those procedures are more invasive and lead to the definitive loss of reproductive ability. In addition to decreasing gender dysphoria, they may also be considered as gynecological cancers prophylaxis. Penile reconstruction surgery is performed by only a few centers and, in addition to being expensive, is associated with frequent unsatisfactory esthetic and functional results.[20]

The legality of sex reassignment surgery was a subject of discussion in Polish criminal law doctrine. This issue is important, because if sex reassignment surgery is to even be considered a potential prerequisite for a legal sex change, it should be legal from the viewpoint of criminal law. As noted by Rejman, criminal responsibility is "autonomous" from other branches of law, i.e., administrative or civil law cannot justify criminally illegal acts.[21]

The most popular way of justifying sex change surgery is to classify it as a medical intervention. It is important to note that views on the legality of medical interventions in criminal law are also divided in Polish criminal law doctrine. While some authors classify them as initially legal, others treat them as a case of criminal law justification. The main controversy behind sex reassignment surgery in Polish criminal legal doctrine concerns the medical character of the aim of the treatment.

The first ruling, which discussed the medical character of sex reassignment surgery, was the decision of the Voivodeship Court for the Capital City of Warsaw, arguing that if the plaintiff's psyche was in danger of permanent and irreversible damage, and he feels like a woman, then there are no contraindications against sex reassignment surgery from the viewpoint of medical ethics.[22] The medical character of the treatment was then reaffirmed by the Supreme Court in 1978.[23] Other decisions made by the Supreme Court in cases regarding transsexualism did not mention the matter of the medical character of the surgery nor the criminal aspects, although one may argue that they were deemed legal implicitly.[24]

However, the opinions of Polish criminal law experts' are divided with regard to both the question of acceptability of sex reassignment surgery and its medical character. Some authors deny its medical character, seeing them as legal nonetheless. The first group of authors, including

notably Rejman and Filar, argue against seeing the surgery as medical intervention (due to the questionable medical character of the surgery).[21,25] This claim is often based on the premise that surgery is only symptomatic treatment and, therefore, cannot heal the patient. According to Rejman, constructed organs are "dummies", unable to perform their biological function.[21] Filar notes that the surgery does not bring back the "correct" gender identification; instead, it exacerbates the pathological state.[26] Other authors denying the medical character of the sex reassignment surgery include Rozental, Gromadzki, Safjan, and others.[27–29] Another idea is to justify this kind of treatment through the institution of necessity; however, the main problem with this line of reasoning seems to be the lack of required immediacy of danger. Other authors propose treating it as an instance of specific extralegal justification.[9]

The aforementioned argumentation lies on the very narrow definition of health. As noted by Przybylska, the definition of 'health' is established in the Constitution of the World Health Organization ("Health is a state of complete physical, mental and social well-being and not merely the absence of disease or infirmity.") and includes the state of mental and social well-being.[30] Even if sex reassignment surgery is considerd as symptomatic treatment, it definitely heads towards improving the mental and social well-being of a patient. It is worth mentioning at this point the high prevalence of depression and incidence of suicides among the transgender population. Studies indicate that up to 22–43% of the transgender population report a history of suicide attempts.[31] Some authors state that sex reassignment surgery, along with the transition process, has a positive and protective impact on mental health and reduces the risk of suicide.[31] The essence of transsexualism could be described as follows: both the mind and the body are healthy on their own, but the essence of the disorder lies in the extreme disparity between them. There is no way of changing the mind (which may be a result of developing a selected sexual identity in the brain), so the only way to deal with this discrepancy is through adaptive treatment. The group of authors who stress the medical character of the treatment includes Daniluk, Leszczyński and Sakowicz.[32] Interestingly enough, the Supreme Court of Poland has generally considered surgery as a prerequisite for legal sex change. In one of its rulings, it stated that legal sex change can be carried out before the surgical sex correction if "the attributes of a newly formed gender are prevailing and that state is irreversible".[33] Also, in a verdict from 1991, the Supreme Court suggested that surgical correction is generally a prerequisite for filing a claim for a legal sex change, because it shows that the sexual identity has a permanent character.[34] The opinion that undergoing a sex reassignment surgery is (de lege lata) or should be (de lege ferenda) a precondition for legal sex change used to be prevalent in the Polish legal doctrine.[25,27,35–37] However, in the newer literature, a number of authors disagree with this statement.[10,38–40]

There are 2 main arguments supporting the claim that sex reassignment surgery should be required for a legal sex change: the argument based on certainty and the argument based on 'social order' (meaning practical consequences, especially those related to family law). The first argument is based on the fact that surgery can be understood as sufficient evidence confirming that one's gender dysphoria is of permanent and irreversible character. Even if we accept that assertion, sufficient does not mean necessary. There are transsexuals who do not want to, or even cannot, as a result of the medical contraindications, undergo this kind of therapy. The reasons may be various (lack of funds is just one). What is more, transsexualism, as a medical category, is a matter of fact, so the opinion of an adequately qualified expert or experts should be proper evidence for the courts in this matter.[39]

The second argument is more multilayered and problematic. There are potential situations in which a person that changed their sex legally, but still has biological organs of the former, may enter a legally troublesome placement. Most of these include family law cases: a marriage of 2 "externally" same sexes, or the conception of a child that would have 2 mothers. Each of these situations, however, requires a separate discussion.[41]

The consequences of adopting a different solution, i.e., the necessity of undergoing surgical therapy before the legal change of sex, are far graver. It would prima facie mean forcing individuals to sterilize themselves. This raises serious concerns from the viewpoint of human rights. This kind of regulation could be classified as incompatible with the constitutional principle of human dignity.[10,40] In the context of international law, such a requirement would explicitly violate Yogyakarta Principles, which is an influential soft law declaration on human rights. Moreover, in recent rulings, *Y.Y. v. Turkey*, and *A.P., Garçon and Nicot v. France*, the European Court of Human Rights declared that the requirement of sterilizing treatment is an infringement of the right to private life, guaranteed in the article 8 of the European Convention on Human Rights.[42,43]

Lack of agreement in Polish legal doctrine concerning the legality of surgical sex correction and the popularity of the thesis that it is a necessary prerequisite for a legal sex change in court proceedings lead to a vicious circle. Physicians may be afraid to perform such a surgery because of criminal responsibility, and courts may require it, nonetheless, as the basis for the ruling.[39] This situation may be especially troublesome for transsexuals, potentially even preventing them from changing their legal sex in Poland or from undertaking medical treatment. Not surprisingly, this may lead to "sex change tourism", not only international, but also of a domestic kind.[10]

Sex assignment and correction of birth certificate

To understand the notion of sex in Polish law, one should start with the procedure of sex assignment. It starts immediately after a child is born. The healthcare facility in which the child is born is obliged to issue a birth card, which includes, i.a., an entry with an alternative: male or female.[44] The Civil Registry Records Act, which regulates this issue, does not provide any term, but it can be inferred from a general rule established in the Administrative Proceedings Code.[45,46] The birth certificate should be, therefore, issued without undue delay, utmost within 7 days of birth, and then the healthcare facility is obliged to deliver it within 3 days to the Civil Registry Office. Parents are also obliged to notify the Civil Registry Office within 21 days of the issuance of the birth certificate. The protocol of notification also includes an entry for sex. Finally, Civil Registry Office shall issue the birth certificate in the date of notification on the basis of birth card and parents' notification protocol. The birth certificate serves as the main legal measure of identifying a child, and is needed in an extensive number of procedures.

The development of transgenderism during a lifetime – from birth to adulthood – is a controversial issue. There is a particularly discussed issue of transgender adolescents who present gender dysphoria similarly to adults, however, before reaching full adolescence. This situation creates an opportunity to start hormonal treatment before the full development of tertiary sexual characteristics. This leads to much better technical treatment results, as the tertiary features of the unwanted sex have not yet developed. However, treatment of transgender youth leads to a potential trap – gender dysphoria in youth does not have to persist into adulthood. The study of Wallien and Cohen-Kettenis showed that gender dysphoria presentment in the pre-pubertal population exists in adulthood only in the minority of subjects.[47] This is why medical treatment before adolescence is generally not recommended; however, The World Professional Association for Transgender Health (WPATH) suggests hormonal suppression of puberty by gonadotropin-releasing hormone (GnRH) analogues in up to Tanner stage II–IV, which allows transgender individuals to temporarily postpone puberty.[48] From the bioethical point of view, such course of action still remains controversial, as GnRH analogues are not free from side effects. Therefore, the legal aspects of the problem are even more complicated.

It may seem that correction of birth certificate could be a valid way for legal sex change in Poland. However, at the moment, the Supreme Court ruled out this way of proceeding and pointed out that article 189 of the Civil Proceedings Code (hereinafter referred to as CPC) stipulates the valid way to proceed.[11,34,49] Nonetheless, the procedure of correcting a birth certificate should be evaluated here as well (since it was legally valid until 1989, and there are important arguments for opting for this procedure). According to article 36 of Civil Registry Record Act, rectification of a civil registry record of this kind shall be performed by the court in non-litigious proceedings. The first case regarding changing the legal sex in Poland was in 1964 and concerned a MTF transsexual who had already undergone surgical correction and filed for the sex marker to be changed on her birth certificate.[45,50] The Voivodeship Court for the Capital City of Warsaw, acting as the court of second instance (the first instance court had dismissed the claim), admitted that such a possibility exists. To determine the sex of a claimant, the Court ascertained that claimant's psyche has primacy, and the decision could not be based solely on the anatomical criterion.[10] The Court also addressed the legal issue of admissibility of the correction of the birth certificate: on the basis of the law applicable at the time, the correction was admissible only if the certificate was initially drawn up recklessly or erroneously. The correction was effective ex tunc. According to the opinion of the Court, the act was drawn up correctly at the outset, since the circumstances justifying its invalidity occurred later – at the moment of the change of sex by the claimant. Therefore, the provisions regarding the correction of the birth certificate were not applied by the Court directly but only by analogy. As noted by Ostojska, this argument contains an inconsistency: if the Court acknowledged the priority of the mental state as crucial for determining the sex, why was the change recognised as the moment of surgical treatment?[10] The process of correcting the birth certificate per analogiam was then confirmed by the Supreme Court in 1978,[23] although it regarded the case of an intersexual person. The Supreme Court stated that, as an exception, a birth certificate can be corrected if the claimant has not yet undergone the surgical correction, once again underlining the importance of the psychological aspect of sex. In addition, the possibility of a legal change of sex was linked to the irreversibility and finality of the factual change of sex, but the latter was not defined, apart from explicit exclusion of the surgical correction criterion.[10] This line of reasoning was approved from the viewpoint of psychiatry and sexology, but was criticised on the legal grounds.[10] Legal doctrine indicates that a civil registry act cannot be corrected if it was drawn up correctly, even by analogy, because that would mean that it is henceforth effective.[25,37] Since it is assumed that transsexualism proceeds throughout one's life, the birth certificate only becomes incorrect and that cannot be the premise to correct it in this way. Even if, as it was pointed out above, we assume that transsexualism is innate, one can argue that we have no valid way to diagnose it at birth, so from a practical point of view the certificate could not be classified as made erroneously. A similar argument was proposed by Ignatowicz. There were extremely rare cases in which an expert opinion stating that transsexualism was innate convinced courts to correct birth certificates, even when this way of proceeding was already generally

proscribed by the ruling of the Supreme Court, as described below.[51] The Supreme Court ascertained in 1989 that the occurrence of transsexualism shall not be a basis for correction of the sex entry in a birth certificate and, moreover, that there was no legal basis at the time for any kind of judicial sex change.[49] Zielonacki instead proposed the general declaratory action from the article 189 of CPC as the legitimate way to proceed.[37] The Supreme Court then affirmed this line of reasoning in 1991, reopening a way for transsexuals to change their legal sex.[34]

Action from Article 189 of Civil Procedure Code

According to Article 189 of CPC, a plaintiff may demand a judicial ascertainment of the existence or nonexistence of a legal relationship or a right if they have legal interest in it. This rule describes a general action and applies to all cases not specified elsewhere (such as, e.g., ascertainment of the nonexistence of marriage). However, as an action in litigious proceedings, it always requires naming a defendant (which is quite problematic, as it will be shown below). A ruling has a declaratory character. According to Zielonacki, gender identity should be classified as a personal right and could be protected by an action to ascertain the correct sex.[37] This view was confirmed by the Supreme Court in 1991, which established the action from Article 189 of CPC as a proper legal solution for the change of sex.[34] This ruling is a result of such proceedings and is effective ex nunc. It is not a basis for the correction of birth certificate, but it suggests that an "additional annotation" should be included, which is an institution from the Civil Registry Record Act – Article 21 of the Act of 1986 (binding at the time), Article 24 of the now binding Act of 2014.[45] What is interesting, the Supreme Court stated that the persistent feeling of belonging to the other sex is a personal right, which is the legal basis for the change of sex; however, generally, this feeling of belonging can be proven only by surgical correction. This raised the question of whether the personal right under protection is the personal feeling of belonging to a sex, or rather the sex per se.[10] As noted by Safjan, the result of the proceedings is the ascertainment of sex as such, and not the personal feeling.[35] In a following ruling in 1995, the Supreme Court did not mention the premise of protection of a personal right and addressed the question of passive legal standing (i.e., who should be sued) in these type of cases.[52] The Court determined that plaintiff's parents are the persons to be sued (or the guardian ad litem, appointed by court, in the case of parents – or one of them – being deceased), denying other propositions posed in the literature, such as the director of the registry office (because they are not related to the plaintiff on the ground of family law). It should be noted, however, that in a few legal systems suing the registrar is the legally binding solution, for example in Hong Kong (see *W v. Registrar of Marriages* (2013). HKCFA 39).[53] Passive legal standing was then reviewed by another ruling of the Supreme Court in 2013 which highlighted the shortcomings of parents as passively legitimized, and indicated that the plaintiff's spouse and children should have passive legal standing in the first place.[54] This solution is currently legally binding.

The Supreme Court indicated the vital reasons against assigning parents passive legal standing. Firstly, passive legal standing in declaratory action from the article 189 of CPC is not chosen arbitrarily, but rather should concern persons that are legally interested in the result of the proceedings. The sex of a child, however, does not affect the legal relationship between parents and the child. Moreover, parents cannot discriminate their children on the basis of sex. If they support their child, then a contradictory action against them is paradoxical and incompatible with the general legal standards of civil proceedings. If they do not accept their child's transsexuality, a court ruling, apart from having no legal impact on their family status, would probably not change their minds whatsoever. Instead, the Supreme Court proposes subjects to whom the plaintiff's sex may be legally relevant. Sex is legally relevant, i.a., in the case of marriage and parenthood. Therefore, passive legal standing in those cases should be ascribed firstly to the spouse and children, since the plaintiff fulfilled their legal and social role as a wife/husband and mother/father. The argument of the Supreme Court goes in the right direction, but it does not concern situations in which the plaintiff is celibate and childless. These cases are the most common.[10] It seems that the source of the problem is the factual, psychophysical state of the plaintiff, not the socio-legal relations of family law. Therefore, non-litigious proceedings that do not require suing any other person seem to be more suitable for this type of case. However, as noted by the Supreme Court, sex has an important role in family law, especially in the case of marriage. On the other hand, it can be argued that divorce as a necessary requirement before the change of legal sex may be a reasonable solution in the case of married plaintiffs, since it avoids the problem of same-sex marriages which are prohibited in the Polish legal system. This kind of prerequisite has been accepted by the European Court of Human Rights in *Parry v. the United Kingdom*, *R. and F. v. the United Kingdom*, and *Hämäläinen v Finland*, and is in fact often used by Polish courts.[10,55,56]

An utmost need for regulation

In the light of the aforementioned issues, it is clear that status of transsexual people in Poland is particularly problematic, especially if they want to change their legal sex. There are no undisputable legal grounds for justifying the surgical treatment, and, what is even more important, the 2 legally plausible civil procedures of sex change do

not provide a proper solution, since they are vitiated with shortcomings that have both a medico-legal and social character. The outdated birth certificate correction procedure may have problematic implications in family law, as it was effective ex tunc. The now-binding procedure through the general declaratory action from 189 CPC requires naming defendants, which is not only questionable from the viewpoint of the principles of civil proceedings, but may provide additional distress for plaintiffs. This situation results in legal uncertainty and may be even seen as violating the human rights of transsexual people, which are, first and foremost, guaranteed in the Constitution of Poland.[40] New legislation which would aim to regulate the issues mentioned above may provide a comprehensive solution to most problems, as well as providing additional positive impact on the situation of transsexuals. This may be a vital point for mental health of transsexual people. Unlike some opinions presented in the Polish doctrine in this context, the law could and should have a therapeutic role to some extent.[25] As noted by the proponents of therapeutic jurisprudence, the practice of law (understood generally as legal procedures and activities of judges, lawyers, etc.) may have serious consequences for the well-being of people who are participants of legal proceedings.[57] This issue is especially important with regard to mental healthcare. Traditionally, therapeutic jurisprudence scholars focused on the issues related mostly to institutionalization and direct influence of legal decision-makers, but it seems that the scope of the therapeutic aspect of law applies to the discussed matter. Designing an adequate procedure for changing the legal sex and regulation of other legally relevant aspects of transsexualism may be helpful in removing the negative stereotypes about transsexualism in the Polish society. It is often pointed out that the act of changing the legal sex is very important in the therapy.

The lack of a specific statutory regulation on the legal sex change procedure for transsexuals may be somehow surprising in the light of the fact Poland was the first country in Europe to have an openly transsexual member of parliament, Anna Grodzka. In 2013, she was representing a group of members of Sejm (lower chamber of Polish parliament) that submitted a bill for gender recognition, which was eventually adopted by the parliament after revisions.[58] However, in 2015, newly elected President Andrzej Duda vetoed the bill and redirected the act to the Parliament. The bill was then discontinued with the end of the term of the Parliament and new election. At the time of writing of this article in February 2019, absolutely no relevant legislative action had been undertaken by the next Parliament.

The bill aimed to solve many of the aforementioned problems.[59] It introduced, i.a., a non-litigious type of proceedings which would not require suing the parents. Moreover, it required the cases to be heard in camera (i.e., non-publicly), and regulated some implications of the sex change for family law and social security. However, the process of medical assessment of transsexualism required in the bill was not flawless; for instance, it did not meet the requirements of WHO. The only reference to medical authorities was the need of providing 2 independent opinions of physicians or of a physician and a certified clinical sexologist, which would confirm the the gender identity is different from the birth-assigned sex. There was not a single word about the clinical criteria needed to be met during the diagnostic process.

Apart from the medico-legal problems, a number of issues connected with transgenderism depend on sociopolitical views.[59] Social issues related to transsexuality seem to be mostly advocated by the LGBT communities and organizations, mostly left-wing-oriented. At the time of the birth of LGBT movement, all of the conditions included in the name – lesbians, gays, bisexuals, and transsexuals – were considered to be diseases and medical conditions. As the medical knowledge progressed along with cultural changes, the first 3 "letters" lost their previous status and became as normal as heterosexuality in Western societies. The continuous fight against discrimination and for equal rights for those individuals is nevertheless still present and is one of the fundamental issues of left-wing political groups. However, if we understand the last "letter" strictly as transsexualism (as defined above), it remains primarily a medical problem, and not only a cultural one. Since Poland is generally a conservative society, there may be little chance of changing and improving the current law for the sake of transsexuals in Poland in the foreseeable future as long as it is advocated only by the left-wing organizations. In addition, knowledge about transsexuality in the Polish society seems to be poor.[60] The co-authors of this article, who are clinical professionals, have personal experience indicating that a crushing majority of medical students have almost no knowledge about the issue. In our personal opinion, the 2 measures that should be taken in order to help the process are as follows: firstly, the transsexual issue should not be advocated solely by the organizations associated with the left-wing political environment, but also by the medical community (e.g., Polish Society of Sexology or Polish Society of Sexual Medicine). Secondly, any serious social awareness campaign could provide vital support. However, it seems to be crucial that this campaign should concentrate on the medical nature of the problem and the troublesome legal issues related to it.

Conclusions

The legal issues related to transsexualism in Poland generate many problems which have direct negative sociomedical consequences. The main source of these problems is the lack of a particular statutory regulations on the procedure of the change of sex, as well as on the legal basis for surgical intervention. The absence of such regulations is striking, especially since a new statutory act is a common postulate formulated in the legal literature. The most

notable drawback of the currently binding judicial procedure regarding legal sex change is the requirement of suing parents, spouse and children. This could be avoided if the change was performed in a non-litigious mode of proceedings, in which the medical criteria by the WHO and an opinion of a strictly regulated team of experts were central factors.

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