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International Day of Pandemic Preparedness, December 27

During its two years, the Covid-19 pandemic has taken a huge number of human lives, leading to major disruptions in the health, economic, and financial sectors around the world with still unforeseeable consequences for all of humanity. Having that in mind, the General Assembly of the United Nations has determined December 27, the birthday of the famous French bacteriologist Louis Pasteur, as the International Pandemic Preparedness Day. The founder wishes to mark this day every year to raise awareness, exchange information, scientific knowledge, best practices, and most quality educational programs on epidemics at the local, regional, national, and global levels as the most effective measures to prevent and respond to epidemics.

Tokom dve godine trajanja, pandemija Covid-19 odnela je ogroman broj ljudskih života, dovela do velikih poremećaja u zdravstvenom, privrednom i finansijskom sektoru širom sveta, sa još uvek nesagledivim posledicama po celo čovečanstvo. Imajući to u vidu, Generalna skupština Ujedinjenih nacija odredila je 27. decembar, dan rođenja čuvenog francuskog bakteriologa Luja Pastera, za Internacionalni dan pripravnosti za pandemije. Želja osnivača je da se ovaj dan obeležava svake godine, sa ciljem podizanja svesti, razmene informacija, naučnog znanja, najbolje prakse i najkvalitetnijih edukativnih programa o epidemijama na lokalnom, regionalnom, nacionalnom i globalnom nivou, kao najefikasnijih mera prevencije i odgovora na epidemije.

Dear Authors, Editors, Peer Reviewers and Readers of the *Vojnosanitetski pregled*,

We thank you for cooperation and support in 2021 and wish you all the best in the upcoming 2022!

Happy Holidays!

Editorial staff of the *Vojnosanitetski pregled*

Dragi autori, urednici, recenzenti i čitaoci *Vojnosanitetskog pregleda*,

Uz zahvalnost na saradnji i podršci u 2021, želimo vam sve najbolje u nastupajućoj 2022. godini!

Srećni praznici!

Redakcija *Vojnosanitetskog pregleda*

ORIGINAL ARTICLES

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Assessment of enthesitis in patients with psoriasis: relationships with clinical features, screening questionnaires results, and quality of life – An ultrasound study

Procena prisustva entezitisa kod bolesnika sa psorijazom: povezanost sa kliničkim karakteristikama, rezultatima *screening* upitnika i kvalitetom života – ehosonografska studija

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Abstract

Background/Aim. Often asymptomatic, enthesitis can be an integral feature of the wide clinical spectrum in psoriasis as well as an early sign of development of psoriatic arthritis (PsA). It may be difficult to clinically recognize enthesitis in patients with psoriasis or distinguish it from other causes of extraarticular pain. Ultrasound (US) expanding use with the development of accurate assessments through standardized US algorithms as the Glasgow Ultrasound Enthesis Scoring System (GUESS) and the Madrid Sonographic Enthesitis Index Scoring System (MASEI) scores made the US the dominant imaging technique in diagnosing enthesitis. The aims of this study were to establish the prevalence of US signs of enthesitis, compare it with screening questionnaires results, and estimate possible connections of US verified enthesitis with quality of life (QOL) of patients with psoriasis without PsA diagnosis. **Methods.** A cross-sectional study was performed on 67 patients with psoriasis who were without systemic therapy. The clinical presence of enthesitis was examined by an experienced rheumatologist, and systemic inflammation was estimated through serum level of C-reactive protein (CRP). The Psoriasis Area Severity Index (PASI) and Body Surface Area-Psoriasis (BSA-PsO) were calculated by a dermatologist. Visual analogue scale (VAS) for pain, screening questionnaires – the Toronto Psoriatic Arthritis Screening (ToPAS), Psoriasis Epidemiology Screening Tool (PEST), Psoriatic Arthritis Screening and Evaluation (PASE), Early Psoriatic Arthritis Screening Questionnaire (EARP), and Psoriasis and Arthrosis Screening Questionnaire (PASQ) – were filled by patients. GUESS and MASEI scores were determined by US. The QOL was estimated by the Dermatology Life Quality Index (DLQI). **Results.** The presence of clinical enthesitis was recorded in 8.7% of patients. According to US signs of enthesitis using GUESS and MASEI scores, only 7% and 2% of patients, respectively, had no sign of enthesitis. Duration of psoriasis and age of subjects were in a significant correlation with GUESS and MASEI scores, while systemic inflammation, VAS value, PASI, and BSA-PsO scores were not. GUESS and MASEI scores significantly correlated with scores of all screening questionnaires as well as with DLQI. **Conclusion.** US can detect subclinical enthesitis better than clinical examination and widely used screening questionnaires, even though the correlations between MASEI and/or GUESS scores and results of screening questionnaires were positive. US examination is important in the multidisciplinary approach in diagnosing and managing psoriasis.

Key words:
arthritis, psoriatic; psoriasis; surveys and questionnaires; tendons; ultrasonography; quality of life.

Apstrakt

Uvod/Cilj. Iako je često asimptomatski, entezitis može biti sastavni deo širokog spektra kliničke prezentacije psorijaze, ali i rani znak ispoljavanja psorijaznog artritisa (PsA). Obično je teško prepoznati prisustvo entezitisa kod bolesnika sa psorijazom, a posebno je teško razlikovati ga od drugih uzroka ekstraartikularnog bola. Ehosonografija

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(ES) je postala sastavni deo ispitivanja reumatoloških bolesnika, a u pogledu dijagnostike entezitisa suverena metoda, sa standardizovanim ES algoritmima kao što su *Glasgow Ultrasound Enthesis Scoring System* (GUESS) i *Madrid Sonographic Enthesitis Index Scoring System* (MASEI) skorovi. Ciljevi ove studije bili su da se ustanovi prevalenca ES znakova prisustva entezitisa, da se uporede ti rezultati sa razultatima *screening* upitnika i da se ispita moguća povezanost entezitisa dokazanog pomoću ES sa kvalitetom života kod bolesnika sa psorijazom, bez dijagnoze PsA. **Metode.** Sprovedena je studija preseka kod 67 bolesnika sa psorijazom koji nisu bili lečeni sistemskom terapijom. Prisustvo klinički manifestnog entezitisa utvrđivano je pregledom reumatologa, sistemska inflamacija je procenjivana na osnovu serumske kocentracije C-reaktivnog proteina (CRP). Indeksi *Psoriasis Area Severity Index* (PASI) i *Body Surface Area-Psoriasis* (BSA-PsO), kao mere zahvaćenosti kože, određivani su od strane dermatologa. Ispitanici su sami popunjavali vizuelnu analognu skalu (VAS) bola i *screening* upitnike za prisustvo PsA – *Toronto Psoriatic Arthritis Screening* (ToPAS), *Psoriasis Epidemiology Screening Tool* (PEST), *Psoriatic Arthritis Screening and Evaluation* (PASE), *Early Psoriatic Arthritis Screening Questionnaire* (EARP) i *Psoriasis and Arthrosis Screening Questionnaire* (PASQ). Skorovi GUESS i MASEI određivani su pomoću ES. Kvalitet života je procenjivan na osnovu *Dermatology Life Quality Index* (DLQI) upitnika. **Rezultati.** Kliničkim pregledom, entezitis je ustanovljen kod 8,7% ispitanika. Na osnovu ES ispitivanja preko GUESS i MASEI skorova, samo 7%, odnosno 2% ispitanika nije imalo znake entezitisa. Trajanje psorijaze i životno doba ispitanika bili su u značajnoj korelaciji sa vrednostima GUESS i MASEI skorova, dok sistemska inflamacija, vrednosti VAS, PASI i BSA-PsO skorova nisu pokazali značajnu povezanost. Skorovi GUESS i MASEI bili su u značajnoj korelaciji sa skorovima svih *screening* upitnika, kao i sa DLQI skorom. **Zaključak.** Primenom ES može se dokazati entezitis češće nego kliničkim pregledom ili pomoću *screening* upitnika, mada je postojala korelacija između MASEI i/ili GUESS skorova sa rezultatima *screening* upitnika. ES je značajna dijagnostička metoda u multidisciplinarnom pristupu dijagnostici i lečenju psorijaze.

Ključne reči:
artritis, psorijazni; psorijaza; ankete i upitnici; tetive; ultrasonografija; kvalitet života.

Introduction

In patients suffering from psoriasis (PsO), a chronic immune-mediated skin disease with a prevalence of 1%–3% in the general population, numerous comorbidities and other related conditions occur more frequently: psoriatic arthritis (PsA), inflammatory bowel disease, anxiety, non-alcoholic fatty liver disease, metabolic syndrome, cardiovascular diseases, and depression [1]. The prevalence of PsA has been estimated to be between 6% and 42% in PsO, and according to systematic review and meta-analysis of 266 observational and clinical studies examining 976,408 patients with PsO, it is 19.7% [2]. Arthritis presentation can vary from subtle manifestations to highly destructive forms. Joint and low back pain, stiffness, and swelling, as well as dactylitis, are the most common symptoms, and they are easily recognized by rheumatologists or dermatologists.

Enthesitis is included in the Classification Criteria for Psoriatic Arthritis (CASPAR) for diagnosing PsA as one of the hallmarks [3]. On the other hand, according to the Group for Research and Assessment of Psoriasis and Psoriatic Arthritis (GRAPPA) recommendations for treatment of PsA, enthesitis is one of the domains that decides the modality of treatment [4]. Similar to other arthritis-related diseases, early treatment of PsA is expected to control joint damage, which usually occurs within the first 2 years of disease [2]. Unrecognized PsA characteristics, especially early signs like enthesitis, can delay the diagnosis of PsA [1]. Bagel and Schwartzman [5] reported that the average diagnostic delay of PsA in a combined population of patients with mild, moderate, and severe psoriasis is about 5 years. Even a 6-month delay in PsA diagnosis can adversely affect structural and functional long-term outcomes. It can be one of the reasons why systematic reviews and meta-analyses estimated an overall prevalence of undiagnosed PsA among PsO patients of 15.5% [6].

In diagnosing enthesitis in PsO, dermatologists and rheumatologists use clinical examination as the first step, by palpation of some entheses included in indices like Leeds Enthesitis Index (LEI) or Spondyloarthritis Research Consortium of Canada Enthesitis Index (SPARCC). It may be difficult to clinically recognize enthesitis in patients with PsO or distinguish it from other causes of extraarticular palpation pain in fibromyalgia, mechanical injury, or comorbid musculoskeletal condition.

Several self-administered screening questionnaires have been developed and validated for identifying patients with PsA: Toronto Psoriatic Arthritis Screening (ToPAS) tool, Psoriasis Epidemiology Screening Tool (PEST), the Psoriatic Arthritis Screening and Evaluation (PASE), and the Early Psoriatic Arthritis Screening Questionnaire (EARP). Some of them (ToPAS, PEST, and EARP) have only questions about joint pain and swelling, especially fingers, wrists, elbows, hips, or dactylitis. These screening questionnaire tools can have limited usefulness in enthesitis diagnosis because of the heterogeneous nature of PsO and PsA. Some other musculoskeletal disorders might be identified as the cause of false-positive or false-negative results. On the other hand, according to the meta-analysis of Iragorri et al. [7], these questionnaires have a sensitivity of 0.65–0.85 and specificity of 0.73–0.87. It was calculated that 13%–35% of patients with PsA cannot be recognized early using these questionnaire tools. Enthesitis as unrecognized distinction PsA is considered important and may be crucial for early diagnosis and treatment of the disease and slow down its progression regarding the role of enthesitis in the pathogenesis of PsA [8]. These facts have contributed to an increase in the

importance of imaging techniques in recent years. Ultrasound (US) is considered more dominant to the diagnosis of enthesitis than magnetic resonance imaging (MRI) because it is cheaper and the assessment through standardized US algorithms is more accurate [9]. Several algorithms have been developed, including the most commonly used and exact Glasgow Ultrasound Enthesis Scoring System (GUESS) and the Madrid Sonographic Enthesitis Index Scoring System (MASEI) as the best standards in standardized enthesitis US assessment [10–12].

The impact of PsO on worse quality of life (QOL) has been well-documented in the clinical practice and literature and, in order to quantify it, numerous simple practical questionnaires for routine clinical use were made, among them, the Dermatology Life Quality Index (DLQI) as the most used, important, and reliable. It was developed by Finlay and Khan [13] in 1994 and Finlay et al. [14], and regarding some modifications of DLQI, a lot of analyses proved the reliability of DLQI.

Besides psoriatic plaques, itching, dandruff, peeling, bleeding, nail changes, other musculoskeletal involvement, as well as arthritis, dactylitis, and enthesitis lead to chronic pain and worsening movement and additionally deteriorate QOL in PsO.

The aims of this study were to establish the prevalence of US signs of enthesitis in patients with PsO without a diagnosis of PsA, to compare it with screening questionnaires results, and to estimate possible connections of US verified enthesitis in PsO patients on their QOL as an independent parameter.

Methods

Patients

This cross-sectional study included participants selected according to the following criteria: diagnosis of PsO without a diagnosis of PsA and volunteer participation. The study sample included 67 subjects with PsO who met the CASPAR classification criteria for the diagnosis (42 males, 25 females) with a mean age of 47.0 years. The mean disease evolution time was 14.9 years. Patients, who met the entry criteria, were informed and gave consent according to the ethical standards of the Helsinki Declaration of 1983 and ICH-GCP. Exclusion criteria included a diagnosis of PsA, osteoarthritis, fibromyalgia or mechanical injury, previous knee, ankle, or elbow surgery, a record of systemic conventional, targeted biologic therapy, or corticosteroid injection into any of the sites to be explored. The Military Medical Academy Ethics Committee approved this study on October 24, 2018. The clinical presence of enthesitis was examined by an experienced rheumatologist. Systemic inflammation was estimated through serum levels of C-reactive protein (CRP). The collection of demographic and case history data was performed by reviewing case notes and treatment records.

Psoriasis Area Severity Index (PASI) and Body Surface Area – Psoriasis (BSA-PsO) were calculated by a dermatologist.

Self-administered screening questionnaires and QOL determination

Visual Analogue Scale (VAS) for pain, ToPAS tool, PEST, PASE, EARP, and the Psoriasis and Arthritis Screening Questionnaire (PASQ) were filled by the patients themselves, and the results were calculated by a dermatologist. QOL was estimated by the Serbian version of the DLQI, also filled by patients. The DLQI consists of ten questions regarding the following topics: symptoms, humiliation, shopping and home care, clothes, social and recreation, sport, work or study, close relationships, gender, and treatment. Each question (scored from 0 to 3) estimates the influence of the skin disorders on the QOL during the previous 7 days. A possible score range is between 0 and 30.

Ultrasound evaluation

The US was conducted by an experienced radiologist, using a Mindray, Resona 7 ultrasound system (Shenzhen Mindray Bio-Medical Electronics Co, China), with a 7–12 MHz linear array transducer, and the MASEI and GUESS indices were calculated.

The MASEI measurement and scoring implies examination of: 1) Inferior pole of the calcaneus: plantar aponeurosis enthesis – plantar aponeurosis structure (0 or 1), plantar aponeurosis thickness > 4.4 mm (0 or 1), inferior pole of calcaneus erosion (0 or 3), inferior pole of calcaneus enthesis calcification (0, 1, 2 or 3), plantar aponeurosis enthesis power Doppler (0 or 3); 2) Superior pole of the calcaneus: Achilles tendon enthesis – Achilles tendon structure (0 or 1), Achilles tendon thickness > 5.29 mm (0 or 1), retrocalcaneal bursitis (0 or 1), posterior pole of calcaneus erosion (0 or 3), posterior pole of calcaneus enthesis calcification (0, 1, 2 or 3), posterior pole of calcaneus power Doppler (0 or 3); 3) Tibial tuberosity: distal patellar ligament enthesis – patellar ligament structure (0 or 1), patellar ligament thickness 0.4 mm (0 or 1), infrapatellar bursitis (0 or 1), tibial tuberosity erosion (0 or 3), tibial tuberosity enthesis calcification (0, 1, 2 or 3), tibial tuberosity enthesis power Doppler (0 or 3); 4) Inferior pole of the patella: proximal patellar ligament enthesis – patellar ligament structure (0 or 1), patellar ligament thickness 0.4 mm (0 or 1), inferior pole of patella erosion (0 or 3), inferior pole of patella enthesis calcification (0, 1, 2 or 3), inferior pole of patella enthesis power Doppler (0 or 3); 5) Superior pole of the patella: quadriceps tendon enthesis: quadriceps tendon structure (0 or 1), quadriceps tendon thickness > 6.1 mm (0 or 1), superior pole of patella erosion (0 or 3), superior pole of patella enthesis calcification (0, 1, 2 or 3), superior pole of patella enthesis power Doppler (0 or 3); 6) Olecranon tuberosity: triceps tendon enthesis – triceps tendon structure (0 or 1), triceps tendon thickness 4.3 mm (0 or 1), olecranon erosion (0 or 3), olecranon enthesis calcification (0, 1, 2 or 3), olecranon enthesis power Doppler (0 or 3).

The total possible score on both sides (12 entheses) is 136. According to international statements, sensitivity of the MASEI is 83.3%, specificity 82.8%, positive predictive value 80.8%, negative predictive value 85.7%, positive likelihood

ratio (LR+) 4.87, negative likelihood ratio (LR-) 0.19. The MASEI score greater than 18 is considered as significant [11].

The GUESS measurement and scoring means examination of: 1) Superior pole of the patella (quadriceps tendon enthesis): quadriceps tendon thickness > 6.1 mm, suprapatellar bursitis, superior pole of patella erosion, superior pole of patella enthesophyte; 2) Inferior pole of the patella (proximal patellar ligament enthesis): patellar ligament thickness > 4 mm, inferior pole of patella erosion, inferior pole of patella enthesophyte; 3) Tibial tuberosity (distal patellar ligament enthesis): patellar ligament thickness > 4 mm, infrapatellar bursitis, tibial tuberosity erosion, tibial tuberosity enthesophyte; 4) Superior pole of the calcaneus (Achilles tendon enthesis): Achilles tendon thickness > 5.29 mm, retrocalcaneal bursitis, posterior pole of calcaneus erosion, posterior pole of calcaneus enthesophyte; 5) Inferior pole of the calcaneus (plantar aponeurosis enthesis): plantar aponeurosis thickness > 4.4 mm, inferior pole of calcaneus erosion, inferior pole of calcaneus enthesophyte; Each item scores one point. The total possible score on both lower limbs is 36 [10].

Statistical analysis

Using IBM SPSS Statistics version 19.0 (SPSS, Chicago, IL, USA), statistical analysis was performed. Categorical variables were presented as frequency. All continuous variables are presented as mean ± standard deviation (SD). The Shapiro-Wilk test was used to test the normality of data distribution. One-way ANOVA and *t*-test for dependent samples were used to investigate differences between groups for parametric variables and χ^2 test for nonparametric variables. The relation between variables was evaluated using the Pearson's coefficient correlation. Observations were considered significant if two-tailed *p* values were below 0.05.

Results

Demographic, clinical data, results of self-administered screening questionnaires, clinical and US enthesitis examination, and QOL questionnaires of the 57 patients with PsO are presented in Table 1.

According to our results, the presence of clinical enthesitis was recorded in 8.7% of the patients. According to US signs of enthesitis using the GUESS score, only 4 out of 57 patients with PsO without a diagnosis of PsA (7%) had no sign of enthesitis indicating high frequency of subclinical or clinical enthesitis in these subjects. The total possible score on both lower limbs is 36, and the average GUESS score was 13 in our sample.

According to the MASEI score, one subject had no sign of US verified enthesitis. The mean value of the MASEI score was high in our subjects (27). As the MASEI score has the cut off value ≥ 18, 75% of our patients with PsO and without a diagnosis of PsA had significant enthesitis with a score of 32.6.

Our analysis suggested that only the duration of PsO and age of subjects were in a significant correlation with the GUESS and MASEI scores, while systemic inflammation (estimated through CRP concentration), the VAS value, PASI, and BSA scores were not in correlation with US enthesitis scores (Table 2).

Table 1

Demographic, clinical data, self-administered screening questionnaires, quality of life (QOL) questionnaires, and ultrasound (US) enthesitis examination in patients with psoriasis (n = 57)

Parameter	Values
Age (years), mean ± SD (range)	47.0 ± 16.5 (14–82)
Male/female, n	42/25
Duration of disease (years), mean ± SD	14.9 ± 12.2 (1–54)
CRP (in referent level/increased), n (%)	59 (88)/8 (12)
PASI, mean ± SD (range)	11.3 ± 8.13 (0–42.6)
BSA-PsO, mean ± SD (range)	25.6 ± 13.4 (2–48)
VAS, mean ± SD (range)	1.1 ± 2.2 (0–7)
ToPAS, mean ± SD (range)	5.6 ± 2.1 (3–12)
PEST, mean ± SD (range)	1.1 ± 1.0 (0–4)
PASE, mean ± SD (range)	28.8 ± 13.5 (15–72)
EARP, mean ± SD (range)	1.8 ± 2.3 (0–9)
PASQ, mean ± SD (range)	2.2 ± 2.3 (0–8)
Presence of clinical enthesitis, n (%)	5 (8.7)
GUESS, mean ± SD (range)	13.0 ± 5.6 (0–22)
MASEI, mean ± SD (range)	27.0 ± 13.0 (0–57)
MASEI > 18 [n = 51 (75%)], mean ± SD (range)	32.6 ± 8.3 (18–57)
DLQI, mean ± SD (range)	10.1 ± 7.1 (0–30)

SD – standard deviation; CRP – C-reactive protein; PASI – Psoriasis Area Severity Index; BSA-PsO – Body Surface Area-Psoriasis; VAS – Visual Analogue Scale; TOPAS – Toronto Psoriatic Arthritis Screening; PEST – Psoriasis Epidemiology Screening Tool; PASE – Psoriatic Arthritis Screening and Evaluation; EARP – Early Psoriatic Arthritis Screening Questionnaire; PASQ – Psoriasis and Arthrosis Screening Questionnaire; GUESS – Glasgow Ultrasound Enthesitis Scoring System; MASEI – Madrid Sonographic Enthesitis Index Scoring System; DLQI – Dermatology Life Quality Index.

Table 2

Pearson's correlation coefficients between clinical characteristics and ultrasound (US) scores in patients with psoriasis (n = 57)

Parameter	GUESS score		MASEI score	
	r	*p*	r	*p*
Age (years)	0.10	ns	0.37	< 0.05
Duration of disease (years)	0.48	< 0.05	0.43	< 0.05
CRP: in referent level/increased	0.08	ns	0.11	ns
PASI	-0.25	ns	-0.16	ns
BSA	-0.15	ns	-0.13	ns
VAS	0.11	ns	0.08	ns

For abbreviations see under Table 1.

All investigated subjects filled the PsA screening questionnaires. Results are presented in Table 3.

When we analyzed paired results of screening questionnaires with US enthesitis scores, both scores (GUESS and MASEI) significantly correlated with scores of all screening questionnaires. The GUESS score was the most correlated with the PASE score, followed by the EARP, PASQ, ToPAS, and in the less manner with the PEST score. In the case of the MASEI score, the best correlation was achieved with the EARP score, followed by the PEST, PASQ, PASE, and finally ToPAS score (Table 4).

We also estimated the effects of US verified enthesitis on QOL, by investigating the relationship between the GUESS and MASEI scores with DLQI scores. In this study, the average DLQI in the patients with PsO was 10.1 ± 7.1.

With the aim to escape confounding effects, we included correlations of age, duration of disease, VAS, CRP concentrations, the PASI, and BSA on DLQI (Table 5). Model

Table 3

Results of psoriatic arthritis (PsA) screening questionnaires filled by patients with psoriasis and without the diagnosis of PsA (n = 57)

Questionnaire	Questions (n)	Cut-off score	Subjects with positive questionnaire results n (%)
ToPAS	12	8	9 (15.7)
PEST	5	3	67 (10.5)
PASE	15	47	10 (17.5)
EARP	10	3	20 (35.0)
PASQ	10	7	6 (10.5)

For abbreviations see under Table 1.

Table 4

Pearson's correlation coefficients between screening questionnaire results and ultrasound (US) scores in patients with psoriasis (n = 57)

Questionnaire	GUESS score		MASEI score	
	r	*p*	r	*p*
ToPAS	0.36	< 0.05	0.33	< 0.05
PEST	0.26	ns	0.36	< 0.05
PASE	0.37	< 0.05	0.37	< 0.05
EARP	0.40	< 0.05	0.42	< 0.05
PASQ	0.35	< 0.05	0.39	< 0.05

For abbreviations see under Table 1.

Table 5

Pearson's correlation coefficients between age, duration of the disease, CRP value and PASI, BSA-PsO, VAS, GUESS and MASEI scores with DLQI score in patients with psoriasis (n = 57)

Parameter	DLQI score	
	r	*p*
Age	-0.01	ns
Duration of disease	-0.07	ns
CRP (in referent level/increased)	0.11	ns
PASI score	0.38	< 0.05
BSA-PsO score	0.35	< 0.05
VAS score	0.41	< 0.05
GUESS score	0.74	< 0.05
MASEI score	0.59	< 0.05
MASEI score < 18	0.65	< 0.05
MASEI score > 18	0.37	< 0.05

For abbreviations see under Table 1.

of linear regression analysis was performed to avoid false results and to confirm the potential relationship between the GUESS and MASEI scores with DLQI scores. It showed that US results are independently connected with the DLQI scores ($R^2 = 0.13$, $^{®} = 4.383$; $p < 0.05$).

Discussion

Enthesitis is part of the CASPAR criteria for PsA and the target for PsA treatment as one of the domains in GRAPPA recommendations. Patients with PsO independently of PsA have a higher prevalence and more severe enthesitis compared to healthy controls [15].

Clinical examination has low sensitivity and specificity, detecting generally swelling and tenderness, and limitations which include a risk of missing clinical or subclinical enthesitis and mimicking other conditions. Polachek et al. [16], using only clinical examination in patients with PsO, identified 15% of subjects with enthesitis as part of possible undiagnosed PsA[16]. In our sample, only 5/57 (8.7%) psoriatic patients with enthesitis were identified by clinical examination (Table 1). Ranza et al. [17] demonstrated a higher frequency (30%) in a cross-sectional study similar to a population-based study from Olmstead County (23%) [18] or Lehtinen et al. [19] (15%). A similar prevalence like in our study was observed in Iceland population-based study (8%) [20].

When the Outcome Measures in Rheumatology (OMERACT) team has defined criteria for US visualized enthesitis: hypoechogenicity, increased tendon penetration thickness, enthesophytes, calcifications, erosions, and Doppler activity, numerous US scores were developed but the GUESS and MASEI scores approved as much reliable and they are most widely used in different clinical trials [21]. They are also applicable for patients with skin PsO alone [22]. When we used the GUESS score, the average value in our subjects was 13 of maximal 36, and 98.2% of them had US signs of enthesitis on at least one point of interest. In literature, by using the GUESS score, signs of enthesitis were found in 90–95.5% of patients with PsO depending on US mode [23, 24]. In the same ULISSE trial, US assessment results were similar in patients with PsA and PsO and significantly higher than in the healthy controls or fibromyalgia population [23]. It was also shown in other studies that the GUESS score was much higher in the patients with PsO without joint and entheseal symptoms compared to age-matched healthy controls. In the study by Gisondi et al. [15], the mean GUESS score was significantly higher in patients with PsO (n = 30) compared with healthy subjects (n = 30): 7.9 vs. 2.9, respectively, while Pistone et al. [22] found a significantly higher the GUESS score in 59 patients with PsO in comparison to 59 patients with other dermopathies.

Using a US MASEI score with a cut-off value of more than 18, we found in the same sample 75% of patients with enthesitis with an average value of 32.6 (maximal score being 36). Eder et al. [25] compared total MASEI score in patients with PsA (n = 50), psoriatic patients (n = 66) and healthy controls (n = 60) [25]. Total MASEI scores were higher in patients with PsA than in those with PsO, with both being higher than in healthy control ($p < 0.0001$, among all groups). The sensitivity of a MASEI score ≥ 18 to correctly classify patients as having PsA was 90% among patients with psoriatic diseases. The specificity was 89% when compared to patients with psoriatic disease (PsA and PsO) [21]. The study of Hamdy et al. [26] included 50 patients with PsO and 20 age- and sex-matched healthy controls. Similar to our results, enthesitis was detected by US in 37 patients (74%) with PsO and 3 (15%) healthy controls with median MASEI scores of 27.8 and 4.3, respectively. In the study by Van der Ven et al. [27], in 542 primary care PsO patients, which were US evaluated, 36% had the MASEI score greater than 18, but some typical structural changes were observed in 95% of PsO patients. On the contrary to our study, 97% of them were treated with systemic therapy (biologics or immunomodulatory drugs).

According to some opinions, US enthesitis scores can predict the development of PsA. Tinazzi et al. [28] performed longitudinal evaluation using repeat ultrasound assessment through the GUESS score in a cohort of 30 cases of PsO for 3.5 years. At follow-up, 23% fulfilled CASPAR criteria, and in the logistic regression analysis, baseline higher values of GUESS score were found to be an independent predictor of the development of PsA. The association between the baseline GUESS score and the development of PsA was strong. In the study by El Miedany et al. [29], 126 psoriatic patients were prospectively evaluated by US at 0, 6, and 12 months for enthesitis, and increased probability for structural progression development in the presence of enthesitis was observed (OR = 3.50). Therefore, they concluded that the presence of enthesitis and higher GUESS score at baseline are predictors of progressive, early PsA [29].

According to our results, the age of a patient and duration of PsO have a significant influence on the presence of enthesitis detected by US, while systemic inflammation (measured as CRP level), pain, body mass index, PASI, and BSA-PsO have no impact on the presence of enthesitis (Table 2). In the study of Eder et al. [25], MASEI enthesopathic changes correlated moderately with age but also with BMI. Gisondi et al. [15] reported the same manner of significant correlation between the GUESS score and aging, as well as BMI. Like in our study, Macıa-Villa and De Miguel [21] did not observe the correlation between th MASEI score and BMI.

Macıa-Villa and De Miguel [21] in the systematic review of the literature concluded that in most studies, the MASEI score did not correlate with CRP levels, except in the study of Falcao et al. [30], which is in concordance with our results.

We found a significant correlation of the MASEI and GUESS scores with the duration of the disease but not with the PASI and BSA_Pso scores. In the study of Gisondi et al. [15], in 30 subjects with PsO, the GUESS score was not correlated either with the duration or severity of the disease according to the PASI and BSA-PsO. In the other analysis of Girolomoni and Gisondi [32], the association between the GUESS score and PASI score was found, but a significant correlation with the disease duration, age, and BMI was not. In the ULISSE study, in 51 patients with PsO, the GUESS

score significantly correlated with the disease duration [15], like in our study, but also with BMI. According to Polachek et al. [16], more pain was associated with US signs of enthesitis, which is not in agreement with our results. Rezvani et al. [33] also found a positive correlation between enthesitis and pain. We can conclude that connections of enthesitis with different variables of PsO are not still clear.

Screening questionnaires (PEST, PASE, ToPAS, PASQ, and EARP) can be used to help during the examination of early identification of signs and symptoms of PsA, although their usefulness is partially limited because of the incapacity to differentiate structural changes, missing subclinical and manifesting enthesitis, especially in patients with central sensitization and/or pain amplification, depression, and comorbid musculoskeletal conditions independent of PsA. Most of these questionnaires, except for the EARP and PEST, have no question about enthesitis, thus their sensitivity and specificity are from 0.66 to 0.85 and from 0.76 to 0.83, respectively. Recent developed Simple Psoriatic Arthritis Screening (SiPAS) questionnaire between five, have one question about pain in heels with sensitivity and specificity of 0.79 and 0.87, respectively [34]. Regarding our results, both US scores (GUESS and MASEI) were in a significant correlation with all screening questionnaires (Table 4). The MASEI score, especially, has a good correlation with EARP and PEST scores (containing question about possible enthesitis) and the GUESS score with PASE (the most detailed) and EARP scores. We confirmed their relative usefulness in detecting subclinical or manifesting enthesitis in PsO.

In this study, the average DLQI in the patients with PsO was 10.1 ± 7.1. It is worse than the mean DLQI (5.9 ± 5.9) from the cross-sectional study of Langenbruch et al. [35] on 1,243 participants with PsO. Among our patients, 63% had a DLQI score in the range from 0 to 1, PsO had no effect on the patient's QOL. The low effect (DLQI 2–5) reported 16% of examinees, moderate effect (DLQI 6–10) 9%, very large or extremely large effect (DLQI 11–20 or 21–30) 10% and 2%, respectively. In the previously mentioned study [35], 21.3% of subjects had DLQI > 10. For example, Finlay and Khan [13] in 52 subjects with PsO reported a DLQI score of 8.9 ± 6.3, higher than in 100 healthy volunteers (0.5 ± 1.1), matched for age and major comorbidities; availability was confirmed using the test-retest method, which is in accordance with our results. In many studies [35, 36], a significant direct correlation between the PASI and the DLQI score was detected, which was also found in our study.

Our study is the first one that showed that a strong correlation between US verified subclinical enthesitis and QOL exists, i.e. between the MASEI and GUESS scores, and on the other hand, the DLQI score in the patients with PsO ($r = 0.59$, $p < 0.001$; $r = 0.74$, $p < 0.001$, respectively). In the available literature, we were unable to find studies that correlated the MASEI and GUESS scores with DLQI in patients with psoriasis. Pain and limitation of movements can be some of the reasons. Moreover, Rezvani et al. [33] found a positive correlation between enthesitis and low QOL in subjects with spondyloarthritis, which was explained by higher pain scores. An analysis of data from the Corrona registry showed that QOL in PsA is significantly worse in patients with enthesitis compared to those without enthesitis, including functional status, patient-reported pain, fatigue, sleep disturbance, working abilities, and experience of overall impairment [6].

Conclusion

US can detect subclinical enthesitis in patients with psoriasis as a component of a wide spectrum of its clinical picture or possible early sign of subsequent development of PsA, better than widely used screening questionnaires, although the correlations between MASEI and/or GUESS scores and results of screening questionnaires were positive. It is widely available and can be used repeatedly. Enthesitis verified by US and other imaging techniques may provide better insight into the effect of psoriasis on their QOL as one of the key outcomes in the comprehensive care of these patients. US examination is important in the multidisciplinary approach in the diagnosis and management of patients with psoriasis.

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Testing of the Serbian version of the Oral Health Impact Profile-14 (OHIP-14) questionnaire among professional members of the Serbian Armed Forces

Testiranje srpske verzije upitnika *Oral Health Impact Profile-14* (OHIP-14) kod profesionalnih pripadnika Vojske Srbije

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Abstract

Background/Aim. The quality of life regarding oral health is a multidimensional concept that shows to which extent oral diseases and disorders impact the quality of life of each individual. One of the most frequently used questionnaires for testing the impact of oral health on the quality of life is the Oral Health Impact Profile-14 (OHIP-14). The aim of this study was to test the short form of the OHIP-14 questionnaire among professional members of the Serbian Armed Forces. **Methods.** A cross-sectional design was applied in this study. The reliability of the questionnaire was determined by Cronbach's coefficient alpha. The validity of the OHIP-14 questionnaire was assessed by the factor analysis. **Results.** A total of 1,611 participants, professional members of the Serbian Armed Forces, with a mean age of 33.5, agreed to participate in the study (the participation rate was 97.4%). The majority of the participants were males (88.8%). The overall Cronbach's alpha coefficient of the OHIP-14 scale was 0.960. The alpha reliability coefficients of all OHIP-14 subscales were between 0.732 and 0.865, indicating that the internal consistency reliability of all subscales was good. The Principal components analysis, same as inspection of the scree plot and parallel analysis supported a one-factor solution for the OHIP-14 scale. **Conclusion.** The OHIP-14 manual is equally reliable for determining the impact of oral health on the quality of life of professional members of the Serbian Armed Forces as it is with the civilian population.

Key words:
military personnel; oral health; serbia; surveys and questionnaires; quality of life.

Apstrakt

Uvod/Cilj. Kvalitet života s obzirom na oralno zdravlje jeste višedimenzionalni koncept koji pokazuje u kojoj meri oralna oboljenja i poremećaji utiču na kvalitet života svakog pojedinca. Jedan od najčešće korišćenih upitnika za testiranje uticaja oralnog zdravlja na kvalitet života jeste *The Oral Health Impact Profile* (OHIP-14). Cilj ove studije je bio testiranje kratke forme upitnika OHIP-14 na profesionalnim pripadnicima Vojske Srbije. **Metode**. Istraživanje je sprovedeno po tipu studije preseka. Pouzdanost upitnika određena je *Cronbach*-ovim alfa koeficijentom. Validacija upitnika određena je faktorskom analizom. **Rezultati.** Ukupno 1 611 učesnika, profesionalnih pripadnika Vojske Srbije, prosečne starosti 33,5 godina, pristalo je da učestvuje u studiji (stopa učešća je bila 97,4%). Većina učesnika su bili muškarci (88,8%). Ukupni *Cronbach*-ov koeficijent na skali OHIP-14 bio je 0,960 što ukazuje na pouzdanost interne konzistencije upitnika. Alfa-koeficijenti pouzdanosti svih podskupova OHIP-14 bili su između 0,732 i 0,865, što ukazuje na to da je pouzdanost unutrašnje konzistencije svih podskupova dobra. **Zaključak.** Uputnik OHIP-14 podjednako je pouzdan za utvrđivanje uticaja oralnog zdravlja na kvalitet života kako profesionalnih pripadnika Vojske Srbije tako i civilnog stanovništva.

Ključne reči:
kadar, vojni; usta, zdravlje; srbija; ankete i upitnici; kvalitet života.

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Introduction

The quality of life related to oral health is an important measure of the disease and the outcome of therapeutic interventions [1]. The state of oral health greatly affects the psychological and physical condition of patients [2]. There are various quality of life indices, general or specific, developed to assess the impact of oral diseases on quality of life. For this purpose, one of the most commonly used questionnaires worldwide is the Oral Health Impact Profile – short version (OHIP–14) [3]. The patient's self-perception about his/her oral health and related life quality are significant in clinical dental practice, dental education and research. It is widely shown that oral conditions can have varied impacts on everyday life [4].

OHIP-14 is the most frequently used instrument to evaluate the effects of intraoral disorders on the perception of the well-being of patients [5]. Derivation and validation of a short form of the oral health impact profile were developed by Slade [6]. The original version contained 49 questions and later it was reduced to 14 questions, keeping the same dimensions: functional limitation, physical pain, psychological discomfort, physical disability, psychological disability, social disability, and inability to conduct day-to-day activities [6]. OHIP-14 was originally written in the English language and so far it has been translated into 15 languages [7–10].

The OHIP-14 has shown great reliability [8, 9]. Lower total results of the OHIP-14 have been significantly linked with better self-evaluation of oral status, reduced need for dental treatment, a larger number of natural teeth and better results of clinical examination [10]. Some reports on the impact of oral health on military personnel are available in the literature [11–13]. However, data on the impact of oral health on the quality of life of professional members of the Serbian Armed Forces have not been published so far. The purpose of this study was to test the short form of the OHIP-14 questionnaire among professional members of the Serbian Armed Forces.

Methods

The study was conducted as an observational cross-sectional study in the territory of the Republic of Serbia during the years 2017–2018. Based on the data from the suitable available literature [11], with the study strength of 0.8 (80%), for the assessment of oral health it is necessary to include at least 1,537 participants. Therefore, the study group included professional members of the Serbian Armed Forces who were examined at the Clinic for Dentistry of the Military Medical Academy and dentist's offices at military barracks in the entire territory of the Republic of Serbia. Parts of the sample, under the principle of the stratified sample, were chosen so they would provide a good assessment at the level of the entire Serbian Army, then for the level of particular regions and cities. The study approval was obtained by the Ethics Committee of the Military Medical Academy in Belgrade (No. 1/15-17). Participation in the study was voluntary, and all participants signed informed consent before doing the survey.

Adults, ≥ 20 years, dentulous persons with ≥ 6 teeth present, able to read, comprehend and respond to the series of questions, willing to undergo a dental examination were included in the study.

Patients with heart murmurs that would require antibiotics prior to dental examination were excluded from the study [14].

A total of 1,654 participants were included in the analysis; the participation rate was 97.4%. Out of the 1,654 respondents, 43 did not return the questionnaire or the questionnaire was not completely filled. Questionnaires with missing data were not included in the analysis. Thus, the final sample consisted of 1,611 subjects.

This questionnaire consists of 14 questions distributed in 7 dimensions of oral impact: functional limitation, physical pain, psychological discomfort, physical disability, psychological disability, social disability and handicap. Each question is evaluated on a Likert scale of 5 points (never = 0, hardly ever = 1, occasionally = 2, fairly often = 3 and very often = 4). The "don't know" option is also present. The questions relate to how often individuals have experienced each problem in the last 12 months [10].

The questionnaire OHIP-14 has been translated from the original English language into the Serbian language in accordance with internationally accepted recommendations [15].

Statistical analysis

The reliability of the OHIP-14 was determined by the internal consistency coefficient, ie. Cronbach's coefficient alpha. The Cronbach's coefficients > 0.70 were considered acceptable, while values ≥ 0.80 were preferable.

The validity of the OHIP-14 was evaluated by Principal components analysis. The Varimax rotation was used for this analysis, with Kaiser Normalization (delta = 0). The factors' importance was assessed according to the Kaiser criterion (all factors with eigenvalues greater than 1.0). Value for the Kaiser-Meyer-Olkin measure of sampling adequacy was 0.966, while the value for Bartlett's test of sphericity was highly significant ($p < 0.001$), which confirmed the adequacy of the choice of the factor analysis. The Cattell`s scree plot (or Kaiser criterion) was used to determine the number of statistically significant factors to keep in the analysis of the principal component.

Parallel Analysis, based on random data generation (using the Monte Carlo simulation technique), was used for determining the number of components or factors to retain from the analysis of the Principal component.

Statistical analyses were performed using the SPSS 20.0 (IBM SPSS Statistics, Chicago, IL, USA).

A *p*-value of < 0.05 was considered statistically significant for all tests.

Results

The majority of participants were males (1,430; 88.8%). The mean age of the participants was 33.5 ± 9.2 years (range

20–59 years). More than half of them (55.2%) had a partner (Table 1).

Table 2 shows that question 5, 'has been self-conscious', seems to have a higher average score than the other items. Concerning internal consistency, all item-total correlations were more than 0.5, indicating good internal consistency. In this case, deleting question 5 does not increase Cronbach's alpha score, thus deletion was not considered.

The alpha reliability coefficients of all OHIP-14 subscales were between 0.732 and 0.865, indicating that the internal consistency reliability of all subscales was good (Table 3). The overall Cronbach's alpha coefficient of the OHIP-14 scale was 0.960.

The Principal Components Analysis with Varimax rotation illustrated the presence of one main component with an eigenvalue greater than 1, explaining 66.5% of the variance (Table 4). The factor loads of each item of the OHIP-14 were only on one factor and were in a high degree (> 0.5). Inspection of the scree plot supported a one-factor solution (Figure 1). The parallel analysis indicated that one component should be retained for the OHIP-14.

Table 1

Baseline characteristics of the study participants (n = 1,611)

Characteristics	Values
Male, n (%)	1,430 (88.8)
Female, n (%)	181 (11.2)
Age (years), mean ± SD (range)	33.5 ± 9.2 (20–59)
Age (years), n (%)	
≤ 20	78 (4.8)
21–30	584 (36.3)
31–40	545 (33.8)
41–50	341 (21.2)
≥ 51	63 (3.9)
Marital status, n (%)	
without partner	722 (44.8)
with partner	889 (55.2)

SD – standard deviation.

Table 2

Descriptive statistics for the OHIP-14 questionnaire

Items of OHIP-14	Mean ± SD	Corrected item (total correlation)	Cronbach's alpha if item deleted
Q1. Had trouble pronouncing some words	0.386 ± 0.667	0.685	0.950
Q2. Felt sense of taste had worsened	0.386 ± 0.658	0.771	0.948
Q3. Had painful aches	0.478 ± 0.733	0.778	0.948
Q4. Found it uncomfortable to eat food	0.558 ± 0.835	0.799	0.947
Q5. Has been self-conscious	0.958 ± 1.251	0.536	0.960
Q6. Felt tensed	0.696 ± 0.973	0.756	0.949
Q7. Diet has been unsatisfactory	0.383 ± 0.670	0.764	0.948
Q8. Had to interrupt meals	0.438 ± 0.701	0.826	0.947
Q9. Found it difficult to relax	0.398 ± 0.675	0.846	0.946
Q10. Has been a bit embarrassed	0.512 ± 0.776	0.813	0.947
Q11. Has been a bit irritable	0.428 ± 0.709	0.795	0.947
Q12. Had difficulty doing usual jobs	0.372 ± 0.650	0.836	0.947
Q13. Felt life, less satisfying	0.448 ± 0.754	0.832	0.946
Q14. Been totally unable to function	0.343 ± 0.611	0.814	0.947

OHIP-14 – Oral Health Impact Profile – short version; SD – standard deviation.

Table 3

Internal consistency of the OHIP-14 subscales

Subscales	Cronbach's coefficient alpha
Functional limitation	0.794
Physical pain	0.816
Psychological discomfort	0.732
Physical disability	0.848
Psychological disability	0.830
Social disability	0.865
Handicap	0.858

OHIP-14 – Oral Health Impact Profile – short version.

Table 4

Factor analysis with Varimax Rotation Method for the OHIP-14 scale items

Items of OHIP-14	Component matrix	Communalities
	1	
Q1. Had trouble pronouncing some words	0.734	0.539
Q2. Felt sense of taste had worsened	0.814	0.663
Q3. Had painful aches	0.813	0.661
Q4. Found it uncomfortable to eat food	0.826	0.682
Q5. Has been self-conscious	0.568	0.323
Q6. Felt tensed	0.775	0.600
Q7. Diet has been unsatisfactory	0.806	0.650
Q8. Had to interrupt meals	0.864	0.746
Q9. Found it difficult to relax	0.883	0.780
Q10. Has been a bit embarrassed	0.843	0.710
Q11. Has been a bit irritated	0.835	0.697
Q12. Had difficulty doing usual jobs	0.878	0.771
Q13. Felt life, less satisfying	0.867	0.751
Q14. Has been totally unable to function	0.860	0.739
% variance	66.5	

OHIP-14 – Oral Health Impact Profile – short version.

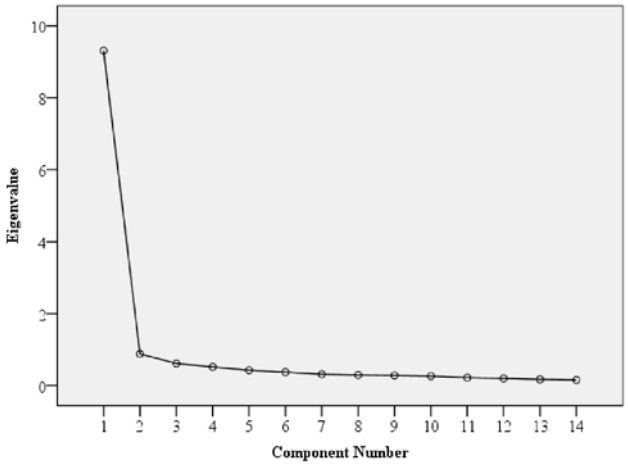

Fig. 1 – The Oral Health Impact Profile-14: scree plot

Discussion

The findings of the present study suggest good reliability and one-dimensional structure for the OHIP-14 scale among professional members of the Serbian Armed Forces.

Questionnaire OHIP-14 has been used in numerous studies for measuring the impact of oral health on the quality of life [8–10]. The OHIP-14-TR (Turkish version of OHIP-14) is a reliable, valid, and comprehensible scale for measuring oral health-related quality of life [16]. So far, the OHIP-14 scale has been applied to the general population, and very rarely in the military population [13, 17]. It is most often used for testing the impact of oral health on the quality of life in people with braces, patients without teeth, and those with orthodontic anomalies or systemic diseases which have manifestations in the oral cavity [18, 19]. In one Belgrade study [7], the adaptation of the OHIP-14 questionnaire was done in the Serbian language in purpose to measure the impact of oral health on the senior citizens' quality of life: in this study, question number 5 was left out ("Have you been self-conscious because of your mouth or dentures?"), because it was incomprehensible to the majority of participants. In contrast to that, this item had Corrected Item-Total Correlation > 0.5 in our study, which points out to the good internal consistency. This difference has occurred most likely due to the large difference in age between tested population groups.

In our study, the questionnaire OHIP-14 showed adequate reliability in the sense of its internal consistency: Cronbach's alpha for OHIP-14 was high (0.960). The coefficients of reliability of all OHIP-14 subscales were between 0.732 and 0.865, which points out that the reliability of internal consistency of all subscales is good. In the study conducted in Nigeria, similar results have been obtained as in our study – Cronbach's alpha for the OHIP-14 was high (0.88) [20]. Good validity and reliability of the OHIP-14 have been determined in the Malesia adult population (the Cronbach's alpha was 0.95) [21]. Moreover, in the assessment of the quality of life in regard to the oral health of patients with cancer of the head and neck, the questionnaire OHIP-14 has shown good internal consistency ($\alpha = 0.861$) [22]. The evaluation of the reliability of the Persian version of the OHIP-14 has also shown good results – the reliability was

excellent (α = 0.954), and Cronbach's coefficient of reliability for all 14 questions in each dimension was more than 70% [23].

In the available literature, we were not able to find the data on validation of the questionnaire OHIP-14 among the military population. Our study showed the presence of one main component with an eigenvalue larger than 1, explaining 66.5% of the variance. Similarly, the Rio de Janeiro study [24] revealed that one factor explained 65.6% of the total variance. Certain studies that had been evaluating the dimensional structure of the OHIP-14 scale presented contradictory results: seven factors in the original study [6], four [25], three [16], two [26], or one factor [24]. Differences in the factor structure of the OHIP-14 questionnaire may be related to the differences in the applied methods, as well as comorbidity and the different sociodemographic characteristics of the respondents in these studies (age, gender, occupation, and education level).

To the best of our knowledge, this is the first study to evaluate the reliability and validity of the OHIP-14 in the Serbian population, and one of the first OHIP-14 validation studies among the members of the armed forces in the world. Furthermore, the strength of this study is that it included a large number of military personnel. However, the study has several limitations. First, there are the known drawbacks of the cross-sectional study design. Secondly, it is necessary to compare the results of this study with the clinical examination of the participants, to get more reliable results. A larger study among middle-aged civilians also needs to be done to compare the results of our study. This survey confirmed that the Serbian version of the questionnaire OHIP-14 has excellent psychometric properties; therefore, it is a reliable instrument.

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Conflict of interest

The authors declare that they have no conflict of interest.

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Sex-specific differences and risk factors for 30-day mortality in acute pulmonary embolism – results from the Serbian University Multicenter Pulmonary Embolism registry

Polno-specifične razlike i faktori rizika od 30-dnevne smrtnosti u akutnoj plućnoj emboliji – rezultati univerzitetskog multicentričnog registra za plućnu emboliju Srbije

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Abstract

Background/Aim. The prediction role of gender in early mortality in patients with acute pulmonary embolism (PE) is still debatable. The aim of the study was to examine sex-specific factors in all-cause 30-day mortality in patients suffering from acute PE. **Methods.** Acute PE subjects (n = 532), 49.6% men, were derived from a "real-life" observational multicenter study. We assessed independent risk factors as predictors for early (one-month) fatal outcome in men, women and total population using univariate Cox regression analysis. **Results.** Age, obesity, hypertension, renal dysfunction, anemia, community-acquired pneumonia, and smoking history presented statistically significant sex-specific differences. One-month mortality was 13.7%, without significant difference in survival based on sex (Log Rank test; $p = 0.324$). Tachycardia at admission [hazard ratio (HR) = 2.61, $p = 0.004$], coronary artery disease (HR = 2.30, $p = 0.047$), immobilization four weeks prior to a PE episode (HR = 2.31, $p = 0.018$) and older age (HR = 1.03, $p = 0.017$) in women, while chronic obstructive pulmonary disease (COPD) (HR = 4.03, $p < 0.001$) and leukocytosis (HR = 1.19, $p < 0.001$) in men significantly increased one-month mortality risk. **Conclusion.** Patient's sex did not prove to be the independent predictor for 30-day mortality in PE patients. We found that tachycardia at admission, older age, coronary artery disease and limb immobilization four weeks prior to PE in women, whereas COPD and elevated leukocyte count in men were associated with higher chance of all-cause early mortality.

Key words:
age factors; coronary disease; immobilization; mortality; pulmonary disease, chronic obstructive; pulmonary embolism; risk factors; sex factors; tachycardia.

Apstrakt

Uvod/Cilj. Uloga pola u predviđanju rizika od ranog smrtnog ishoda kod obolelih od akutne plućne embolije (PE) je nedovoljno razjašnjena. Cilj rada bio je da se utvrde polno-specifični činioci za 30-dnevnu smrtnost od svih uzroka ukupno kod obolelih od akutne PE. **Metode.** Studija po tipu "real-life" opservacionog multicentričnog istraživanja je obuhvatila 532 obolelih od akutne PE (49,6% muškaraca). Primenom univarijantne Cox regresione analize ispitali smo prediktivnu vrednost nezavisnih faktora rizika od ranog (jednomesečnog) smrtnog ishoda kod muškaraca, žena i svih bolesnika ukupno. **Rezultati.** Razlike specifične prema polu bolesnika pokazale su se u uzrastu, gojaznosti, arterijskoj hipertenziji, bubrežnoj slabosti, anemiji, vanbolničkoj pneumoniji i pušačkom statusu. Stopa smrtnosti u prvih mesec dana je iznosila 13,7%, bez značajne razlike u preživljavanju zavisno od pola bolesnika (Log Rank test, $p = 0,324$). Stepen rizika (*hazard ratio* – HR) od 30-dnevne smrtnosti bio je značajno povišen kod žena koje su imale tahikardiju na prijemu (HR=2,61; $p = 0,004$), koronarnu arterijsku bolest (HR = 2,30; $p = 0,047$), imobilizaciju unutar četiri nedelje pre epizode PE (HR = 2,31; $p = 0,018$) i stariji uzrast (HR = 1,03; $p = 0,017$), dok je povišen stepen rizika kod muškaraca bio udružen sa hroničnom op

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struktivnom bolešću pluća (HOBP) (HR = 4,03; $p < 0,001$) i leukocitozom (HR = 1,19; $p < 0,001$). **Zaključak.** Pol bolesnika se nije pokazao kao nezavisni prediktor 30-dnevne smrtnosti kod bolesnika sa PE. Utvrdili smo da su kod žena sa PE, tahikardija na prijemu, uzrast bolesnika, koronarna bolest i imobilizacija nogu četiri nedelje pre PE, a kod muškaraca sa PE, prisustvo HOBP i leukocitoze, povezani sa povećanim rizikom od ranog smrtnog ishoda.

Ključne reči:
životno doba, faktor; koronarna bolest; imobilizacija; mortalitet; pluća, opstruktivne bolesti, hronične; pluća, embolija; faktori rizika; pol, faktor; tahikardija.

Introduction

Pulmonary embolism (PE) is a partial or total occlusion of pulmonary arterial circulation usually by a clot migrating from deep veins resulting in various clinical scenarios – from asymptomatic state to hemodynamic instability, cardiogenic shock and death. PE and deep venous thrombosis (DVT) are referred as venous thromboembolism (VTE) with annual incidence 23-69/100,000 adults [1]. Mortality rate is high – around 30% when left untreated, while 10–15% of hospital-treated PE patients die in the first 1–3 months [1, 2]. Higher fatality rates are derived from registries than from randomized clinical trials [3]. Severity of clinical presentation, numerous patient and setting-related factors influence the outcome [4]. Therefore, according to the 2014 European Society of Cardiology Guidelines (2014 ESC) [4] model based on the simplified Pulmonary Embolic Severity Score (sPESI) [5], the presence of hypotension and right ventricular dysfunction (positive myocardial biomarkers and/or echocardiographic signs) in patients are stratified into four risk groups as having low, intermediate-low, intermediate-high or high risk for 30-day mortality. The respective all-cause mortality rates are 0.5%, 6.0%, 7.7% and 22% [6].

The essential patient assessment for anticoagulant therapy and/or thrombolysis starts with weighing bleeding and mortality risks. Many validated prediction rules have been used for this purpose and several have incorporated female/male gender in scoring, ie. the original PESI score for mortality risks and the VTE-bleed score for VTE patients on stable anticoagulation [7, 8]. However, there are controversies whether sex can impact the outcome following acute PE. One large research on ~280,000 acute PE hospitalizations in the United States found higher in-hospital mortality in females [9]. An opposite finding was derived from the U.S. National Center of Health Statistics which demonstrated that men consistently had a higher fatality rate in all racial and age strata following acute PE during 1979–1998 [10]. Also, a Japanese study on PE patients treated during 1951–2000 found that male mortality was higher [11].

The aim of this study was to determine possible sex-related risk factors influencing the outcome in acute PE patients and to evaluate if sex independently predicts 30-day mortality. The differences in clinical presentation of PE based on sex had previously been reported on a smaller sample of our patients [12].

Methods

This is an ongoing "real-life" observational multicenter study on PE patients initiated in 2012 as the *Serbian University Pulmonary Embolism Registry* (SUPER). These study data were collected retrospectively until 2018 from medical electronic records. The patients were detected by discharge code I26 of the standard International Classification of Diseases, the 10th edition. The authors gathered, maintained and extracted data to the SUPER. They were responsible for interpreting the data and composing the article.

The study population included 532 acute PE patients (49.6% men). Clinicians confirmed PE diagnosis mostly by positive computed-tomography pulmonary angiogram (CTPA), and in few patients by an intermediate-high probability nuclear pulmonary perfusion scintigraphy or by an autopsy finding of pulmonary thrombi. We studied only patients treated for PE. Vital parameters and levels of blood glucose, C-reactive protein (CRP), serum creatinine, leukocyte count and B-type natriuretic peptide (BNP) plasma concentration were recorded on the day of clinical suspicion for PE, i.e. admission, when the treatment decision was made. Creatinine clearance was calculated by Cockcroft-Gault equation [13]. Pneumonia was defined as the appearance of pulmonary infiltrates in the first 48h from admission requiring antibiotic treatment. Anemia was determined with hemoglobin upper limit of normal of 125 g/L and 115 g/L for men and women, respectively. Transthoracic echocardiography examinations had been performed at the bedside or in a specialized office before treatment decisions were made. Right ventricular systolic pressure (RVSP) was estimated using tricuspid regurgitation method [14].

Based on the sPESI (0 or more) presence of hypotension and right ventricular dysfunction (positive myocardial biomarkers and/or echocardiographic signs), all the patients were stratified into four risk groups as having high, intermediate-high, intermediate-low or low risk for one-month mortality [4, 5].

Depending on the risk-stratification group, the patients received treatment with weigh-adjusted subcutaneous low-molecular-weight heparin, unfractionated heparin intravenously or reperfusion. Reperfusion included conventional ESC fast and slow systemic thrombolysis protocols as well as local catheter thrombolysis [4]. Fast protocol used intravenous application of 100 mg alteplase in 2 hours or streptokinase (1,500,000 IJ in 2 h). The slow protocol presented 24 h-continuous intravenous infusion of alteplase 5 mg/h or streptokinase 100,000 IJ/h. In patients with intermediate-high risk PE with the increased risk of bleeding, we performed local catheter thrombolysis. We used ultrasound-assisted catheter thrombolysis (USACT) using the EKOS system (EKOS Corporation, BTG International Group Company) with 1–2 mg/h of alteplase with a dose range of 12–50 mg. We applied the higher doses in patients with a longer duration of PE symptoms, higher thrombus burden and younger age.

We grouped the patients according to sex and presented their characteristics as frequencies with mean values ± standard deviation (SD). Menopausal status was unknown and to avoid sex hormonal differences we analyzed two age-specific groups: < 55 years or older. The age was also assessed using age tertiles in men and women. We calculated statistical significance in difference between sex characteristics with the Pearson's, χ^2 or Student's *t*-test. The main outcome in the study population was all-cause 30-day mortality. We defined mortality as death from any cause within 30 days since the index date of hospital admission. Using univariate Cox regression analysis, we assessed the influence of each risk factor as an independent predictor for all-cause one-month mortality within the entire and gender-specific population. We calculated hazard ratios (HR) with 95% confidence intervals (CI). We applied multivariate Cox regression analysis to estimate HR adjusted for age, smoking status and body mass index (BMI). We looked at the four risk-groups as variable in the scale where the highest value had the highest risk (from 1 to 4). All these tests were two-sided and probability (*p*) value was of less than 0.05 determined statistical significance. We used Kaplan-Meier analysis with Log rank test to compare survival between genders. The analyses were performed with Statistical Package for Social Sciences, version 20.

Every patient had signed an informed consent for proposed medical measures at the admission, as well as before each particular non-standard diagnostic or therapeutic procedure in accordance with national ethical standards and Helsinki declaration.

Results

The study population characteristics according to gender are summarized in Table 1. Arterial hypertension, anemia and creatinine clearance < 60 mL/min were more frequent in women, while pneumonia in the first 48 hours and smoking history proved more common in men. However, during their hospital stay, women were more often intubated and mechanically ventilated due to respiratory failure than men. Incidence rates of other comorbidities were not gender-specific. A BNP level of > 100 pg/mL was statistically more frequent in women than in men (72.4% vs. 57.7%, respectively; $p = 0.003$). Regarding therapy options, more than half of the patients treated with thrombolytic agents received slow systemic protocols, with

Table 1

Characteristics of the pulmonary embolism patients at the admission according to gender

Characteristic	Women (n = 268)	Men (n = 264)	*p*
Age (years), mean ± SD	65.06 ±16.557	58.18 ± 15.764	0.000
Age older than 55 years, n (%)	209 (78.3)	165 (62.5)	0.000
Obesity (BMI > 30 kg/m^2), n (%)	71 (30.5)	39 (16.6)	0.002
Arterial hypertension, n (%)	155 (57.8)	121 (45.8)	0.006
DVT at admission, n (%)	142 (55.7)	142 (56.6)	0.841
Previous DVT or PE, n (%)	34 (12.8)	42 (16.0)	0.137
COPD, n (%)	25 (9.3)	30 (11.4)	0.441
DM, n (%)	50 (18.7)	38 (14.4)	0.179
Malignancy, n (%)	39 (14.6)	27 (10.2)	0.130
Chronic heart failure, n (%)	39 (14.6)	27 (10.2)	0.130
Coronary disease, n (%)	24 (9.2)	28 (11.2)	0.454
Stroke, n (%)	21 (7.9)	18 (6.8)	0.635
Major surgery three weeks prior PE, n (%)	30 (11.6)	21 (8.4)	0.226
Immobilization four weeks prior PE, n (%)	40 (15.0)	30 (11.4)	0.218
Drug predisposing to bleeding, n (%)	78 (29.4)	85 (32.3)	0.473
Systolic blood pressure, n (%)	120.03 (26.92)	122.78 (24.65)	0.221
Heart rate, n (%)	101.38 (23.18)	101.15 (23.83)	0.912
Smoking, n (%)	22 (9.0)	64 (26.1)	0.000
Anemia, n (%)	100 (37.5)	61 (23.3)	0.000
Blood glucose (mmol/L), n (%)	8.25 (4.11)	7.90 (3.94)	0.325
Creatinine clearance < 60 mL/min, n (%)	88 (36.7)	56 (23.5)	0.002
Pneumonia in 48 hours from admission, n (%)	43 (16.1)	65 (24.6)	0.015
Leukocyte count, n (%)	11.95 (9.50)	10.81 (4.24)	0.081
C-reactive protein, n (%)	73.12 (79.24)	77.72 (78.80)	0.512
sPESI > 0, n (%)	180 (68.4)	201 (75.3)	0.080
RVSP > 40 mmHg, n (%)	169 (67.6)	149 (62.6)	0.247
BNP > 100 pg/mL, n (%)	139 (72.4)	112 (57.7)	0.003
Thrombolysis, n (%)			
fast systemic protocol	24 (29.3)	21 (23.1)	0.281
slow systemic protocol	47 (57.3)	48 (52.7)	
local catheter lysis	11 (13.4)	20 (22.0)	
Reanimation during hospital stay, n (%)	35 (13.9)	26 (10.4)	0.225
Mechanical ventilation during hospital stay, n (%)	34 (13.6)	20 (8.0)	0.044
Length of stay (days), mean ± SD	11.98 ± 8.00	10.91 ± 7.15	0.129

SD – standard deviation; BMI – body mass index; DVT – deep venous thrombosis; PE – pulmonary embolism; COPD – chronic pulmonary obstructive disease; sPESI – simplified Pulmonary Embolism Severity Index; RVSP – right ventricular systolic pressure; BNP – brain natriuretic peptide; DM – diabetes melitus.

no difference between gender for all four thrombolytic protocols. Mean length of stay (LOS) was 10.91 days for men and 11.98 days for women ($p = 0.129$). Mean LOS was similar in all four risk groups – low, intermediate-low, intermediate-high and high risk patients – 10.56 ± 6.05 days, 12.01 ± 7.39 days, 11.75 ± 7.90 days, and 11.16 ± 9.46 days, respectively.

During the first month of the treatment, 73 (13.7%) of patients died without any statistically significant difference between women and men (41 women, 32 men, $\chi^2 = 1.134$; $p = 0.287$). All of these fatal outcomes occurred during the in-hospital treatment. The majority of deceased patients (86.3%) were in the high and intermediate-high risk group. Mortality rate was the highest on the first hospital day (32.9%), out of which 70.8% patients were in high, 20.8% in intermediate-high, 8.3% in intermediate-low and 0.0% in low risk group. Total population mortality rate was 6.0%, 5.8%, 1.7% and 0.2% in high, intermediate-high, intermediate-low and low risk patients, respectively. Observing closely within the risk groups, death within 30 days occurred in 41.6% of high-risk patients, 15.3% of intermediate-high-risk patients, 7.1% of intermediate-low-risk patients and 0.8% of low-risk patients.

We assessed the influence of each independent predictor for all-cause 30-day mortality for three groups – all-patient population, separately for men and women, using univariate Cox regression analysis, and after adjusting for age, BMI and smoking status using multivariate Cox regression analysis (Table 2, A–C). Regardless of the group observed, a patient having any of the following factors – reanimation or mechanical

Table 2

Risk factors for 30-day mortality in all patients (A), men (B) and women (C) with acute pulmonary embolism (PE)

(A) All patients (n = 532)

Risk factor	*p*	Unadjusted-HR (95% CI)	*p*	Adjusted-HR* (95% CI)
Age	0.001	**1.026 (1.010-1.043)**		
Body mass index > 30 kg/m^2	0.393	0.760 (0.406–1.425)		
Smoking	0.432	0.784 (0.427–1.439)		
Arterial hypertension	0.470	1.186 (0.747–1.883)	0.256	1.561 (0.724-3.368)
DVT at presentation	**0.000**	**2.767 (1.580–4.846)**	**0.027**	**2.422 (1.107–5.300)**
Previous DVT	0.195	0.576 (0.250–1.328)	0.207	0.395 (0.093–1.674)
COPD	**0.022**	**2.015 (1.106–3.671)**	0.502	0.505 (0.069–3.717)
Chronic heart failure	**0.024**	**1.893 (1.087–3.295)**	0.114	1.942 (0.853–4.422)
Diabetes mellitus	**0.034**	**1.778 (1.044–3.028)**	**0.004**	**3.068 (1.426–6.601)**
Malignancy	0.073	1.704 (0.951–3.052)	0.135	1.858 (0.825–4.187)
Coronary artery disease	**0.007**	**2.317 (1.265–4.246)**	**0.010**	**3.031 (1.308–7.025)**
Stroke	**0.000**	**3.083 (1.658–5.734)**	0.057	2.472 (0.974–6.269)
Drugs	**0.001**	**2.235 (1.412–3.539)**	**0.005**	**2.821 (1.363–5.840)**
Heart rate > 100 bpm	**0.012**	**1.828 (1.141–2.931)**	**0.001**	**4.121 (1.776–9.561)**
Systolic BP < 100 mmHg	**0.000**	**4.886 (3.070–7.778)**	**0.003**	**2.994 (1.457–6.153)**
Anemia	0.388	1.238 (0. 762–2.011)	0.226	1.545 (0.764–3.125)
Glycaemia	**0.000**	**1.096 (1.053–1.140)**	**0.000**	**1.148 (1.084–1.215)**
Creatinine clearance (mL/min)				
> 60	**0.000**	**1.00[a]**	**0.008**	**1.00[a]**
< 60		**4.774 (2.864–7.960)**		**3.097 (1.336–7.180)**
Pneumonia	0.084	1.573 (0.940–2.631)	0.271	1.676 (0.668–4.210)
Leukocyte count	**0.012**	**1.021 (1.004–1.037)**	0.403	1.012 (0.984–1.089)
C–reactive protein	**0.000**	**1.006 (1.004–1.008)**	**0.001**	**1.006 (1.002–1.010)**
sPESI score				
0	**0.000**	**1.00[a]**	**0.011**	**1.00[a]**
> 0		**15.073 (3.697–61.455)**		**13.532 (1.815–100.864)**
RVSP > 40 mmHg	**0.000**	**4.352 (1.980–9.565)**	**0.004**	**8.592 (1.985–37.184)**
BNP > 100 pg/mL	**0.000**	**5.491 (2.182–13.822)**	**0.003**	**6.929 (1.975–24.315)**
Thrombolysis protocol				
fast systemic		**1.00[a]**		**1.00[a]**
slow systemic	**0.001**	**0.264 (0.123–0.564)**	**0.002**	**0.197 (0.069–0.559)**
local catheter	**0.011**	**0.073 (0.010–0.551)**	**0.040**	**0.111 (0.014–0.900)**
Reanimation	**0.000**	**17.647 (11.003–28.302)**	**0.000**	**37.811 (16.297–87.725)**
Mechanical ventilation	**0.000**	**20.893 (12.583–34.692)**	**0.000**	**77.120 (28.490–208.755)**
Surgery prior PE				
up to 3 weeks		1.00[a]		1.00[a]
3 weeks – 6 months	0.986	1.010 (0.308–3.311)	0.665	0.717 (0.160–3.223)
no	0.357	1.483 (0.641–3.428)	0.678	0.799 (0.277–2.304)
Immobilization four weeks prior PE	**0.004**	**2.197 (1.276–3.781)**	0.357	1.529 (0.619–3.780)
Risk groups for 30–day mortality				
low risk		**1.00[a]**		1.00[a]
intermediate–low	**0.033**	**9.520 (1.206–75.142)**	0.903	55,820.5 (0.000→∞)
intermediate–high	**0.003**	**20.701 (2.826–151.648)**	0.900	75,264.3 (0.000→∞)
high	**0.000**	**67.097 (9.164–491.271)**	0.891	22,8437.8 (0.000→∞)

Table 2 (continued)

(B) Men (n = 264)

Risk factor	*p*	HR (95% CI)
Age	0.058	1.023 (0.999–1.048)
Body mass index > 30 kg/m^2	0.196	0.387 (0.092–1.632)
Smoking	0.303	0.652 (0.289–1.472)
Arterial hypertension	0.660	1.168 (0.584–2.336)
DVT at presentation	**0.013**	**3.128 (1.274–7.684)**
Previous DVT	0.081	0.170 (0.023–1.246)
COPD	**0.000**	**4.031 (1.907–8.522)**
Chronic heart failure	0.083	2.028 (0.911–4.514)
Diabetes mellitus	0.142	1.875 (0.811–4.335)
Malignancy	0.117	2.033 (0.837–4.941)
Coronary artery disease	0.063	2.343 (0.954–4.062)
Stroke	**0.018**	**3.159 (1.215–8.211)**
Drugs	**0.027**	**2.187 (1.094–4.374)**
Heart rate > 100 bpm	0.563	1.227 (0.613–2.457)
Systolic BP < 100 mmHg	**0.000**	**8.365 (4.025–17.384)**
Anemia	0.108	1.819 (0.877–3.773)
Glycaemia	**0.003**	**1.098 (1.032–1.169)**
Creatinine clearance (mL/min)		
> 60	**0.000**	**1.00[a]**
< 60		**5.135 (2.426–10.868)**
Pneumonia	0.316	1.466 (0.694–3.095)
Leukocyte count	**0.000**	**1.186 (1.108–1.270)**
C–reactive protein	**0.000**	**1.007 (1.004–1.010)**
sPESI score		
0	**0.020**	**1.00[a]**
> 0		**38.550 (1.77–835.59)**
RVSP > 40 mmHg	**0.006**	**7.705 (1.821–32.610)**
BNP >100 pg/mL	**0.005**	**8.201 (1.916–35.094)**
Thrombolysis protocol		
fast systemic	**0.003**	**1.00[a]**
slow systemic		**0.168 (0.852–0.546)**
local catheter		**No male patients died with local catheter**
Reanimation	**0.000**	**19.138 (9.240–39.636)**
Mechanical ventilation	**0.000**	**25.689 (11.984–55.068)**
Surgery prior PE		
up to 3 weeks		1.00[a]
3 weeks–6 months	0.859	0.851 (0.142–5.091)
no	0.969	1.024 (0.311–3.375)
Immobilization four weeks prior to PE	0.142	1.947 (0.801–4.730)
Risk groups for 30–day mortality		
low risk		
intermediate–low		1.00[a]
intermediate–high	0.075	3.134 (0.893–10.999)
high	**0.000**	**13.070 (3.799–44.964)**

Table 2 (continued)

(C) Women (n = 268)

Risk factor	*p*	HR (95% CI)
Age	**0.017**	**1.029 (1.005–1.053)**
Body mass index > 30 kg/m^2	0.767	0.895 (0.432–1.857)
Smoking	0.647	1.115 (0.582–2.135)
Arterial hypertension	0.667	1.148 (0.613–2.149)
DVT at presentation	**0.010**	**2.576 (1.255–5.286)**
Previous DVT	0.791	1.135 (0.445–2.891)
COPD	0.635	0.752 (0.232–2.437)
Chronic heart failure	0.133	1.808 (0.835–3.915)
Diabetes mellitus	0.151	1.658 (0.831–3.310)
Malignancy	0.340	1.457 (0.673–3.154)
Coronary artery disease	**0.047**	**2.296 (1.011–5.214)**
Stroke	**0.009**	**2.955 (1.306–6.685)**
Drugs	**0.006**	**2.346 (1.270–4.336)**
Heart rate > 100 bpm	**0.004**	**2.609 (1.351–5.038)**
Systolic BP < 100 mmHg	**0.000**	**3.178 (1.721–5.865)**
Anemia	0.743	0.897 (0.468–1.718)
Glycaemia	**0.001**	**1.092 (1.037–1.150)**
Creatinine clearance (mL/min)		
> 60	**0.000**	**1.00[a]**
< 60		**4.459 (2.193–9.068)**
Pneumonia	0.101	1.815 (0.890–3.703)
Leukocyte count	0.505	1.008 (0.984–1.034)
C–reactive protein	**0.001**	**1.006 (1.001–1.009)**
sPESI score		
0	**0.008**	**1.00[a]**
> 0		**6.888 (1.663–28.529)**
RVSP > 40 mmHg	**0.024**	**2.966 (1.151–7.646)**
BNP >100 pg/mL	**0.035**	**3.616 (1.094–11.947)**
Thrombolysis protocol		
fast systemic		1.00[a]
slow systemic	0.064	0.383 (0.139–1.058)
local catheter	0.201	0.960 (0.032–2.061)
Reanimation	**0.000**	**16.153 (8.653–30.156)**
Mechanical ventilation	**0.000**	**17.753 (8.959–35.177)**
Surgery prior PE		
up to 3 weeks		1.00[a]
3 weeks – 6 months	0.842	1.176 (0.237–5.828)
no	0.227	2.439 (0.636–6.724)
Immobilization four weeks prior PE	**0.018**	**2.311 (1.158–4.613)**
Risk groups for 30–day mortality		
low risk		1.00[a]
intermediate–low	0.070	7.098 (0.855–58.961)
intermediate–high	**0.017**	**11.539 (1.540–86.444)**
high	**0.001**	**30.292 (4.015–228.559)**

Unadjusted and adjusted hazard ratios (HR) with 95% confidence interval (CI) were calculated using univariate and multivariate Cox regression model. *Adjusted for age, body mass index, and smoking. DVT – deep venous thrombosis; COPD – chronic obstructive pulmonary disease; BP – blood pressure; sPESI – simplified Pulmonary Embolism Severity Index; RVSP – right ventricular systolic pressure; BNP – brain natriuretic peptide; [a] – reference value.
Bolded values are statistically significant.

ventilation during hospitalization, systolic blood pressure < 100 mmHg, creatinine clearance < 60 mL/min, BNP level >100 pg/mL, RVSP > 40 mmHg, DVT at presentation, or using drugs predisposing to bleeding, had significantly increased chances of fatal outcome in the first month of the treatment. There was no difference in the all-cause mortality in men with admission heart rate > 100 beats/min comparing to men with heart rate < 101 beats/min (13.4% vs. 10.9%, respectively; $p = 0.576$). Still, women with the heart rate at admission > 100 beats/min had higher all-cause mortality rate within 30 days than women with lower heart rate (21.9% vs. 9.3%, respectively; $p = 0.006$) (Table 3). The same was for PE-cause mortality at 30 days. Elevated glycaemia and CRP level only slightly contributed to the 30-day mortality risk in each cohort. Simplified PESI score

> 0 appeared to be a strong predictor of fatality, especially in men, but with a wide confidence interval. PE presentation with previous stroke or elevated leukocyte count were found as mortality predictors, but lost the significance after adjusting for age, BMI and smoking. Tachycardia [in women HR 2.609 (1.351–5.038); $p = 0.004$] and coronary artery disease (CAD) [in women HR 2.296 (1.011–5.214); $p = 0.047$] influenced the outcome in all except the male group. The patients with chronic heart failure (CHF) and diabetes mellitus (DM) had 80% increase in mortality risk, but did not reach sex-specific statistical significance in predicting mortality due to their distribution between men and women. Age only slightly increased the chance of dying in the all-patient and female population. Extremity immobilizations within four weeks prior to PE appeared as independent predictors of early mortality in women [HR = 2.31 (1.16–4.61); $p = 0.018$]. Male patients with chronic obstructive pulmonary disease (COPD) ran the elevated risk of one-month mortality with HR of 4.03 (95% CI = 1.91–8.52; $p < 0.001$). Comparing with fast systemic thrombolysis, slow systemic protocols correlated with a reduction in 30-day mortality risk of up to 80% in men and all-patient cohort. We perceived a greater mortality risk reduction in patients treated with local catheter thrombolysis comparing to systemic protocols. Mortality risk in the all-patient group gradually decreased starting from the high, intermediate-high, and intermediate-low to the low-risk patients PE, but with wide confidence intervals. After adjusting for age, BMI and smoking, all confidence intervals reached zero value. Women classified in the intermediate-high or high-risk group bore significantly elevated mortality risk, whereas the increased chance of dying in men was found only in the high-risk group.

Table 3

All-cause death within 30 days in women and men according to heart rate at admission

Sex	Heart rate (bpm)		Total	Chi-square test (p)
	< 101	> 100		
Men	15/137	17/127	32/264	0.576
Women	13/140	28/128	41/268	0.006

bpm – beats per minute.

There was a statistically significant difference in the distribution of mortality rate in male patients according to tertiles of age ($\chi^2 = 9.104$; $p = 0.011$) (Figure 1). Men were dying more in their "third age" (67-88 years of age). Women who died in the third age tertiles (75 years or more) were more often high and intermediate-high-risk patients comparing to the age-matched men, but we could not perform a valid statistical analysis due to the small subgroup number of deceased patients. One-month survival within intermediate-risk group and high-risk group was not significantly different between men and women (Table 4).

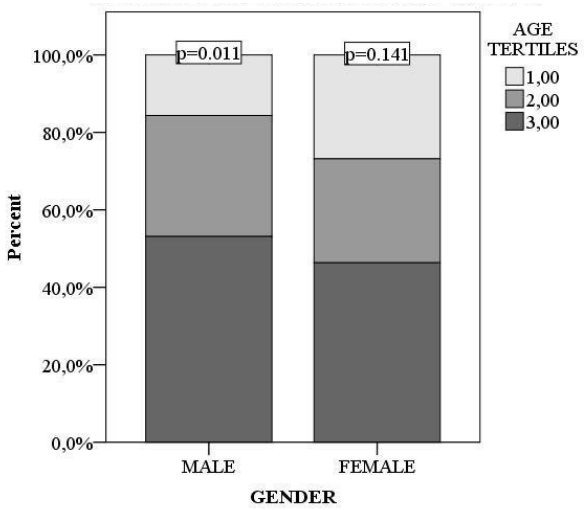

Fig. 1 – One-month mortality rate according to sex by age tertiles [(men: 1st tertile – 19–53 years; 2nd tertile – 54–66 years; 3rd tertile – 67–88 years); women: 1st tertile – 17-62 years; 2nd tertile – 63–74; 3rd tertile – 75–92 years)].

According to Kaplan Meier curve and Log rank test we recognised no significant sex-specific difference in survival time in the 30-day period (Log Rank test; $\chi^2 = 0.971$, $p = 0.324$) (Figure 2).

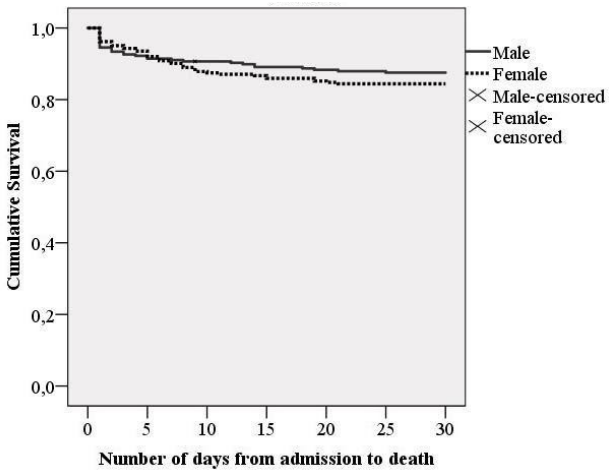

Fig. 2 – Kaplan-Meier one-month survival curve according to sex. Log rank test (Mantel-Cox) $\chi^2 = 0.971$, $p = 0.324$.

Table 4

One-month mortality based on the patient sex within intermediate-risk and high-risk groups

Death	Risk groups					
	intermediate-high			high		
	male	female	p	male	female	p
No	86 (86.9)	86 (82.7)	0.408	18 (52.9)	27 (62.8)	0.384
Yes	13 (13.1)	18 (17.3)		16 (47.1)	16 (37.2)	
Total	99 (100.0)	104 (100.0)		34 (100.0)	43 (100.0)	

Values are expressed as number (percentage).

Discussion

Management of acute PE patients depends primarily on the clinical presentation, based on the 2014 ESC model [4], but also on the patient and setting-related predisposing factors for thrombosis and bleeding. In this study, we assessed the sex-specific risk factors for all-cause 30-day mortality. Our total 30-day mortality rate was 13.7%, which corresponds to the previous bibliography data [1, 2]. Survival time in both patient sexes was almost identical (Log Rank test; $\chi^2 = 0.962$, $p = 0.327$). The first hospital day was fatal in one third of deceased patients, which additionally stresses the importance of prompt mortality risk stratification and appropriate treatment. The mean length of stay matched the mean LOS reported in one Italian real-life study [15].

According to the 2014 ESC model for 30-day mortality risk stratification [4], 77 (14.5%) of patients of our study population were classified as having high, 203 (38.1%) intermediate-high, 126 (23.7%) intermediate-low and 126 (23.7%) low risk, similar to the published research by Becattini et al. [6]. Interestingly enough, we discovered one-month mortality rate to be very high among high-risk (41.6%) and intermediate-high-risk patients (15.3%) in comparison with Becattini et al. [6] (22% and 7.7%, respectively). Comparing to women, we did not find the increased risk of dying in men of intermediate-high risk group, probably due to different sex-specific distribution in the low-risk group (no male patients died in the low-risk category). Some additional analysis of our population is necessary to discover the causes of higher death rate in these specific strata of patients.

We found that the heart rate of more than 100 beats/min increased the chance of death in the one-month period in women and in the total study population after adjusting for age, BMI and smoking status. Masotti et al.[15] reported that sPESI score had better predictive ability of all-cause in-hospital mortality in women than in men. In our study, no sex-specific differences were noticed in terms of sPESI score, even though it incorporates heart rate > 110 beats/min. Perhaps analyzing antiarrhythmic drug usage, ie. beta-blockers, would be of interest to assess its potential impact on the PE clinical presentation and prognosis. The increase in admission glycaemia level only slightly raised the chances of early death in our population. Scherz et al. [16] found that cumulative probability of 30-day mortality increased with the rise in blood glucose level at admission in PE patients without diabetes, but not in diabetic PE population. Nevertheless, we did not compare blood glucose level between diabetics and non-diabetics. We must note that the well-known risk factors for developing PE – surgery, previous DVT, malignancy, pneumonia, arterial hypertension and obesity – were not determined as mortality predictors in our study. In the International Cooperative Pulmonary Embolism Registry (ICOPER) study, malignancy was one of the best predictors of death both in men and women [3].

The population of advanced age were more frequently women, especially those older than 55 years, as previously reported [12], contrary to the ICOPER study where the elders were more often men [3]. Women had higher incidence of arterial hypertension related to age. Natural aging process results in atherosclerosis progression and development of multiple cardiovascular diseases. Paradoxically, women's death rate was not significantly rising with age, as we would expect and as we found in the men of the "third age". So, what protected women from PE in younger age? A hypothetical explanation could be linked to female hormonal protective mechanisms in reproductive period [17], but human trials did not investigate the impact of endogenous estrogen levels on venous thrombosis risk. Some findings suggest that lower endogenous estradiol levels in perimenopausal women were associated with higher levels of plasminogen activator inhibitor-I (PAI-I) and tissue plasminogen activator and higher cardiovascular risk [18]. Correspondingly, as established in thromboelastographic studies, the whole blood coagulability trend increases from men, from non-pregnant to pregnant women [19]. On the other hand, what contributed to fatal outcome in younger women remains uncertain. This requires certain further analyses of all factors based on the age groups in the future.

We found higher BMI in women, as expected [12], which may be coupled with lower physical activity and consequent VTE. Adiposity produces significantly higher levels of estrogen, fibrinogen, prothrombin, CRP, plasminogen activator inhibitor-I (PAI-I) and microparticles which all in part assist in the clot formation [20, 21]. Nonetheless, venous thrombosis risk can not be estimated only basing it on the BMI since metabolic profile substantially differs in people having identical BMI depending on adipose tissue distribution [20]. Unfortunately, we could not provide more information on the socioeconomic state and lifestyle. As previously mentioned, obesity was not connected with increased mortality rate in our population.

Interestingly enough, COPD increased the chance of fatal outcome in men fourfold ($p < 0.001$). Borrero et al. [22] also found chronic lung disease associated with higher mortality rate in men. Leukocyte count at admission and CRP levels did not differ considerably. Opposite to the fact that the female population more often required mechanical ventilation, men were more frequently smokers and had pneumonia in the first two days from admission. Community-acquired pneumonia did not influence mortality rate, but could have aggravated the clinical course of COPD patients. Considering that we could not extract the indications for mechanical ventilation from the Registry, we may only hypothesize that the underlying condition was heart failure as women presented more often with elevated BNP. Yet, higher levels of BNP could partly be a result of the lower estimated creatinine clearance measured in women. Naturally, women being older already had renal dysfunction as creatinine clearance declines over lifetime [13]. All these parameters: mechanical ventilation, elevated BNP level and creatinine clearance < 60 mL/min, were found to predict one-month fatality in both sexes. Women had lower admission hemoglobin level, as expected according to the anemia global prevalence sex distribution [23], but that did not increase their mortality risk. Other publications on the acute PE

patients also listed women to be more aged, with lower creatinine clearance and hemoglobin level [9, 15].

The patients treated with slow systemic thrombolysis protocols and local catheter-directed thrombolysis had better survival rate comparing to those receiving fast systemic protocols. This could be explained by the disease severity (i.e. cardiogenic shock) when the fast protocol as a salvage therapy is mandatory. However, we did not assess these protocols by patient risk-stratification groups.

Apart from the aforementioned and retrospective nature of the study, there are several more limitations in the analysis. Weighing mortality risk demanded some additional severity stratification of some conditions, such as COPD, chronic heart failure and malignancy stage. Echocardiographic parameters of right ventricular function were not always fully available (ie. tricuspid annular plane systolic excursion, pulmonary valve acceleration time, right atrium area, etc.) and we limited statistical analysis to the RVSP value alone. Equally important, the database currently could not supply more facts about the left ventricular heart function, i.e. left ventricular diastolic function. Echocardiography is of special interest in intermediate risk groups as these patients require closer monitoring and timely recognition of potential hemodynamic compromise with prompt and repetitious assessment for introducing thrombolysis in treatment [4]. Furthermore, we did not compare patients by medications and their dosage, ie. thrombolytic agents and anticoagulation (unfractioned heparin, low-molecular-weight heparin and direct oral anticoagulants).

Conclusion

We found that significant differences in sex-specific characteristics of the PE population were advanced age, presence of comorbidities – obesity, arterial hypertension, renal insufficiency, anemia, pneumonia and smoking history. Tachycardia, age, coronary artery disease and limb immobilization four weeks prior to PE in women, whereas COPD and elevated leukocyte count in men were associated with higher chance of all-cause 30-day mortality. Nevertheless, patient's sex alone did not predict the outcome. More research on mortality risk factors should help improve recognition and management of PE patients, especially those in high and intermediate-high risk of early mortality.

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Physical activity and eating habits of students of the University of Belgrade: An epidemiological study

Fizička aktivnost i navike u ishrani studenata Univerziteta u Beogradu: epidemiološka studija

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Abstract

Background/Aim. Numerous studies have indicated that university students exhibit a variety of risky health behaviors, such as the lack of regular physical activity and unhealthy eating habits. Due to lack of information about students' lifestyles in Serbia, we designed a study with the following aims: 1) to investigate physical activity and eating habits variations of students according to gender, year of study and attended faculty; 2) to estimate the relationship between their physical activity and eating habits. **Methods.** The survey method was used. Data were collected in the 2016/2017 school year during the spring semester. The sample was randomly stratified (4,019 students of the University of Belgrade – 2,284 males), which corresponded to about 5% of the whole student population of the University of Belgrade. For data collection, two standardized questionnaires were used: International Physical Activity Questionnaire (IPAQ) and Youth/Adolescent Food Questionnaire (YAQ). **Results.** Male students were significantly more highly and moderately active than female students [3.15 vs. 2.80 days, respectively; $p = 0.003$, effect size (ES) = 0.651]. Correspondingly, female students were less active during the day (326 vs. 305 min; $p = 0.044$, ES = 0.232). Interaction between factors revealed that the most active on a weakly basis were fourth-year male students of the natural sciences (4.20 days), while on a daily basis, the most active were first-year female students (161 min). The most sedentary ones were third-year female students of medical sciences (475 min of sitting per day). Significant correlations were found between the duration of intense physical activity and consuming water, vegetables, citrus fruits, integral bread, and cereals. **Conclusion.** Female students were more prone to sedentary behavior, while older students were more active and aware of the importance of physical activity. Furthermore, the nature of studies can influence the lifestyle. Female and older students were more concerned about diets. Additionally, students who were more physically active had healthier eating habits.

Key words:
attitude to health; exercise; feeding behavior; healthy lifestyle; serbia; students; sex factors; surveys and questionnaires.

Apstrakt

Uvod/Cilj. Brojne studije su pokazale da studenti pokazuju niz rizičnih zdravstvenih ponašanja, kao što su nedostatak redovne fizičke aktivnosti i nezdrave navike u ishrani. Zbog nedostatka informacija o životnom stilu studenata u Srbiji, dizajnirali smo studiju sa sedećim ciljevima: 1) ispitati fizičku aktivnost i varijacije u ishrani studenata u zavisnosti od pola, godine studija i upisanog fakulteta; 2) proceniti odnos između njihove fizičke aktivnosti i navika u ishrani. **Metode.** Korišćen je metod anketiranja. Podaci su prikupljeni u školskoj 2016/2017. godini tokom letnjeg semestra. Uzorak je nasumično stratifikovan (4 019 studenata Univerziteta u Beogradu – 2 284 muškarca), što je odgovaralo približno 5% od ukupne studentske populacije Univerziteta u Beogradu. Za prikupljanje podataka korišćeni su upitnici Međunarodni upitnik fizičke aktivnosti (IPAQ) i Upitnik za hranu za mlade/adolescente (YAQ). **Rezultati.** Studenti muškog pola su bili statistički značajno više umereno i intenzivno aktivniji od studentkinja [3,15 vs. 2,80 dana, $p = 0,003$; *effect size* (ES) = 0,651]. U skladu s tim, studentkinje su bile manje aktivne tokom dana (326 vs. 305 min; $p = 0,044$; ES = 0,232). Najaktivniji na sedmičnom nivou bili su studenti četvrte godine prirodno-matematičkih nauka (4,20 dana), dok su na dnevnom nivou to bili studenti prve godine (161 min).

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Studentkinje treće godine medicinskih nauka provodile su najviše vremena sedeći (475 min dnevno). Značajne korelacije su utvrđene između intenzivne fizičke aktivnosti i konzumacije vode, povrća, citrusnog voća, integralnog hleba i žitarica. **Zaključak.** Studentkinje su bile sklonije sedentarnom načinu ponašanja, a stariji studenti su bili aktivniji i svesniji važnosti fizičke aktivnosti. Takođe, priroda studija može imati uticaja na životni stil. Studentkinje i stariji studenti su se više brinuli o načinu ishrane. Studenti koji su bili fizički aktivniji imali su zdravije navike u ishrani.

Ključne reči:
stav prema zdravlju; vežbanje; ishrana, navike; zdravlje, način života; srbija; studenti; pol, faktor; ankete i upitnici.

Introduction

Physical activity and diet are very important aspects of lifestyle that influence the risk of serious diseases, such as cancer [1] and coronary heart disease [2]. The World Health Organization (WHO) recommends a minimum of 150 min of aerobic physical activity of moderate-intensity or at least 75 min of the activity of vigorous-intensity throughout the week, for adults aged 18–64 years [3]. Numerous significant positive correlations have been found between low levels of physical activity and various health outcomes, such as cardiovascular diseases [4], obesity and diabetes [5], and even impaired cognitive performance [6]. Although it has been shown that children and youth are usually most active [7], there is evidence that the decelerating trend is present in the population that is physically active with advancing age [8]. The major decline of this trend occurs during young adulthood [9, 10]. Additionally, the dietary habits of young adults are affected by the fast-food market and, consequently, obesity and overweight are increased among them [11].

The population that is especially important for this concern are students, since the youth transiting from school to university has many health implications [12]. Numerous studies have indicated that university students exhibit a variety of risky health behaviors, such as the lack of regular physical activity [13–16], along with unhealthy eating habits [12, 17, 18]. Different results have been obtained regarding the students of different ages [17, 19, 20], gender [17, 21] and those that attended faculty [21–23]. Furthermore, previous studies have revealed different physical activity patterns and eating habits between students from different geographical areas, such as Europe [12], the USA [17, 24], Africa [18, 25], Japan and Korea [26], etc. Moreover, the differences were revealed among the different parts of Europe, such as the Mediterranean [22, 23, 27, 28], Central [29, 30] and Scandinavian part [31]. Nevertheless, there are only a few studies on physical activity and eating habits of students from the Balkan countries [20, 21, 28, 32]. The authors of one cross-sectional study, tried to investigate the relationship between the body mass index (BMI) and eating habits of 1,624 students of the University of Belgrade. Their results revealed that every fourth male student was overweight, and that students' BMI did not correlate with the frequency of taking breakfast [32].

Due to the lack of information on students' lifestyles in Serbia, we designed a study with the purpose of investigating their physical activity and eating habits. Therefore, the aim of the present study was to evaluate physical activity and eating habits of students from the University of Belgrade. Moreover, the specific aims were to describe and examine physical activity and eating habits variations according to gender, year of study and attended faculty, as well as to investigate the relationship between physical activity patterns and eating habits.

Methods

The present study had a cross-sectional design; the survey research method was used.

Participants

Students from all scientific groups from the University of Belgrade were included in the study. The University of Belgrade was selected, because it is the oldest and the largest educational institution in Serbia. At the beginning of the research process, we recruited 4,200 undergraduate students. Nevertheless, 4,019 students regularly filled out questionnaires, and, therefore their answers were further analyzed. The sample of the present study symbolized a representative sample, because it corresponded to about 5% of the whole student population of the University of Belgrade. Moreover, the same percentage of students (5%) from each faculty participated in the research. Therefore, the present study had a randomly stratified sample.

Out of all the students who had participated in the research, 42.4% (n = 1,703) were studying socio-humanistic sciences (S-HS), 33.4% (n = 1,344) technical-technological sciences (T-TS), 14.1% (n = 566) medical sciences (MS), and 10.1% (n = 406) natural-mathematical sciences (N-MS). Moreover, 21.7% (n = 874) were students of the first year, 29.8% (n = 1,197) of the second year, 25.3% (n = 1,016) of the third year, and 23.2% (n = 932) students of the fourth (ie. last) year of the studies.

Note that physical education students were excluded from the study. The reason was the nature of the classes and the students who enroll in them. Namely, monitoring the level of physical activity and eating habits of 'regular' students would be somewhat confounded by monitoring physical education students, who are supposed to be more aware of the importance of physical activity and healthy eating habits. The criteria for selecting the participants were as follows: students of undergraduate/integrated academic studies, attending faculties from certain educational and scientific fields in each year of study, voluntariness and successful completion of the questionnaires. The study was conducted in accordance with the Declaration of Helsinki

and all participants signed an informed consent approved by the Institutional Review Board (No. 02-766/19-1).

Instruments

Two standardized questionnaires were used in the present study: the International Physical Activity Questionnaire (IPAQ) [33] and Youth/Adolescent Food Questionnaire (YAQ) [34]. The IPAQ involved a total of 7 questions and assessed the overall level of physical activity by collecting information on the number of days and duration of low, moderate and high-intensity physical activity, as well as the duration of sedentary behavior during the working days in the last 7 days. On the other hand, the YAQ included a total of 28 questions related to eating habits (in terms of the type and frequency of consumed food) of young people and adolescents in the previous 6 months.

Procedure

The data were collected in the 2016/2017 school year, during the spring semester (from April to June 2017). Data collection was conducted at each faculty individually during regular classes. Researchers handed out the questionnaires and explained the procedure to all participants. Additionally, students were informed that their participation was voluntary and that their responses would be held in strict confidence. They filled out the questionnaires and returned them straight away. The questionnaires were completed in half an hour, on average.

Statistical analyses

In order to analyze the assessed data, we used Statistical Package for the Social Sciences (SPSS) for Windows, version 25 (IMB Corporation, Chicago, IL, USA). Descriptive statistics of the prevalence of certain students' life habits, such as frequency and level of physical activities along with the use of certain diets, were presented as percentage values, as well as, means and standard deviations. The significance of the differences between the groups of physical activity variables was tested by multiple analysis of variance (ANOVA) with Bonferroni post-hoc comparison in order to analyze differences in physical activity indicators by gender, year of study and attended faculty. Cohen's *d* was also calculated with ANOVA, where the effect sizes (ES) 0.2, 0.5, and above 0.8 were considered as small, medium and large, respectively [35]. In order to interpret eating habits by the YAQ, we applied Cronbach's alpha (α), which has shown high internal consistency of the Likert scale ($\alpha > 0.75$). Subsequently, comparisons between the different genders were tested by the Mann-Whitney *U* test, while factors like the year of study and attended faculty were tested by independent samples of the Kruskal-Wallis test. In order to assess the relationship between two variable groups (ie. physical activity and eating habits), we initially created two groups regarding WHO physical activity recommendations (the group that meets and that does not meet the recommendations; see the Introduction part). In addition, we applied the χ^2 test on two categorical variables (nutrition question and physical activity group). Furthermore, we used the Spearman correlation analyses. The level of significance was set *a priori* at $p < 0.05$.

Results

Physical activity

Multivariate ANOVA revealed the gender, year of study and attended faculty as significant factors. Moreover, the interaction between the factors like the year of study and the faculty was also significant ($p < 0.05$). Additional application of the Bonferroni post-hoc test revealed the direction of the assessed differences. Table 1 shows the differences in physical activity patterns between male and female students.

Table 1

Physical activity and sedentary behavior variables labeled as average values (IPAQ questionnaire)

Variables	Mean ± SD	*p*-value	ES
High physical activity (days)			
male	3.15 ± 0.58	0.003	0.651
female	2.80 ± 0.49		
High physical activity per day (min)			
male	111 ± 68	0.267	0.099
female	105 ± 52		
Moderate physical activity (days)			
male	3.56 ± 0.96	0.010	0.280
female	3.31 ± 0.82		
Moderate physical activity per day (min)			
male	86 ± 23	0.788	0.100
female	84 ± 16		
Low physical activity (days)			
male	4.89 ± 1.65	0.919	0.011
female	4.87 ± 1.89		
Low physical activity per day (min)			
male	159 ± 54	0.054	0.335
female	144 ± 33		
Sedentary behavior per day (min)			
male	305 ± 88	0.044	0.232
female	326 ± 93		

IPAQ – International Physical Activity Questionnaire; SD – standard deviation; ES – effect sizes.

The results revealed that students of the fourth year were on average more highly active than students of the first and second year, during the day (124 min, 100 min, 103 min, respectively; $p = 0.028$, ES = 1.205 and $p = 0.049$, ES = 1.227, rspectively). Moreover, the oldest students were more moderately active during the day than the

students of the second and third year (103 min, 69 min, 73 min, respectively; $p = 0.001$, ES = 1.446 and $p = 0.005$, ES = 1.116, respectively). Similarly, the first-year students were more moderately active in comparison with the students of the second and third year throughout the day (99 min, 69 min, 73 min, respectively; $p = 0.004$, ES = 2.208 and $p = 0.022$, ES = 1.518, respectively), while there was no significant difference between first- and fourth-year students. Regarding low physical activity on a weekly basis, first-year students were more active than third-year students (5.23 days vs. 4.49 days, respectively; $p = 0.003$, ES = 1.048). Conversely, students of the third year were less active throughout the day than first-year students, as well as the second-year students (174 min, 144 min, 139 min , respectively; $p = 0.034$, ES = 0.524 and $p = 0.004$, ES = 1.227, respectively). Lastly, second-year students spent significantly more time during the day in sedentary behavior, in comparison to fourth-year students (334 min vs. 291 min, respectively; $p = 0.012$, ES = 0.501).

Regarding the attended faculty, the results revealed that S-HS students were weekly less highly active than N-MS, MS and T-TS students (2.43 days, 3.34 days, 2.92 days, 3.56 days, respectively; $p = 0.000$, ES = 4.775; $p = 0.038$, ES = 2.419 and $p = 0.000$, ES = 5.777, respectively). Moreover, they were daily less highly active than MS and T-TS students (94 min, 117 min, 120 min, respectively; $p = 0.046$, ES = 1.835 and $p = 0.000$, ES = 3.571, respectively). Similarly, S-HS students were less moderately active on a weekly basis than N-MS and T-TS students (3.20 days, 3.77 days, 3.64 days, respectively; $p = 0.010$, ES = 0.669 and $p = 0.001$, ES = 0.516, respectively). Surprisingly, S-HS students were the most moderately active on a daily basis and significantly more active than T-TS students (98 min vs. 69 min; $p = 0.001$, ES = 1.636). Although there were no significant differences among groups, S-HS students were the most active on a weekly basis (5.01 days), while N-MS students were the most active on a daily basis (175 min). T-TS students spent the most time during the day in sedentary behavior, even significantly more than S-HS (331 min vs. 294 min, respectively; $p = 0.007$, ES = 0.564).

The interaction between factors revealed that the most highly active on a weekly basis were fourth-year male students of N-MS (4.20 days), while the most active on a daily basis were first-year female N-MS students (161 min). The most sedentary ones were third-year female students of MS (475 min of sitting per day).

Eating habits

Students reported eating 3.03 (± 1.38) times a day on average. Regarding consumption of vegetables, most students (35.4%) reported eating them once a weak; there were no differences between male and female students. Note that around 30% of them consumed vegetables 2–4 times a week, while only 0.6% of students were eating vegetables on a daily basis. Nevertheless, female students reported more frequent consumption of citrus fruits than male students did ($p = 0.003$). They consumed it between one (38.7%) and 2–4 times a week (21.6%). Furthermore, the Mann-Whitney *U* test revealed that male students consumed lamb or pork meat more often (26.5% of them consuming 2–4 times a week) than female students did (31.6% less than once per week; $p = 0.002$).

Second-year students reported a significantly more frequent intake of vegetables than third-year students ($p = 0.043$), while it was the other way around when it comes to citrus ($p = 0.039$) and other fruits ($p = 0.004$). Correspondingly, second-year students were drinking fruit juices (28% of students less than once per week) less frequently than the students of the first, third and fourth year of studies ($p = 0.041$; $p = 0.028$; $p = 0.001$, respectively). The youngest students were drinking water more often than third and fourth year students did ($p = 0.001$; $p = 0.014$, respectively). On the other hand, they reported a more regular intake of full-fat dairy products than third-year students did ($p = 0.032$). Over 11% of first-year students eat these products more than 2 times a day. Furthermore, the oldest students reported the most frequent consumption of fish and seafood (25.9% of third-year and 24.3% of fourth-year students eat fish and seafood 2–4 times a week), significantly more than second-year students did ($p = 0.015$; $p < 0.01$, respectively). Attention-grabbing fact is that first- year students reported the most frequent consumption of candies (significantly more than second-year students, $p = 0.031$) and fried food (more than second-year, $p = 0.010$ and third-year students, $p = 0.039$). Consequently, they wanted to change their eating habits the most, while that trend was descending with the older students.

MS students drank water more often than S-HS and N-MS students ($p = 0.004$; $p = 0.038$, respectively). Surprisingly, MS students reported more frequent consumption of full-fat dairy products, putting salt in food, and consequently, they wanted to change their eating habits more than the rest of the students.

Correlation between physical activity and eating habits of examined students is presented in Table 2.

The Spearman correlation analysis revealed a significant correlation between the time that the students were highly active during the week and their consumption of vegetables ($r = 0.112$, $p < 0.01$). Moreover, these students reported frequently eating citrus fruits ($r = 0.069$, $p < 0.01$), integral bread and cereals ($r = 0.108$, $p < 0.01$), surprisingly candies ($r = 0.113$, $p < 0.01$) and drinking water ($r = 0.098$, $p < 0.01$). Consequently, they did not want to change their eating behavior ($r = -0.069$, $p < 0.01$). Contrariwise, students with sedentary behavior more frequently put salt in their food ($r = 0.043$, $p < 0.01$), consumed potatoes ($r = 0.046$, $p < 0.01$), margarine ($r = 0.085$, $p < 0.01$), white flour products ($r = 0.032$, $p = 0.04$) and commercial beverages ($r = 0.064$, $p < 0.01$). These students also reported eating less integral bread and cereals ($r = -0.058$, $p < 0.01$), drinking less water ($r = -0.035$, $p = 0.029$) and wanting to change their eating habits ($r = -0.041$, $p < 0.01$).

Table 2

Relationship between students' nutrition choice (YAQ questionnaire) and daily physical activity level (regarding WHO recommendations)

Food choice	χ^2 value	df	*p*-value
Green leafy vegetables	18.293	4	0.001
Broccoli, cauliflower, cabbage	8.708	4	0.069
Carrot	27.483	5	0.000
Potatoes (not chips, French fries)	17.772	4	0.001
Other vegetables (not including those mentioned above)	3.024	5	0.696
Beans, green beans, lentils	9.296	5	0.098
Citrus fruits (not counting 100% juices)	4.999	4	0.287
Other fruits (not including citrus fruits)	23.800	4	0.000
100% fruit juices	9.667	5	0.085
Plain water (not including other beverages)	23.813	5	0.000
Full fat dairy products (milk, hard cheese, butter, ice cream)	29.421	5	0.000
Low fat dairy products (e.g. skimmed milk, yogurt, young cheese)	32.000	5	0.000
Eggs	3.439	4	0.487
Lamb, pork or mutton	30.254	4	0.000
Meat products (e.g. sausages, salami, hot dogs, bacon)	30.436	4	0.000
Chicken or turkey	19.396	4	0.001
Fish and seafood (not fried but boiled, baked or preserved)	6.231	4	0.183
Margarine	16.792	4	0.002
White flour products (eg. white bread, white rice)	11.224	4	0.024
Integral bread and cereals (oats, brown rice, groats, barley)	2.428	4	0.658
Sweets (muffins, donuts, cakes, pastries)	15.805	4	0.003
Commercial beverages (eg. cola beverages, sweetened teas, soda beverages; not counting dietary unsweetened beverages)	24.246	5	0.000
Fried foods	52.092	4	0.000
Frequency of salting food	7.212	5	0.205
Frequency of breakfast	8.130	4	0.087
Frequency of daily meals	19.855	5	0.001
Desire to change diet	23.203	4	0.000
Ability to change diet	35.561	5	0.000

YAQ – Youth/Adolescent Food Questionnaire; WHO – World Health Organization.

Discussion

Regarding physical activity, students were, on average, highly active for three days a week. Moreover, male students were significantly more highly and moderately active than female students. This is in line with the Croatian students, where males exercised more than females (4.4 h/week vs. 1.6 h/week) [28]. Correspondingly, female students spent significantly more time in sedentary behavior during the day. These findings are in accordance with the results of a few studies [36, 37], but not with the study, in which male students, despite being more active, reported spending more time in sedentary behavior [24]. Furthermore, the results revealed that fourth-year students were the most highly and moderately active during the day, while third-year students were mostly involved in low daily physical activities. The most sedentary ones were second-year students. Similar results were found at the University of Novi Sad, where, in addition to the higher activity of older students, 56.4% of students were physically active, while 52.1% of students spent 2 h and more in front of the TV and computer [20]. These results suggest that students' awareness of the importance of physical activity is perhaps changing over time. Thus, the results are very encouraging, especially because they are not in line with the decelerating trend with advancing age, which is present in the population regarding physical activity [8–10]. Additionally, the results showed that students of S-HS were the least highly active throughout the week and during the day. Likewise, they were least moderately active during the week, but surprisingly, the most moderately active on a daily basis. We are sure, that the results would be somewhat different, if the physical education students were included in this group. In order to assess objective information about the students' level of physical activity, we excluded the mentioned students. Nevertheless, the most highly and moderately active were the students of N-MS. Students of T-TS mostly showed sedentary behavior. We can only speculate that the main reason for these findings was the nature of the studies (ie. a lot of drawing and table paperwork that requires sitting).

Students reported eating 3.03 times per day, which is comparable with the study of Colić Barić et al. [28] who showed that Croatian students had 2.4 meals and 1.3 snacks per day. That fact points out a slightly "healthier" lifestyle.

The results of interaction between factors also confirmed the previous results. Namely, the most highly active on a weakly basis were fourth-year male students of N-MS (4.20 days a week on average), while the most active on a daily basis were first-year female students of N-MS (161 min per day). The most sedentary ones were third-year female students of MS (475 min of sitting per day). This can be explained by the fact that MS are considered as the "hardest" studies at the university, and consequently, they

require a lot of learning time. Therefore, it could affect the physical activity patterns and lifestyle of an individual.

Unlike for physical activity, female students were more focused on eating habits than male students. Specifically, they reported frequent consumption of citrus fruits, while male students ate lamb or pork meat more often. Similar results were found in population of medical students of the University of Bialystok [29] and also in Greek students, where males were more physically active, while females had healthier eating habits [21]. Moreover, in the study of Lowry et al. [17], female students were more likely to use diet, than physical activity for weight control.

In accordance with the physical activity lifestyle patterns, first-year students reported the most frequent consumption of candies, fried food and full-fat dairy products. Accordingly, they had the greatest desire to change their eating habits, while that trend was descending with age. This is understandable, given the fact that the oldest students ate fish and seafood significantly more than the other students did.

At first glance, MS students showed more responsibility regarding eating habits, since the results revealed that they drank water more often than the rest of the students. Suprisingly, however, they reported more frequent eating of full-fat dairy products, putting salt in food, and, consequently, they had the greatest desire to change their eating habits than the rest of the students. Similar findings were made in Greece with medical students whose regular diet contained excessive quantities of saturated fat, cholesterol and sodium [22]. Conversely, medical students from the Beijing University reported exceptionally healthy eating habits [26]. It seems that cultural and traditional environment has a greater impact than the attending faculty regarding eating habits.

In order to investigate the relationship between physical activity patterns and eating habits, we used the χ^2 test and additionally the Spearman correlation analysis. The χ^2 test revealed that the group that meets WHO recommendations for daily level of physical activity consumed more green vegetables, carrots, potatoes, fruits, drank plain water, full and low dairy products, meat products, margarine and surprisingly white flour products, commercial beverages and fried foods. In addition, they had a higher frequency of daily meals, but also wanted to change their diet. The Spearman correlation analysis revealed significant correlations between the time being highly active and consuming water, vegetables, citrus fruits, integral bread, and cereals. Surprisingly, highly active students reported frequent candy consummation. Expectedly, a significant negative correlation existed with the attitude to change their eating behavior. Conversely, significant positive correlations were obtained between the sedentary time and eating salty food, consuming potatoes, margarine, white flour products and commercial beverages. Moreover, negative correlations were revealed regarding drinking water, eating integral bread, cereals, and the attitude to change eating habits. Note that, regardless of the relatively small correlation coefficients, the observed relationships between physical activity and eating habits were significant, which means that the probability of obtaining such a correlation by chance is minor. On the other hand, note that the nature of compared variables (ordinal vs. categorical) contributed to the obtained results. Thus, it must be taken into account, at least as a qualitative parameter of the analysis.

Limitations

The main drawback of the present study was the fact that only questionnaires were used to assess the level of physical activity and eating habits of the students. There are objective methods for assessing the level of physical activity, such as accelerometry [38], pedometry [39] and double-labeled water [40]. Hence, a directive for future research could be perhaps, using one of the objective methods in order to assess the more precise level of physical activity of the student population.

Besides, it should be noted that the Spearman correlation coefficients provided us with more qualitative than quantitative information, because of the nature of the compared variables (ordinal scale vs. categorical scale). Thus, the results should be interpreted bearing this in mind.

Conclusion

The results of the present comprehensive epidemiologic study on a large representative sample, revealed very important information on the physical activity and eating habits of the academic elite in Serbia. The main results regarding physical activity showed that female students were more likely to incline sedentary behavior, older students were more aware of the importance of physical activity, and that the nature of studies can be connected to students' lifestyles. Regarding eating habits, female students were more concerned about diets. Moreover, older students showed more responsibility regarding this issue. Additionally, correlation analyses showed that students, who were more physically active, had healthier eating habits.

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Mathematical procedure for prediction of dental anxiety in children with inherited bleeding disorders

Matematička procedura za predviđanje dentalne anksioznosti kod dece sa urođenim poremećajima koagulacije

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Abstract

Background/Aim. The dental anxiety in children is commonly measured applying the Dental Anxiety Scale (DAS) and the level is statistically treated. However, there are no studies on prediction of development of dental anxiety. The aim of this study was to develop a mathematical procedure for dental anxiety prediction based on the statistical values. **Methods.** Mathematical relation between the dental anxiety and aging was developed using the interpolation method based on the statistical values. It was supposed that the anxiety level is a polynomial function of the age. The obtained function was tested on the healthy children and those with inherited bleeding disorders. By mathematical fitting procedure using statistical data, the analytical relation for prediction of dental anxiety as a function of aging was developed. **Results.** According to prediction function, it was found that for healthy population the dental anxiety has the tendency to increase quite slowly or to remain almost constant after the age of 18. In the patients with inherited bleeding disorders, the DAS score would increase even after age of 18. The treatment of the anxiety in children with inherited bleeding disorders is optimal at about the age of 12 when they become aware of their illness. **Conclusion.** The introduced prediction function gives the tendency of DAS score development which enables timely adequate action in reducing dental anxiety. The predicted DAS score may be used as a control measure of oral health.

Key words:
blood coagulation disorders, inherited; forecasting; child; dental anxiety; models, theoretical.

Apstrakt

Uvod/Cilj. Dentalna anksioznost kod dece meri se primenom skale dentalne anksioznosti (DAS), a nivo se statistički obrađuje. Međutim, ne postoje istraživanja koja predviđaju razvoj dentalne anksioznosti. Cilj rada je bio da se razvije matematička procedura za predviđanje dentalne anksioznosti na osnovu statističkih podataka. **Metode.** Matematička relacija između dentalne anksioznosti i godina starosti je razvijena korišćenjem interpolacione metode statističkih veličina. Pretpostavljeno je da je nivo anksioznosti polinomijalna funkcija godina starosti. Izračunata funkcija je testirana na zdravoj deci i na deci sa urođenim poremećajima krvarenja. Postupkom matematičkog prilagođavanja statističkih podataka, razvijena je analitička relacija za predikciju dentalne anksioznosti kao funkcije životne dobi. **Rezultati.** Na osnovu funkcije predviđanja zaključeno je da je porast dentalne anksioznosti kod zdrave populacije veoma spor ili nepromenljiv posle 18. godine života. Kod dece sa koagulopatijom, vrednosti DAS skora rastu i posle 18. godine života. Lečenje anksioznosti kod dece sa urođenim poremećajima krvarenja je optimalno u uzrastu od 12 godina, kada ona postaju svesna svoje bolesti. **Zaključak.** Navedena funkcija predviđanja pokazuje tendenciju razvoja vrednosti DAS skora koja omogućava pravovremeno lečenje u cilju smanjenja dentalne anksioznosti. Predviđena vrednost DAS skora može se koristiti kao kontrolni parameter oralnog zdravlja.

Ključne reči:
poremećaji koagulacije, nasledni; predviđanje; deca; anksioznost, stomatološka; modeli, teorijski.

Introduction

Most people have some anxiety about going to the dentist, a sense of uneasiness, exaggerated or unfound worries or fears [1]. Dental anxiety is a common global problem and has been studied: in Australia [2], in Europe (Great Britain [3], France [4], Finland [5], Portugal [6], Ukraine [7], Turkey [8]), in Asia (Jordan [9], Iran [10], Saudi Arabia [11], Chi

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na [12], Taiwan [13], Malaysia [14], India [15], Pakistan [16], Sri Lanka [17]), in Africa (Ghana [18]), and etc. In presented papers, the results on dental anxiety are quite different. It is found that significant number of factors like age, gender, education, socioeconomic status (number of siblings in family, for example), parental/maternal anxiety, ethnicity and culture, previous dental and medical experiences, frequency of visits to dentists, clinical environment, personal traits and psychological status are crucial for the dental anxiety [19].

People develop dental anxieties for many different reasons. When researchers interview patients, a few common themes emerge: pain, feeling of helplessness and loss of control when staying still in the dental chair, embarrassment and negative past experience connected with pain or discomfort during previous dental procedures. In a survey by the British Dental Health Foundation, 36% of those who did not see a dentist regularly said that fear was the main reason [20].

Dental anxiety is a problem, which appears to develop mostly in childhood [21] and adolescence [22, 23] and is straightening with ages: in student ages [24], also in middle years [25] and even in old years [26]. Published studies [27, 28] suggest that just under half of children report low to moderate general dental anxiety and between 10% and 20% of them report extremely high levels of dental anxiety (e.g. dental phobia). Parents whose children constantly fail to attend the children dental appointments report that their children's dental anxiety is one of the influencing factors for their avoidance behavior [29].

Dental anxiety is the prime reason discouraging children to receive appropriate dental treatment [30, 31]. Rantavuori et al. [32] reported about various factors which cause dental fear among children. Folayan and Idehen [33] found that the information has an effect on dental anxiety and behaviour ratings in children. The role of child personal characteristics [34], family related factors [35], affect of birth order [36], previous childhood traumatic experiences, life events, and parental bonding [37], parental rearing style [38], the fathers' mediating role in parental transfer of fear [39] have important influence on the dental anxiety. It is found that the congenital hemorrhagic chronic disease causes an additional dental anxiety in children [40], due to the tendency of increased bleeding during and after dental treatment [41], which can be modified in blood phobia [42]. Dogan et al. [43] studied the anxiety during dental treatment among boys with hemophilia. It was found that pain is a predictor for dental fear and anxiety for male children with hemophilia and healthy ones.

In this paper a mathematical procedure for the Dental Anxiety Scale (DAS) score prediction based on the statistical values is developed. The investigation occupied 80 children. The study group (SG) included children with inherited bleeding disorders (hemophilia A, hemophilia B, Morbus Von Villebrand, etc). The control group (CG) included healthy children. The results allowed the comparison of anxiety trends in both groups of children.

Methods

The most often applied anxiety and pain measure in dentistry is the DAS which was introduced fifty years ago [44]. Since that time it is modified [45] and the text is translated into some foreign languages, for example, Turkish [46], Chinese [47], etc. In our investigation DAS for children is given in Serbian.

The questionnaire consisted of 4 questions pertaining to dental treatment situations to which the respondents answered how they felt in a given situation, by circling the number in front of the offered answers (1 = not anxious, 2 = slightly anxious, 3 = fairly anxious, 4 = very anxious, 5 = extremely anxious).

Thus, responses are scored from 1 to 5 points, with a higher score indicating more anxiety. The questions were the following: 'How anxious are you feeling at the moment when you need to go to a dentist?', 'How anxious are you feeling waiting for your turn in the waiting room of a dentist?', 'How anxious are you feeling in the dental chair waiting on the treatment?' and 'How anxious are you feeling in the dental chair when your dentist is examining your teeth?'

The total number of points on the test ranges from 4 points (no anxiety) to 20 points (extreme fear) which is the maximal achievable score. A score between 4 and 8 showed no anxiety, 9–12 moderate anxiety, 13 and 14 high anxiety and between 15 and 20 showed severe anxiety. In the study, respondents were divided by age into children younger than 6 years (with primary dentition), 6–12 years (with mixed dentition) and older than 12 years (with permanent dentition).

The obtained data were statistically analyzed using the Wilcoxon signed rank test. In addition, the descriptive statistics methods, the measure of central tendency (arithmetic mean), the measure of variability (standard deviation) and the frequency (proportion) for attributive features were applied for analysis. Means and standardized deviations were used to compare the current data with existing norms. By mathematical fitting procedure using statistical data, the analytical relation for prediction of dental anxiety as a function of aging was developed.

The investigation occupied 80 children who entered the Dental Clinics of Vojvodina in Novi Sad (Serbia): 40 of them have the inherited bleeding disease (IDB) (study group – SG) and 40 were healthy children (control group – CG).

In the SG, 35 participants identified as males and 5 as females, while in the CG there were 32 males and 8 females. The children up to 18 years were retrieved. Child's health-related information was collected through a questionnaire. The obtained data about the underlying disease of children with IBDs is given in Table 1. The questionnaire gives the information about the type of the IBD, severity of the disease, reasons for receiving coagulation factors and of presence of coagulation inhibitors.

It was found that among the children in the SG the most were those with haemophilia A (n = 26) and there were 7 with haemophilia B, 6 with Morbus Von Willebrand and 1 with rare coagulopathy. It was shown that in 31 children, concentrated coagulation factors were used for therapeutic

Table 1

Data about the inherited bleeding disorder in the study group of patients

Data	Haemophilia A	Haemophilia B	Morbus Von Willebrand	Rare coagulopathy
	n (%)	n (%)	n (%)	n (%)
Number of children	26 (65)	7 (17.5)	6 (15)	1 (2.5)
Severity of disease				
mild	4 (10)	2 (5)	44 (10)	0 (0)
moderate	4 (10)	0 (0)	1 (2.5)	1 (2.5)
sever	18 (45)	5 (22.5)	1 (2.5)	0 (0)
Previous transfusions	5 (12.5)	0 (0)	1 (2.5)	0 (0)
Number of actions with FVIII/IX				
0	4 (10)	3 (7.5)	3 (7.5)	1 (2.5)
1–3	12 (30)	11 (2.5)	3 (7.5)	0 (0)
> 3	10 (25)	3 (7.5)	0 (0)	0 (0)
Reasons to receive coagulation factor concentrates				
prophylactic	8 (20)	1 (2.5)	0 (0)	0 (0)
therapy	18 (45)	6 (15)	6 (15)	1 (2.5)
Presence of coagulation inhibitors	3 (7.5)	0 (0)	0 (0)	0 (0)

purposes, while for prophylactic reasons they were given in 9 patients (Table 1).

Coagulation factor inhibitors were identified in 3 patients with severe haemophilia A, representing a frequency of 7.5% of the total sample. If an analysis was performed on the number of patients with severe haemophilia A, the presence of inhibitors was detected in 16.7% of patients.

For better understanding of the dental anxiety of children with inherited bleeding disorder, an additional questionnaire was applied.

This is questionnaire that was filled in by the CG and SG subjects, and the values obtained were compared to determine whether there was an increased level of dental anxiety in patients with congenital coagulopathies. For comparison of results for the SG and CG, the χ^2 test, which gives the dependence of the individual pairs of observed attribute characteristics, was applied. The *t*-test based on the difference between the mean values in the numerical characteristics was applied for gender difference data analyses. The probability level of $p < 0.05$ was selected for statistical significance.

It was assumed that DAS score variation during the time is a continual function. It was also assumed that the DAS score is the polynomial type function of ages (A):

$$DAS = \sum_{i=0}^{n} a_i A^i DAS = \sum_{i=0}^{n} a_i A^i \quad \text{Eq. (1)}$$

where $a_i a_i$ are constants. Parameters $a_i a_i$ in Eq.(1) had to be determined using the DAS score values for certain ages. The number of terms i which is included into Eq. (1) depends on the number of statistical data for ages. Thus, for 'n' known values of DAS score obtained for 'n' ages, 'n' equations of the form (1) with i = n terms was written.

Solving the system of 'n' linear algebraic equations the constants a_i (i =1,2,..,n) were obtained. The higher the number of the known DAS score, the higher the number of terms in Eq. (1) and the function of prediction (1) was more appropriate.

If there are three statistical data $(DAS)_6(DAS)_6$, $(DAS)_{12}(DAS)_{12}$ and $(DAS)_{12}(DAS)_{12}$ for ages 6, 12 and 18, respectively, the DAS score has three terms:

$$DAS = a_0 + a_1 A + a_2 A^2$$
$$DAS = a_0 + a_1 A + a_2 A^2$$

Eq. (2)

where

$$a_0 = 3(DAS)_6 + 3(DAS)_{12} - 2(DAS)_{18}$$
$$a_0 = 3(DAS)_6 + 3(DAS)_{12} - 2(DAS)_{18}$$

Eq. (3)

$$a_1 = (5(DAS)_{18} - 8(DAS)_{12} - 5(DAS)_6)/12$$
$$a_1 = (5(DAS)_{18} - 8(DAS)_{12} - 5(DAS)_6)/12$$

Eq. (4)

$$a_2 = (2(DAS)_{12} - (DAS)_{18} + (DAS)_6)/72$$
$$a_2 = (2(DAS)_{12} - (DAS)_{18} + (DAS)_6)/72$$

Eq. (5)

which satisfied the relations

$$(DAS)_6 = a_0 + 6a_1 + 36a_2$$
$$(DAS)_6 = a_0 + 6a_1 + 36a_2$$

Eq. (6)

$$(DAS)_{12} = a_0 + 12a_1 + 144a_2$$
$$(DAS)_{12} = a_0 + 12a_1 + 144a_2$$

Eq. (7)

$$(DAS)_{18} = a_0 + 18a_1 + 324a_2$$
$$(DAS)_{18} = a_0 + 18a_1 + 324a_2.$$

Eq. (8)

The analytical result was tested on the healthy children and those with coagulopathy.

Results

The results of an additional questionnaire are shown in Table 2. Using the methodology presented in the previous section, the average age of children in the SG and CG was compared. It was found that the average age in the SG was 11.5 ± 5.6 and in the CG, it was 10.1 ± 4.3 years.

It is concluded that there was no main statistical difference in gender (χ^2 = 0.827, p > 0.05), nor in age, in total sample (t = 1.467, p > 0.05) and in separate groups (χ^2 = 2.259, p > 0.05).

It was seen that there was no statistical difference between the CG and SG (p < 0.05, Mann-Whitney test). Using suggestion of Sonbol et al. [48] and Alpkilic et al. [49], the stratification on the DAS scores was made by using the dependence on the oldness divided into three groups: children with primary dentition (< 6 years), with mixed dentition (6–12 years) and permanent dentition (> 12 years). In Table 3, results of DAS scores for the CG and SG in primary dentition are presented.

The score was obtained using the answers on all four questions separately (DAS1, DAS2, DAS3 and DAS4) and finding the total DAS score. For the primary dentition we calculated the following: DAS 1 (U = 95.0, z = 0.129, p = 0.45, p > 0.05, Mann-Whitney test), DAS 2 (U = 95.0, z = 0.129, p = 0.45, p > 0.05, Mann-Whitney test), DAS 3 (U = 13.5, z = 1.16, p = 0.13, p > 0.05, Mann-Whitney test), DAS 4 (U = 10.5, z = 0.38, p = 0.35, p > 0.05, Mann-Whitney test), and DAS total (U = 10.5, z = 0.38, p = 0.35, p > 0.05, Mann-Whitney test).

DAS scores in mixed dentition for the CG and SG are given in Table 4 [DAS 1 (U = 134.0, z = 0.14, p = 0.44, p > 0.05, Mann-Whitney test), DAS 2 (U = 162.0, z = 1.179, p = 0.12, p > 0.05, Mann-Whitney test), DAS 3 (U = 148.0, z = 0.663, p = 0.262, p > 0.05, Mann-Whitney test), DAS 4 (U = 160.5, z = 1.123, p = 0.13, p > 0.05, Mann-Whitney test), DAS total (U = 131.0, z = 0.03, p = 0.49, p > 0.05, Mann-Whitney test)].

There were no values on the SG and CG for which the statistical difference was evident.

Results of DAS scores in the CG and SG for patients in permanent dentition are given in Table 5 [DAS 1 (U = 159.0, z = 2.159, p = 0.01, p < 0.05, Mann-Whitney test), DAS 2 (U = 149.0, z = 1.566, p = 0.06, p > 0.0 5, Mann-Whitney test), DAS 3 (U = 157.5, z = 2.095, p = 0.01, p < 0.05, Mann-Whitney test), DAS 4 (U = 154.5, z = 1.501, p = 0.06, p >

Table 2

Dental experiences of the study group of patients

Data	Haemophilia A	Haemophilia B	Morbus Von Willebrand	Rare coagulopathy
	n (%)	n (%)	n (%)	n (%)
Has a dentist	18 (45)	5 (12.5)	2 (5)	1 (2.5)
Bad experience with the dentist because of the underlying disease	4 (10)	0 (0)	0 (0)	0 (0)
Prolonged bleeding after tooth extraction	7 (17.5)	1 (2.5)	0 (0)	0 (0)
Bleeding when brushing teeth	14 (35)	4 (10)	5 (12.5)	1 (2.5)

Table 3

Dental Anxious Scale (DAS) scores in the study group (SG) and control group (CG) of children with primary dentition

Group	DAS 1	DAS 2	DAS 3	DAS 4	DAS
	mean values ± SD				
SG	4.16 ± 1.60	4.33 ± 1.21	4.83 ± 0.40	4.83 ± 0.40	18.16 ± 2.99
CG	4.66 ± 0.57	4.33 ± 0.57	4.33 ± 0.57	4.66 ± 0	18.00 ± 2.00

Table 4

Dental Anxious Scale (DAS) scores in the study group (SG) and control group (CG) of children with mixed dentition

Group	DAS 1	DAS 2	DAS 3	DAS 4	DAS
	mean values ± SD				
SG	2.61 ± 1.04	1.84 ± 1.51	2.30 ± 1.31	2.61 ± 1.26	9.38 ± 3.92
CG	2.60 ± 0.94	2.40 ± 1.31	1.95 ± 0.99	2.10 ± 1.11	9.05 ± 3.34

Table 5

Dental Anxious Scale (DAS) scores in the study group (SG) and control group (CG) of children with permanent dentition

Group	DAS 1	DAS 2	DAS 3	DAS 4	DAS
	mean values ± SD				
SG	2.89 ± 0.90*	2.00 ± 1.23	2.83 ± 1.50*	2.72 ± 1.74	10.44 ± 4.52*
CG	2.08 ± 0.90*	1.17 ± 0.39	1.58 ± 0.99*	1.50 ± 0.67	6.41 ± 2.23*

***statistically significant difference between the SG and CG (p < 0.05, Mann-Whitney test).**

0.05, Mann-Whitney test), and DAS total (U = 164.0, z = 2.37, $p = 0.008$, $p < 0.05$, Mann-Whitney test)].

Based on the previous results, the DAS score distribution for the CG and SG are shown in Table 6. The obtained results showed that the dental anxiety was statistically higher for the SG than for the CG for the first DAS1, second DAS2 and fourth DAS4 scores, and also the total DAS score, except for DAS2 score where was no statistically significant difference between two groups [DAS 1 (U = 983.0, z = 1.76, $p = 0.03$, $p < 0.05$, Mann-Whitney test), DAS 2 (U = 908.0, z = 1.03, $p = 0.14$, $p > 0.05$, Mann-Whitney test), DAS 3 (U = 1103.0, z = 2.91, $p = 0.001$, $p < 0.05$, Mann-Whitney test), DAS 4 (U = 1075.0, z = 2.64, $p = 0.003$, $p < 0.05$, Mann-Whitney test), DAS total (U = 1,067.0, z = 2.56, $p = 0.004$, $p < 0.05$, Mann-Whitney test)]. It was found that up to 12 years, there was no difference in dental anxiety between the two groups of patients. However, for older children, the dental anxiety was much higher for patients with coagulopathy.

The result could be explained with the fact that older patients with IBD are aware of possible effects and are afraid of bleeding during dental intervention, especially of application of local anesthesia or extraction therapy. It was found that even 70% of patients with IBD needed hematological preparation before dental intervention and it was extremely high percentage.

Based on the statistically obtained values, the suggested analytical prediction procedure was applied. The interpolation DAS score – age functions, which give the quantitative measure of dental anxiety during time, were calculated.

Using the data for DAS1, DAS2, DAS3, DAS4 and total DAS scores, the corresponding DAS score – time history diagrams are plotted in Figure 1. In Figure 1a, the DAS1 and total DAS scores and in Figure 1b, the DAS2, DAS3 and DAS4 scores for the SG and CG as aging functions are plotted.

Using the statistical values for the total DAS score, the interpolation functions DAS_{CG} and DAS_{SG} for the CG and SG, respectively, were calculated as

$$DAS_{CG} = 33.4 - 3.100\,A + 0.089A^2$$
$$DAS_{CG} = 33.4 - 3.100\,A + 0.089A^2,$$
$$DAS_{SG} = 36.5 - 3.875A + 0.135A^2$$
$$DAS_{SG} = 36.5 - 3.875A + 0.135A^2$$

Eq. (9)

where A is aging.

The relations were of parabolic type with the minimum which was different for the SG and CG: the minimal value of DAS score was smaller for the CG than for the SG. From Figure 1a, it is evident that the total values of DAS score are approximately equal for children up to 12 years, but after that there is a strong difference in fear level in children with IBD in comparison to healthy children.

Namely, the dental anxiety decreased after the age of 12 years in healthy children, while in children with IBD it increased since approximately of the age of adolescence. The

Table 6

Averaged Dental Anxious Scale (DAS) scores in the study group (SG) and control group (CG)

Group	DAS 1	DAS 2	DAS 3	DAS 4	DAS
	mean values ± SD				
SG	3.05 ± 1.19*	2.45 ± 1.51	3.05 ± 0.70*	3.10 ± 1.59*	11.65 ± 5.10*
CG	2.52 ± 1.13*	2.08 ± 1.30	2.02 ± 1.21*	2.12 ± 1.28*	8.78 ± 4.18*

***statistically significant difference between the SG and CG ($p < 0.05$, Mann-Whitney test); SD – standard deviation.**

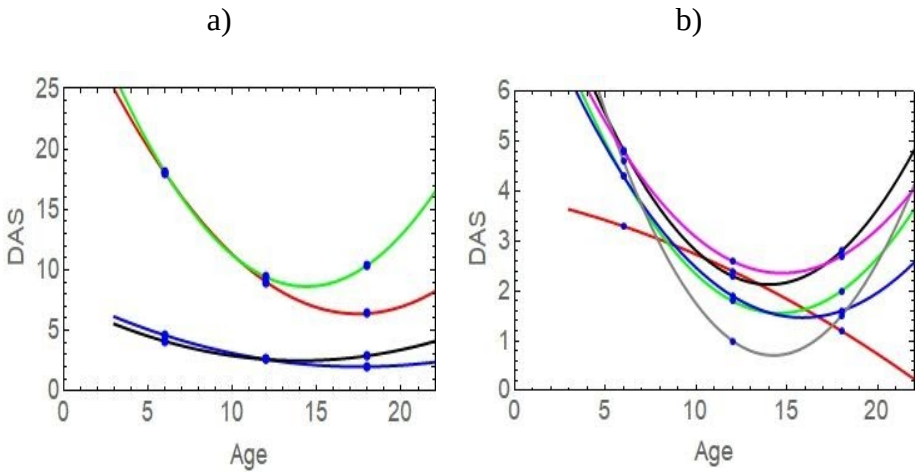

Fig. 1 – a) Dental Anxious Scale (DAS) – Age curves for the control group (CG) (red line) and study group (SG) (green line); DAS1 – Age curves for the CG (blue line) and SG (black line); b) DAS2 – Age curves for the CG (red line) and SG (green line); DAS3 – Age curves for the CG (blue line) and SG (black line); DAS4 – Age curves for CG (grey line) and SG (magenta line).

prediction is that after the age of 18 years, the dental anxiety in patients with coagulopathy would increase, while in healthy persons, the increase would be quite small tending to a constant level.

In Figure 1a, the DAS1 scores for the CG and SG as aging functions are plotted. It is seen that the scores in early childhood are similar for both groups of children. However, increasing the aging, difference in DAS1 score is also increasing: for persons in the CG the dental anxiety remains at an almost constant level, while for those in the SG it has the tendency of increase. Analytical relations which describe variation of DAS1 score are:

$$\begin{matrix}(DAS1)_{CG} = 8.0 - 0.683A + 0.019A^2 \\ (DAS1)_{CG} = 8.0 - 0.683A + 0.019A^2 \\ (DAS1)_{SG} = 7.4 - 0.7A + 0.025A^2 \\ (DAS1)_{SG} = 7.4 - 0.7A + 0.025A^2\end{matrix}, \quad \text{Eq. (10)}$$

where A is aging. The prediction is that for both groups of patients after their age of 18 years, the DAS1 score would tend to a constant value. Analyzing the DAS2 score for the CG (red line) and the SG (green line), it is obvious that the curves are quite different (Figure 1b).

Tendency of curve for the CG was to decrease in time, while for the SG it was to increase.

Mathematical description of the curves are:

$$\begin{matrix}(DAS2)_{CG} = 3.9 - 0.075A - 0.004A^2 \\ (DAS2)_{CG} = 3.9 - 0.075A - 0.004A^2 \\ (DAS2)_{SG} = 9.5 - 1.091A + 0.037A^2 \\ (DAS2)_{SG} = 9.5 - 1.091A + 0.037A^2\end{matrix}, \quad \text{Eq. (11)}$$

Based on Eq. (3), the prediction of the anxiety score would be calculated. Due to the result, it is obvious that the time spent in the waiting room of dentist has different influence on the SG and CG.

DAS3 score for the CG (blue line) and the SG (black line) curves are shown in Figure 1b.

Comparing the curves:

$$\begin{matrix}(DAS3)_{CG} = 8.8 - 0.925A + 0.029A^2 \\ (DAS3)_{CG} = 8.8 - 0.925A + 0.029A^2 \\ (DAS3)_{SG} = 10.4 - 1.179A + 0.04A^2 \\ (DAS3)_{SG} = 10.4 - 1.179A + 0.04A^2\end{matrix}, \quad \text{Eq. (12)}$$

it is obvious that they have the same character. Both curves are of parabolic type where with aging the increase of anxiety occurs. The gradient of the score increase was approximately equal, but the initial anxiety in children with IBD was higher. Finally, in Figure 1b, the DAS4 scores for the CG (gray line) and the SG (magenta line) as aging functions are plotted.

The analytical description of curves is:

$$\begin{matrix}(DAS4)_{CG} = 12.3 - 1.625A + 0.057A^2 \\ (DAS4)_{CG} = 12.3 - 1.625A + 0.057A^2 \\ (DAS4)_{SG} = 9.3 - 0.941A + 0.032A^2 \\ (DAS4)_{SG} = 9.3 - 0.941A + 0.032A^2\end{matrix} \quad (13)$$

Both curves have the same property: the minimal value is for 14 years. After that, the DAS4 score increases for both groups. However, the DAS4 score for the SG is higher than for the CG, but with tendency of slower increase than for the CG.

Discussion

Haemophilia and other IBDs are very rare diseases, probably this is the reason why there is a little literature about dental anxiety and dental fear in this group of patients.

For the prediction of dental anxiety in our study, we used the age as a predictor. The same predictor was used in the study of Lee et al. [13]. They found out that level of dental fear and clinical anxiety have different predictors, but age and cooperativeness in the first dental visit are important predictors for dental fear and clinical anxiety. The other predictors were maternal dental fear, unbearable pain during the first dental visit and the visiting dentist in a regular dental clinic.

Findings of the present study indicate that there is a high prevalence of dental anxiety among the children. After statistical analyses of the DAS score, it is obtained that the score depends on the general health and age of children included into the investigation.

This study showed that age differences have an influence on dental anxiety and fear. Namely, the level of DAS score difference depends on the oldness of children. The same results were found out in other studies about dental anxiety [19–22, 26].

In the age up to 6 years (children with primary dentition) and age group of 6–12 years (children with mixed dentition), the DAS score for children with inherited bleeding disorders and healthy children is almost equal. The highest difference in the score value is obtained in children older than 12–18 years. In our study dental anxiety decreased after the age of 12 in healthy children, while in children with IBDs dental anxiety increased since the age of 18.

Kakkar et al. [21] in their study of prevalence of dental anxiety in 10–14 years old children also found high prevalence of dental fear in the same age group, and that the dental fear score decreases with increasing age and experience of the children. The influence of the age could be explained by the immature psychological development of the children.

The difference in the level of dental anxiety of the SG and CG at the age of 12–18 years could be explained with the Poti et al. [40] study of subjective experience of living with haemophilia in 20 young adults with haemophilia, aged 11–25 years. Transition from adolescence to adulthood is a complex process, especially for patients with chronic disease like haemophilia. Reasons for that are problems imposed by the disease and by the therapy, the assumption of responsibility for care and progressive awareness that the disorder will accompany them throughout their life. While in childhood, parents are personally responsible for their child and treatment, at that age, the patient becomes progressively more independent and needs to transfer personal responsibility from parental to self care. This taking of responsibility proves dif-

ficult for young people, confirming poor adherence to treatment in adolescence, a common issue in haemophilia. Vika et al. [42], in their study about relationship of dental anxiety and fear of blood, injury and injections among 18-years olds in Norway, showed that 20% of subjects were classified as having dental anxiety, and 11% of adolescents with dental anxiety reported a high probability of avoiding necessary dental treatment when a dental injection was needed.

Higher level of dental anxiety in children with IBD aged 12–18 years could be explained with awareness of possible bleeding during and after the dental procedures.

In our study, a significantly higher degree of dental anxiety was found in the overall sample in patients with IBDs, the SG compared to the CG. The DAS score of children with IBDs in the SG compared with that of healthy children in the CG is obtained to be higher. Namely, children with bleeding disorder, expect anxiety due to dental treatment, have an additional fear caused by bleeding during or after dental intervention.

Dogan et al. [43] in their study about dental anxiety related to the first dental intervention for children with haemophilia aged 7–12 years and healthy controls, did not find significant difference in level of dental anxiety between the groups. But, in both group, the dental anxiety score was higher in children who had experienced dental pain before the treatment.

For patients with haemophilia and other bleeding disorders access to dental treatment is an issue, resulting with neglected dental health and urgent treatment need. Therefore, appropriate prevention and dental treatment is required in this group of children to prevent them to develop dental anxiety.

Conclusion

Mathematical procedure for DAS score prediction based on the statistical values is developed. The analytical results allowed the comparison of anxiety trends in both groups of children: healthy and those with IBDs.

We concluded that dental anxiety in both groups of children was lower in the older age than in younger one (minimum is for ages of adolescence). The gradient of dental anxiety increase in time would be more significant in the SG patients than in the CG group. The dental anxiety in the healthy patients after 18 years is predicted to be of moderate level, while for those with IBDs it tends to severe anxiety.

This may be due to increased exposures over time allowing children to develop a tolerance to treatment, and therefore have less anxiety as they age. Childhood dental anxiety is not only distressing for the child and their family but is also associated with poor oral health outcomes and an increased reliance on costly specialist dental services.

The analytical prediction, based on the statistical data, is that the dental anxiety would increase in time in both groups of patients, those with IBDs and healthy. The predicted DAS score may be used as a control measure of oral health.

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Gender-specific differences in the anthropometric characteristics of the distal femur and proximal tibia condyles

Polno specifične razlike u antropometrijskim karakteristikama donjeg okrajka butne kosti i gornjeg okrajka golenjače

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Abstract

Background/Aim. In the course of identification, skeletal remains are used to determine the gender, age, and height of the body. The pelvic bone, skull, and femur were commonly used to determine gender; however, modern radiology techniques have enabled the use of other bones of the skeletal system (all long bones, scapula, clavicle, metacarpal and metatarsal bones, vertebras, ribs, etc.). The aim of this study was to determine whether certain anthropometric characteristics of the distal femur and proximal tibia are indicative of gender differences. **Methods.** The respective research has been carried out between 2011 and 2014 at the Institute of Pathology and Forensic Medicine and Institute of Radiology of the Military Medical Academy in Belgrade on 203 subjects (152 men and 50 women), between 11 and 63 years of age (35.50 ± 12.98). Diagnostic magnetic resonance imaging (MRI) imaging of the living persons' knees was used. Measures taken included the longest mediolateral diameter of the distal femur condyle, the mediolateral diameter of the proximal tibia condyle and the diameter of the proximal tibia intercondylar eminence. Descriptive statistics and the Student's *t*-test were used for statistical analyses of data. **Results.** The mediolateral diameter of the distal femur in men was from 7.70 cm to 9.70 cm (8.80 ± 0.39 cm), and in women from 6.60 cm to 8.50 cm (7.62 ± 0.39 cm). The mediolateral diameter of the proximal tibia in men was from 7.20 cm to 9.30 cm (8.09 ± 0.38 cm), and in women from 5.90 cm to 8.00 cm (7.04 ± 0.36 cm). The mediolateral diameter of the proximal tibia intercondylar eminence in men was from 1.00 cm to 2.30 cm (1.44 ± 0.21 cm), and in women from 0.90 cm to 2.00 cm (1.33 ± 0.21 cm). The measures obtained showed a gender-specific statistically significant difference. **Conclusion.** The mediolateral diameters of the distal femur condyle, proximal tibia condyle and proximal tibia intercondylar eminence are indicative of gender-specific differences and may be used in the procedure of determining gender based on skeletal remains.

Key words:
anthropometry; femur; sex factors; tibia.

Apstrakt

Uvod/Cilj. U toku postupka identifikacije osoba, skeletni ostaci se koriste za utvrđivanje pola, životnog doba i visine tela. Za utvrđivanje pola do sada su najčešće korišćene karlična kost, lobanja i butna kost, ali su savremene radiološke tehnike omogućile da se koriste i druge kosti skeletnog sistema (sve duge kosti, lopatica, ključna kost, metakarpalne, metatarzalne kosti, kičmeni pršljenovi, rebra i dr.). Cilj ovog rada je bio da se utvrdi da li su određene antropometrijske karakteristike donjeg okrajka butne kosti i gornjeg okrajka golenjače specifične za pol. **Metode.** Retrospektivno istraživanje sprovedeno je od 2011. do 2014. godine u Institutu za patologiju i sudsku medicinu i Institutu za radiologiju Vojnomedicinske akademije u Beogradu na 203 ispitanika (152 muškarca i 50 žena), životnog doba od 11 do 63 godina (35,50 ± 12,98). Korišćeni su snimci kolena živih osoba, urađeni u dijagnostičke svrhe magnetnom rezonancom (MRI). Mereni su najduži mediolateralni promer u predelu kondila donjeg okrajka butne kosti, mediolateralni promer u predelu kondila gornjeg okrajka golenjače i promer interkondilarne eminencije gornjeg okrajka golenjače. Za statističku analizu podataka korišćene su metode deskriptivne statistike i Studentov *t*-test. **Rezultati.** Pokazano je da mediolateralni promer donjeg okrajka butne kosti muškaraca iznosi od 7,70 cm do 9,70 cm (8,80 ± 0,39 cm), a žena od 6,60 cm do 8,50 cm (7,62 ± 0,39 cm). Mediolateralni promer gornjeg okrajka golenjače muškaraca je u rasponu od 7,20 cm do 9,30 cm (8,09 ± 0,38 cm), a žena od 5,90 cm do 8,00 cm (7,04 ± 0,36 cm). Mediolateralni promer interkondilarne

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eminencije gornjeg okrajka golenjače muškaraca je bio od 1,00 cm do 2,30 cm (1,44 ± 0,21 cm), a žena od 0,90 cm do 2,00 cm (1,33 ± 0,21 cm). Ustanovljenim merama je pokazana statistički značajna razlika specifična za pol. **Zaključak.** Mediolateralni promeri u predelu kondila donjeg okrajka butne kosti, gornjeg okrajka golenjače i interkondilarne eminencije gornjeg okrajka golenjače pokazuju polnu specifičnost i mogu se koristiti u postupku utvrđivanja pola na osnovu skeletnih ostataka.

Ključne reči:
antropometrija; femur; pol, faktor; tibija.

Introduction

Forensic medicine is an interdisciplinary science that uses forensic anthropometry in its scope of work. Forensic anthropometry is a forensic anthropology discipline dealing with the identification and analysis of human skeletal remains with the help of different metric techniques [1, 2]. The skeletal remains are often the only material that can be used to determine the identity of a person, and thus they are used to determine the gender, age, and height of the body [3]. Pelvic bones [4, 5] and skulls [6], but also the long bones [7, 8], scapula, clavicle [9], metacarpal [10] and metatarsal bones, vertebras and ribs [1] are most frequently used to determine gender. Each identification procedure based on skeletal remains starts with a detailed description of individual bones, their anthropometric characteristics, the existence of signs of diseases, recent or old fractures and injuries [1]. The development of radiology and forensic anthropology brought about the use of various techniques for the analyses of anthropometric characteristics of the bones, allowing the visualization and precise measuring of certain bone elements on the bodies during the autopsy, on skeletal remains after exhumation, but also living persons. Radiologic knee examinations of living persons may be used to analyze the anthropometric characteristics of the distal femur and proximal tibia [11]. So far, several anthropometric characteristics of the distal femur and proximal tibia have been found that are gender-specific [12].

The aim of this research was to determine whether the following anthropometric characteristics – the mediolateral diameter of the distal femur condyle, the mediolateral diameter of the proximal tibia condyle and the diameter of the proximal tibia intercondylar eminence can be indicative of gender differences.

Methods

This retrospective study was carried out at the Institute of Pathology and Forensic Medicine and the Institute of Radiology of the Military Medical Academy in Belgrade using the archived materials covering the period from November 2011 until September 2014. The research included 203 subjects (152 men and 50 women), from 11 to 63 years of age. The average age of the subjects was 35.50 ± 12.98 years. Images in electronic form, made during the magnetic resonance imaging (MRI) examination of patients' knees were used for this research. Radiologic examinations of patients using MRI were done as part of the clinical examinations and diagnostic procedures of various painful knee conditions, including fractures and tumorous changes. Diagnostic MRI examinations were done using the GE SIGNA HDX-3T device, while the analyses included the distal femur and proximal tibia. During the anteroposterior examination, the diameters were measured in accordance with the standards of anteroposterior measuring: the mediolateral diameter of the distal femur condyle, the mediolateral diameter of the proximal tibia condyle and the diameter of the proximal tibia intercondylar eminence (Figure 1).

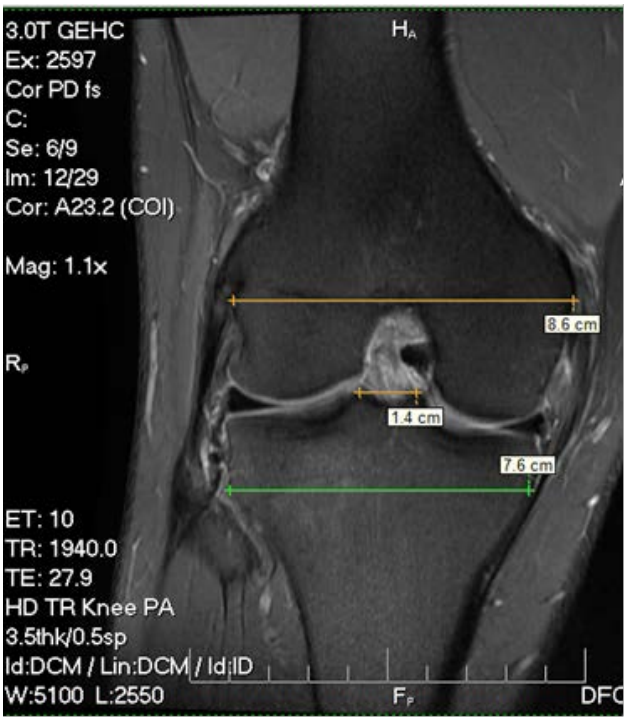

Fig. 1 – Magnetic resonance imaging examination of the knee: the longest mediolateral diameter of the distal femur condyle (orange), the longest mediolateral diameter of the proximal tibia condyle (green) and the longest diameter of the proximal tibia intercondylar eminence (orange).

Statistical analysis of the data obtained from this research was done using the statistical software IBM SPSS Statistics Version 23 by applying standard statistical methods of descriptive statistics (mean value ± standard deviation). The results were statistically analyzed using the parametric test (Student's *t*-test). The statistical significance level was $p < 0.05$.

Results

Statistical analyses of the data showed that the mediolateral diameter of the distal femur in men ranged from 7.70 cm to 9.70 cm (8.80 ± 0.39 cm), and in women from 6.60 cm

to 8.50 cm (7.62 ± 0.39 cm) (Figure 2). The mediolateral diameter of the proximal tibia in men ranged from 7.20 cm to 9.30 cm (8.09 ± 0.38 cm), and in women from 5.90 cm to 8.00 cm (7.04 ± 0.36 cm) (Figure 3). The mediolateral diameter of the proximal tibia intercondylar eminence in men ranged from 1.00 cm to 2.30 cm (1.44 ± 0.21 cm), and in women from 0.90 cm to 2.00 cm (1.33 ± 0.21 cm) (Figure 4).

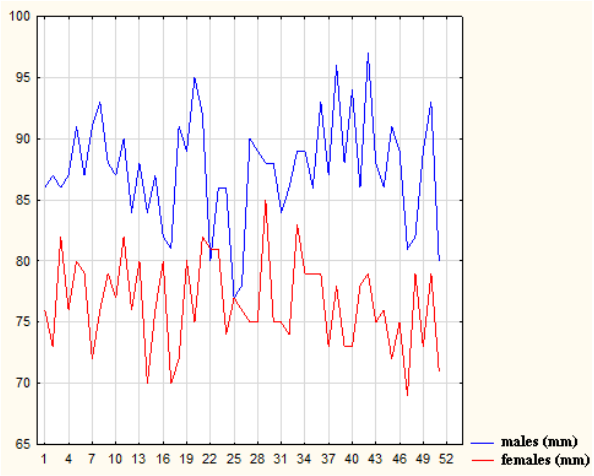

Fig. 2 – Mediolateral diameter of the distal femur condyle.

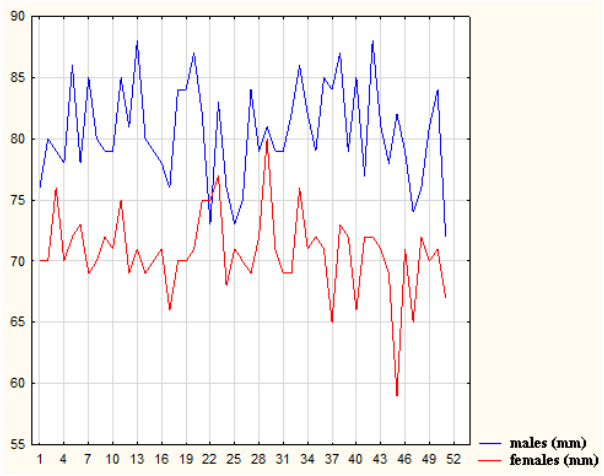

Fig. 3 – Mediolateral diameter of the proximal tibia condyle.

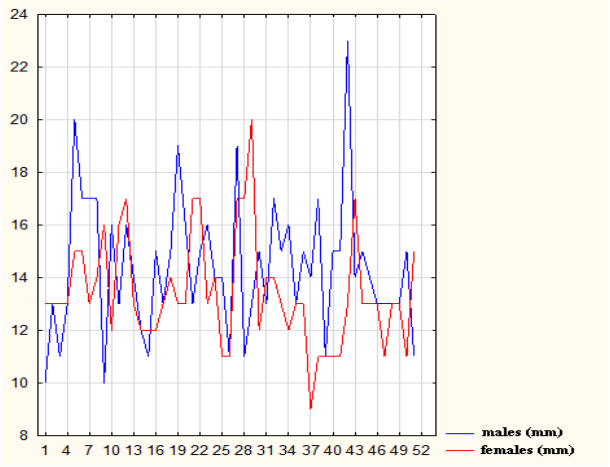

Fig. 4 – The proximal tibia intercondylar eminence diameter.

The statistical analyses using the parametric test – Student's *t*-test showed a statistically significant difference in all of the analyzed parameters, in particular the mediolateral diameter of the distal femur between men and women ($p < 0.0001$), the mediolateral diameter of the proximal tibia between men and women ($p < 0.0001$) and the mediolateral diameter of the proximal tibia intercondylar eminence between men and women ($p = 0.024$).

Discussion

The research showed a statistically significant differences in the mediolateral diameter of the distal femur, the mediolateral diameter of the proximal tibia and the mediolateral diameter of the proximal tibia intercondylar eminence between men and women in the analyzed sample. The diameters in the area of medial and lateral condyles of the distal femur in the population of India are smaller compared to our analyzed sample and measure up to 71.5 ± 2.5 mm in men and 65.1 ± 3.1 mm in women. The knee images made using computed tomography (CT) were used for the analyses of these diameters and it has been established that there are statistically significant differences in diameters in the area of medial and lateral condyles of the distal femur, as well as in diameters in the area of medial and lateral condyles of the proximal tibia between men and women [13]. The research of the femur diameter in the population of Yemen, done by using MRI images of femurs, used to measure the diameter of the medial and lateral condyles, intercondylar height and width, has shown a statistically significant difference in all measured diameters between men and women ($p < 0.001$) [14]. By virtue of measurements using a digital osteometer, gender-specific differences in the diameter of femurs were found in the population of Bulgaria, and the diameters that have proven to be the most gender-specific are the maximal length and bicondylar length of the femur [3]. By using a

CT during the autopsy, gender-specific differences were found in the relationship between the volume of the femur, tibia and fibula and their length in the population of Japan [15]. Additionally, by using the CT, differences between mediolateral and anteroposterior diameters of distal femur were found in various populations of the same race (Malaysia, India, China) with the lowest values for both genders in the population of India [13, 16]. By using the CT and MRI, a statistically significant difference in the mediolateral diameter of the distal femur was noticed in women from the population of China and in Caucasian women, but not in the anteroposterior diameter. The measured values of the mediolateral and anteroposterior diameter of the proximal tibia are smaller in women from the population of China compared to the Caucasian women, but larger in men from the population of China compared to Caucasian men. However, this has no statistical significance [17]. Using the CT on the population of Turkey, it has been found that, from 13 measured femur parameters, the greatest gender-specific difference is found in bicondylar length, neck length and mediolateral subtrochanteric width [18]. By using the combination of standard osteometric measuring and digital radiography to determine the bicondylar angle, it has been found that a gender-specific, statistically significant difference in bicondylar diameter exists in the population of Bengal [11]. By using 3D images of femurs of cadavers or patients undergoing surgery in the population of Korea, by measuring a total of 28 parameters, gender-specific differences were found in the majority of parameters, while population-specific differences were found for 14 parameters [19]. Using the standard osteometric measuring of the tibia on the cadavers from the population of the Mediterranean (Greece, Spain, Italy), gender-specific differences have been found that have statistically greater significance for the population of Greece in terms of tibia length and distal epiphyseal width, for the population of Spain in terms of tibia length and proximal epiphyseal width and for the population of Italy in terms of distal and proximal epiphyseal width [20].

Conclusion

The mediolateral diameter of the distal femur condyle, the mediolateral diameter of the proximal tibia condyle and the diameter of the proximal tibia intercondylar eminence are indicative of gender-specific differences in the population of Serbia and may be used in the procedure of determining gender based on skeletal remains. Since differences and specific characteristics have been found among different populations, it is clear that the research of population-specific anthropometric characteristics has to continue.

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Prevalence of vitamin D3 deficiency in patients with type 2 diabetes and proteinuria

Zastupljenost deficita vitamina D3 kod bolesnika sa dijabetesom melitusom tip 2 i proteinurijom

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Abstract

Background/Aim. Vitamin D3 plays an important role in glucose metabolism, with influence on insulin secretion and sensitivity. Low grade inflammation is present in patients with diabetes type 2 and it is a known fact that vitamin D has an anti-inflammatory effect. Vitamin D deficiency is particularly pronounced in patients with diabetic nephropathy. Vitamin D3 levels during the year are associated with seasonal changes primarily influenced by UV radiation. The aim of the study was to examine the prevalence of vitamin D3 deficiency in patients with diabetic nephropathy. **Methods.** Patients with type 2 diabetes and diabetic nephropathy were included in the study after the vitamin D3 levels were established. The results were classified according to a lower limit level given for each month being reduced or normal values. For the purpose of further research, patients with low vitamin D3 levels were divided into two groups, study and control group, each including 45 patients. The study group received cholecalciferol at the dose necessary to achieve the intended optimal vitamin D3 blood level of 90–100 nmol/L. **Results**. At the beginning of the study, vitamin D3 value for all patients with vitamin D3 deficiency (n = 90) was 45.1 ± 15.6 nmol /L. Vitamin D3 deficiency in the study sample (n = 109) was found in 82.56% of participants, while the normal values of vitamin D3 were found in 17.43% of patients. There is a statistically significant difference in the deviation of vitamin D3 levels from the lower normal values in the whole group of subjects between winter and summer, with the deviation being more pronounced in summer. There is no gender difference in these values, although in both men and women there is a more pronounced deviation in summer. **Conclusion**. Vitamin D3 deficiency is significantly represented in patients with type 2 diabetes and diabetic nephropathy.

Key words:
cholecalciferol; deficiency; diabetes mellitus; prevalence; proteinuria; seasons.

Apstrakt

Uvod/Cilj. Vitamin D3 ima važnu ulogu u metabolizmu glukoze, a ispoljava se kroz uticaj na insulinsku sekreciju i senzitivnost. Upala niskog stepena je prisutna kod bolesnika sa dijabetesom tip 2, a poznato je da vitamin D3 ima značajno antiinflamatorno dejstvo. Nedostatak vitamina D3 je posebno izražen kod bolesnika sa dijabetesnom nefropatijom. Nivo vitamina D3 je tokom godine uslovljen sezonskim promenama, prvenstveno uticajem UV zračenja. Cilj rada je bio da se utvrdi prevalencija deficita vitamina D3 kod bolesnika sa dijabetesnom nefropatijom. **Metode**. Ispitanici sa dijabetesom tipa 2 i dijabetesnom nefropatijom su uključeni u studiju nakon određivanja vrednosti vitamina D3. Rezultati su klasifikovani kao normalni ili sniženi, u odnosu na donju granicu normalnih vrednosti koja je data za svaki mesec u godini. Za potrebe daljeg istraživanja bolesnici sa sniženim nivoom vitamina D3 podeljeni su u dve grupe: studijsku i kontrolnu grupu, svaka sa po 45 bolesnika. Studijska grupa ispitanika je dobijala holekalciferol u dozi potrebnoj za postizanje planiranog optimalnog nivoa vitamina D3 od 90–100 nmol/L u krvi. **Rezultati**. Vrednost vitamina D3 kod svih ispitanika sa deficitom vitamina D3 (n = 90) na početku istraživanja je iznosila 45,1 ± 15,6 nmol/L. Zastupljenost bolesnika sa deficitom vitamina D3 je u ispitivanom uzorku (n = 109) bila 82,56%, dok su normalne vrednosti vitamina D3 imalo 17,43% ispitanika. Utvrđena je statistički značajna razlika u odstupanju nivoa vitamina D3 od donjih normalnih vrednosti između zimskog i letnjeg perioda u celoj grupi ispitanika, pri čemu je odstupanje bilo izraženije u letnjem periodu. Nije utvrđena razlika tih vrednosti između muškaraca i žena, mada je kod

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oba pola odstupanje bilo izraženije u letnjem periodu. **Zaključak**. Deficit vitamina D3 je značajno zastupljen kod bolesnika sa dijabetesnom nefropatijom.

Ključne reči:
vitamin d; nedostatak; dijabetes melitus; prevalenca; proteinurija; godišnja doba.

Introduction

The prevalence of vitamin D3 deficiency is more common in type 2 diabetes mellitus (T2DM) patients compared to the healthy population [1]. Studies have indicated that vitamin D deficiency is a risk for the development of T2DM [2]. Patients with T2DM with vitamin D3 deficiency are at a higher risk of developing nephropathy [3]. Moreover vitamin D3 deficiency increases with the progression of diabetic nephropathy (DN) so that serum 25-hydroxyvitamin D [25(OH)D] appears to be a favourable inverse predictor of DN progression [4]. A significant association between vitamin D deficiency and glycemic control has been also reported [5]. Vitamin D3 is involved in glucose metabolism by improving insulin secretion and sensitivity [6]. Vitamin D3 produces these effects by increasing intracellular free calcium and insulin receptor transcription [7]. These paracrine effects are manifested by its action on vitamin D3 receptors (VDRs), which are widely expressed on various cell types, including pancreatic beta cells [8]. Vitamin D3 also exhibits other characteristics that directly or indirectly affect the expression of the sequelae of diabetes disease. It was found that vitamin D has a beneficial effect on reducing proteinuria, hence it is expected that its action can slow down the progression of DN. The reduction of proteinuria is considered to be an important predictive factor regarding the future outcome of renal function [9]. The indirect mechanism of the beneficial effect of vitamin D3 in diabetic patients is manifested by reducing inflammation. There are indications that diabetes is a condition of low-grade chronic inflammation, the so-called state of "metaflamation". "Metaflamation" is a form of low-grade systemic and chronic inflammation that occurs in metabolic diseases [10]. Its effect on lipid parameters was also investigated. A cross-sectional study of a large number of subjects indicated an association between high values of 25(OH)D and low values of total cholesterol, low density lipoprotein (LDL)-cholesterol, high values of high density lipoprotein (HDL)-cholesterol, and low triglycerides [11]. However, when longitudinal studies were performed, it was not confirmed that the change in the value of 25(OH)D from deficiency to sufficiency has a positive effect on the lipid profile. Namely, an increase in total and HDL-cholesterol, without changes in LDL-cholesterol and triglycerides, was found [12]. In some other studies positive results have been reported. In a study by Ramiro-Lozano and Calvo-Romero [13] a statistically significant reduction in total cholesterol levels and an insignificant trend of decreasing values of LDL-cholesterol, non-HDL cholesterol and triglycerides and no change in the value of HDL-cholesterol were found. In addition, vitamin D deficiency affects bone metabolism. In such conditions, the initial compensatory mechanism is increased secretion of parathyroid hormone (PTH), which stimulates the kidneys to increase phosphate and decrease calcium excretion.

The level of vitamin D3 varies during the year so that serum 25(OH)D concentrations are strongly associated with the exposure to ultraviolet (UV) light [14]. Because of seasonal variations which are very pronounced in temperate climates, 25(OH)D concentrations are highest in late summer and early autumn and lowest in late winter and early spring [15]. Therefore, in order to more accurately assess vitamin D3 deficiency in temperate climates, it is important to define its lower limit values depending on the time of year, which can significantly affect the decision on the need for supplementation and the required dose for its correction.

The aim of the study was to determine the prevalence of vitamin D3 deficiency in patients with T2DM and proteinuria, taking into account its seasonal variations, as well as to examine the influence of vitamin D3 deficiency and its replenishing on some parameters of bone metabolism.

Methods

This non-randomised controlled clinical trial was conducted in the General Hospital in Subotica, Serbia. The 24-week study included patients followed for T2DM with nephropathy defined with proteinuria > 150 mg/24 h, who were treated and followed at the outpatient Clinics for Nephrology and Diabetes. The population included in the study lived in the wider geographic region of Subotica, located at 46 °6 '0"north latitude and 19° 40' 0.01" east longitude, with a pronounced Pannonian-continental climate. The study was conducted and lasted from May 2018 to November 2019. Criteria for inclusion in the study were fulfilled: if patients aged 18 to 75 years with body weight (BW) was > 50 kg and body mass index (BMI) in the range of 18 to 35 kg/m^2 and if patients were treated for T2DM for at least 5 years, were on an antidiabetic diet or oral hypoglycemics, and had satisfactory glycoregulation in the period before enrolment in the study (the target HbA1 values were < 7% based on the criteria for the prevention of microvascular complications); patients with proteinuria > 150 mg/24 h and creatinine clearance > 30 mL/min/1.73 m^2 chronic renal failure (CRF) grade 4 which according to the National Kidney Foundation Kidney Disease Outcomes Quality Initiative (NFK DOQI) is a stage in which a permanent vascular approach is prepared] and in whom another cause of proteinuria was excluded (glomerulonephritis, amyloidosis, malignancies, systemic lupus erythematosus,…); patients who did not use vitamin D supplementation or its analogues for at least 3 months, who did not use dietary calcium, did not use corticosteroid therapy and did not have a history of other kidney diseases and current urinary tract infection; patients who if using antihy-

pertensive therapy, used angiotensin-converting enzyme inhibitors (ACEI) or angiotensin II receptor blockers (ATB) and who were on this therapy for at least 3 months prior to the enrolment in a study with satisfactorily regulated arterial tension (values around 140/90 mmHg). The exclusion criteria were as follows: lack of cooperation (irregular intake of prescribed therapy, non-attendance at the scheduled outpatient check-ups ...); development of a disease or condition during the examination that could affect the implementation of diagnostic methods; pregnancy; the will of the respondents to no longer participate in this survey.

After the initial screening phase, 90 patients were selected for the study, and were divided into two groups, study (experimental) and control group, each consisting of 45 patients. The study group received cholecalciferol, and the control group received their standard therapy. The lower limit of normal vitamin D values for each patient was determined on the basis of seasonally defined limits for the required level of vitamin D3 given by months of the year, and according to their gender. For the assessment of vitamin D status and for the purpose of this study, values of seasonally defined limits for the required level of vitamin D3 were adapted to our climate conditions (Table 1).

For optimal values of vitamin 25(OH)D3, the level of 90–100 nmol/L was determined. Vigantol® (MERCK KGaA) 20,000 IU/mL in the form of oral drops (500 IU of vitamin D in one drop) was used. The number of cholecalciferol drops was determined on the basis of the difference between the level of vitamin D in the patient's serum and the set optimal levels. The number of drops was increased/decreased in men by changing the winter-summer period by 2 drops, and in women by 1 drop. The dose of cholecalciferol was reduced if the concentration of calcium in the urine in two consecutive controls exceeded the value of 7.5 mmoL/24 hours and in the serum a value greater than 2.6 nmol/L. Subjects in both groups were monitored for 6 months for sedimentation rate, complete blood count, serum and 24-hour urine calcium, phosphorus, alkaline phosphatase, C-reactive protein (CRP), fibrinogen, albumin, proteinuria in 24-hour urine and glomerular filtration rate (GFR) – at the beginning of the study (control examination I), after two (control examination II), four (control examination III) and six months (control examination IV), and lipid status (total cholesterol, triglycerides, LDL-cholesterol, HDL-cholesterol, atherosclerosis risk factor – FAKRIZ and atherosclerosis index – INDART), HbA1c values in both groups at the beginning (control examination I) and at the end of the study (control examination IV) and $25(OH)D_3$ level only in the study group at the end of the study (control examination IV). The $25(OH)D_3$ was determined by the chemiluminescence method with acridinium ester-CMIA on the Abbott Architect I 1000 Immunochemical Analyser of MEDLAB laboratory (accreditation number: 03–008, with the accepted requirements prescribed by SRPS ISO 15189: 2014). Abbot tests were used. For blood sampling vacutainers and vacutainer needles Becton Dickinson, ref 367955 were used. The samples were sent on the same day to a central laboratory and were analysed within 6 hours.

Ethical aspects

The study design was approved by the local Ethics Committee. Each participant in the study signed a consent form.

Statistical analysis

Using the International Business Machines Corporation (IBM) Statistical Package for Social Sciences (IBM SPSS) version 20 and STATISTICA version 11 data were analysed. Qualitative data were presented in the form of numbers and percentages while quantitative data with parametric distribution were presented in the form of means, standard deviations (SD) and ranges. The whole tests were two sided. When the *p* value was less than 0.05, it was considered statistically significant; when it was less than 0.001 it was highly statistically significant, and greater than or equal to 0.05 it was statistically insignificant. The relationship between the two continuous variables was determined by quantitative correlation measures (Pearson's correlation coefficient). The results were interpreted and explained. The difference between the mean values of all observed variables at monitored time intervals of the applied therapy (I–IV) at the beginning and end of the study was analysed using the *t*-test and the *Z* test.

Table 1

Seasonally defined limits for the required level of vitamin D3

Month	Minimum level of 25-(OH)D3 (nmol/L)			
	> 50		> 80 female	
	male	female	male	female
July	81	65	131	105
August	87	69	137	109
September	87	71	137	111
October	79	67	129	107
November	69	62	119	102
December	59	57	109	97
January	52	52	102	92
February	50	50	100	90
March	50	50	100	90
April	53	51	103	91
May	61	54	111	94
June	71	60	121	100

Results

From 109 patients who were screened for the study, 19 of them had normal levels of vitamin D3. Thus, the prevalence of patients with vitamin D3 deficiency in the study sample was 82.56%, while the normal values of vitamin D3 were found in 17.43% of the subjects, out of which 10 (52.63%) were men and 9 (47.36%) women.

The clinical and biochemical characteristics of the patients in the experimental and control group are given in Table 2.

The distribution of vitamin D3 values in all study patients with deficient and normal vitamin D3 values, in total and by gender, is shown in Tables 3 and 4.

The average normal levels of vitamin D3 during follow-up by months and by gender in the examined patients are shown in Figure 1.

The normal levels of vitamin D3 by months of follow-up with respect to the lower limit values for women and men are shown in Figure 2.

The average vitamin D3 levels by month, in total and by gender, for patients enrolled in the study and control groups are shown in Figure 3.

The average values of vitamin D3 level per month for all patients in the study and control groups with respect to gender are given in Figures 4 and 5.

Vitamin D3 values measured in patients in both groups (n = 90) at the beginning were 45.1 ± 15.6 nmol/L, in the study group, the mean value was 42.8 ± 15.22 nmol/L and in the control group it was 47.02 ± 16.06 nmol/L. There was a statistically significant difference between deviations in vitamin D3 levels to the lower limit of normal, between summer and winter in the study and control groups together (p = 0.038), as well as in the study group (p = 0.03), where the difference to normal values of this vitamin was higher in summer. The summer period is defined as the period from

Table 2

Clinical and biochemical characteristics of patients in the study and control group

Variables	Study group (n = 45)	Control group (n = 45)	*p*
Mean age (years), M/F	63/65	66.5/63	0.512
BMI (kg/m^2), mean ± SD	29.936 ± 4.392	29.192 ± 4.278	0.418
Duration of diabetes (years), mean ± SD	8.46 ± 4.679	8.52 ± 4.095	0.94
Systolic pressure (mmHG), mean ± SD	130.9 ± 11.324	128.51 ± 10.224	0.296
Diastolic pressure (mmHg), mean ± SD	79.77 ± 5.999	79.88 ± 5.486	0.921
Antihypertensive therapy (n), yes / no	43/2	40/5	
monotherapy	9	8	
dual therapy	20	18	
triple therapy	14	14	
ACEI or ATB (n), yes/no	40/5	37/8	
Oral antidiabetics (n), yes/no	42/3	43/2	
monotherapy	27	20	
dual therapy	15	25	
Hypolipemics (n), yes/no	18/27	23/22	
statins	15/30	18/27	
fibrates	3/42	5/40	
Sedimentation rate (mm/h), mean ± SD	18 ± 14.69	12.9 ± 10.376	0.043
CRP (mg/L), mean ± SD	6.19 ± 7.856	4.11 ± 5.099	0.668
Fibrinogen (g/L), mean ± SD	4.048 ± .979	3.71 ± 0.755	0.88
Albumin (g/L), mean ± SD	43.8 ± 4.145	44.84 ± 5.143	0.306
Calcium (s) (mmol/L), mean ± SD	2.42 ± 0.135	2.426 ± 0.109	0.837
Phosphorus (s) (mmol/L), mean ± SD	1.097 ± 0.164	1.041 ± 0.216	0.132
Alkaline phosphatase (U/L), mean ± SD	76.59 ± 72.09	72.09 ± 21.01	0.733
HbA1c (mmol/moL), mean ± SD	47.8.57 ± 5.076	47.592 ± 5.486	0.331
Cholesterol (mmol/L), mean ± SD	5.352 ± 1.164	5.48 ± 1.119	0.565
Triglycerides (mmol/L), mean ± SD	2.091 ± 1.401	1.830 ± 0.825	0.296
HDL (mmol/L), mean ± SD	1.171 ± 0.297	1.23 ± 0.267	0.201
LDL (mmol/L), mean ± SD	3.42 ± 0.905	3.443 ± 0.960	0.806
FACRIZ, mean ± SD	4.74 ± 1.125	4.547 ± 1.032	0.186
INDART	2.962 ± 0.811	2.834 ± 0.852	0.190
GFR (mL/min), mean ± SD	100.782 ± 33.76	105.31 ± 46.452	0.500
24 h proteinuria (g), mean ± SD	0.683 ± 1.446	0.680 ± 1.161	0.335
Calcium(u) (mmoL/24 h), mean ± SD	3.069 ± 1.496	5.342 ± 3.151	0.00
Vitamin D3 (nmol/L), mean ± SD	43.2 ± 15.082	47.02 ± 16.069	0.198
No. of drops of cholecalciferol (mean ± SD)/ (IU)	4.72 ± 1.448/237	–	

M – males; F – females; BMI – body mass index; ACEI – angiotensin converting enzyme inhibitors; ATB – angiotensin receptor blockers; CRP – C-reactive protein; HDL – high density lipoprotein; LDL – low density lipoprotein; FACRIZ – risk factor for atherosclerosis; INDART – index of atherosclerosis; GFR – glomerular filtration rate [GFR (mL/min) = Cu*Vu(mL)/Cp*1,440 min (C – concentration, V – Volume, u – urine, p – plasma)]; (s) – serum; (u) – urine; IU – international unit.

Table 3

Vitamin D3 levels (nmol/L) in subjects with low values of vitamin D3

Month	All subjects (SG + CG)		Males			Females		
	vitamin D3	(n)	vitamin D3	lower limit value/month	(n)	vitamin D3	lower limit value/month	(n)
January	32.88 ± 10.82	7	36.87 ± 11.2	52	2	27.56 ± 9.4	52	2
February	31.18 ± 6.17	8	31.92 ± 6.56	50	3	29.96 ± 6.6	50	1
March	31.53 ± 12.6	6	31.53 ± 18.9	50	1	34.36 ± 4.1		2
April	38.95 ± 6.75	6	41.13 ± 8	53	1	36.76 ± 5.9	51	2
May	46.28 ± 13.81	6	53.77 ± 4.52	61	1	37.9 ± 15.5	54	2
June	38.05 ± 11.65	6	52.1	71	1	35.24 ± 10.5	60	2
July	53.7 ± 15.1	5	53.42 ± 17.5	81	2	55.1	65	1
August	44.97 ± 19.14	9	53.83 ± 19.35	87	3	33.9 ± 13.5	69	2
September	57.35 ± 16.14	10	53.82 ± 20.24	87	2	60.88 ± 12.5	71	3
October	61.29 ± 13.94	10	57.7 ± 17.54	79	1	66.67 ± 2.4	67	3
November	46.128 ± 8.9	8	52.46 ± 6	69	1	43.85 ± 9.13	62	2
December	46.94 ± 9.31	9	51.27 ± 7.6	59	2	43.48 ± 10.95	57	3
Total	45.1 ± 15.6	90	47.75 ± 15.89		20	42.57 ± 14.98		25

SG – study group; CG – control group.

Table 4

Vitamin D3 levels (nmol/L) in subjects with normal reference values of vitamin D3

Month	All subjects (SG + CG)		Males		Females	
	vitamin D3	(n)	vitamin D3	(n)	vitamin D3	(n)
January	67.2	1			67.2	1
February	57.6	1			57.6	1
March						
April	82.2	1			82.2	1
May	60	1			60	1
June						
July	72.1	1			72.1	1
August	88.25 ± 1.2	2	88.25 ± 1.2	2		
September	93.43 ± 20.11	3	113.1	2	83.6 ± 15.13	1
October	85.2 ± 6.7	5	89.53 ± 3.17	2	78.75 ± 4.88	3
November	81.0 ± 3.2	3	81.0 ± 3.2	3		
December	64	1	64	1		
Total	80.47 ± 13.36	19	86.52 ± 12.38	10	73.75 ± 11.53	9

SG – study group; CG – control group.

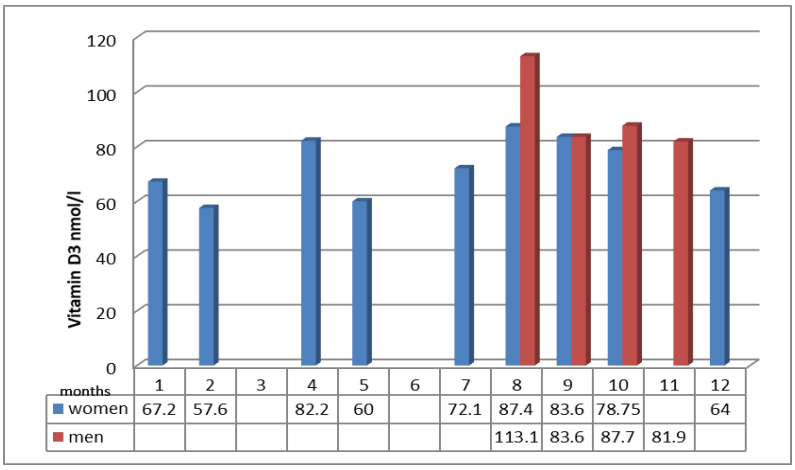


Fig. 1 – The average normal vitamin D3 levels in patients with diabetes mellitus type 2 and diabetic nephropathy during the year from January to December.

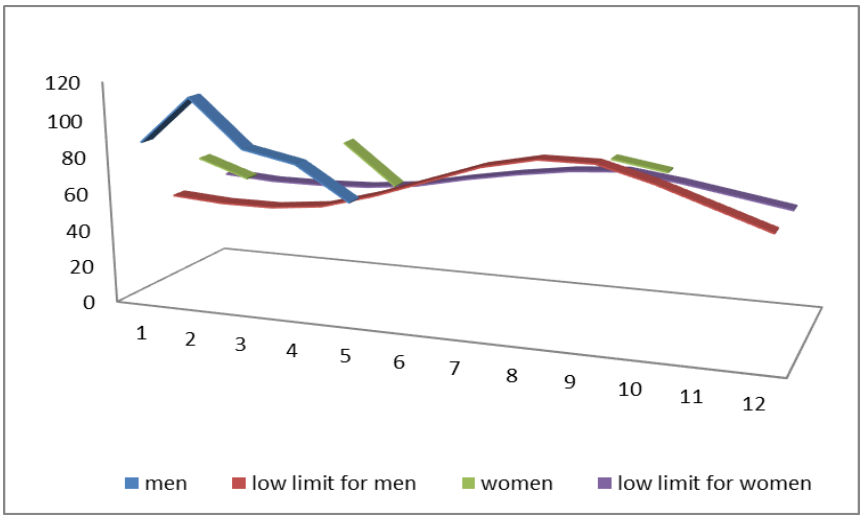
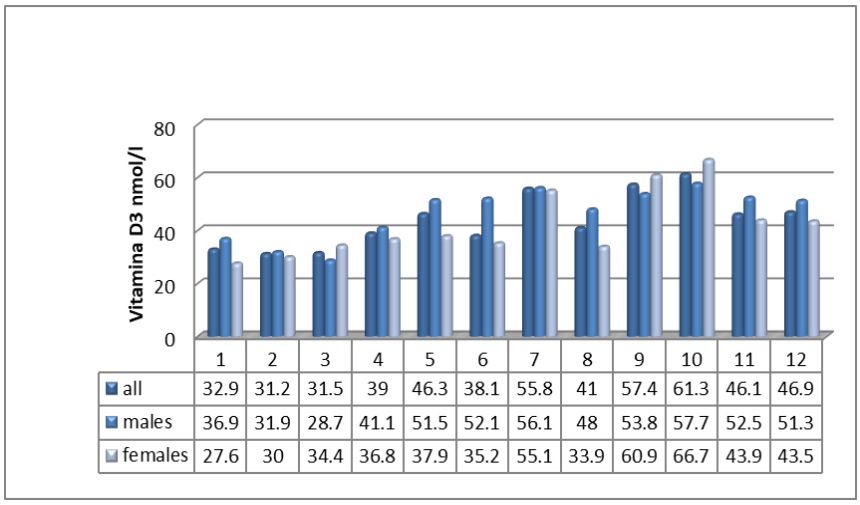
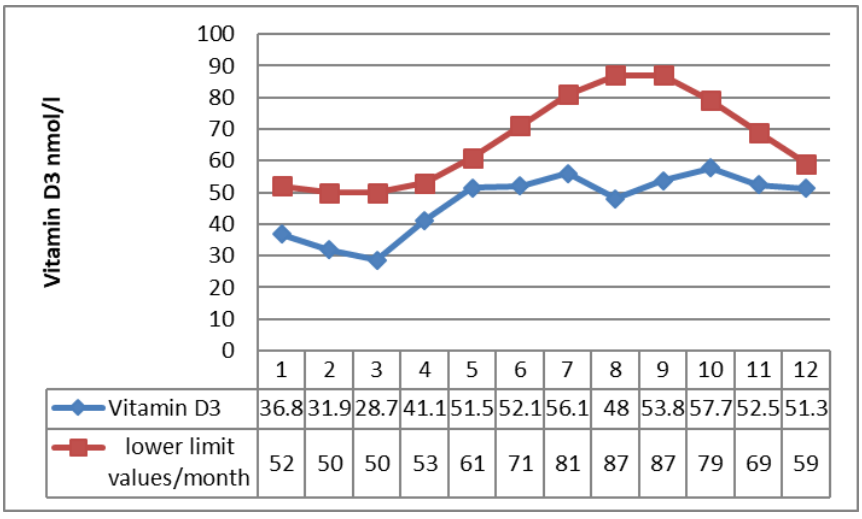


Fig. 2 – The average normal levels of vitamin D3 (nmol/L) in relation to the lower limit (ordinate) in men and women during the year from January to December (abscissa).



	1	2	3	4	5	6	7	8	9	10	11	12
all	32.9	31.2	31.5	39	46.3	38.1	55.8	41	57.4	61.3	46.1	46.9
males	36.9	31.9	28.7	41.1	51.5	52.1	56.1	48	53.8	57.7	52.5	51.3
females	27.6	30	34.4	36.8	37.9	35.2	55.1	33.9	60.9	66.7	43.9	43.5

Fig. 3 – The average vitamin D3 deficiency levels per month during the year, from January to December, in both the study and control groups.



	1	2	3	4	5	6	7	8	9	10	11	12
Vitamin D3	36.8	31.9	28.7	41.1	51.5	52.1	56.1	48	53.8	57.7	52.5	51.3
lower limit values/month	52	50	50	53	61	71	81	87	87	79	69	59

Fig. 4 – The average values of vitamin D3 deficiency during the year in men from month 1 (January) to 12 (December)

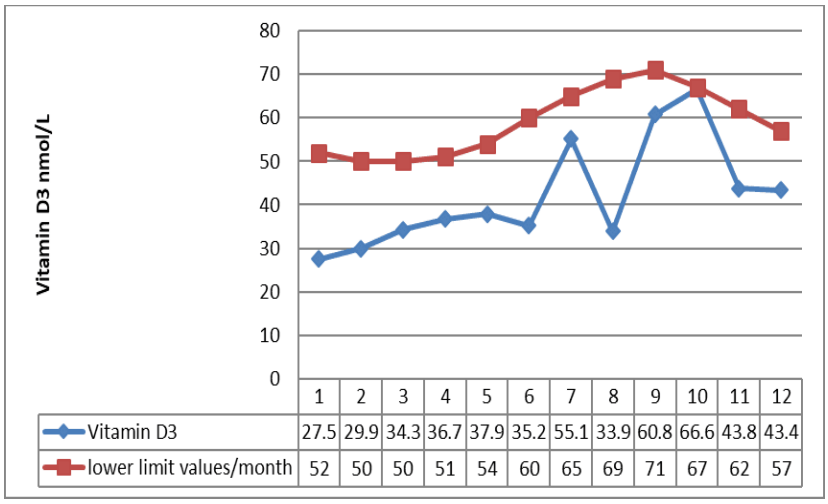


Fig. 5 – The average values of vitamin D3 deficiency during the year in women from month 1 (January) to 12 (December).

April to the end of September and the winter period from November to March. In the control group, there was also a difference between the values of vitamin D3 and the lower limit values for this vitamin, which was more pronounced in summer but this difference was not statistically significant ($p = 0.449$). There was also a difference in vitamin D3 values by gender, which was not statistically significant ($p = 0.21$) and was more pronounced during the summer period in men.

In relation to some parameters of bone metabolism, the following results were obtained: a significant positive correlation in the study group existed between serum vitamin D3 levels and serum calcium levels at the beginning ($r = 0.303$; $p = 0.043$) and at the end of the study ($r = 0.312$; $p = 0.49$), alkaline phosphatase activity at the beginning ($r = 0.298$; $p = 0.047$) and serum phosphorus levels ($r = 0.343$; $p = 0.030$) at the end of the study. There was a variable relationship between the values of vitamin D3 and calcium in urine ($r = 0.109$; $p = 0.491$ and $r = -0.033$; $p = 0.836$, respectively), with the fact that in the initial phase of the increase in the value of vitamin D3 there was a positive correlation and in the later phase it was negative one. The analysis of the relationship between glomerular filtration rate (GFR) and monitored parameters showed a statistically significant correlation only with urinary calcium ($r = 0.46$; $p < 0.01$). Alkaline phosphatase activities in the study group showed a statistically significant difference between control examination I (at the beginning of the study) and III (after four months) ($t = 2.091$; $p = 0.043$) and between control examination I (at the beginning of the study) and IV (after six months) ($t = 2.389$; $p = 0.022$), while in the control group there was no statistically significant difference (Figure 6).

There was a statistically significant difference in the values of serum calcium in the study group between control examination I (at the beginning of the study) and II (after two months) ($t = -3.894$; $p = 0.00$) as well as between control examination I (at the beginning of the study) and III (after four months) ($t = -2.027$; $p = 0.049$) and control examination I (at the beginning of the study) and control examination IV (after six months) ($t = -2.624$; $p = 0.012$), while between control examination II (after two months) and III (after four months), the value of the difference was at the limit of statistical significance ($t = 1.964$; $p = 0.056$). In the control group,

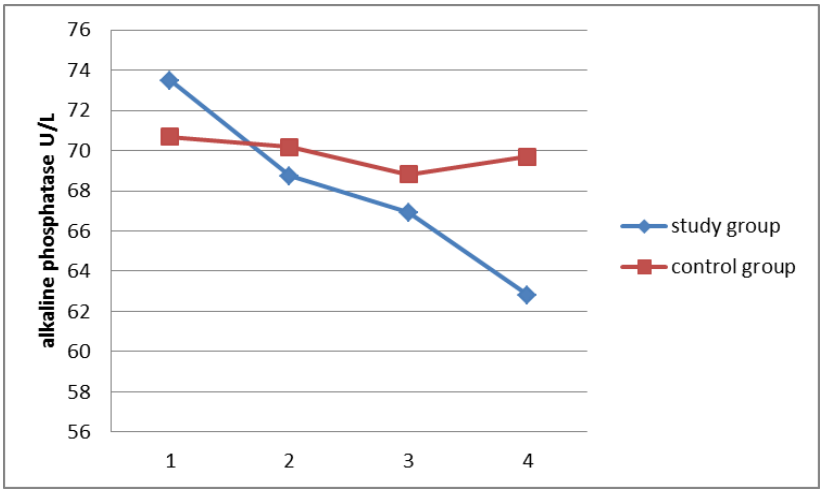


Fig. 6 – Average values of alkaline phosphatase in the both groups. 1 –at the beginning of the study; 2 – after two months; 3 – after four months; 4 – after six months.

there were no statistically significant differences in serum calcium levels among between control examnations (Figure 7).

The value of calcium in urine in the initial phase of the study in the study group increased and a statistically significant difference was found between control examination I (at the beginning of the study) and II (after two months) (t = 3.387; p = 0.002), as well as between control examination I (at the beginning of the study) and III (after four months) when the increase in calcium levels was less pronounced (t = 2.664; p = 0.011). Furthermore, the follow-up calcium values were slightly increased but without a statistically significant difference. The average values of calcium in urine remained in the reference range. The average value of calcium in urine in the control group was higher than the value in the study group, without significant changes among control examinations during the follow-up (Figure 8).

Discussion

It is known that, there is an increased frequency of vitamin D3 deficiency in the patients with T2DM [16]. Lower levels of vitamin D3 have also been found to be strongly correlated with a higher prevalence of microvascular complications such as nephropathy and retinopathy [17]. This study is, to our knowledge, the first one which took into account seasonally defined limit values for this vitamin, by months of the year and by gender of patients, when determining vitamin D levels.

The vitamin D3 value at the beginning of the study in all included patients in the study and control groups (n = 90) was 45.1 ± 15.6 nmol/L. In a study by Majiti and Lochan [18], the value of vitamin D3 level was 49.6 ± 8.9 nmol/L in the group of subjects older than 45 years. The difference in the

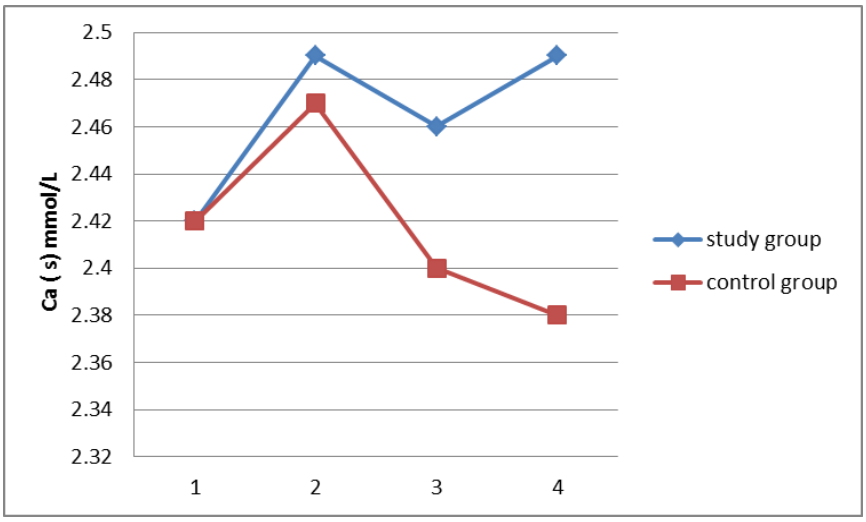


Fig. 7 – Average serum calcium values during the follow-up in both groups. 1 –at the beginning of the study; 2 – after two months; 3 – after four months; 4 – after six months.

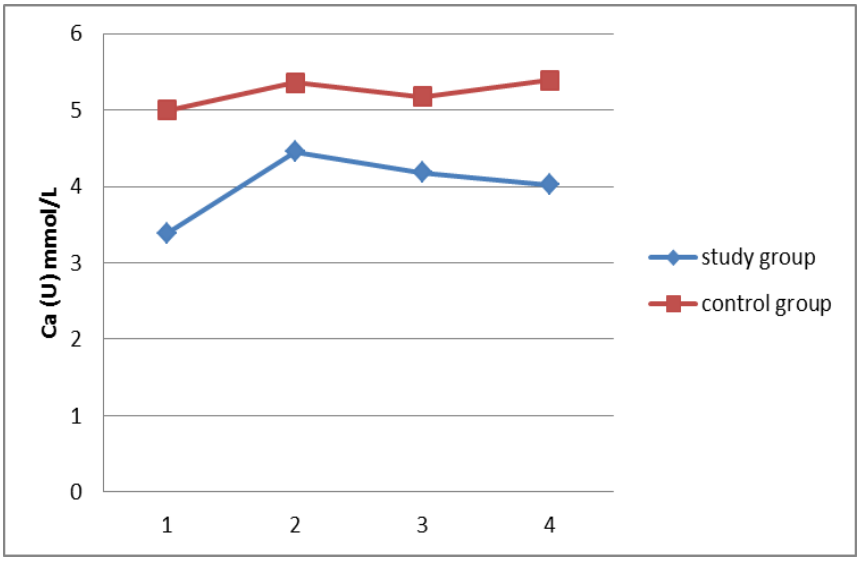


Fig. 8 – Average levels of calcium in the urine during the follow-up in both groups. 1 –at the beginning of the study; 2 – after two months; 3 – after four months; 4 – after six months.

obtained results in these studies can be explained by the different age structure of the study participants.

The prevalence of vitamin D3 deficiency in the study sample of 109 patients was 82.56%, while the normal vitamin D3 values were found in 17.43% of the subjects, out of which 10 (52.63%) were men and 9 (47.36 %) women. These results were obtained using the classification of vitamin D3 levels as reduced and normal values, which was performed according to the data of Bolland et al. [19] and modified according to our geographical conditions (Table 1). This data shows the lower normal limits for vitamin D3 for each month of the year, for men and women separately. This seasonally adapted classification for vitamin D3 deficiency is not commonly used in the patient's follow-up. Better insight into the comparison with the results of other authors can be obtained if the results of this study are expressed through classification according to which the normal values of vitamin D3 are determined by values of > 75 nmol/L, insufficiency with values between 45 and 75 nmol/L and vitamin D3 deficiency as < 45 nmol/L. Based on these criteria, we found vitamin D3 levels to be within the normal range in 18 (16.51%) patients in our sample, while in 48 patients (44.03%) we found insufficiency and in 43 (39.44%) patients, deficiency of vitamin D3.

Vitamin D3 deficiency is known to be more pronounced in diabetic patients than in the healthy population. In a study by Bayani et al. [20], among diabetic patients with a mean age of 51.2 ± 7.98 years, the mean value of vitamin D3 levels was 46.75 ± 25.5 nmol/L, while in the group of healthy subjects with a mean age of 50.6 ± 7.73 years, it was 61.5 ± 33.75 nmol/L. Among diabetic patients, vitamin D3 level was found to be within the reference range in 10.3%, deficiency in 64.2%, and insufficiency in 25% of the patients. The difference in the proportion of patients with vitamin D3 deficiency and insufficiency between our patient group and the cited author group can be partly explained by the difference in the age of diabetic patients, taking into account that expressiveness of vitamin D3 deficiency increases with age [21], also with different climatic conditions (the Pannonian-continental climate in Serbia and the mountainous-continental in northern Iran) as well as their diet. Due to the proximity of the Caspian Lake, it is possible that the fish diet in this population is higher, while the intake of fatty meat, as a nutritional source of vitamin D, is limited in our population.

Some other studies show results that are comparable to our results. In a study of Aljabri [22], patients with DN were found to have the following prevalence of vitamin D status: normal value was found in 17%, deficiency in 55.6% and insufficiency in 27.3% of patients. The mean age of patients was 54.4 ± 16.5 years. In our group of patients who were older than the patients in the mentioned study, the insufficiency (39.44%) was more represented and the deficiency (44.03%) of vitamin D3 was less present.

Because patients were enrolled in the study during the twelve months on a monthly basis, the difference between the deviations in vitamin D levels to the lower limit of normal for each month of the year was analysed on admission to the study. Patients were divided into two periods according to the time of enrolment into the study: summer and winter. The summer period is defined as the period from April to the end of September and the winter period from November to the end of March. A statistically significant difference was found between winter and summer values in the whole sample of participants with vitamin D3 deficiency, as well as in the study group of patients, with the deviation towards the normal values of this vitamin being greater in summer. In the control group, a difference between the measured values of vitamin D3 and the lower limit of normal values was also found, which was more pronounced in summer, and compared to gender, more pronounced in men, but none of the obtained differences was statistically significant. Our results do not correspond to the results of Carnevale et al. [23] who found the presence of vitamin D3 deficiency in 17.8% of subjects in the whole sample in winter and 2.2% of participants in summer, while in women, the deficiency was registered in 27.8% in winter and 3.4% in summer. In men, no difference was found in vitamin D deficiency in summer and winter. In this study, a single cut-off value for vitamin D3 deficiency was set at 75 nmol/L, and in these subjects vitamin D3 was determined twice a year, in February and in August. The study included healthy young adults (men average age 39.4 ± 7 years and women average age 36.9 ± 6.4 years) who are likely to have different dietary habits and more common outdoor activities resulting from good health. It is known that vitamin D synthesis in the skin under the influence of UV rays contributes significantly to its concentration during the summer. It was also found that the skin ability to synthesise vitamin D decreases with age [24].

Recent studies have suggested that there is a difference in vitamin D3 levels in women and men. Seasonally adjusted lower limit values for vitamin D3 have been defined and are different in men and women [15]. This difference is explained by the different BMI between genders [25], differences in lifestyle, cultural and religious factors that are associated with exposure to sunlight, physical activity, and the use of skin protection against UV damage [26]. Gender-related differences in vitamin D metabolism are also of importance [27].

In our patients, no difference was found in the level of vitamin D3 in relation to gender: the mean value at the study enrolment was 42.78 ± 15.37 nmol/L for women and 43.14 ± 15.1 nmol/L for men (p = 0.413). The patients were older middle-aged (men = 64.98 ± 7.58 years and women = 64.19 ± 7.05 years), and no difference was found between the mean BMI in men (29.09 ± 4.0 kg/m^2) and in women (30.07 ± 4.7 kg/m^2) (p = 0.345). The exposure to sunlight through outdoor activities was reduced due to age and associated diseases, and dietary habits in the winter allow more nutritional intake of vitamin D, resulting in a lower vitamin D3 deficit in winter than in summer.

The impact of vitamin D3 deficiency and its supplementation on some parameters of bone metabolism is of importance for monitoring and analysis. It is known that the vitamin D3 deficiency reduces the intestinal absorption of calcium by 15%–30%, which increases the level of serum parathyroid hormone (PTH) [28]. Alkaline phosphatase, which is important for bone formation and mineralisation, is usually

elevated in response to the action of PTH leading to stimulation of osteoblastic activity.

Vitamin D deficiency, however, is most commonly associated with normal serum calcium and phosphate values, high normal or elevated PTH values, normal or elevated alkaline phosphatase activity values, and low 24-hour urinary calcium excretion. Hypocalcemia and hypophosphatemia are rarely seen in patients with severe and long-term vitamin D deficiency [29]. In the study group of patients who used cholecalciferol, serum calcium and phosphate increased as a sign of an increase in vitamin D values, while a decrease in the value of alkaline phosphatase was registered as an expression of suppression, i.e. normalisation of previously elevated PTH values. Heaney et al. [30] found that maximal renal calcium absorption in men occurs when 25(OH)D levels are 70 nmol/L to 90 nmol/L, which otherwise represent the cut-off value for the onset of PTH suppression.

The mechanism by which vitamin D supplements potentially increase the risk of hypercalciuria has not been fully elucidated. Although data suggest that vitamin D supplements increase the risk of hypercalcemia by increasing intestinal calcium absorption, episodes of hypercalciuria are not correlated with hypercalcemia [31]. In a study by Tacheri et al. [32], an increase in calcium in urine from 3.74 mmoL/24 h to 5.7 mmoL/24 h was registered [32], while in our study after the initial increase in calcium urinary values from 3.34 mmoL/24 h to 4.5 mmoL/24 h, a stagnation of its values was registered with a slight decrease by the end of monitoring. In this research, it was concluded that despite the increase in calciuria during supplementation with vitamin D, it was not correlated with the increase in vitamin D values or changes in PTH values [32]. It has been suggested that other, predominantly dietary factors may be associated with hypercalciuria. Thus, in 24 h urine, a significant increase in the value of excreted urea was registered due to increased protein intake, and natrium and sucrose were also found [31]. In our study, a statistically significant correlation was found between vitamin D3 and calcium levels, only in serum, not in urine, at the beginning and end of the follow-up.

PTH levels are increased in patients with vitamin D deficiency, and it is expected that its values decrease during supplementation. A decrease in serum PTH could lead to an increase in urinary calcium due to a decrease in calcium resorption in the renal tubules [32]. The absence of this phenomenon can be explained in two ways. First, the tightly regulated conversion of 25(OH)D to $1.25(OH)_2D_3$ under the action of 25-hydroxyvitamin D-1α-hydroxylase (CYP27B1) limits the synthesis of the active form of vitamin D3 and thus prevents excessive intestinal calcium resorption. Second, the increased reabsorption of calcium in the intestines will not result in an increase in calciuria due to additional calcium deposition in the bones in order to restore their mineral content [33].

The limitation of this study is in the relatively small number of monitored patients as well as the lack of PTH values, which was attempted to be compensated by the use for the first time, to our knowledge, a seasonally defined limit values for vitamin D3, by months of the year and by gender of patients, and a detailed analysis of other bone parameters.

Conclusion

Based on our results and in consideration of the natural seasonal variation in vitamin D3 levels, it may be concluded that there is a significant deficiency in vitamin D3 during the whole year in patients with T2DM and nephropathy. Since patients with T2DM lasting more than 5 years are most often elderly, a deficiency of this vitamin can be expected due to its reduced synthesis in the skin which becomes thinner, due to obesity in which vitamin D is deposited in adipose tissue, due to reduced outdoor activity as a consequence of associated comorbidities, as well as due to the altered diet recommended for these patients - which does not contain a sufficient nutritional source of vitamin D. Significant majority of these patients have vitamin D deficiency and therefore its supplementation is recommended due to its multiple beneficial effects. The safety profile of serum and urine calcium values during long-term use is good, a slight reduction in calciuria was found during therapy. Deviations from normal values are more pronounced in the summer. Individual treatment with vitamin D3 supplements is required at intermittent intervals, which should be adjusted according to the diet and exposure to sunlight.

Conflict of interest

The authors have no conflict of interest to declare. There was no outside funding for the study.

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Indicators of the effectiveness of the healthcare financing system in the Western Balkan countries – critical analysis

Pokazatelji efikasnosti sistema finansiranja zdravstvene zaštite u zemljama Zapadnog Balkana – kritička analiza

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Abstract

Background/Aim. The main objective of the health system is to preserve and improve the general level of health of the population. Every country is making considerable efforts to ensure a sustainable healthcare financing system that would enable the qualitative realization of basic social security rights, rights to healthcare. The aim of the study was to determine the difference between the health system and the concepts of financing through the critical analysis of the system/model and indicators of financing health care in the Western Balkan countries. **Methods.** An overview of the current state of the health care system in the Western Balkan countries was based on data collected from sources such as the World Bank, World Health Organization, United Nations Development Programme (UNDP) reports, health ministries, finance ministries and statistical institutes of all countries in the analysis. Following the classification of the data, some categories were created to identify differences and similarities between the funding methods used in the Western Balkan countries. The analysis was performed by measuring the effect of healthcare funding on variables by measuring performance. Because it is impossible to measure the relationship between variables in a single regression analysis model, several regression functions were used for accurately determining the relationship results. **Results.** The two indicators: a total expenditure on health services and institutions as a percent of gross domestic product (GDP), and health expenditure *per capita* shows weak positive correlation ($p = 0.3$) indicating that a higher amount of GDP *per capita* does not have a positive impact on the percentage of health expenditure in the Western Balkan countries observed. Despite differences in expenditures, all countries had a relatively similar funding method with different regulation that has impact on effectiveness of health system and resources used. **Conclusion.** The health sector in the Western Balkans is characterized by a lack of adequate administrative resources, legislation and regulations, as well as significant constraints in securing the necessary budget. Considering the resources devoted to the health sector in the Balkan countries, it can be said that the authorities in these countries do not see the health system as an important pillar of the country's development, as they do not devote sufficient financial resources to ensure the functioning of the health system.

Key words:
balkan peninsula; economics, medical; health care costs; health care sector; models, theoretical.

Apstrakt

Uvod/Cilj. Glavni cilj zdravstvenog sistema je očuvanje i poboljšanje opšteg nivoa zdravlja stanovništva. Svaka država ulaže značajne napore da osigura održiv sistem finansiranja zdravstvene zaštite koji bi omogućio kvalitativnu realizaciju osnovnih prava stanovništva na socijalno osiguranje, tj. prava na zdravstvenu zaštitu. Cilj istraživanja bio je da se utvrdi razlika između sistema i koncepata finansiranja kroz kritičku analizu sistema/modela i pokazatelja finansiranja zdravstvene zaštite u zemljama Zapadnog Balkana. **Metode.** Pregled trenutnog stanja sistema zdravstvene zaštite u zemljama Zapadnog Balkana zasnovan je na podacima prikupljenim iz izvora Svetske banke, Svetske zdravstvene organizacije, izveštaja Programa Ujedinjenih nacija za razvoj, ministarstava zdravlja, ministarstava finansija i zavoda za statistiku svih analiziranih zemalja. Nakon klasifikacije podataka, kreirane su neke kategorije da bi se identifikovale razlike i sličnosti između metoda finansiranja u zemljama Zapadnog Balkana. Analiza je urađeno merenjem efekata finansiranja zdravstvene zaštite na promenljive, merenjem učinka. Kako je nemoguće izmeriti odnos između promenljivih u jednom modelu regresione analize, u studiji je korišćeno

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nekoliko regresionih funkcija kako bi se tačno utvrdili rezultati odnosa. **Rezultati**. Dva pokazatelja – ukupni izdaci za zdravstvene usluge i ustanove, kao procenat bruto domaćeg proizvoda (BDP), i zdravstveni izdaci po glavi stanovnika pokazali su slabu, pozitivnu korelaciju (p = 0,3), što ukazuje na to da veći iznos BDP po glavi stanovnika nema pozitivan uticaj na procenat troškova za zdravstvo u posmatranim zemljama Zapadnog Balkana. Uprkos razlikama u troškovima, sve zemlje su imale relativno slične načine finansiranja sa različitom regulativom koja utiče na efektivnost zdravstvenog sistema i resursa koji se koriste. **Zaključak**. Zdravstveni sektor na Zapadnom Balkanu karakteriše nedostatak adekvatnih administrativnih resursa, zakonodavstva i propisa, kao i značajna ograničenja u osiguravanju potrebnog budžeta. Uzimajući u obzir resurse posvećene zdravstvenom sektoru u zemljama Zapadnog Balkana, može se reći da vlasti u tim zemljama ne vide zdravstveni sistem kao važan stub razvoja zemlje, jer ne izdvajaju dovoljno finansijskih sredstava za osiguranje funkcionisanja zdravstvenog sistema.

Ključne reči:
balkansko poluostrvo; ekonomija, medicinska; zdravstvena zaštita, troškovi; zdravstvena zaštita, pružanje usluga; modeli, teorijski.

Introduction

Modern healthcare systems differ the most in the methods of raising funds for health care, as well as in the payment methods of health care providers. Healthcare costs vary from country to country depending on its development. They are measured by the issue of *per capita* health supplies or as a percentage of total national income. The sources of financing the healthcare system are: state budget – general and specific taxes, insurance fund – compulsory health insurance (contributions), voluntary/private insurance (insurance premiums), participation (personal participation of the health insurer in the costs of using the health service), full price of the service (private practice) and donations and voluntary contributions from institutions, groups and individuals. The issue of defining healthcare financing involves not only the method of payment, but also the persons contributing to its payment, how the beneficiaries and providers are involved in the transaction and how much is spent on healthcare. Consequently, the way the health sector is financed is quite sensitive, as it can be a deciding factor for the various implications of the overall health care system.

The healthcare system must provide physically, geographically and economically accessible, integrated (vertical connection of primary, secondary, tertiary level and horizontal connection in the system and in relation to the local community) and high quality health care (continuous improvement of the quality of health care and the right of beneficiaries' physician choice and awareness), personal development of employees working in a healthcare system, sustainability of financing, decentralization of management and financing of healthcare, and placement of citizens at the center of the healthcare system and protection.

Given the demographic trends present throughout Europe, including the countries of the Western Balkan, and especially the increase in the proportion of the elderly, it is a fact that a larger number of individuals require some health care. Also, the advancement in the field of medicine requires the application of new and more expensive treatments, including new medicines and modern equipment. All this implies, in the long run, an increase in costs and the need for greater investment in the health care system.

The healthcare system in the Western Balkan countries is currently facing a number of issues related to health care financing [1]. In particular, some of the major financial problems that have accompanied the healthcare system in this region are the funding methods used in financing health activities and the attitude of the authorities in these countries regarding the performance and quality of health care. Regardless of the decision makers, those who bear the costs of the health sector are citizens of the Western Balkan countries whose social protection is deteriorating due to denial of access to quality health services [1].

The aim of the study was to determine the difference between the healthcare system and the concepts of financing through the critical analysis of the system/model and indicators of financing healthcare in the Western Balkan countries.

Methods

The current state of the healthcare system in the Western Balkan countries, was based on data from reliable and credible sources such as the World Bank, the World Health Organization, United Nations Development Programme (UNDP) reports, health ministries, finance ministries and statistical institutes of all countries and desk analysis was done; the narrative was presented as background in the text above. Variables proven to be important for cross-country financial comparisons are total health expenditure – total expenditure on health services and institutions as a percentage of each country's gros domestic product (GDP) in the Western Balkans and *per capita* health expenditure – total *per capita* expenditure of each country in the region for one specific year (2017).

Since it was impossible to measure the relationship between variables in a single regression analysis model, several regression functions were used in the study to accurately determine the relationship results. Statistical analysis was performed with SPSS version 20.0 statistic software package. The Kolmogorov-Smirnov statistics was used to assess the normality of the distribution of scores. A non-significant result (p value of more than 0.05) indicates normality. Since both variables had a normal distribution

($p > 0.05$), the dependence between them was determined using Pearson's correlation coefficient.

Results

Albania is a Balkan country whose health sector is funded by a combination of general tax, payroll tax, compulsory health insurance and voluntary health insurance expenditures, out-of-pocket payments and various domestic donors. Among them, the Ministry of Health and the Health Insurance Institute play the most important financial role. It has been shown that Albania has managed to increase its economic development, but the health sector is still significantly underdeveloped [1–3]. According to a report published by the World Bank, many indicators suggest that Albanian health care has progressed in recent decades, but other sources indicate that its health sector is not in a favorable position relative to Southeast European countries. According to World Health Statistics, published by the World Health Organization in 2017, total health expenditure in Albania was 6.9% of the total GDP (Figure 1), while *per capita* health expenditure was USD 520 (Figure 2), one of the lowest in the region [4].

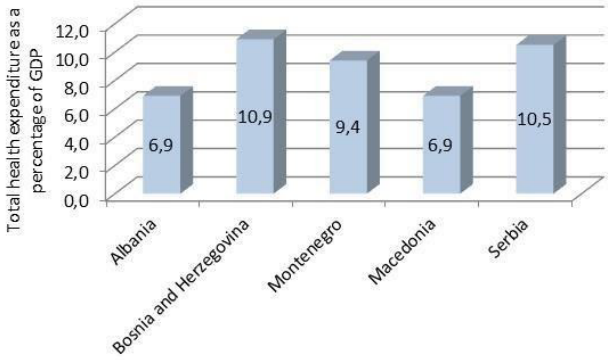

Fig. 1 – Total health expenditure as a percentage of gross domestic product (GDP) [4].

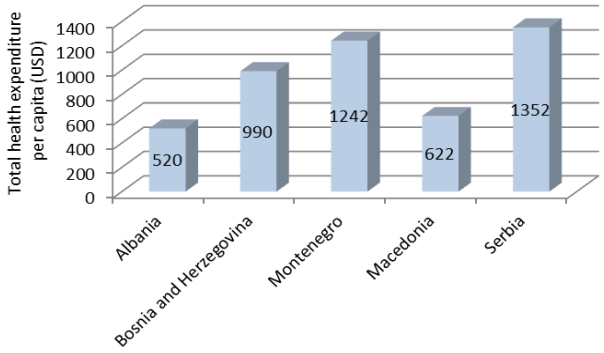

Fig. 2 – Total health expenditure *per capita* [4].

Due to low public expenditure on health care, out-of-pocket expenditures are high, accounting for 56.1% of total health expenditure and 99.8% of total private expenditure (Figure 3). High levels of payments from the Treasury are causing serious implications for "equity, poverty and the health sector". Moreover, a World Bank publication classifies healthcare quality in Albania as low, mainly because human capital remains isolated and unable to receive training to improve their skills [5].

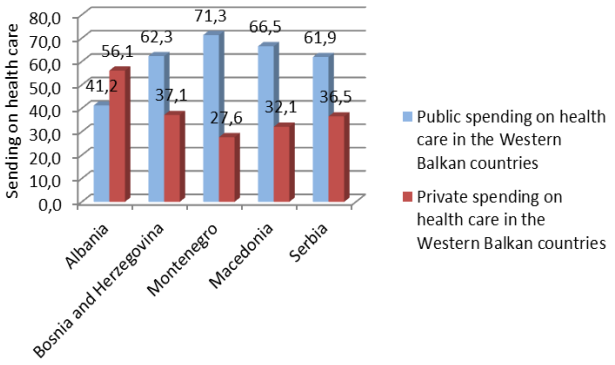

Fig. 3 – Public and private spending on health care in the Western Balkan countries [4].

Bosnia and Herzegovina is funded by compulsory national health insurance, state budget, private contribution and donations. The health system in Bosnia and Herzegovina suffers from inefficient administrative management because the system faces a large number of unnecessary staff due to the different socio-economic situations between the entities and the cantons [6, 7]. Moreover, a report published by WHO shows that the entire economy of Bosnia and Herzegovina is burdened by the effects of an unsustainable financial system in the health sector. WHO statistics showed the financial state of the health system in Bosnia and Herzegovina in 2017, where the total health spending was about 10.9% (Figure 1) of GDP, while *per capita* spending in the same year was $990 (Figure 2). In addition, statistics show that private health care expenditure accounts for 38.7% of the total spending, and that 100% of private expenditure is funded out-of-pocket [4].

In Macedonia, health care is funded through a combination of public and private funds. The Health Insurance Fund (HIF) is funded by the payroll tax, the pension fund, the unemployment fund and the government budget, while out-of-pocket payments consist of most private expenditures. According to a report released by the Ministry of Health, financial management in the health sector is quite poor due to the lack of training of the individuals needed. Basically, this report noted the absence of incentives to control the financial sector in healthcare, and is supported by patients and doctors, who do not report ill-treatment [8, 9].

As a result of poor financial management in the health care system, Apostolska and Tozija [10] argue that high out-of-pocket payments will continue to increase, thus increasing social inequalities between classes of people regarding health services. Total health sector expenditures in Macedonia in 2017 amounted to 6.9% of GDP (Figure 1), and *per capita* health care expenditures amounted to $622 (Figure 2). It is also important to note that out-of-pocket expenditures account for 33% of total expenditures and 99.1% of private expenses [4].

Montenegro is a country where the health sector is funded through mandatory health contributions, general government funds, out-of-pocket payments and donors. According to the development plan of the Ministry of Health in Montenegro, the country has experienced positive steps, but due to poor socio-economic conditions in the country, Montenegro's health is lagging behind compared to EU countries [11, 12]. Furthermore, the WHO World Health Statistics report showed that total health expenditure in Montenegro in 2017 was 9.4% of the total GDP (Figure 1), while per capita health expenditure was $1242 (Figure 2) [4].

The same report further explains that public expenditures are only 71.3% of total expenditures and 28.3% are private expenditures. Out-of-pocket payments include 26% of total health expenditure and 91% of private expenditure. High levels of payment out-of-pocket are some negative signals that the health care system is not functioning properly.

The health system in Serbia is funded by public and private contributions. The Republicn Health Insurance fFund (RHIF) is funded by mandatory contributions and is one of the key sources of financing for the health sector. Healthcare in Serbia is also funded by the state budget and out-of-pocket payments, which consist of almost all private expenditure and donations [13]. WHO statistics showed that the total health spending in Serbia in 2017 was 10.5% of GDP (Figure 1), while per capita spending was $1,352 (Figure 2) [4]. The same statistics also showed a high level of out-of-pocket payments; namely, 35% of total health expenditure and 92.2% of private expenditure, accounting for 38.1% of total expenditure. Out-of-pocket payments can easily create financial blockages and reduce the use of health prevention services due to the high cost of healthcare services [14].

Results of the Kolmogorov-Smirnov statistics are given in Table 1. It assesses the normality of the distribution of scores. A non-significant result ($p > 0.05$) indicates normality. Since both variables have a normal distribution ($p > 0.05$), the dependence between them was determined using Pearson's correlation coefficient (Table 2).

Table 1

Results of the Kolmogorov-Smirnov test for normality

Parameter	GDP/*capita* (USD)	Health expenditure (% of GDP)
Average value	4,868	8.92
Standard deviation	921.61	1.92
Kolmogorov-Smirnov Z	0.396	0.566
p	0.998	0.906

GDP – gross domestic product.

Table 2

Pearson's correlation coefficients (*r*) between GDP and health expenditure

Parameter	GDP		Health expenditure	
	r	*p*	*r*	*p*
GDP	1	-	0.3	0.624
Health expenditure	0.3	0.624	1	-

GDP – gross domestic product.

The value of Pearson's correlation coefficient ($r = 0.3$) shows a weak, positive correlation between the two observed variables, indicating that a higher amount of GDP *per capita* does not have a positive impact on the percentage of health expenditure in the Western Balkan countries observed.

Discussion

Authorities in Bosnia and Herzegovina, Serbia and Montenegro consider the health care system to be very important because they have allocated a relatively large portion of their GDP to secure the health care system in their countries. In 2017, health systems in Bosnia and Herzegovina, Serbia and Montenegro accounted for 10.9%, 10.5% and 9.40% of their total GDP respectively. Knowing this, it can be said that, as a percentage, these countries are on par with many developed countries and even have a higher share of health expenditure than them. On the other hand, there are countries with lower overall costs such as Albania and Macedonia, which are categorized with similar levels of expenditures, namely between 6.70% and 6.90%. Compared to other countries, this low percentage of total costs can serve as a key factor in determining the quality and performance of healthcare.

Focusing only on this variable and keeping everything constant, it can be implied that Bosnia and Herzegovina, Serbia and Montenegro should have their health systems competitive with developed countries, as they are on par with developed EU countries in terms of total health expenditure as a percentage of GDP, while other countries with lower health expenditures should have a less developed health care system because they are quite lagging behind compared to other countries in terms of this variable.

The Balkan countries are considered to have the Bismarck's and Beveridge's system of healthcare financing, but with significant changes in the overall funding methods. Basically, three major financial sources are recognized in the Balkans: Social Security Fund (mandatory contribution as payroll tax), government revenue (from the total budget), and out-of-pocket payments (direct payments by the service user). Nonetheless, voluntary health insurance and donor funding are other financial sources for the health sector in the region, which can be explained as voluntary payments by individuals to avoid catastrophic healthcare costs and payments offered as donations by various organizations.

The Western Balkans is a geopolitical region comprising: Albania, Bosnia and Herzegovina, Macedonia, Serbia and Montenegro. The Western Balkans covers an area of 196,047 km^2 with a population of 21.5 million. The Western Balkan countries have been in the process of transition for the last twenty years. While still a major challenge for the whole region for institutional and structural reform, positive macroeconomic characteristics are evident in the region. At the beginning of this century, the countries of the region recorded the highest economic growth since the beginning of transitional changes. The growth was mainly due to the rapid expansion of consumption and investment-financed loans, and one of the important drivers of progress

was foreign direct investment. However, the problem is that capital inflows in the region are mainly concentrated in several countries (EU candidate countries) and in the most attractive sectors by country (telecommunications; oil, gas and electricity production; food production; steel production and tourism).

In Albania, the health care system is generally public, while private practice has little market share. Albanian law guarantees equal access to health care for all citizens. Albania's public health service is the main provider of health services, health promotion, prevention, diagnosis and treatment for the Albanian population. Primarily, the Albanian Government finances the state health system. Other sources of funding include contributions from qualified employers, employees and the self-employed (a certain percentage of their salaries or income are deducted) and contributions to the insurance scheme [2, 3]. However, poverty in Albania is quite common and only a small number of people can afford such contributions [1]. As a result, many citizens do not receive necessary medical assistance and medication for their illnesses. The failure to raise a significant amount of contributions means that Albania's health care system relies heavily on charitable assistance for medical supplies and medicines.

The existence of catastrophic health care costs is a concern. Disastrous healthcare expenditures not only impose a higher risk of poverty for people seeking healthcare, but can also impose barriers to access to healthcare. Albanian authorities need to give serious consideration to reducing the total out-of-pocket payments, which amount to nearly 60% of the country's total health care expenditure. This is best achieved by ensuring the efficiency and attractiveness of formal health care financing mechanisms (general tax revenue and health insurance). Although improving the efficiency of such mechanisms requires better coordination and allocation of resources, attractiveness could be enhanced by adopting a contribution and participation structure to better reflect revenue sharing. Measures such as exemptions or subsidies for vulnerable groups have already proven effective in reducing catastrophic payments in other countries [15].

Conversely, the complete healthcare system in Bosnia and Herzegovina is characterized by marked fragmentation as it is organized differently in the Federation of Bosnia and Herzegovina, Republic of Srpska and Brčko District of Bosnia and Herzegovina. Viewed through organizational structure and management, it is realized through 13 completely different subsystems, at the level of entities, cantons in the Federation of Bosnia and Herzegovina and Brčko District, which greatly complicates the way health care services are provided, increases the costs of management and coordination and has a poor impact on the rationality of healthcare operations, primarily viewed through the prism of inadequate utilization of economies of scale [1].

The health sector of the Federation of Bosnia and Herzegovina is composed of a network of as many as 11 health ministries (10 cantonal and one federal), 11 health insurance institutes (10 cantonal and Federal health insurance and reinsurance institutes) and 11 public health institutes [6, 7].

When it comes to financing healthcare, it is mainly financed by compulsory health insurance contributions; namely, health insurance contributions from wages, salaries contribution paid by the employer, health insurance contributions paid by pension beneficiaries, farmers' contributions to the unemployed and other categories. In addition, each canton has its own Health Insurance Institute, which bears responsibility for financing health services at its own level. Although the law provides for other forms of financing (cantonal budget, Federation, donations, income of health institutions, participation, etc.), contribution financing is a major source of health revenue [6, 7].

The public health system in Bosnia and Herzegovina, with its current funding model, is clearly not capable of keeping up with the needs, expectations and habits of the population in terms of health services. The fact is that population expectations, demand and need for health services have also been increasing for a long period, mainly because health care is one of the most valuable and significant forms of personal consumption. Also important is the fact that the financing of the public health care system in the Federation of Bosnia and Herzegovina is not subject to a single regulation, but differs by canton. Only the calculation of the base and the rate of contribution for employees at the employer (12.5% at the expense of employees and 4% at the expense of the employer) is uniquely regulated, while the base and rate of contribution for other categories of population are defined differently based on decisions of cantonal assemblies. Therefore, cantonal health insurance institutions are in different financial positions (depending on the number of employees and average gross salary), which has a direct impact on the scope and categories of rights offered to policyholders [7].

Healthcare financing in Montenegro is based on the principles of Bismarck's social health insurance, which is funded by contributions to categories defined by law. According to the latest available data, more than 95% of the population is covered by this insurance. The missing funds for the functioning of the health system and the needs of healthcare are provided from the state budget. These funds relate to the payment of salaries of employees in public health institutions, as well as to the financing of the activities of the Ministry of Health, which implies a mixed financing system, and especially if it is kept in mind that the current legal solutions (Budget Law, Treasury system) are more appropriate to the system budget financing healthcare than insurance system. The minimum additional funding for healthcare financing in Montenegro comes from the personal participation of health care beneficiaries (participation), other payments and donations [11, 12].

The method of payment for healthcare institutions takes the form of budget financing by item. The Fund, based on the Decision on the allocation of funds of the Fund for the current year, allocates funds to health institutions intended for earnings, material costs, medicines and medical devices,

capital expenditures, etc. Health institutions know in advance the monthly amount of funds that the Fund will transfer to them and make payments within the available financial means, and due to the lack of funds to cover all the needs, they report outstanding liabilities. In Montenegro, there is no specific contribution rate for injuries at work and occupational diseases, as in some other countries in Europe, where employers pay special rates of contribution to insure employees from injuries at work and occupational diseases. This type of income differs and is contingent on the amount of risk expenditure [11]. The implementation of the said contribution rate is certainly one of the potential sources of additional funding.

In Macedonia, there are two types of health insurance under the Health Insurance Act: compulsory and voluntary insurance for some forms of health care. Mandatory health insurance has been established for all Macedonian citizens in order to provide social security and healthcare and exercise certain rights in the event of illness or injury and other health care rights set out in the Health Insurance Act. Compulsory health insurance is based on the principles of obligation and universal coverage, solidarity, equity and efficient use of funds in accordance with the law. This means that every insured person can use health services (basic covered by compulsory health insurance) and unlimited health insurance when needed. On the other hand, there is an obligation to all employees and other insurance carriers to continuously pay health insurance contributions. The contribution rate is the same for all employees, regardless of salary or income, or the frequency and amount of health care services used in a health insurance account. The principles of solidarity and fairness are mandatory [8, 9].

Some specific risks and services, which are not covered by compulsory health insurance, should be provided by the employers of certain groups of workers. Compulsory health insurance is a major source of health care revenue. The HIF income is used to fund programs for which the HIF is responsible. Health insurance costs for those who are not enrolled in the program, who are not insured by fund, and their healthcare costs are covered by the state budget. Direct contributions from employers and health insurance workers were 59.4% of the Fund's total revenues in 2017. In addition, their retirement and unemployed contributions include components used for health insurance for retirees, the unemployed, the disabled or social security recipients. These amounts, which amount to about 36.1% of the HIF's income, are paid out of state funds for pensions, unemployment and other social programs. The Fund's revenue from the general budget in 2017 was 0.4%. The Ministry of Finance establishes budgets for the Ministry of Health vertical programs and examines and approves the budget for the HIF [8].

The healthcare system in Serbia is constituted to provide access to all health services for the entire population. Insurance coverage covers all employed persons, pensioners, self-employed persons and farmers who make contributions. In addition, the state budget provides funds for health insurance for the unemployed, internally displaced persons and refugees. The special health insurance coverage system applies to the military, civilians in the military and retirees of the armed forces, as well as their family members and dependents. Healthcare financing in Serbia is a combination of Bismarck and Beveridge model. Basically, the financing of the healthcare system is based on the compulsory health insurance provided by the contributions (10.3% rate), which is the basis of the Bismarck model. On the other hand, for the persons who are not covered by compulsory health insurance (uninsured persons, refugees and internally displaced persons), financing from the budget of the Republic is provided, which is a characteristic of the Beveridge model. Therefore, healthcare financing in Serbia is characterized solely by the public source of financing, as it is largely financed from contributions and from the budget of the Republic [13, 14].

The most important source of financing the healthcare system in Serbia is the Republic HIF (RHIF). Within the public sector of healthcare financiers in Serbia, it was found that the predominant financier was RHIF with a share of 91.2% in 2007 and 93.6% in 2017. Consequently, the payment of the RHIF largely determines the public provision of services. Part of the public financing of health services is also provided by the Ministry of Health, through regional and local governments, the Ministry of Defense, the Ministry of Justice and the Military Health Insurance [13].

As mentioned above, there are four commonly used health financing methods in the Balkans. These four methods of financing healthcare are through direct contribution from the country's budget, health contributions (HIF), direct payments from patients and through donations. In addition to these general healthcare financing methods, many of them are subdivided into specific sources of health care financing. For example, contributions from the state budget can be collected through different types of taxes, while HIF contributions can be collected as a fixed amount for each worker or as a percentage of workers' pay. It is important to note that there is no country that depends solely on one way of financing healthcare, but in all countries, there is a combination of different ways of financing to ensure that there is sufficient budget for health services and to (conditionally) ensure the effective use of funding methods [15–18].

Taxation as a way of financing the health function is a way when certain authorities are responsible for collecting different taxes through different means than citizens operating in that country. These taxes create the country's budget, which allocates part of the budget to different ministries for different purposes. In this case, the Ministry of Health is responsible for receiving part of the budget earmarked for health, and it is the authorities that prioritize the projects and decide how the money will be allocated within the sector. Another way to finance healthcare is through HIF contributions, which are similar to the taxation method. As in the previous methods, HIF contributions are paid by contributors in two forms, in some places they are paid as a fixed amount by each worker, while in others they are paid as a percentage of wages, which means that the

higher the salary, the greater the contribution in absolute value. Unlike the method of taxation, HIF contributions from people operating in a particular country are not classified in the state budget category, but are directly categorized into the health budget separately [15–18].

Another important way of financing healthcare is the category of direct payments by patients. This category is part of private health spending because people pay directly for the health care services they use, without involving any third party in the transaction process. Direct payments, also known as pocket payments, refer to the process when patients visit healthcare facilities and pay directly for the services they use at those facilities. This method is widely used, especially in less developed countries, and is also common in the Balkans. Another method of financing, which is categorized under the umbrella of private expenditure, is through private health insurance. Through this method, patients purchase health insurance packages before needing medical services. Then, in case patients need medical services, they are covered by a third party, as an insurance company that pays for medical services for a patient who has already purchased health insurance. The next form of healthcare funding is through donations. This method occurs when an organization, whether internal or external, offers financial support to a country's healthcare sector. The grants are generally dedicated to less developed countries because they lack adequate financial resources to properly fund the health sector, and as a result, different organizations are constantly ready to assist different countries in establishing and maintaining their health systems [15–18].

Each of the explained ways of financing health care has a positive and negative effect on the health sector of a country. It can not be said that a particular method produces certain result in each country, since there are many other factors affecting country’s health care. Moreover, countries have different needs and priorities, so one method may be most suitable for one country, but not for another.

The issue of defining healthcare financing involves not only the method of payment, but also the persons contributing to its payment, how users and providers are involved in the transaction, and how much is spent on healthcare. Accordingly, the way the health sector is financed is quite sensitive as it can be a deciding factor for the various implications across the healthcare system.

The decision on how to pay for healthcare services is not only an individual issue, but also a matter for society as a whole. Potential alternatives to health sector financing are through public and private expenditures. Public spending refers to general tax revenues collected at different levels. Some countries may even introduce a special tax only to finance the health sector, while other countries only differentiate the fund from the overall state budget. Public expenditure is mainly focused on the well-being of the poor by allowing them access to health services. Businesses suffer large public expenditures because they face double costs, once they pay for their health care treatments and once they pay higher taxes to secure sufficient funds for public health expenditures. In addition, public expenditures in the healthcare system reduce the level of efficiency by reducing competition between public and private healthcare providers [17]. Competition is generally reduced by the fact that, through higher public expenditures, people receive more services in public health facilities; in this case, the readiness of physicians to work in the private sector is reduced. In a study by Jakovljevic et. al. [14], according to purely economic criteria, most institutions responsible for providing public sector services in middle-income economies in Southeast Europe show more than modest performance, which is in complete agreement with the results of this study.

All countries have a similar status in terms of quality and performance of the healthcare sector. Therefore, there is a tendency to believe that increased health expenditure in a country may not lead to improvements in the quality and impact of health care. In their book on whether more money translates into better health, Irvine et al. [18] argue that the question of whether higher costs lead to better health quality and performance is far more complex than it seems, and that the relationship between health costs and health quality is very complex to measure. They have concluded that financial resources are very important and affect many factors that determine a country's health quality; however, they argue that more money does not always lead to better quality of health due to mismanagement or misallocation of resources.

It is important to decide effectively how to finance the health sector in the country, because according to Thomson et al. [16], an efficient system minimizes the losses associated with raising and paying out income. However, countries decide at the individual levels which system best fits the strategies of the country and its citizens. Regardless of the type of financing of the health sector, all countries need to adjust their alternative to financing to achieve three basic principles: increase revenues to provide individuals with planned health care packages that provide health and financial protection against catastrophic medical costs caused by illnesses and injuries in a fair, efficient and financially sustainable manner; managing this revenue to pool health risks equally and effectively; ensure that payment or purchase of health services is done in a manner that is allocative and technically efficient [5].

Fundamentally, these are the main goals of providing an effective way of financing the health sector. Whether these goals are achieved, depends on the economic development and sustainability of the health sector itself.

Conclusion

The healthcare sector in the Western Balkans is currently facing a number of questions regarding health care financing. In particular, some of the major financial problems that have accompanied the health sector in this region are the methods used in financing health activities and the attitude of the authorities in these countries towards health performance and quality. Regardless of the decision-makers, those who bear the costs are the citizens of the Western Balkan countries whose social well-being is

deteriorating as a result of denial of access to quality health services.

Considering that a large part of healthcare activities is financed by private expenditure, especially payments from one's own pocket, the methods of financing healthcare in the Western Balkans are considered inappropriate for the region. In most cases, because of the poverty rate in the region, which is higher than in other countries, it can be said that out-of-pocket payments as a method of financing health care create obstacles for society to access health services. Due to such payment methods, most people living in the Western Balkans do not receive the necessary medical treatment because they are constantly faced with payment obstacles that impede their full access to health services.

Given the resources devoted to the health sector in the Western Balkan countries, it can be said that the authorities in these countries do not see the healthcare system as an important pillar of the country's development because they do not devote sufficient financial resources to ensure the proper functioning of the health care system. Although these countries have experienced economic growth over the years, the budget for health care has not changed in proportion to economic growth; instead, there was a very small increase relative to economic growth. This negligence on the health sector has caused inadequate functionality of the whole system in most Western Balkan countries. The consequence of such action may be considered to be poor performance of actors involved in healthcare, and especially because of the low budget, health systems in the Western Balkan countries have lost a lot of human capital in public health institutions, or their impact has been adversely affected by not having sufficient incentives to be fully dedicated to the health sector in general.

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Genome-wide association study of mitochondrial DNA in Chinese men identifies seven new susceptibility loci for high-altitude pulmonary oedema

Ispitivanjem udruženih genoma mitohondrijske DNK kod Kineza prepoznaje se sedam novih lokusa za visinski edem pluća

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Abstract

Background/Aim. High-altitude pulmonary oedema (HAPE), which normally occurs at altitudes higher than 3,000 m, is a potentially fatal disease due to hypoxia. The role of mitochondrial genomes in determining an individual's susceptibility to HAPE has not been determined yet. However, a number of genetic polymorphisms have recently been found to be overrepresented in HAPE patients. The majority of published genome-wide association studies have investigated only a small number of top-ranking single-nucleotide polymorphisms (SNPs)/genes by the overview of nuclear DNA and considered each of the identified SNPs/genes independently. Little research has been conducted on mitochondrial genomes in relapsing HAPE patients by genome-wide association studies. **Methods.** To identify biological pathways important to HAPE occurrence, we examined approximately 500,000 SNPs genome-wide from 10 unrelated cases of relapsing HAPE and we compared the SNPs in these cases with those in the Chinese in Beijing, China population (45 controls) to discover the association between genotypes and HAPE susceptibility among the mitochondrial function-related genes. We used the FUMA platform to expand those SNPs to selected candidate SNPs. **Results.** A total of 369 candidate SNPs, 4 lead SNPs, 4 genomic risk loci and 5 mapped genes were obtained. The 7 mapped genes were ADAMTS9-AS2, NEK1, CLCN3, C4orf27(HPF1), RP11-219J21.2, ANKRD26 and YME1L1. **Conclusion.** This study confirms the association of ADAMTS9-AS2, NEK1, CLCN3, C4orf27(HPF1), RP11-219J21.2, ANKRD26 and YME1L1 with HAPE, which may provide future targets for the treatment of this disease.

Key words:
genetic techniques; genome; hypoxia; polymorphism, genetic; pulmonary oedema; pulmonaty edema of mountaineers.

Apstrakt

Uvod/Cilj. Visinski edem pluća [*high-altitude pulmonary oedema* (HAPE)], koji se obično javlja na visinama većim od 3 000 m usled hipoksije, potencijalno je smrtonosna bolest. Uloga mitohondrijskih genoma u određivanju podložnosti pojedinca na HAPE nije određena. Međutim, nedavno je otkriveno da je veliki broj genetskih polimorfizama prekomerno zastupljen kod bolesnika sa HAPE. Većina objavljenih studija vezanih za genom istraživala je samo mali broj najčešćih jednonukleotidnih polimorfizama [*single-nucleotide polymorphisms* (SNPs)/gena] pregledom nuklearne DNK i razmotrila svaki od identifikovanih SNPs/gena nezavisno. Malo istraživanja je sprovedeno na mitohondrijskim genima kod bolesnika sa recidivantnim HAPE. **Metode.** Da bi se identifikovali biološki putevi važni za pojavu HAPE, ispitano je približno 500 000 SNPs genoma iz 10 nepovezanih slučajeva recidivantnih HAPE, i ti SNPs su upoređeni sa onima iz populacije Kineza, stanovnika Pekinga (n = 45; kontrolna grupa) kako bi se utvrdila povezanost između genotipova i osetljivost na HAPE među genima koji se odnose na funkciju mitohondrija. Korišćena je platforma FUMA u cilju proširenja lepeza SNPs na odabrane SNPs kandidate. **Rezultati.** Ukupno je dobijeno 369 SNPs kandidata, 4 vodeća SNPs, 4 lokusa genomskog rizika i 5 mapiranih gena. Mapirano je ukupno 7 gena: ADAMTS9-AS2, NEK1, CLCN3, C4orf27 (HPF1), RP11-219J21.2, ANKRD26 i IME1L1. **Zaključak.** Studija potvrđuje povezanost ADAMTS9-AS2, NEK1, CLCN3, C4orf27 (HPF1), RP11-219J21.2, ANKRD26 i IME1L1 sa HAPE, što obezbeđuje buduće ciljeve za lečenje te bolesti.

Ključne reči:
genetičke tehnike; genom; hipoksija; polimorfizam, genetički; plućni edem; plućni edem planinara.

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Introduction

High-altitude pulmonary oedema (HAPE) is a kind of pulmonary oedema that occurs primarily in the hypoxic environment at high altitude. HAPE occurs mostly among the residents of low-lying areas who enter the plateau for the first time or when the inhabitants of the plateau enter the higher-altitude areas. The incidence rate is 0.4~2%. Because HAPE has acute onset, rapid progress and causes considerable harm to the body, if the treatment is not timely, it can develop to coma or even death in a relatively short period of time, which seriously threatens life and health [1–4]. HAPE has an obvious susceptibility tendency.

Previous studies have shown that there are significant individual differences in susceptibility to HAPE in the same high-altitude hypoxia environment [5, 6]. Accumulated evidence has suggested that a large number of genetic factors are associated with genetic susceptibility to HAPE, including nitric oxide synthase 3 *(*NOS3), cytochrome b-245 (CYBA), angiotensin converting enzyme (ACE), surfactants A1 and A2, and hypoxia-inducible factor-1 (HIF-1) [5–8]. The genetic analysis of these studies was based on an overview of nuclear DNA. However, the role of mitochondria and their genomes is an area of genetic investigation that has been neglected.

Mitochondria are organelles that produce energy in aerobic cells and contain their own genome. Maintaining a sufficient quantity of mitochondrial DNA (mtDNA) in specific tissues is essential for cell viability. Therefore, many common human diseases, such as cancer [9, 10], cardiomyopathy [11] and liver disease [12], are associated with changing mtDNA levels. In a previous study, we sequenced the mtDNA of *Ochotona curzoniae* (Chinese red pika) and identified 15 novel mtDNA-encoded amino acid changes, including 3 in the subunits of cytochrome c oxidase. These amino acid substitutions may modulate mitochondrial complexes and electron transport efficiency during cold weather conditions and hypoxia adaptation [7]. In another study, we found that the sperm mtDNA copy number for those living at a high altitude (5,300 m) for one month was significantly higher than for those at the lower altitude (1,400 m) or in donors who had been living at the 5,300-m altitude for 1 year [13]. Anyway, the association between mitochondria and HAPE occurrence has not been confirmed.

In addition, with the emergence of genome-wide linkage disequilibrium (LD)–based marker panels and improvements in high-throughput genotyping technology, genome-wide association studies (GWAS) have become feasible [14]. GWAS can systematically survey the whole genome for causal genetic variants for complex traits/diseases and is a powerful tool for dissecting the genetic basis for HAPE. Combining the modest association signals in the GWAS data with the information on biological pathways and networks, the emerging pathway-based approaches can be designed to utilize the GWAS data to a greater extent and are likely to yield new insights into HAPE aetiology.

To identify the important aetiology mechanism of HAPE occurrence more systematically and comprehensively, we used a novel pathway-based GWAS to approximately 871,166 single-nucleotide polymorphisms (SNPs) from 10 unrelated reoccurrence HAPE, which is different from the other studies based on GWAS [15]. Those studies chose the patients appearing for only one time, which cannot demonstrate that these patients have HAPE susceptibility compared with the data of the Chinese in Beijing, China (CHB). Although these patients did not go to high-altitude areas, the incidence rate of HAPE is too low (0.4~2%) to affect CHB as a control group; therefore, we investigated the association between mtDNA function-related genes and HAPE susceptibility.

Methods

Patients and controls

Relapsing HAPE patients (n = 10) were recruited from the Han ethnic group in China. We compared the allele frequency of HAPEs with the CHB population (control = 45) to exclude 185,646 SNPs with minimum allele frequency (MAF) < 0.01. The SNPs with the last successful assay were 673,843. The recurrent HAPE patients consisted of 10 individuals (25.01 ± 10.70 years old) who had at least two episodes of HAPE, as determined by the standard diagnostic criteria [16], including cough and dyspnea at rest, with pulmonary rales, cyanosis, and patchy shadows detected using chest X-ray. Relapsing HAPE patients and controls were unrelated to each other and matched gender and age. This study was approved by the Ethics Committee of the Third Military Medical University in China.

Isolation of DNA

The samples of HAPE patients were collected before using drugs; the venous blood (2 mL) was collected from HAPE patients and healthy controls and placed in EDTA-anticoagulation tubes, which were stored at -80 °C prior to analysis. Genomic DNA was extracted from peripheral blood according to the introduction of Omega DNA extraction kits (Omega, USA). Genomic DNA was tested using gel electrophoresis on a 0.8% agarose gel stained with ethidium bromide.

Genotyping

Affymetrix Genome Wide SNP 6.0 arrays were used following the protocol supplied by the manufacturer (Affymetrix, Santa Clara, CA) at Capital Bio Corporation (Beijing, China). Briefly, 250 ng of genomic DNA was digested with Nsp and Sty enzymes, ligated with specific adaptors, and amplified by polymerase chain reaction (PCR) using the kit primers. The amplicons were purified and quantified. The products were fragmented and labeled, followed by hybridization to the array chips at 48 °C for 16–18 h. Excess unhybridized products were washed and

followed by scanning with a GeneChip Scanner 3000 (Affymetrix, Santa Clara, CA [19481479]). Genotypes were called using the Affymetrix BRLMM algorithm as implemented in the Genotyping Console software (Affymetrix, Santa Clara, CA). All samples had BRLMM call rates greater than the 95% cutoff. We used default parameters for the Birdseed algorithm (version 2) to determine genotypes for all samples (Affymetrix, Santa Clara, CA, USA). Genotypic data were analysed using the Affymetrix Genotyping Console 3.1 (Affymetrix) and included all autosomes but excluded the X and Y chromosomes and mitochondrial genome. Firstly, we performed principal components analysis based on genetic distances as previously described between HAPEs (n = 10) and controls (n = 45). We tested 871,166 SNPs, out of which 177,502 SNPs failed. Then, we compared the allele frequency of HAPEs with the CHB population to exclude 185,646 SNPs with MAF < 0.01. The SNP with the last successful assay was number 673,843.

Statistical analysis

Allele frequencies between the patient and control groups were compared using the χ^2 test. A stringent p value < 5×10–8 was considered significant for GWAS. We used Haploview 4.2 (http://www.broadinstitute.org/haploview) to create a Manhattan plot of p values from the GWAS study. A quantile-quantile (QQ) plot of p values from GWAS was created using R project (http://www.r-project.org). We used the FUMA platform (http://fuma.ctglab.nl/tutorial) to analyse GWAS results and selected SNPs of $p < 10^{-8}$, which was of the GWAS significance [17].

Results

In the GWAS, we genotyped a total of 871,166 SNPs, and 673,843 (77.35%) of SNPs were successfully genotyped. We ranked genotyped SNPs based on the strength of association using the allelic association test. Nominally significant results were detected for 1,558 SNPs ($p < 5 \times 10–8$) (Table 1). This analysis indicates that HAPE patients are genetically similar to the ones from the combined CHB population. HapMap populations provide context for the patterns of variation observed among these populations. Genotyping data yielded an average call rate of 96.6%, and apparent inheritance errors in trio samples were detected in < 0.2% of all SNPs. A Manhattan plot was generated for the SNPs in patients with recurrent HAPE in Figure 1. A quantile-quantile (QQ) plot for association results is provided in Figure 2 for all SNPs. The group of SNPs that slightly deviated from a diagonal straight line in the QQ plot are considered to reflect SNPs with weak genetic effects, and from the plot, it seems that there is not gross inflation of false-positive results derived from genotyping errors. We used the FUMA platform to expand those of SNP $p < 5 \times 10^{-8}$ to SNPs that included their linkage disequilibrium ($r^2 \geq 0.6$). Having imported the data into FUMA, we chose the East Asian population (EAS, consistent with the GWAS population), selected the SNP minimum allele frequency (MAF ≥ 0.01) and r^2 (minimum $r^2 \geq 0.6$). A total of 369 candidate SNPs, 4 lead SNPs, 4 genomic risk loci and 5 mapped genes were obtained. The 7 mapped genes were ADAMTS9-AS2, NEK1, CLCN3, C4orf27(HPF1), RP11-219J21.2, ANKRD26 and YME1L1 (Table 2).

Table 1

Significantly different SNPs between 10 recurrent HAPE cases and 45 Hapmap CHB subjects in the first stage

SNP ID	Chromosome	Position	Band	Allele A	Allele B	min_P_Chi	HWE	MAF
rs4353667	2	162025114	q24.2	A	G	4.099E-19	0.940	0.011
rs509193	13	101618897	q33.1	C	G	2.021E-16	0.572	0.078
rs890527	3	142257543	q23	A	T	3.287E-15	0.879	0.022
rs12593141	15	25878695	q13.1	C	T	6.838E-15	0.402	0.111
rs744306	3	186272442	q27.2	A	G	1.632E-14	0.693	0.056
rs9470449	6	37055364	p21.2	A	G	2.140E-14	0.939	0.012
rs4810414	20	42306337	q13.12	C	G	5.139E-14	0.122	0.133
rs10016530	4	184061978	q35.1	A	C	4.779E-13	0.940	0.011
rs8010479	14	80195033	q31.1	C	T	4.779E-13	0.940	0.011
rs2505465	10	26080532	p12.1	A	G	5.504E-13	0.693	0.056
rs12796975	11	132811275	q25	C	T	3.196E-12	0.755	0.044
rs7948049	11	98403015	q22.1	A	C	3.620E-12	0.701	0.189
rs2904699	8	17135169	p22	A	G	3.672E-12	0.362	0.100
rs7929194	11	62269326	q12.3	C	T	4.884E-12	0.318	0.159
rs10075708	5	35582672	p13.2	A	G	5.540E-12	0.940	0.011
rs9364178	6	168952425	q27	A	G	1.235E-11	0.693	0.056
rs3785499	17	17355942	p11.2	A	G	2.244E-11	0.879	0.022
rs7523787	1	94103203	p22.1	A	G	2.927E-11	0.456	0.100
rs6471504	8	96060736	q22.1	C	T	3.137E-11	0.502	0.222
rs1992305	7	41347571	p14.1	C	G	3.419E-11	0.000	0.022
rs9668938	12	9405128	p13.31	A	G	3.419E-11	0.000	0.022
rs8046088	16	77670982	q23.1	A	T	3.419E-11	0.000	0.500
rs1484545	3	641971	p26.3	A	G	3.819E-11	0.940	0.011
rs7199767	16	81560851	q23.3	C	G	3.950E-11	0.879	0.022

Table 1 (continued)

SNP ID	Chromosome	Position	Band	Allele A	Allele B	min_P_Chi	HWE	MAF
rs1536688	9	16119553	p22.3	A	G	4.179E-11	0.000	0.500
rs2132766	4	78019649	q21.1	C	T	5.684E-11	0.001	0.044
rs4707773	6	93740627	q16.1	A	C	6.125E-11	0.708	0.233
rs2253804	17	45710559	q21.33	A	G	6.770E-11	0.201	0.144
rs3780410	9	4588116	p24.2	C	G	6.838E-11	0.000	0.022
rs907425	8	57038845	q12.1	A	G	9.168E-11	0.675	0.239
rs6020381	20	48277755	q13.13	A	C	1.169E-10	0.578	0.244
rs13379947	15	59972093	q22.2	A	G	1.269E-10	0.996	0.211
rs4799715	18	29531002	q12.1	C	T	1.465E-10	0.701	0.189
rs803302	1	25328122	p36.11	A	G	1.880E-10	0.000	0.022
rs11577001	1	192870487	q31.3	C	T	1.880E-10	0.000	0.022
rs4428669	8	22951725	p21.3	A	T	1.880E-10	0.000	0.022
rs784814	14	47539712	q21.3	C	T	1.880E-10	0.000	0.022
rs16967738	17	37799793	q21.2	A	G	1.880E-10	0.000	0.022
rs7275393	21	40817980	q22.2	G	T	1.880E-10	0.000	0.022
rs11860414	16	13097760	p13.12	C	T	2.257E-10	0.000	0.023
rs6705908	2	238098704	q37.3	A	G	2.998E-10	0.227	0.151
rs17024521	1	120268277	p12	C	G	3.761E-10	0.000	0.033
rs9498354	6	149804544	q25.1	A	G	3.761E-10	0.000	0.033
rs13258727	8	16617623	p22	G	T	3.761E-10	0.000	0.033
rs497022	10	85442083	q23.1	C	T	3.761E-10	0.000	0.033
rs11051790	12	32132279	p11.21	C	G	3.761E-10	0.000	0.033
rs2941948	16	77117341	q23.1	C	G	3.761E-10	0.000	0.033
rs907661	1	117548617	p13.1	A	T	3.761E-10	0.940	0.011
rs2581409	1	112577867	p13.2	A	G	3.761E-10	0.940	0.011
rs10776807	1	109757679	p13.3	A	G	3.761E-10	0.940	0.011
rs12127734	1	102738259	p21.1	C	T	3.761E-10	0.940	0.011
rs1931256	1	95930004	p21.3	A	C	3.761E-10	0.940	0.011
rs6420974	1	86496645	p22.3	A	C	3.761E-10	0.940	0.011
rs6424623	1	79258910	p31.1	A	T	3.761E-10	0.940	0.011
rs12121720	1	75159525	p31.1	C	T	3.761E-10	0.940	0.011
rs10157120	1	52983476	p32.3	A	G	3.761E-10	0.940	0.011
rs7525612	1	47664398	p33	C	T	3.761E-10	0.940	0.011
rs41524944	1	44894612	p34.1	C	T	3.761E-10	0.940	0.011
rs2816602	1	43040557	p34.2	C	T	3.761E-10	0.940	0.011
rs2182111	1	29637387	p35.3	A	T	3.761E-10	0.940	0.011
rs2746535	1	17264939	p36.13	C	T	3.761E-10	0.940	0.011
rs16862547	1	19316539	p36.13	C	T	3.761E-10	0.940	0.011
rs6703014	1	151806944	q21.3	A	G	3.761E-10	0.940	0.011
rs10752607	1	152983427	q21.3	C	T	3.761E-10	0.940	0.011
rs6702567	1	157784484	q23.2	A	G	3.761E-10	0.940	0.011
rs1288913	1	161882823	q23.3	C	T	3.761E-10	0.940	0.011
rs4987357	1	167932764	q24.2	C	T	3.761E-10	0.940	0.011
rs12117954	1	170933444	q24.3	G	T	3.761E-10	0.940	0.011
rs539038	1	189048657	q31.2	A	G	3.761E-10	0.940	0.011
rs613232	1	209836516	q32.3	C	T	3.761E-10	0.940	0.011
rs714214	1	228825228	q42.2	C	T	3.761E-10	0.940	0.011
rs4658949	1	230014942	q42.2	A	C	3.761E-10	0.940	0.011
rs6665236	1	246060280	q44	A	G	3.761E-10	0.940	0.011
rs4852883	2	72708531	p13.2	C	T	3.761E-10	0.940	0.011
rs262501	2	63712161	p15	A	G	3.761E-10	0.940	0.011
rs6751340	2	54041121	p16.2	A	G	3.761E-10	0.940	0.011
rs17389310	2	42343095	p21	C	G	3.761E-10	0.940	0.011
rs13416119	2	42316434	p21	A	G	3.761E-10	0.940	0.011
rs17024325	2	39845266	p22.1	C	G	3.761E-10	0.940	0.011
rs4648234	2	37191174	p22.2	A	G	3.761E-10	0.940	0.011
rs12104627	2	35364483	p22.3	A	T	3.761E-10	0.940	0.011
rs11893869	2	106032330	q12.2	A	G	3.761E-10	0.940	0.011
rs260711	2	108923531	q13	C	T	3.761E-10	0.940	0.011
rs17783857	2	140102541	q22.1	C	G	3.761E-10	0.940	0.011
rs10185178	2	171064520	q31.1	A	G	3.761E-10	0.940	0.011
rs3914402	2	174296267	q31.1	C	G	3.761E-10	0.940	0.011

Table 1 (continued)

SNP ID	Chromosome	Position	Band	Allele A	Allele B	min_P_Chi	HWE	MAF
rs12989588	2	194838617	q32.3	A	G	3.761E-10	0.940	0.011
rs16842071	2	201639975	q33.1	A	G	3.761E-10	0.940	0.011
rs11902586	2	213683899	q34	C	G	3.761E-10	0.940	0.011
rs11898042	2	220596890	q35	A	G	3.761E-10	0.940	0.011
rs6431283	2	233888576	q37.1	C	T	3.761E-10	0.940	0.011
rs10175460	2	231048405	q37.1	A	G	3.761E-10	0.940	0.011
rs10933609	2	241092142	q37.3	A	G	3.761E-10	0.940	0.011
rs6548631	3	79729007	p12.3	C	G	3.761E-10	0.940	0.011
rs9847658	3	70073539	p14.1	A	C	3.761E-10	0.940	0.011
rs755358	3	62509509	p14.2	C	T	3.761E-10	0.940	0.011
rs9830403	3	27938612	p24.1	C	G	3.761E-10	0.940	0.011
rs778044	3	10255233	p25.3	C	T	3.761E-10	0.940	0.011
rs352748	3	6615700	p26.1	C	G	3.761E-10	0.940	0.011
rs1144107	3	101924406	q12.2	C	T	3.761E-10	0.940	0.011
rs2056534	3	115966848	q13.31	A	G	3.761E-10	0.940	0.011
rs13326852	3	121649170	q13.33	C	T	3.761E-10	0.940	0.011
rs6769033	3	137066778	q22.2	C	T	3.761E-10	0.940	0.011
rs344076	3	158035479	q25.31	C	T	3.761E-10	0.940	0.011
rs2566339	3	159791569	q25.32	C	T	3.761E-10	0.940	0.011
rs16846456	3	174240032	q26.31	C	T	3.761E-10	0.940	0.011
rs6788878	3	178926662	q26.32	G	T	3.761E-10	0.940	0.011
rs10002498	4	47623342	p12	C	G	3.761E-10	0.940	0.011
rs5743591	4	38479523	p14	C	G	3.761E-10	0.940	0.011
rs13105862	4	36976442	p14	C	T	3.761E-10	0.940	0.011
rs41339448	4	19206250	p15.31	A	G	3.761E-10	0.940	0.011
rs13148734	4	63013453	q13.1	A	G	3.761E-10	0.940	0.011
rs313139	4	127754207	q28.1	C	G	3.761E-10	0.940	0.011
rs1201202	4	152060202	q31.3	A	G	3.761E-10	0.940	0.011
rs1594869	4	158681812	q32.1	A	G	3.761E-10	0.940	0.011
rs17628308	4	171106945	q33	A	G	3.761E-10	0.940	0.011
rs2173826	4	170922763	q33	A	G	3.761E-10	0.940	0.011
rs17057309	4	172849798	q34.1	C	T	3.761E-10	0.940	0.011
rs17074536	4	184417378	q35.1	C	T	3.761E-10	0.940	0.011
rs4862023	4	183246608	q35.1	A	C	3.761E-10	0.940	0.011
rs6879532	5	23092333	p14.3	A	G	3.761E-10	0.940	0.011
rs17295893	5	14125258	p15.2	C	T	3.761E-10	0.940	0.011
rs10472006	5	56791259	q11.2	C	T	3.761E-10	0.940	0.011
rs158342	5	55661090	q11.2	A	C	3.761E-10	0.940	0.011
rs10057147	5	53473290	q11.2	A	G	3.761E-10	0.940	0.011
rs255233	5	56633746	q11.2	C	T	3.761E-10	0.940	0.011
rs6896756	5	66947893	q13.1	C	T	3.761E-10	0.940	0.011
rs11959381	5	75724016	q13.3	C	T	3.761E-10	0.940	0.011
rs16902631	5	86679983	q14.3	A	T	3.761E-10	0.940	0.011
rs2963029	5	108782510	q21.3	C	G	3.761E-10	0.940	0.011
rs4272129	5	124365847	q23.2	C	T	3.761E-10	0.940	0.011
rs7707878	5	126011942	q23.2	A	C	3.761E-10	0.940	0.011
rs3861854	5	141280553	q31.3	C	T	3.761E-10	0.940	0.011
rs1432672	5	143945814	q32	C	T	3.761E-10	0.940	0.011
rs10037531	5	156738482	q33.3	A	G	3.761E-10	0.940	0.011
rs4868935	5	164941974	q34	A	G	3.761E-10	0.940	0.011
rs10462997	5	169942958	q35.1	C	T	3.761E-10	0.940	0.011
rs10067345	5	171183175	q35.1	A	G	3.761E-10	0.940	0.011
rs10039715	5	173603095	q35.2	C	T	3.761E-10	0.940	0.011
rs3129704	6	30342679	p21.33	C	T	3.761E-10	0.940	0.011
rs7767176	6	28033346	p22.1	C	T	3.761E-10	0.940	0.011
rs10484632	6	20755639	p22.3	A	C	3.761E-10	0.940	0.011
rs13206084	6	16653930	p22.3	A	G	3.761E-10	0.940	0.011
rs11969660	6	14503352	p23	A	G	3.761E-10	0.940	0.011
rs6919114	6	10780583	p24.2	A	G	3.761E-10	0.940	0.011
rs3804481	6	6577398	p25.1	A	G	3.761E-10	0.940	0.011
rs2110903	6	107679904	q21	G	T	3.761E-10	0.940	0.011
rs3757302	6	108478901	q21	C	G	3.761E-10	0.940	0.011

Table 1 (continued)

SNP ID	Chromosome	Position	Band	Allele A	Allele B	min_P_Chi	HWE	MAF
rs6913809	6	113957665	q22.1	A	C	3.761E-10	0.940	0.011
rs6569290	6	123195382	q22.31	A	G	3.761E-10	0.940	0.011
rs12110924	6	118674618	q22.31	C	G	3.761E-10	0.940	0.011
rs12205922	6	128127367	q22.33	A	G	3.761E-10	0.940	0.011
rs9480356	6	156948860	q25.3	A	G	3.761E-10	0.940	0.011
rs10486806	7	40468520	p14.1	A	G	3.761E-10	0.940	0.011
rs12536300	7	33159362	p14.3	A	G	3.761E-10	0.940	0.011
rs17675986	7	29077382	p15.1	A	T	3.761E-10	0.940	0.011
rs10251505	7	7221014	p21.3	A	G	3.761E-10	0.940	0.011
rs1207867	7	78239513	q21.11	A	G	3.761E-10	0.940	0.011
rs7802018	7	94898249	q21.3	A	G	3.761E-10	0.940	0.011
rs1558005	7	100936342	q22.1	A	G	3.761E-10	0.940	0.011
rs10252737	7	101486484	q22.1	A	C	3.761E-10	0.940	0.011
rs13231181	7	103979084	q22.1	C	T	3.761E-10	0.940	0.011
rs10261618	7	136853662	q33	A	C	3.761E-10	0.940	0.011
rs4335058	7	132550141	q33	A	C	3.761E-10	0.940	0.011
rs851734	7	146993038	q35	C	G	3.761E-10	0.940	0.011
rs6967282	7	150538127	q36.1	A	G	3.761E-10	0.940	0.011
rs2101138	8	26186805	p21.2	C	G	3.761E-10	0.940	0.011
rs2410675	8	20915740	p21.3	G	T	3.761E-10	0.940	0.011
rs369240	8	55686306	q12.1	C	T	3.761E-10	0.940	0.011
rs35711827	8	76793565	q21.11	G	T	3.761E-10	0.940	0.011
rs1448676	8	92396335	q21.3	A	C	3.761E-10	0.940	0.011
rs16870588	8	104706458	q22.3	C	G	3.761E-10	0.940	0.011
rs3018507	8	103347864	q22.3	C	G	3.761E-10	0.940	0.011
rs7826950	8	134980387	q24.22	A	C	3.761E-10	0.940	0.011
rs10088738	8	139205255	q24.23	A	G	3.761E-10	0.940	0.011
rs17247766	9	33098605	p13.3	G	T	3.761E-10	0.940	0.011
rs1885170	9	17554267	p22.2	C	T	3.761E-10	0.940	0.011
rs13285034	9	74559353	q21.13	A	T	3.761E-10	0.940	0.011
rs10993086	9	95990540	q22.32	G	T	3.761E-10	0.940	0.011
rs10441773	9	107233498	q31.2	C	T	3.761E-10	0.940	0.011
rs12553905	9	121402295	q33.1	C	T	3.761E-10	0.940	0.011
rs16929767	9	129113684	q33.3	A	T	3.761E-10	0.940	0.011
rs3011286	9	134883811	q34.13	C	T	3.761E-10	0.940	0.011
rs2643955	10	29197524	p11.23	G	T	3.761E-10	0.940	0.011
rs11015156	10	26863974	p12.1	G	T	3.761E-10	0.940	0.011
rs661882	10	27808089	p12.1	A	G	3.761E-10	0.940	0.011
rs17465850	10	17812128	p12.33	A	G	3.761E-10	0.940	0.011
rs12358414	10	3707846	p15.2	C	T	3.761E-10	0.940	0.011
rs17501883	10	44506780	q11.21	A	C	3.761E-10	0.940	0.011
rs11001982	10	78468130	q22.3	A	G	3.761E-10	0.940	0.011
rs17334741	10	90168436	q23.31	A	C	3.761E-10	0.940	0.011
rs11597377	10	121733856	q26.12	C	T	3.761E-10	0.940	0.011
rs17594946	10	122702917	q26.12	A	C	3.761E-10	0.940	0.011
rs12412522	10	122789916	q26.12	C	T	3.761E-10	0.940	0.011
rs2818393	10	133792619	q26.3	A	G	3.761E-10	0.940	0.011
rs4755364	11	34249101	p13	A	G	3.761E-10	0.940	0.011
rs1482734	11	23211390	p14.3	A	T	3.761E-10	0.940	0.011
rs7939809	11	13862425	p15.2	C	G	3.761E-10	0.940	0.011
rs12807017	11	9635721	p15.4	A	G	3.761E-10	0.940	0.011
rs17704641	11	60939964	q12.2	C	T	3.761E-10	0.940	0.011
rs3017605	11	61017594	q12.2	A	C	3.761E-10	0.940	0.011
rs632280	11	78178911	q14.1	G	T	3.761E-10	0.940	0.011
rs7121003	11	86964252	q14.2	A	C	3.761E-10	0.940	0.011
rs4512880	11	86955572	q14.2	A	G	3.761E-10	0.940	0.011
rs655922	11	100153283	q22.1	G	T	3.761E-10	0.940	0.011
rs522819	11	100460929	q22.1	A	G	3.761E-10	0.940	0.011
rs7113906	11	101758880	q22.2	C	T	3.761E-10	0.940	0.011
rs1375423	11	104601723	q22.3	A	T	3.761E-10	0.940	0.011
rs1902238	11	106468971	q22.3	C	T	3.761E-10	0.940	0.011
rs7122110	11	120527150	q23.3	A	G	3.761E-10	0.940	0.011

Table 1 (continued)

SNP ID	Chromosome	Position	Band	Allele A	Allele B	min_P_Chi	HWE	MAF
rs11216478	11	117016434	q23.3	A	G	3.761E-10	0.940	0.011
rs41507249	11	122112574	q24.1	C	T	3.761E-10	0.940	0.011
rs583194	11	125456998	q24.2	C	T	3.761E-10	0.940	0.011
rs10894844	11	133952614	q25	C	T	3.761E-10	0.940	0.011
rs17472165	12	26494853	p11.23	C	T	3.761E-10	0.940	0.011
rs3863355	12	25850114	p12.1	C	T	3.761E-10	0.940	0.011
rs4350408	12	22043980	p12.1	G	T	3.761E-10	0.940	0.011
rs11045107	12	20220645	p12.2	G	T	3.761E-10	0.940	0.011
rs6487064	12	20226964	p12.2	G	T	3.761E-10	0.940	0.011
rs16915116	12	19186252	p12.3	A	T	3.761E-10	0.940	0.011
rs12307636	12	9512800	p13.31	C	T	3.761E-10	0.940	0.011
rs1805731	12	8986493	p13.31	A	G	3.761E-10	0.940	0.011
rs7312896	12	662066	p13.33	C	T	3.761E-10	0.940	0.011
rs9325199	12	70273227	q21.1	A	C	3.761E-10	0.940	0.011
rs310836	12	76001666	q21.2	C	T	3.761E-10	0.940	0.011
rs4143188	12	81326916	q21.31	A	C	3.761E-10	0.940	0.011
rs10777572	12	92977940	q22	A	T	3.761E-10	0.940	0.011
rs9669774	12	113260569	q24.21	C	G	3.761E-10	0.940	0.011
rs17441172	12	117352644	q24.23	C	T	3.761E-10	0.940	0.011
rs7298854	12	125553390	q24.32	A	C	3.761E-10	0.940	0.011
rs10847172	12	125560866	q24.32	A	G	3.761E-10	0.940	0.011
rs9314935	13	28583729	q12.3	A	G	3.761E-10	0.940	0.011
rs9548515	13	38338848	q13.3	A	C	3.761E-10	0.940	0.011
rs2503454	13	46987969	q14.2	A	G	3.761E-10	0.940	0.011
rs12429341	13	47347285	q14.2	A	G	3.761E-10	0.940	0.011
rs17060868	13	61588183	q21.31	A	C	3.761E-10	0.940	0.011
rs9516058	13	91762201	q31.3	A	G	3.761E-10	0.940	0.011
rs9514865	13	107995471	q33.3	C	T	3.761E-10	0.940	0.011
rs6650482	13	111970835	q34	A	G	3.761E-10	0.940	0.011
rs7160516	14	43848866	q21.3	A	G	3.761E-10	0.940	0.011
rs10484082	14	51162516	q22.1	C	T	3.761E-10	0.940	0.011
rs17107847	14	78091511	q24.3	G	T	3.761E-10	0.940	0.011
rs6574673	14	81183387	q31.1	G	T	3.761E-10	0.940	0.011
rs6574612	14	80473827	q31.1	C	T	3.761E-10	0.940	0.011
rs4905612	14	97248380	q32.2	A	G	3.761E-10	0.940	0.011
rs4924188	15	35766234	q14	A	G	3.761E-10	0.940	0.011
rs8041819	15	50401611	q21.2	A	G	3.761E-10	0.940	0.011
rs11858794	15	57498627	q22.2	A	G	3.761E-10	0.940	0.011
rs1912049	15	61942659	q22.31	G	T	3.761E-10	0.940	0.011
rs7171610	15	63227145	q22.31	A	T	3.761E-10	0.940	0.011
rs11630776	15	76234845	q25.1	C	G	3.761E-10	0.940	0.011
rs9944345	16	49976666	q12.1	C	G	3.761E-10	0.940	0.011
rs2058673	16	45580279	q12.1	A	G	3.761E-10	0.940	0.011
rs12597729	16	49769655	q12.1	C	G	3.761E-10	0.940	0.011
rs13332434	16	58632433	q21	A	C	3.761E-10	0.940	0.011
rs16957304	16	65892470	q22.1	C	T	3.761E-10	0.940	0.011
rs9935976	16	85593861	q24.1	C	T	3.761E-10	0.940	0.011
rs6540041	16	85961876	q24.2	A	T	3.761E-10	0.940	0.011
rs1015218	17	20673956	p11.2	C	G	3.761E-10	0.940	0.011
rs7503902	17	59833749	q23.3	A	G	3.761E-10	0.940	0.011
rs12150174	17	62856936	q24.2	C	T	3.761E-10	0.940	0.011
rs6501586	17	68456801	q25.1	A	T	3.761E-10	0.940	0.011
rs4006794	17	69999430	q25.1	C	T	3.761E-10	0.940	0.011
rs610541	18	11960809	p11.21	C	T	3.761E-10	0.940	0.011
rs566559	18	5960704	p11.31	C	G	3.761E-10	0.940	0.011
rs9646461	18	4075990	p11.31	A	G	3.761E-10	0.940	0.011
rs2846834	18	861268	p11.32	C	T	3.761E-10	0.940	0.011
rs1623716	18	30665290	q12.1	A	C	3.761E-10	0.940	0.011
rs1790534	18	30663569	q12.1	A	G	3.761E-10	0.940	0.011
rs11873775	18	24417919	q12.1	C	T	3.761E-10	0.940	0.011
rs654975	18	58418480	q21.33	G	T	3.761E-10	0.940	0.011
rs1704816	18	62280193	q22.1	C	T	3.761E-10	0.940	0.011

Table 1 (continued)

SNP ID	Chromosome	Position	Band	Allele A	Allele B	min_P_Chi	HWE	MAF
rs12962239	18	73493166	q23	A	G	3.761E-10	0.940	0.011
rs12981996	19	20342025	p12	A	T	3.761E-10	0.940	0.011
rs16996008	19	19226400	p13.11	A	G	3.761E-10	0.940	0.011
rs6511939	19	14545425	p13.12	A	G	3.761E-10	0.940	0.011
rs11672838	19	14948335	p13.12	C	T	3.761E-10	0.940	0.011
rs7003	19	14486790	p13.12	C	T	3.761E-10	0.940	0.011
rs12983312	19	10190245	p13.2	C	T	3.761E-10	0.940	0.011
rs407743	19	6593417	p13.3	C	G	3.761E-10	0.940	0.011
rs1558133	19	1253965	p13.3	C	T	3.761E-10	0.940	0.011
rs8112607	19	38162646	q13.11	C	G	3.761E-10	0.940	0.011
rs1661906	19	58201490	q13.41	A	T	3.761E-10	0.940	0.011
rs6510101	19	62999086	q13.43	G	T	3.761E-10	0.940	0.011
rs6042568	20	1418343	p13	C	T	3.761E-10	0.940	0.011
rs13041282	20	29836903	q11.21	G	T	3.761E-10	0.940	0.011
rs2868093	20	42397212	q13.12	C	T	3.761E-10	0.940	0.011
rs6073310	20	42139597	q13.12	A	G	3.761E-10	0.940	0.011
rs928072	20	48368185	q13.13	A	G	3.761E-10	0.940	0.011
rs6020818	20	48926335	q13.13	C	G	3.761E-10	0.940	0.011
rs1980424	21	15164448	q11.2	A	G	3.761E-10	0.940	0.011
rs13048221	21	14381307	q11.2	A	G	3.761E-10	0.940	0.011
rs551680	21	39876578	q22.2	A	G	3.761E-10	0.940	0.011
rs2535708	22	16564169	q11.21	A	G	3.761E-10	0.940	0.011
rs7293008	22	27772666	q12.1	C	T	3.761E-10	0.940	0.011
rs3730114	22	24421306	q12.1	C	T	3.761E-10	0.940	0.011
rs17834914	22	45605985	q13.31	A	G	3.761E-10	0.940	0.011
rs8137937	22	45846062	q13.31	C	G	3.761E-10	0.940	0.011
rs243505	7	148066272	q36.1	A	G	4.008E-10	0.360	0.267
rs7119096	11	127453448	q24.3	C	T	4.429E-10	0.649	0.122
rs7872136	9	85091738	q21.32	A	G	4.597E-10	0.939	0.011
rs4584989	2	108686189	q13	C	T	4.597E-10	0.939	0.011
rs4378452	12	109988416	q24.11	A	G	4.597E-10	0.939	0.011
rs8130198	21	42503393	q22.3	C	T	4.597E-10	0.939	0.011
rs7909124	10	97709510	q23.33	C	G	5.641E-10	0.939	0.012
rs17261573	2	80528623	p12	C	G	5.868E-10	0.996	0.211
rs6762195	3	126740626	q21.2	C	T	7.214E-10	0.726	0.278
rs11199331	10	122174433	q26.12	A	T	8.142E-10	0.290	0.244
rs6854931	4	6828065	p16.1	A	G	1.034E-09	0.940	0.011
rs6720335	2	233540064	q37.1	A	G	1.128E-09	0.000	0.044
rs41453247	14	54982693	q22.3	A	G	1.128E-09	0.000	0.500
rs581459	1	36147697	p34.3	C	T	1.129E-09	0.848	0.222
rs250238	5	50302287	q11.1	A	C	1.129E-09	0.502	0.222
rs16992471	19	4591295	p13.3	A	C	1.129E-09	0.502	0.222
rs241301	1	227029050	q42.13	C	T	1.276E-09	0.103	0.289
rs2078330	16	73137556	q22.3	C	T	1.276E-09	0.859	0.289
rs8100750	19	55775407	q13.33	C	T	1.462E-09	0.667	0.178
rs7221423	17	78551921	q25.3	C	T	1.561E-09	0.130	0.078
rs8118315	20	4109500	p13	C	T	1.573E-09	0.000	0.489
rs17483466	2	111513929	q13	A	G	1.880E-09	0.940	0.011
rs9878562	3	53864028	p21.1	C	T	1.880E-09	0.000	0.033
rs31745	5	140400408	q31.3	A	G	1.880E-09	0.000	0.033
rs1778894	9	125595350	q33.2	A	C	1.880E-09	0.940	0.011
rs10501627	11	86029148	q14.2	A	C	1.880E-09	0.000	0.033
rs568739	11	127565639	q24.3	A	G	1.880E-09	0.940	0.011
rs7142084	14	91892784	q32.12	C	T	1.880E-09	0.000	0.033
rs11854845	15	69688499	q23	A	G	1.880E-09	0.940	0.011
rs11806573	1	62591934	p31.3	A	C	1.880E-09	0.940	0.011
rs473223	1	54896976	p32.3	A	G	1.880E-09	0.940	0.011
rs12066062	1	149925647	q21.3	C	T	1.880E-09	0.940	0.011
rs11583867	1	183984337	q25.3	A	G	1.880E-09	0.940	0.011
rs2867890	1	203736379	q32.1	A	G	1.880E-09	0.940	0.011
rs12731771	1	202027279	q32.1	C	T	1.880E-09	0.940	0.011
rs11118935	1	206171611	q32.2	A	G	1.880E-09	0.940	0.011

Table 1 (continued)

SNP ID	Chromosome	Position	Band	Allele A	Allele B	min_P_Chi	HWE	MAF
rs12074002	1	209897308	q32.3	C	G	1.880E-09	0.940	0.011
rs2965012	1	216853172	q41	G	T	1.880E-09	0.940	0.011
rs6696165	1	242834795	q44	C	T	1.880E-09	0.940	0.011
rs11125521	2	54205862	p16.2	A	T	1.880E-09	0.940	0.011
rs1403450	2	45696779	p21	C	T	1.880E-09	0.940	0.011
rs908679	2	22283114	p24.1	A	G	1.880E-09	0.940	0.011
rs1983376	2	17289515	p24.2	A	C	1.880E-09	0.940	0.011
rs11889931	2	106141807	q12.2	C	T	1.880E-09	0.940	0.011
rs13021341	2	144247607	q22.2	C	T	1.880E-09	0.940	0.011
rs1113988	2	168059681	q24.3	A	C	1.880E-09	0.940	0.011
rs3914752	2	170833364	q31.1	A	C	1.880E-09	0.940	0.011
rs10179515	2	212255007	q34	C	G	1.880E-09	0.940	0.011
rs1082901	3	77834657	p12.3	A	G	1.880E-09	0.940	0.011
rs1502616	3	59505361	p14.2	C	T	1.880E-09	0.940	0.011
rs9845785	3	31504110	p23	C	G	1.880E-09	0.940	0.011
rs17015506	3	24956816	p24.2	A	G	1.880E-09	0.940	0.011
rs17036852	3	12518475	p25.1	A	G	1.880E-09	0.940	0.011
rs9864656	3	137126228	q22.2	C	T	1.880E-09	0.940	0.011
rs7639012	3	155697801	q25.2	G	T	1.880E-09	0.940	0.011
rs16832690	3	183003503	q26.33	A	T	1.880E-09	0.940	0.011
rs17513709	4	40496876	p14	G	T	1.880E-09	0.940	0.011
rs6831500	4	17810438	p15.32	C	T	1.880E-09	0.940	0.011
rs17592868	4	68897521	q13.2	C	T	1.880E-09	0.940	0.011
rs3792662	4	95689234	q22.3	C	G	1.880E-09	0.940	0.011
rs10517681	4	159059047	q32.1	A	C	1.880E-09	0.940	0.011
rs11723043	4	189744112	q35.2	C	T	1.880E-09	0.940	0.011
rs16901423	5	31715101	p13.3	A	G	1.880E-09	0.940	0.011
rs13362111	5	33328915	p13.3	C	G	1.880E-09	0.940	0.011
rs7734697	5	7469304	p15.31	A	T	1.880E-09	0.940	0.011
rs2897554	5	81311997	q14.2	C	T	1.880E-09	0.940	0.011
rs41459348	5	94239098	q15	C	T	1.880E-09	0.940	0.011
rs10477915	5	107955270	q21.3	C	T	1.880E-09	0.940	0.011
rs10042652	5	141636901	q31.3	G	T	1.880E-09	0.940	0.011
rs10072565	5	166242667	q34	A	G	1.880E-09	0.940	0.011
rs9313568	5	171344886	q35.1	A	C	1.880E-09	0.940	0.011
rs6867969	5	172157416	q35.1	C	T	1.880E-09	0.940	0.011
rs13156607	5	168832565	q35.1	C	T	1.880E-09	0.940	0.011
rs9475536	6	56008167	p12.1	C	G	1.880E-09	0.940	0.011
rs513248	6	53546485	p12.1	A	G	1.880E-09	0.940	0.011
rs7766333	6	25070202	p22.2	A	G	1.880E-09	0.940	0.011
rs6900027	6	10760336	p24.2	A	G	1.880E-09	0.940	0.011
rs10455706	6	71345716	q13	C	T	1.880E-09	0.940	0.011
rs10944336	6	88718737	q15	C	T	1.880E-09	0.940	0.011
rs9489754	6	98342750	q16.1	A	C	1.880E-09	0.940	0.011
rs4377817	6	115194976	q22.1	C	T	1.880E-09	0.940	0.011
rs17250161	6	153849770	q25.2	C	G	1.880E-09	0.940	0.011
rs1737317	6	163709828	q26	A	G	1.880E-09	0.940	0.011
rs856588	7	46703840	p12.3	C	T	1.880E-09	0.940	0.011
rs11979904	7	38684422	p14.1	A	C	1.880E-09	0.940	0.011
rs10257031	7	35907991	p14.2	A	C	1.880E-09	0.940	0.011
rs2098273	7	36484536	p14.2	C	T	1.880E-09	0.940	0.011
rs17457143	7	20559116	p15.3	C	G	1.880E-09	0.940	0.011
rs3807573	7	5636086	p22.1	C	T	1.880E-09	0.940	0.011
rs6463483	7	5497369	p22.1	C	T	1.880E-09	0.940	0.011
rs6460734	7	71597254	q11.22	C	G	1.880E-09	0.940	0.011
rs4730058	7	104347376	q22.1	C	T	1.880E-09	0.940	0.011
rs706561	7	136925970	q33	C	G	1.880E-09	0.940	0.011
rs17667159	7	156988826	q36.3	A	C	1.880E-09	0.940	0.011
rs17595134	8	40076812	p11.21	C	G	1.880E-09	0.940	0.011
rs7822050	8	72730829	q13.3	C	T	1.880E-09	0.940	0.011
rs16938568	8	74209396	q21.11	C	T	1.880E-09	0.940	0.011
rs16874193	8	107268534	q23.1	A	C	1.880E-09	0.940	0.011

Table 1 (continued)

SNP ID	Chromosome	Position	Band	Allele A	Allele B	min_P_Chi	HWE	MAF
rs2799753	9	38475256	p13.1	A	T	1.880E-09	0.940	0.011
rs7021837	9	13844176	p23	A	G	1.880E-09	0.940	0.011
rs10959547	9	11110180	p23	C	G	1.880E-09	0.940	0.011
rs35613585	9	74634393	q21.13	C	G	1.880E-09	0.940	0.011
rs1330288	9	74626903	q21.13	A	G	1.880E-09	0.940	0.011
rs12686427	9	88530367	q21.33	C	T	1.880E-09	0.940	0.011
rs4314720	9	112411728	q31.3	C	T	1.880E-09	0.940	0.011
rs41407147	9	121795392	q33.1	G	T	1.880E-09	0.940	0.011
rs12554146	9	133317958	q34.13	A	T	1.880E-09	0.940	0.011
rs2797468	10	29197311	p11.23	A	C	1.880E-09	0.940	0.011
rs16926660	10	26523271	p12.1	C	T	1.880E-09	0.940	0.011
rs11256585	10	10468085	p14	C	T	1.880E-09	0.940	0.011
rs1005907	10	4863106	p15.1	A	G	1.880E-09	0.940	0.011
rs4881163	10	3395755	p15.2	C	G	1.880E-09	0.940	0.011
rs12242220	10	49698112	q11.22	C	T	1.880E-09	0.940	0.011
rs17500631	10	52297578	q11.23	G	T	1.880E-09	0.940	0.011

SNPs – single-nucleotide polymorphisms; HAPE – high-altitude pulmonary oedema; CHB – Chinese in Beijing, China; HWE – Hardy-Weinberg Equilibrium; MAF – minimum allele frequency.

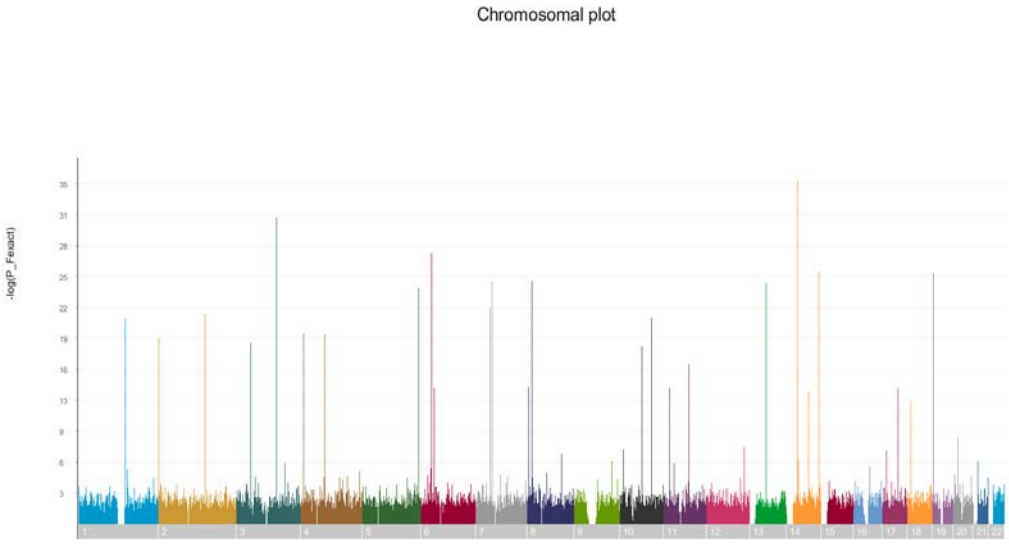

Fig. 1 – Manhattan plot for the whole single-nucleotide polymorphisms (SNPs) in recurrent high-altitude pulmonary oedema (HAPE) subjects of Chinese Han descent. Demonstrating the distribution of *p* values of the Fisher's exact test in the whole genome under four genetic models of allele, genotype, recessive and dominant. The horizontal axis is the physical position of each SNP, and the vertical axis is the negative logarithm of the *p* value.

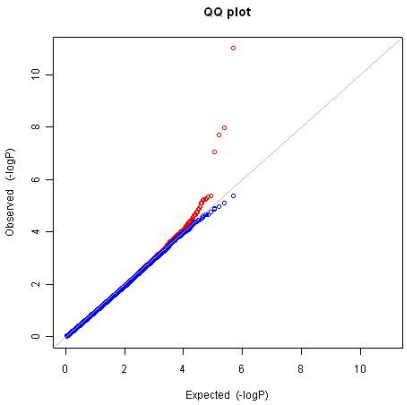

Fig. 2 – Quantile-quantile (QQ) plot for association results of the first-stage analysis (red plots are the cases for all loci, and blue plots are the cases after removing the significant locus).

Table 2

Main effects of tested SNPs on HAPE risk by FUMA

Symbol gene	Chromosome	Start	End	Strand	Type
ADAMTS9-AS2	3	64670585	64997143	1	antisense
NEK1	4	170314426	170533780	-1	protein-coding
CLCN3	4	170533784	170644824	1	protein-coding
C4orf27	4	170650616	170679104	-1	protein-coding
RP11-219J21.2	8	25634195	25634972	1	lncRNA
ANKRD26	10	27280843	27389421	-1	protein-coding
YME1L1	10	27399383	27444195	-1	protein-coding

SNPs – single-nucleotide polymorphisms; HAPE – high-altitude pulmonary oedema.

Discussion

We performed a GWAS to identify susceptibility genes and risk variants for HAPE in Chinese populations. Seven novel candidate genes have emerged from our staged association analyses. Specifically, NEK1, CLCN3, C4orf27, ANKRD26 and YME1L1 are protein-coding genes, and ADAMTS9-AS2 and YME1L1 are RNA genes.

ADAMTS9-AS2 (ADAMTS9 antisense RNA 2) is located at the positive strand of chromosome 3 (chr3: 64, 684, 935-65, 053, 439) with a length of 2.258 kb and is classified as an lncRNA. ADAMTS9-AS2 is an antisense transcription of ADAMTS9. ADAMTS plays important roles in connective tissue organization, coagulation, inflammation, arthritis, and angiogenesis and is regulated by the tissue inhibitor of metalloproteinase 3 gene (TIMP3) [18, 19]. Moreover, it has been shown that in the Japanese population TIMP3 was associated with HAPE susceptibility [20–22]. TIMP plays a key role in the physiological turnover of the extracellular matrix (ECM) by closely regulating the activity of matrix metalloproteinase (MMP). TIMP3 is the only TIMP closely integrated with ECM. The balance between MMP and TIMP plays an important role in maintaining the integrity of healthy tissues. The disturbance of the TIMP/MMP system is related to various pathological conditions of the lungs, including pulmonary inflammation, oedema, emphysema and fibrosis, among which the loss of ECM integrity is the main feature [23]. Our results, together with those of previous studies, suggest that the balance between MMPs and TIMPs plays an important role in the pathogenesis of HAPE.

Chloride voltage-gated channel 3 (ClC-3) is a protein-coding gene. Among its related pathways are ion channel transport and transport of glucose and other sugars, bile salts and organic acids, metal ions and amine compounds [24]. This protein plays a role in both acidification and transmitter loading of GABAergic synaptic vesicles and in smooth muscle cell activation and neointima formation [25]. This protein is required for lysophosphatidic acid (LPA)-activated Cl^- current activity and fibroblast-to-myofibroblast differentiation. Dai et al. [26] observe that ClC-3 in rat hypertensive lung and heart is a novel upregulation. These researchers also suggest that upregulation of ClC-3 is an adaptive response of the inflamed pulmonary artery. ClC-3 may be associated with the adaptability of the pulmonary artery to the plateau environment in HAPE.

Ankyrin repeat domain 26 (ANKRD26) is a protein-coding gene. Diseases associated with ANKRD26 include thrombocytopenia 2 and platelet disorder, familial, with associated myeloid malignancy. There is a case reporting that ANKRD26-related thrombocytopenia resulting in lower-limb deep vein thrombosis was complicated by pulmonary embolism [27]. NIMA-related kinase 1 (NEK1) is a protein-coding gene. Diseases associated with NEK1 include short-rib thoracic dysplasia 6 with or without polydactyly and amyotrophic lateral sclerosis. NEK1 is involved in DNA damage checkpoint control and proper DNA damage repair [28]. In response to injury that includes DNA damage, NEK1 phosphorylates VDAC1 to limit mitochondrial cell death [28]. YME1L1 (YME1-like 1 ATPase) is a protein-coding gene. Diseases associated with YME1L1 are spastic paraplegia 7, autosomal recessive and include optic atrophy 11 [29]. Gene Ontology (GO) annotations related to this gene include metalloendopeptidase activity. This protein is localized in the mitochondria and can functionally complement a YME1 disruptant yeast strain. It is proposed that this gene plays a role in mitochondrial protein metabolism and could be involved in mitochondrial pathologies [30]. ATP-dependent metalloprotease, which catalyses the degradation of folded and unfolded proteins with a suitable degron sequence in the mitochondrial intermembrane region [31], takes a big part in regulating mitochondrial morphology and function by cleaving OPA1 at position S2, giving rise to a form of OPA1 that promotes maintenance of normal mitochondrial structure and mitochondrial protein metabolism [31–33]. C4orf27 (also known as histone PARylation factor 1) (HPF1) is a protein-coding gene [34]. C4orf27 acts as a cofactor for serine ADP-ribosylation by conferring serine specificity on PARP1 and PARP2: this protein interacts with PARP1 and PARP1 and is able to change amino acid specificity towards serine [35]. However, ANKRD26, NEK1, YME1L1 and C4orf27 in HAPE remain unknown and require additional studies.

This study has several limitations. The small size of this study does not provide sufficient power for a conclusive analysis of the association. We hope that collaboration with other researchers with access to more HAPE patients will lead to the identification of gene(s) responsible for HAPE. Controls are not known to have traveled to high-altitude regions. We believe that only 0.5–2% of the population experienced HAPE after ascending to high-altitude regions. Considering the rarity of HAPE, we deem that all of these people can be used as healthy controls.

Conclusion

In summary, we provide evidence for the contribution of ADAMTS9-AS2, NEK1, CLCN3, C4orf27 (HPF1), RP11-219J21.2, ANKRD26 and YME1L1 to the pathogenesis of HAPE in Chinese populations. This prioritized gene deserves further evaluation to improve the understanding of HAPE genetics.

Acknowledgments

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Conflict of interest

The authors declare that they have no competing interests.

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Autopolymerized poly(methyl methacrylate) reinforced with aluminum trioxide nanoparticles

Autopolimerizovani poli(metilmetakrilat) ojačan nanočesticama aluminijum trioksida

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Abstract

Background/Aim. Mechanical properties, most significantly flexural strength of cold polymerized acrylic dental materials, used for denture reparation are lower compared to the equivalent hot polymerized materials. This paradox can be rectified by the application of alumina nanoparticles, which was the aim of this work. **Methods.** The liquid component of the commercial autopolymerized denture reline resin was modified with 0.05%, 0.2% and 1.5% (wt) 13 nm hydrophobic Al_2O_3. These mixtures, along with the unmodified liquid, were mixed with the powder component to form test specimens. Flexural modulus and strength were tested, while the results were statistically evaluated by the one-way ANOVA analysis followed by Tukey's test. Differential scanning calorimetry, scanning electron microscopy and energy dispersive X-ray analysis were performed to assess the heat and fracture surface features. **Results.** A statistically significant increase in flexural modulus was obtained only for 0.2% nanoparticle content, while flexural strength was significantly increased for specimens modified with 0.05% and 0.2% nanoparticles. Moreover, the rise of nanoparticle content to 1.5% contributed the formation of agglomerates, giving unsatisfactory mechanical properties. Also, the rise in glass transition temperature was noted for the most effective 0.05 and 0.2% Al_2O_3 contents. **Conclusion.** The 0.2% 13 nm Al_2O_3 loading is the most effective in improving the tested mechanical properties of cold polimerized poly(methyl methacrylate) reline resin.

Key words:
acrylates; calorimetry, differential scanning; denture rebasing; elasticity; materal testing; nanoparticles; polymethyl methacrylate; stress, mechanical.

Apstrakt

Uvod/Cilj. Mehaničke osobine, u najvećoj meri savojna čvrstoća, hladno polimerizovanih akrilata za reparaturu proteza, manja je u odnosu na toplo polimerizovane akrilate. Taj paradoks može biti rešen upotrebom nano čestica, što je bio cilj ovog rada. **Metode.** Tečna komponenta komercijalnog autopolimerizujućeg polimetilmetakrilata za podlaganje proteza modifikovana je sa 0,05%, 0,2% i 1,5% (wt) hidrofobnim Al_2O_3 nanočesticama promera 13 nm, testiranih merenjem zeta potencijala. Te mešavine, zajedno sa nemodifikovanom tečnom komponentom, pomešane su sa prahom kako bi se dobili uzorci za ispitivanje. Izvršeno je ispitivanje savojnog modula elastičnosti i čvrstoće, uz primenu jednostruke analize varijanse (ANOVA) sa *Tukey* testom. Toplotne karakteristike su ispitane diferencijalnom skenirajućom kalorimetrijom, dok su površinska svojstva prelomljenih površina ispitana skenirajućim elektronskim mikroskopom i energetskom disperzionom analizom. **Rezultati.** Statistički značajno povećanje savojne čvrstoće dobijeno je samo kod uzoraka sa 0,2% nanočestica, dok je to u slučaju savojnog modula elastičnosti nađeno kod uzoraka sa 0,05% i 0,2% nanočestica. Povećanje sadržaja nanočestica na 1,5% doprinosilo je pojavi aglomerata, što je negativno uticalo na mehaničke osobine. Povećanje temperature ostakljivanja postignuto je kod najefikasnijih koncentracija nano Al_2O_3 od 0,05% i 0,2%. **Zaključak.** Za najveći stepen povećanja mehaničkih osobina hladno-polimerizujućeg polimetilmetakrilata potreban je sadržaj od 0,2% nanočestica Al_2O_3 promera 13 nm.

Ključne reči:
akrilati; kalorimetrija, diferencijalno skenirajuća; zubna proteza, podlaganje; elastičnost; materijali, testiranje; koloidi; polimetilmetakrilat; stres, mehanički.

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Introduction

Poly(methyl methacrylate) (PMMA) has been used traditionally as a shatterproof replacement for glass due to its convenient properties, such as transparency, which results in a broad array of applications, including aircraft canopies, protective goggles, automobile running lights, as well as construction panels and dentures [1]. Today, around 90% of all dentures are made from PMMA [2, 3]. However, PMMA also has disadvantages, the most notable being insufficient ductility and strength, which leaves room for further improvement [4]. Mechanical properties of denture base can be increased in a number of ways. The addition of metal wire and unidirectional glass fibers can significantly increase the strength of dentures [5]. Moreover, ultra-high-molecular-weight polyethylone (UHMWPE) was used to reinforce the denture [6]. An increased flexural modulus and reduced flexural strength were obtained by adding rigid rod polymer fillers [7]. ZrO_2 and Al_2O_3 particulate fillers [8, 9], as well as the combination of these two filler types [10–12] were used, with a profound increase in strength and toughness.

PMMA is used for denture reline resins as well, but they are cold, rather than hot polymerized. This material is used for providing better retention of removable prostheses in cases of alveolar resorption and denture reparation in case of crack or fracture [13]. The mechanical properties of cold polymerized denture reline resin is lower as a result of a limited time for mixing the liquid and powder component, after which the radical polymerization commences. This results in an increased unconverted monomer content [14, 15]. The unconverted monomer acts as a microvoid, which weakens the material. There are several methods to decrease the monomer content, i.e., to increase the mechanical properties of denture reline resins. Post heat treatment in hot water was suggested by Lamb et al. [16] or by microwave treatment [17, 18]. A different approach is to add a certain amount of nanoparticles, aimed at reducing the mobility of polymer chains in the vicinity of the nanoparticle. An array of well-distributed nanoparticles throughout the material causes the formation of a reinforcing field increasing the materials mechanical properties [19].

The aim of this study was to test the effect of hydrophobic alumina (Al_2O_3) nanoparticles on mechanical properties (flexural modulus and flexural strength) of autopolymerized PMMA denture reline resin.

Methods

The basic material used was a PMMA denture reline resin designated as Simgal® (Galenika, Zemun, Serbia). This material consists of powder and liquid, while the mixing ratio was kept at 2:1 in weight, respectively. The liquid component was modified with AEROXIDE Alu C 13 nm Al_2O_3 (Evonik, Essen, Germany), having a specific surface area of 85–115 m^2/g. The following contents in the liquid phase were used: 0.05%, 0.2% and 1.5% (wt). The control specimen was left untreated. To obtain the correct amount of nanoparticles for mixing, the analytical balance with an accuracy of 0.0001 g was used (Adventurer Pro Ohaus, Parsippany, NJ). The mixing was done in a magnetic stirrer MM-530 (Tehtnica, Zelezniki, Slovenia) for 10 min. Zetasizer Nano ZS (Malvern Instruments, Malvern, UK) analyzer was used to determine the size of the particles in the liquid component (Figure 1). The unmodified and modified liquid components were then mixed with the powder component and the obtained mixture was poured into square Al-alloy molds. The specimens were obtained by mechanical cutting with cooling and the abrasive papers were used to get the final specimen shape and size (prismatic, 6 × 2.5 × 45 mm). To determine the flexural modulus of elasticity and flexural strength, Toyoseiki AT-L-118B (Toyoseiki, Tokyo, Japan) universal tensile testing machine was used. Three point bend test was used, with crosshead speed of 50 mm/min. The distance between the supports was 40 mm. The flexural modulus of elasticity was calculated by using the following equation (1):

$$E = \frac{\Delta F l^4}{4 \Delta d b h^3} \quad (1)$$

where l is the distance between the supports [mm], Δd is the displacement range [mm] for a testing load range ΔF [N], b is specimen width [mm] and h is specimen height [mm].

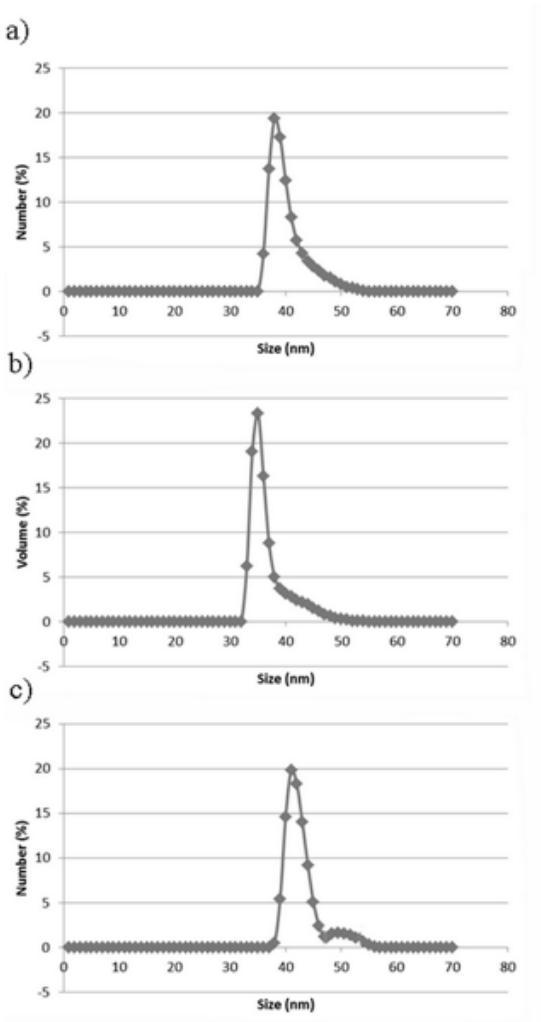

Fig. 1 – Particle distribution after mixing with liquid phase.

The flexural strength was calculated by using the equation (2):

$$\sigma = \frac{3Fl}{2bh^2} \text{ V} \quad (2)$$

where F is maximum force [N], l is the distance between the supports [mm], b is specimen width [mm] and h is specimen height [mm]. Five specimens were used for each of the following sample groups: the control group (unmodified), the group with 0.05%, 0.2% and 1.5% (wt) nanoparticles. One-way analysis of variance (ANOVA) followed by Tukey's test with the significance value of $p < 0.05$ was used. The tests were performed by using Minitab 16 software.

To determine the thermal properties of obtained materials, differential scanning calorimetry (DSC) analysis was performed. Q20 (TA Instruments, New Castle, DE) DSC device was applied, in the temperature range from 60 °C to 160 °C, with a scan rate of 10 °C/min. Furthermore, fracture surfaces were examined by JSM-6460LV (JEOL, Tokyo, Japan) scanning electron microscope (SEM), operating at 25 kV. The specimens were previously coated with gold, using the SCD-005 (Bal-tec/Leica, Wetzlar, Germany) device. To examine certain fracture surface features, energy-dispersive X-ray spectroscopy (EDX) was used (Oxford Instruments INCA Microanalysis system).

Results and discussion

The flexural modulus, flexural strength and standard deviations of tested materials, along with letter indicators of statistical significance between the results are given in Figures 2 and 3. It can be seen that the highest flexural modulus

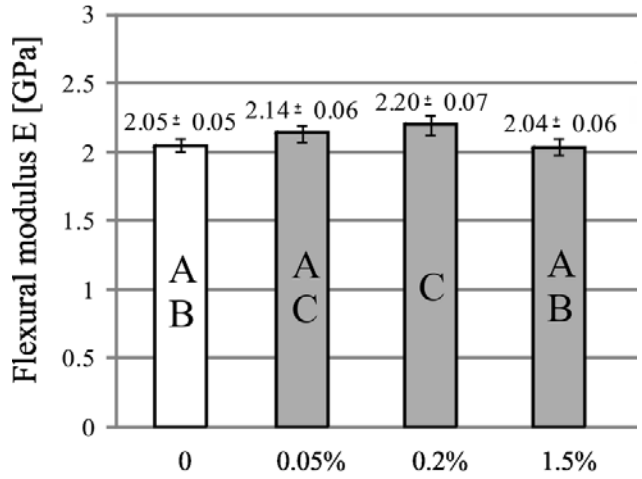

Fig. 2 –Flexural modulus and standard deviations of tested materials (different letters indicate statistically significant differences at a level of 95%).
GPa – gigapascals.

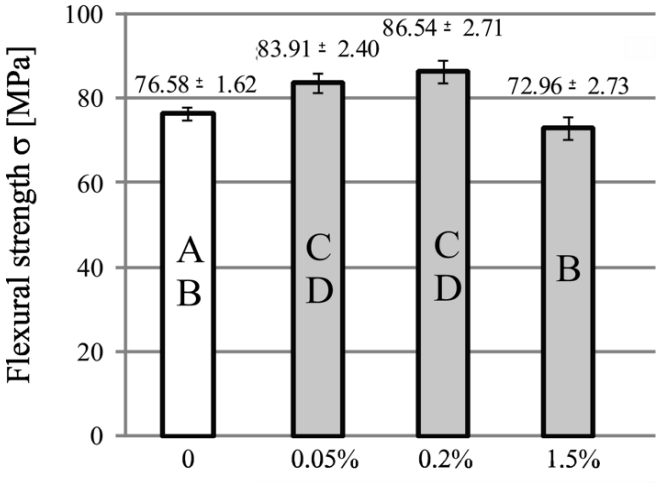

Fig. 3 –Flexural strength and standard deviations of tested materials (different letters indicate statistically significant differences at a level of 95%).
MPa – megapascals.

and strength were obtained for the specimen modified with 0.2% of Al_2O_3 nanoparticles. Both mechanical properties were significantly different from the control specimen. Specimens obtained with 0.05% nanoparticles also had a higher flexural modulus and strength; however, only in terms of strength, the difference was statistically significant. On the other hand, in specimens modified with 1.5% nanoparticles, the obtained values were lower than those of the control specimen.

The results of the DSC analysis are shown in Figure 4. The glass transition temperature (T_g) in the specimen modified with 0.05% and 0.2% nanoparticles was higher than that in the control specimen. This is the result of the layer surrounding the nanoparticles, where the mobility of the polymer chains is reduced compared to the unmodified material [19, 20]. In the specimen modified with 1.5% nanoparticles, T_g was the same as in the control specimen, probably due to a relatively thin modified layer and the limited amount of this modified material surrounding the nanofiller. This was obtained in spite of a higher addition of nanoparticles, because agglomeration occurs. This can be correlated both to the results obtained with zeta sizer, the distribution of nanoparticle size in liquid component (Figure 1) and mechanical properties shown in Figures 2 and 3. Namely, as a larger amount of polymer is immobilized, the modulus of elasticity and strength are higher, as in specimens modified with 0.05% and 0.2% of nanoparticles. This is also in accordance with a larger amount of smaller particles in the liquid component prior to mixing of the liquid and powder of the cold polymerized PMMA, as shown when Figures 1a and 1b are compared to Figure 1c. Lower mechanical properties of the specimens modified with 1.5% of Al_2O_3 are the result of larger particles (agglomerates) in the liquid phase before mixing, which, along with the results of DSC analysis indicate that the elevated agglomeration remained in the polymerized material (Figure 4).

Fracture surfaces of flexural strength testing specimens are shown in Figure 5. It can be seen that there are river marks present, typical for brittle fracture. Additionally, in Figure 5a and particularly in Figure 5c, spherical structures can be noticed. These structures are powder prepolymerized PMMA particles that are bonded by the polymerized MMA from the liquid component of the material. The crack path passing between the PMMA powder particles indicates that there is a significant difference between the strength of the liquid-component originated matrix and powder particles. In the specimen modified with 0.2% of nanoparticles, the crack path does not avoid powder particles, indicating a smaller difference between local mechanical properties between the matrix and spherical particles. That means, the cold polymerized matrix mechanical properties in the specimen modified with 0.2% nanoparticles are higher than those in the control specimen and the specimen modified with 1.5% nano Al_2O_3.

The results of the EDX analysis for the agglomerate particle and the surrounding material are shown in Figure 6. It can be seen that the analyzed agglomerate clearly can be identified as aluminum oxide. The analyzed particle size is

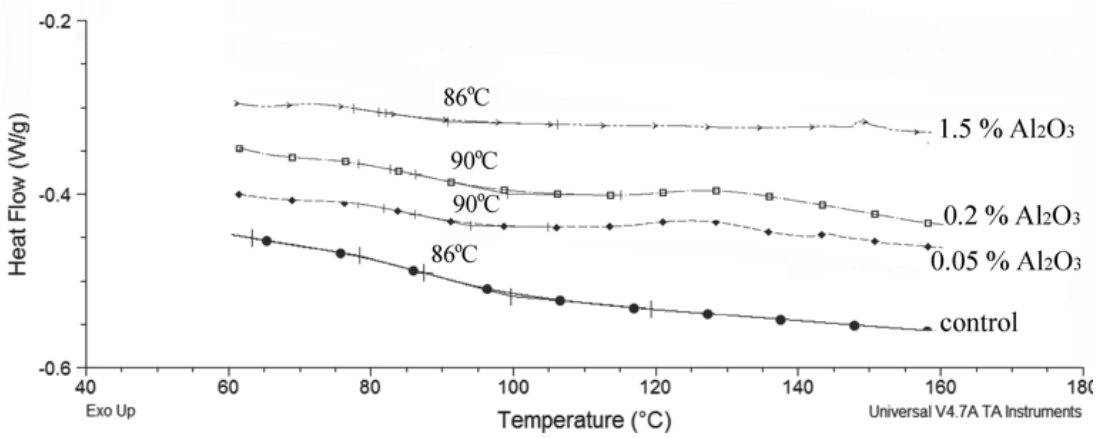

Fig. 4 – Differential scanning calorimetry (DSC) analysis results.

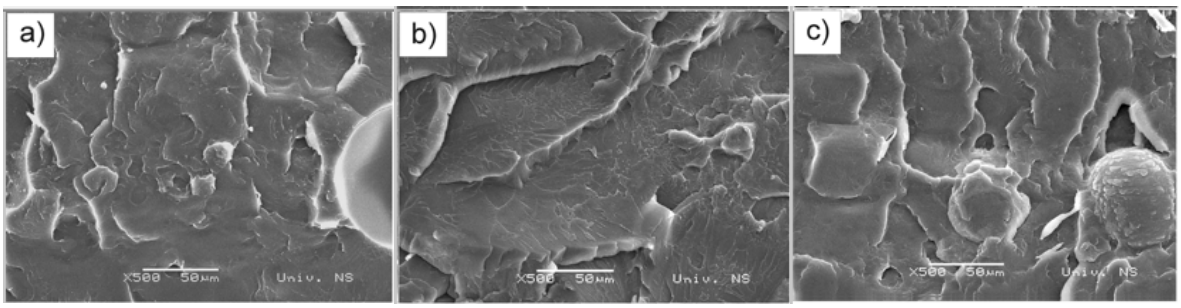

Fig. 5 – Scanning electron microscope (SEM) images of fractured surfaces: a) the control specimen; b) the specimen modified with 0.2% nanoparticles; c) the specimen modified with 1.5% nanoparticles.

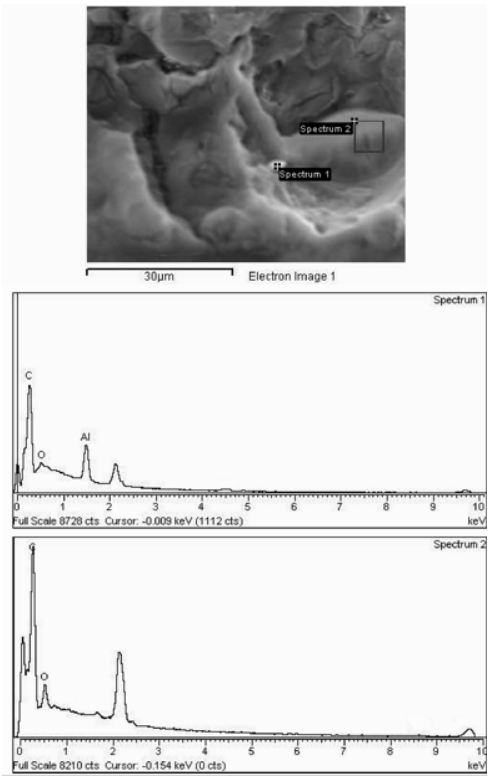

Fig. 6 – Energy-dispersive x-ray spectroscopy (EDX) analysis of agglomerate (spectrum 1) and the surrounding material (spectrum 2) of the specimen modified with 1.5% nanoparticles.

several orders of magnitude larger than nanoparticles introduced in the liquid component, as well as the measured particles in the liquid component (39–55 nm). The particle shown in Figure 6 can be classified as a relatively large agglomerate, which can explain the mechanical behavior of the material tested. As shown in Figures 2 and 3, the specimen modified with 1.5% nanoparticles has a lower flexural modulus and strength compared with the control specimen. Such results can be explained by the existence of large agglomerates that negate the effect of a relatively small volume of material surrounding the particles with reduced mobility of polymer chains. Namely, the agglomerates are collectives of nanoparticles, due to the existence of Van der Waals forces between them. As the agglomerates fracture under load, the stress is transmitted to the surrounding material, causing unstable crack propagation, weakening the material [4, 21].

Conclusion

The incorporation of 13 nm hydrophobic Al_2O_3 nanoparticles into the cold polymerized PMMA is beneficial for improving flexural modulus and flexural strength. The most effective content is 0.2% Al_2O_3, with a statistically significant rise in flexural modulus and strength compared to the control specimen. The nanoparticle loading of 1.5% proved to decrease mechanical properties compared to the control, unmodified specimens. The main reason for this behavior is the agglomeration of nanoparticles, creating particles several orders of magnitude larger, that contribute to the decrease in mechanical properties through the mechanism of fracture and unstable crack propagation.

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SHORT COMMUNICATION

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Two-grade metabolic tumor tissue assessment using positron emission tomography in prediction of overall survival in glioblastoma patients

Dvostepena metabolička procena tumorskog tkiva u predviđanju ukupnog preživljavanja bolesnika sa glioblastomom izvedena pozitronsko emisionom tomografijom

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Abstract

Background/Aim. Although considered rare, gliomas cause morbidity and mortality disproportionate to their incidence. The aim of the study was to determine whether pre and post-therapeutic metric values, derived from the FDG PET/CT maximal standardized uptake value (SUV_{max}) and calculated ratios between tumor and normal brain tissue, may provide a predictive/prognostic biomarker information in estimating overall survival of glioblastoma patients. **Methods.** In 26 out of 31 patients with glioblastoma treated with standard Stupp protocol after maximal safe reductive surgery, we performed a baseline 18F-FDG PET/CT examination before commencing combined and concomitant chemotherapy/radiotherapy (pre-therapy FDG PET/CT) and a second examination three months after the therapy completion (post-therapy FDG PET/CT). Two-graded SUV_{max} values and a calculated ratio of uptake in tumor-to-normal-tissue (T/N ratio) value, divided into two grades by the calculated cut-off value, were measured in all patients at both pre- and post-therapy FDG PET/CT studies. Data sets were statistically analyzed by the Kaplan-Meier survival test and Log-rank was calculated, with the level of confidence determined at $p < 0.05$. **Results.** Pre-therapy FDG PET/CT two-grade T/N ratio value and both pre- and post-therapy FDG PET/CT derived two-grade SUV_{max} values had a strong predictive impact on overall survival of glioblastoma patients. **Conclusion.** Based on two-grade SUV_{max} and T/N ratio values assessment, FDG PET/CT could provide valuable predictive survival information in glioblastoma patients and serve as a selection tool for identifying patients at higher risk from worse outcomes and shorter survival time.

Key words:
brain neoplasms; glioblastoma; positron-emission tomography; prognosis; survival analysis; tomography, x-ray computed.

Apstrakt

Uvod/Cilj. Mada su retki, gliomi izazivaju morbiditet i mortalitet nesrazmeran njihovoj incidenci. Cilj rada bio je da se utvrdi da li pre- i postterapijske metričke vrednosti koje proizilaze iz FDG PET/CT maksimalnih standardizovanih vrednosti preuzimanja (SUV_{max}) i izračunatih odnosa između tumora i normalnog moždanog tkiva (T/N odnos) mogu obezbediti prediktivne/prognostičke biomarkerske informacije u proceni ukupnog preživljavanja bolesnika sa glioblastomom. **Metode.** Kod 26 od 31 ispitanika sa glioblastomom, lečenih Stupp protokolom nakon maksimalne reduktivne hirurgije, načinjen je bazni 18F-FDG PET/CT pregled pre početka kombinovane i istovremene hemoterapije/radioterapije (preterapijski FDG PET/CT), a potom i drugi pregled tri meseca nakon završetka terapije (posttera

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pijski FDG PET/CT). Vrednosti SUV_{max} i izračunati T/N odnos, podeljeni u dva razreda izračunatom vrednošću razdela, mereni su kod svih bolesnika FDG PET/CT pregledima, pre i posle terapije. Skupovi podataka statistički su analizirani Kaplan-Meier-ovim testom preživljavanja i izračunat je Log-rank sa nivoom pouzdanosti utvrđenim na $p < 0.05$. **Rezultati.** Preterapijski FDG PET/CT dvostepeni T/N odnos i dvostepeni SUV_{max}, izveden iz FDG PET/CT pregleda pre i posle terapije, imali su snažan prediktivni uticaj na ukupno preživljavanje ispitanika sa glioblastomom. **Zaključak.** Na osnovu procene vrednosti dvostepenog SUV_{max} i T/N odnosa, FDG PET/CT omogućava dobijanje vrednih prediktivnih informacija o ukupnom preživljavanju bolesnika sa glioblastomom i stoga može poslužiti kao selekciona metoda za identifikaciju bolesnika pod povišenim rizikom od lošijeg ishoda bolesti i kraćeg vremena preživljavanja.

Ključne reči:
mozak, neoplazme; glioblastom; tomografija, pozitron-emisiona; prognoza; preživljavanje, analiza; tomografija, kompjuterizovana, rengdenska.

Introduction

Even though high-grade central nervous system gliomas are considered rare, with an incidence rate of five to six cases *per* 100,000 individuals, they cause morbidity and mortality disproportionate to their incidence.

High-grade gliomas represent the most common, but a heterogeneous group of adult intra-axial brain tumors with a median overall survival of 15 months in the patient subgroup treated with maximal safe tumor resection, concomitant radiation/chemotherapy, and adjuvant chemotherapy [1, 2].

Despite many recent efforts to develop multimodal approaches for optimizing combinations of surgery, radiation, and chemotherapy for high-grade gliomas, especially glioblastomas, disappointingly, survival rates remain nearly unchanged. Variability in clinicopathological tumor behavior complicates the combination and timing of multimodal treatment approaches, responses, and finally, outcomes [2–5].

Magnetic resonance imaging (MRI) is the primary clinical imaging modality at all disease stages in glioblastoma, ranging from the primary evaluation, presurgical planning, early postsurgical evaluation of residual tumor presence, radiotherapy planning, surveillance during chemotherapy, and recurrence detection. Objective and standardized MRI-based criteria for response assessment in neurooncology (RANO) have been developed and initially introduced for clinical trials in brain tumors [6].

Molecular imaging by use of positron emission tomography and computerized tomography (PET/CT) is an established and broadly used method in oncology [7], and for a certain time, is being increasingly used to supplement MRI in the clinical management of high-grade gliomas [8, 9]. Recent evidence-based recommendation by the PET-RANO working group and European Association of Neuro-Oncology (EANO) on the clinical use of different radiotracers, such as 2-deoxy-2-[18F] fluoro-D-glucose (FDG), radiolabeled amino acids like [11C-methyl]-methionine (MET), 2-[18F] fluoroethyl)-L-tyrosine (FET), and 3,4-dihydroxy-6-[18F] fluoro-L-phenylalanine (FDOPA), are providing convincing pieces of evidence of PET imaging additional value in high-grade gliomas and glioblastoma patients management [8], starting from differentiation of the glioma grade [7, 8], guidance of stereotactic biopsies [10, 11], the definition of target volume for radiation dose escalation, and differentiation of recurrent tumor from radiation necrosis [12, 13].

Though FDG PET/CT has an established role in glioblastoma patient management, it is still not routinely incorporated into neuro-oncological practice as a reliable prognostic indicator of outcome, mostly due to the inconsistent results of studies evaluating pre-therapy and/or post-therapy PET/CT findings [14–20].

Therefore, the aim of this study was to determine whether pre- and post-therapeutic metric values, derived from the FDG PET/CT maximal standardized uptake value (SUV_{max}) and calculated ratios between tumor and normal brain tissue, may provide a predictive/prognostic biomarker information in estimating overall survival (OS) of glioblastoma patients.

Methods

The prospectively designed study included overall 31 patients with histopathological verified glioblastoma treated with standard Stupp protocol (temozolomide 75 mg/m^2 daily, together with radiotherapy 60 Gy/30 fractions over 6 weeks, followed by six cycles of adjuvant temozolomide continued after radiotherapy completion) after maximal safe reductive surgery. Apart from regular MRI check-ups, the design of the study included the first baseline 18F-FDG PET/CT examination before the commencement of combined and concomitant chemotherapy and radiotherapy (pre-therapy FDG PET/CT), and a second examination three months after the completion of chemotherapy and radiotherapy (post-therapy FDG PET/CT). Out of 31 patients, 26 patients completed the study requirements and underwent the second post-therapy FDG PET/CT examination after the concomitant chemoradiation. In all of the patients, pre-therapy FDG PET/CT was performed within five to seven days before the commencement of combined concomitant therapy, and the post-therapy FDG PET/CT was done three months after the completion of radiotherapy treatment.

The institutional Ethical Board approved the study, and the patients' informed consent was obtained.

The 18F-FDG PET/CT exams were performed on Siemens Biograph 64 True Point PET/CT scanner (Siemens, Erlangen, Germany), and after 4–6 hours of fasting, all patients were intravenously injected with 185 MBq (5 mCi). After an uptake period of around 30–45 min in a dim room, a

one-bed position of 10 min acquisition period with a non-contrast-enhanced CT scan for attenuation correction was done. Scans were visualized and analyzed on the Leonardo Siemens workstation independently by two experienced readers.

With a region-of-interest area covering the surgically reduced tumor bed and brain tissue at exactly the same level in the contralateral brain hemisphere, SUV_{max} value and calculated ratio of uptake in tumor-to-normal-tissue (T/N ratio) value were determined in all patients at both pre- and post-therapy FDG PET/CT studies.

The two-grade semiquantitative assessment was applied by using both calculated SUV_{max} and T/N ratio cut-off values, calculated as median ± standard deviation (SD) value. The cut-off value for the pre-therapy FDG PET/CT exam was calculated as 1.1, dividing the patients into the hypermetabolic group (T/N ratio value higher than 1.1) and the hypometabolic group (T/N ratio value equal to or lower than 1.1), while the median T/N ratio cut-off value for the post-therapy PET/CT was calculated as 1.2. Patients were also divided into two groups regarding the SUV_{max} grading system, including the hypermetabolic group (SUV_{max} value higher than 8.96 for pre-therapy FDG PET/CT and 9.43 for post-therapy FDG PET/CT) and the hypometabolic group (SUV_{max} value equal to or lower than 8.96 and 9.43 for pre and post-therapy FDG PET/CT, respectively). Observed by the visual uptake two-grade grading system, the hypermetabolic group had tumor bed uptake higher than cortex uptake and hypometabolic group uptake equal to or lower than the cortex uptake. Both of the latter grouping systems resulted in a consistent division of patients into two almost identical and fully comparable patient groups.

Patients were clinically followed-up, and their OS as a study endpoint was registered. OS was determined as a period from surgical biopsy and maximal safe reduction date to the date of death.

Regarding OS, patients were divided into two groups: low survival rate group (OS < 12 months) and high survival rate group (OS > 12 months).

OS was compared to prognostic factors that included age, sex, pre-therapy SUV_{max}, post-therapy SUV_{max}, initial pre-therapy T/N ratio value, and post-therapy T/N ratio value after combined and concomitant therapy.

Data sets were statistically analyzed by using SPSS Statistics for Windows, version 16.0 (SPSS Inc. Chicago, Ill, USA) that included descriptive statistics, univariate (ANOVA), and multivariate analysis; both Cox regression and Linear regression were used to test variables including age, sex, pre-therapy SUV_{max}, post-therapy SUV_{max}, pre-therapy T/N ratio value, and post-therapy T/N ratio value. The Kaplan-Meier survival test with Log-rank test was also performed, and the median values ± SD of the SUV_{max} and the T/N ratio value were used to distinguish between two survival groups, with the level of confidence determined at $p < 0.05$.

Results

Out of 31 patients, 26 patients [17 (65.4%) men and 9 (34.6%)] women, age range from 30 to 75 years (mean: 56.04, and median ± SD: 59 ± 13.63) who fulfilled the study design requests and underwent both pre- and post-therapy FDG PET/CT examinations were included in the study. Five out of 31 patients were lost to follow-up, being unable to finish the whole therapy course or due to death, and, therefore, excluded.

The OS period ranged from 5 to 55 months (mean 15, median ± SD: 11.5 ± 11.86 months), and regarding OS values, 12 patients were included in the low survival rate group (OS < 12 months) and 14 in the high survival rate group (OS > 12 months).

For the pre-therapy PET/CT examination, SUV_{max} values ranged from 1.47 to 16.24 (mean: 6.33 and median ± SD: 5.71 ± 3.25) and for the post-therapy PET/CT examination, SUV_{max} values ranged from 3.53 to 12.6 (mean: 7.03 and median ± SD: 6.67 ± 2.76).

Pre-therapy FDG PET/CT T/N ratio value ranged from 0.19 to 1.64 (mean: 0.77 and median ± SD: 0.77 ± 0.35), and post-therapy FDG PET/CT T/N ratio value ranged from 0.15 to 2.73 (mean 0.82 and median ± SD: 0.75 ± 0.53).

Calculated SUV_{max} and T/N ratio cut-off values by both pre-therapy and post-therapy FDG PET/CT examinations divided the patients into hypometabolic and hypermetabolic groups, as shown in Table 1.

Survival curves were constructed with the Kaplan-Meier method (Figure 1), showing statistically significant difference between both hypo- and hypermetabolic SUV_{max}

Table 1

Descriptive statistics for the Kaplan-Meier overall survival in pre-therapy FDG PET/CT SUV_{max} and T/N ratio two-grade assessed hypometabolic and hypermetabolic groups

Parameter	n	Mean OS	SD	95% CI		Median OS	SD	95% CI		Min OS	Max OS
				lower	upper			lower	upper		
SUVmax											
hypometabolic	18	24.5	4.65	15.38	33.62	16	3.16	9.8	22.19	5	55
hypermetabolic	8	12.16	1.17	9.85	14.47	12	1.67	9.4	15.28	7	15
overall	26	21.18	3.65	13.98	28.39	15	1.82	11.43	18.56	5	55
T/N ratio											
hypometabolic	18	24.5/23.36	4.65/4.36	15.38/14.82	33.62/31.9	16	3.16/2.47	9.8/11.1	22.19/20.8	5	55
hypermetabolic	8	12.28/12.4	1.11/1.59	10.1/9.28	14.46/15.5	12	1.66/2.96	8.74/6.2	15.25/17.8	7	11
overall	26	21.18	3.67	13.98	28.39	15	1.82	11.43	18.56	5	55

OS – overall survival (in months); SD – standard deviation; CI – confidence interval; Min – minimal; Max – maximal; SUVmax – maximal standardized uptake value; T/N – uptake in tumor-to-normal-tissue; **FDG – 2-deoxy-2-[18F] fluoro-D-glucose; PET – positron emission tomography; CT – computed tomography.**

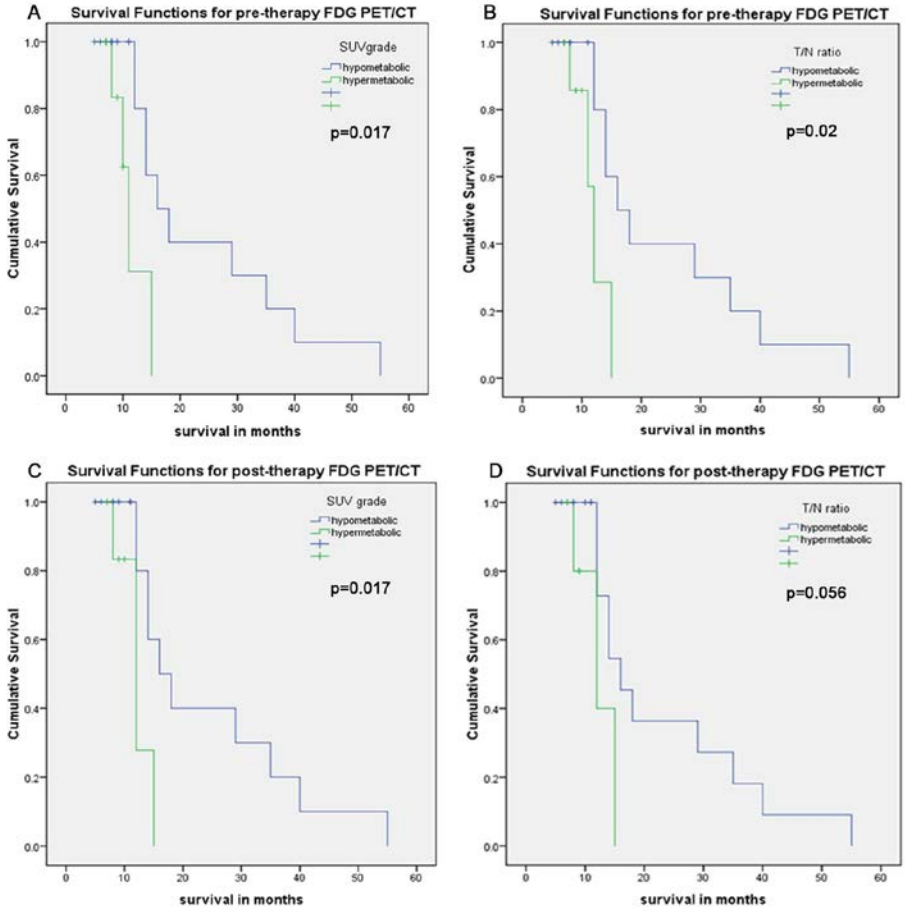

Fig. 1 – Kaplan-Meier survival curves showing overall survival in hypometabolic and hypermetabolic groups of patients in the function of pre-therapy FDG PET/CT SUV_{max} grading (A) and T/N ratio (B), and in the function of post-therapy FDG PET/CT SUV_{max} grading (C) and T/N ratio (D) [post-therapy SUV_{max} and pre-therapy T/N ratio: $p < 0.05$; log-rank for post-therapy T/N ratio: $p > 0.05$ ($p = 0.056$)].
For abbreviations see under Table 1.

grading, and hypo- and hypermetabolic T/N ratio value, obtained by pre-therapy FDG PET/CT exam ($p < 0.05$), and the same significant difference for post-therapy FDG PET/CT SUV_{max} grading ($p < 0.05$), but for the post-therapy T/N ratio value was just at the edge of the border of significance ($p = 0.056$).

Though by the ANOVA and multivariate analysis for any of the prognostic factors tested, no statistical significance was established, apart from the near-to-significant result of post-therapy T/N ratio value on survival ($p = 0.077$), with linear regression test, we found that age was a significant factor influencing survival ($p = 0.049$). Cox regression analysis demonstrated that gender and age were statistically significant variables influencing OS and, also, indicated pre-therapy T/N ratio near-to-significant influence on survival ($p = 0.05$) (Table 2).

Table 2

Impact of prognostic factors on overall survival in ANOVA analysis, Linear and Cox regression analysis with 95% confidence interval (CI) estimate for the odds ratio [95% CI for Exp(B)]

Prognostic factor	ANOVA *p*-value	Linear regression *p*-value	Cox regression	95% CI for Exp (B)
Gender	0.281	0.222	0.048	1.015–79.197
Age	0.471	0.049	0.023	1.018–1.28
Pre-therapy SUV_{max}	0.428	0.713	0.505	0.387–1.597
Post-therapy SUV_{max}	0.652	0.402	0.600	0.514–1.469
Pre-therapy T/N	0.203	0.511	0.050	1.009–5.772
Post-therapy T/N	0.077	0.903	0.789	0.003–80.049

SUVmax – maximal standardized uptake value; T/N – uptake in tumor-to-normal-tissue.

Table 3

Percentage of overall survival after 12, 24, and 36 months for SUV_{max} and T/N ratio value determined hypometabolic and hypermetabolic groups of patients

Group of patients	Overall survival (%)		
	12 months	24 months	36 months
Hypometabolic group	55.5	22.2	16.6
Hypermetabolic group	50.0	0	0

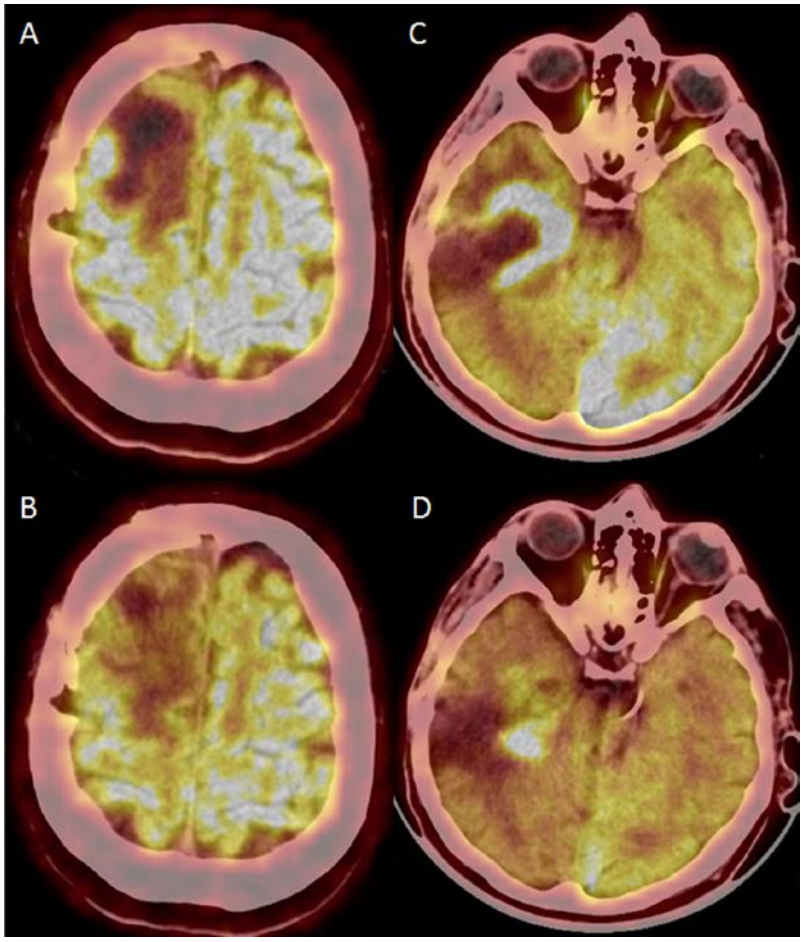

Fig. 2 – Two-grade SUV_{max} and T/N ratio assessment on pre-therapy (A) and post-therapy (B) fused FDG PET/CT scans enabled the distinction of a 47-year-old male patient with 55-month overall survival into the hypometabolic group, and on pre-therapy (C) and post-therapy (D) fused FDG PET/CT axial scans classified a 60-year-old male patient in the hypermetabolic group with a 9-month overall survival time. For abbreviations see under Table 1.

Calculated OS for the hypometabolic group was 55.5% after 12 months, and for the hypermetabolic group, it was 50% (Table 3), with the longest survival period of 15 months in the hypermetabolic group and 55 months in the hypometabolic group (Figure 2).

Discussion

Several prospective and retrospective studies already suggested the possible predictive value of FDG PET/CT in patients with glioblastoma [14–17, 21–25].

So far, prognostic factors predicting survival in glioblastoma patients, such as age, glioma grade, genetic and molecular biomarker status, tumor location, the extent of surgery, and concomitant therapy were recognized and/or accepted [26–31].

Yet, the prognostic value of FDG PET/CT remains controversial, and even though some studies demonstrated an inverse correlation of direct FDG uptake with survival [18], we were not able to confirm such results.

Some of the published studies, such as the study of Colavolpe et al. [32], indicate the pretreatment FDG PET/CT as an

independent prognostic factor of survival by using the a T/N ratio rather than a five grade scale SUV_{max} measurement only as an apparently more quantitative, precise and reliable method.

A study by De Witte et al. [14] showed that two-grade semiquantitative metabolic assessment could be used as a predictive factor of OS, reporting significantly shorter survival with increased metabolic grading. The same was confirmed in a meta-analysis by Zhang et al. [33].

Our results are aligned with the results of these studies, indicating that two-grade, hypo/hypermetabolic SUV_{max} grading, and a T/N ratio inversely correlate with OS. In patients with hypermetabolic SUV_{max} and T/N ratio values, OS was significantly decreased, while in the patients with hypometabolic SUV_{max} and T/N ratio values, OS was increased.

Based on our results, we differentiated two possible predictive metabolic biomarker factors for identifying glioblastoma patients under risk of significantly decreased OS. First one, pre-therapy FDG PET/CT derived T/N ratio value higher than calculated cut-off T/N ratio value of 1.1, hence considered hypermetabolic, which correlate to the results of the Leiva-Salinas et al. [34] study, who have calculated a cut-off T/N ratio value of 2.0 or 2.5 as a predictive factor of shorter survival, and the second one, pre-therapy FDG PET/CT derived hypermetabolic SUV_{max} grading value, also correlating to the results determined in other studies [33].

We have confirmed that age and gender are significant prognostic factors of survival, with a meaningful impact on glioblastoma patients' OS that coincides with several previously performed studies [26–28].

Since pre-therapy FDG PET/CT two-grade T/N ratio value, together with pre-therapy and post-therapy FDG PET/CT derived two-grade SUV_{max} values emerged as a strong predictive factor of survival, we are of the opinion that differentiation of the glioblastoma patients into hypo- or hypermetabolic groups by using FDG PET/CT appears to be relevant in predicting OS.

Variety in the strength of statistical significance impact on survival between pre-therapy and post-therapy T/N ratio FDG PET/CT exams implies that the changes during glioblastoma irradiation and chemotherapy are diminishing the metabolic differences between the tumor and normal brain tissue, making them both less observable and less easily detectable.

In our opinion, one of the limitations was that all patients included in our study underwent FDG PET/CT after the surgical biopsy with maximally safe tumor reduction. We believe that this could be a restraining factor in our study, in comparison to the studies where FDG PET/CT was done in the patients without or prior to surgical treatment, that may improve the SUV_{max} value significance as a prognostic factor of survival [32], leading us to infer that it may be highly recommendable to perform FDG PET/CT in glioblastoma patients before the surgical treatment.

Another limitation could be the absence of delayed FDG PET/CT studies, which, as indicated in several investigations [34, 35], could improve the T/N ratio values and thus improve the definition of cut-off value. Study dependence on subjectivity and reasonably decreased reproducibility level is caused by operator-dependent manual selection of the region of interest, instead of automatic segmentation, as a limitation resulting from a lack of availability of adequate software solutions at the time of the study.

The same should be stated for the limited number of patients belonging to the T/N ratio and SUV_{max} grading hypermetabolic group; we firmly believe that further investigation on a higher number of included patients could result in potential improvement of currently obtained statistical significance.

As already stated, new PET tracers, other than FDG, do show good performance in detection, tumor delineation, and tumor grading and are promising for the wider acceptance of PET/CT diagnostic methods in neurooncology [7, 8, 20, 24].

Amino acid transporters tracers 11C-MET, 18F-FET, or 18F-FDOPA are considered to provide the insight into treatment response associated with long term outcome [36–39], enabling redirection of the patients to new radiotherapy planning concepts, called radiotherapy dose painting [40], or in the case of 18F-FET, potentially facilitating pseudoprogression differentiation in glioblastoma patients [41].

However, as highlighted in the article of Albert et al. [8], one of the major restraints for amino acid PET is their availability, since there are significantly fewer centers using them routinely in everyday practice in comparison to many centers using FDG, and the second one, probably even more important would be limited health insurance companies reimbursement.

Therefore, even with all limitations of FDG PET/CT, the fact that prognostic assessment in glioblastoma patients does not require optimal detection performance but adequate prognostic information [32], we could conclude that FDG PET/CT can be incorporated in everyday clinical neurooncological practice.

Conclusion

Performed before the commencement of combined therapy, FDG PET/CT could provide valuable predictive survival biomarker information in glioblastoma patients, based on proposed two-grade SUV_{max} and T/N ratio values assessment, and serve as a selection tool for identifying patients at higher risk for the worse outcome and shorter survival time.

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Conflict of interest

The authors declare no conflict of interest.

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CASE REPORTS

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Mediastinal ectopic thyroid tissue as diferential diagnostic problem: A case report

Ektopično tkivo štitaste žlezde u medijastinumu kao diferencijalno dijagnostički problem

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Abstract

Introduction. Mediastinal ectopic thyroid tissue (ETT) represents a rare entity. Clinically, it can manifest with thyroid gland dysfunction or with symptoms and signs caused by a compressive effect on the surrounding structures, but in most cases it is an asymptomatic condition and incidental finding. All pathologic processes, including malignancy that can occur in the orthotopic thyroid gland can also develop in the ETT. **Case report.** We presented a case of a 17-year-old female with incidentally found mediastinal ETT. Besides ETT, the patient had an orthotopic thyroid gland and was euthyroid. During follow-up, mild compressive symptoms developed. Magnetic resonance imaging examination showed a non-significant increase of the mediastinal mass volume, but due to its morphological changes, a suspicion of another etiology was raised. A discrepancy between the positive technetium-99m pertechnetate and negative 131 iodine radionuclide imaging of the mediastinal mass was highly suspicious for malignancy. Surgery was performed and the pathologist confirmed that it was a colloid goiter in the mediastinal ETT. **Conclusion.** Mediastinal ectopic thyroid tissue should be taken into account in the differential diagnosis of the mediastinal tumor mass. An increase in the size of the mediastinal ETT, development of compressive symptoms or suspected malignant alteration require surgical treatment.

Key words:
congenital abnormalities; diagnosis, differential; histological techniques; mediastinal neoplasms; thyroid gland.

Apstrakt

Uvod. Ektopično tireoidno tkivo (ETT) u medijastinumu predstavlja redak entitet. Klinički se može manifestovati simptomima poremećaja funkcije štitaste žlezde ili znacima kompresije okolnih organa, ali najčešće protiče asimptomatski i otkriva se slučajno. Svi patološki poremećaji, koji mogu nastati u ortotopičnoj štitastoj žlezdi, uključujući i malignitet, mogu nastati i u ETT. **Prikaz bolesnika.** Prikazana je 17-godišnja bolesnica sa slučajno otkrivenim medijastinalnim ETT koja je, pored ETT, imala ortotopičnu tireoideu i bila eutireoidna. Tokom praćenja razvili su se blagi simptomi kompresije. Nalaz magnetne rezonance ukazao je na beznačajno uvećanje medijastinalne mase, ali je diferencijalno dijagnostički posumnjano na drugu etiologiju opisane promene. Protivrečnost između pozitivnog nalaza medijastinalne mase ustanovljenog snimanjem tehnecijum-99m pertehnetatom i negativnog nalaza ustanovljenog snimanjem primenom ^{131}joda ukazivao je na mogući malignitet. Bolesnica je operisana, odstranjena joj je medijastinalna masa i patohistološki je potvrđena koloidna struma u medijastinalnom ETT. **Zaključak.** Ektopično tkivo štitaste žlezde u medijastinumu mora se uzeti u obzir prilikom razmatranja diferencijalne dijagnoze medijastinalnih tumorskih masa. Porast medijastinalnog ETT, pojava simptoma kompresije ili sumnja na malignu alteraciju zahtevaju hirurško lečenje.

Ključne reči:
anomalije; dijagnoza, diferencijalna; histološke tehnike; medijastinum, neoplazme; tireoidna žlezda.

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Introduction

Ectopic thyroid tissue (ETT) is a rare congenital anomaly that develops during the migration of the thyroid angle from the floor of the primitive foregut to its final position on the anterior neck between 2nd and 4th tracheal cartilage rings. The prevalence of the ETT is 1 case per 100,000–300,000 people [1], while autopsy studies show the prevalence of 7%–10% in the population. ETT can coexist with or without a normal localized thyroid gland.

The anatomical locations of the ETT can be various: lingual, sublingual, submandibular, lateral cervical space, carotid space, axillar, endotracheal, mediastinal, pulmonary, cardiac, duodenum, stomach, pancreas, porta hepatis, adrenal glands, ovaries even iris and pituitary gland [1–4]. The most common ectopic location is lingual in about 90% of cases [1, 2].

Clinical presentation of the ETT includes both hyper- and hypo-thyroidism, thyroiditis and symptoms caused by compression effect of the ectopic tissue, but it can also be asymptomatic and therefore an incidental finding.

Beside the tests of the thyroid function, imaging methods such as ultrasound, computed tomography (CT), magnetic resonance imaging (MRI), radionuclide thyroid imaging and biopsy, also have an important role in the diagnostic algorithm of the ETT.

Rare locations, functional and morphologic changes in the ectopic tissue can represent a challenge in differential diagnosis, as in this case.

Case report

We presented a 17-year-old female with an incidental finding of two nodules in the left lobe of the thyroid gland on the neck. Ultrasound examination was performed because of repeated sore throats. Further diagnostic procedures included thyroid gland scintigraphy performed with technetium-99m pertechnetate scintigraphy (Figure 1), fine needle aspiration biopsy (FNAB) of the nodules and computed tomography (CT) of the neck and thorax. Thyroid gland scintigraphy with technetium-99m pertechnetate showed normal radionuclide uptake by the gland and uptake by the ETT in the upper mediastinum on the left side. Soft tissue mass just below the left lobe of the thyroid gland, at the level of the *apertura thoracis superior*, without compressive effect on the trachea was described on the CT scan, suspected to be an ETT. Thyroid function was normal, while the additional findings included two hypodense nodules, one in both thyroid gland lobes, with diameter less than 10 mm. Performed FNAB consisted of a benign follicular nodule.

One year later, the patient was examined by an endocrinologist. Ultrasound of the neck showed small cysts (less than 5 mm) in both thyroid gland lobes while the nodules were the same as on the previous examination. Thyroid function was normal (Table 1). Due to the appearance of intermittent pain and feel of pressure in the lower part of the neck, the patient underwent an MRI scan. The MRI scan showed a well-circumscribed soft tissue paratracheal mass

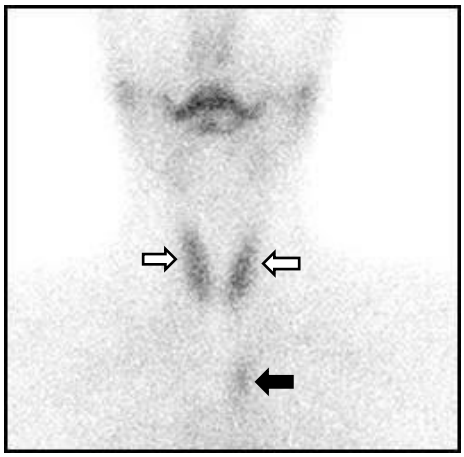

Fig. 1 – Thyroid gland scintigraphy performed with technetium-99m pertechnetate showing normal radionuclide uptake by the gland (white arrows) and uptake by the ectopic thyroid tissue in the upper mediastinum on the left side (black arrow).

Table 1

Results of laboratory examinations before and after surgery

Parameters	Before	After	Reference interval
Free thyroxin (pmol/L)	18.64	17.44	9.0–19.0
Free triiodothyronine (pmol/L)	5.01	5.29	2.6–5.7
Thyroid-stimulating hormone (IU/L)	1.02	1.13	0.35–4.94
Calcitonin (pg/mL)	2.78	n.t.	1.4–78
Anti-TPO antibodues (IU/mL)	< 10.0	n.t.	< 5.6
Anti-Tg antibodies (IU/mL)	< 20.0	n.t.	< 4.1

TPO - thyroid peroxidase; Tg – thyroglobulin; n.t. – not tested.

on the left side (approximately measured 18 × 14 × 30 mm), with inhomogeneous postcontrast enhancement and mild compressive effect on the trachea (Figure 2). Differential diagnosis included ETT, but also teratoma and parathyroid adenoma. Repeated scintigraphy with ^{131}I showed a normal image of the thyroid gland but this time, there was no uptake by the soft tissue mass in the mediastinum (Figure 3). The patient underwent left-sided cervicotomy in general anesthesia, and the surgeon removed the whole mass that was not attached to the thyroid gland. The pathologist confirmed that it was a colloid goiter in the ectopic thyroid gland tissue (Figure 4). After the surgery, thyroid function remained normal (Table 1) and the patient was asymptomatic.

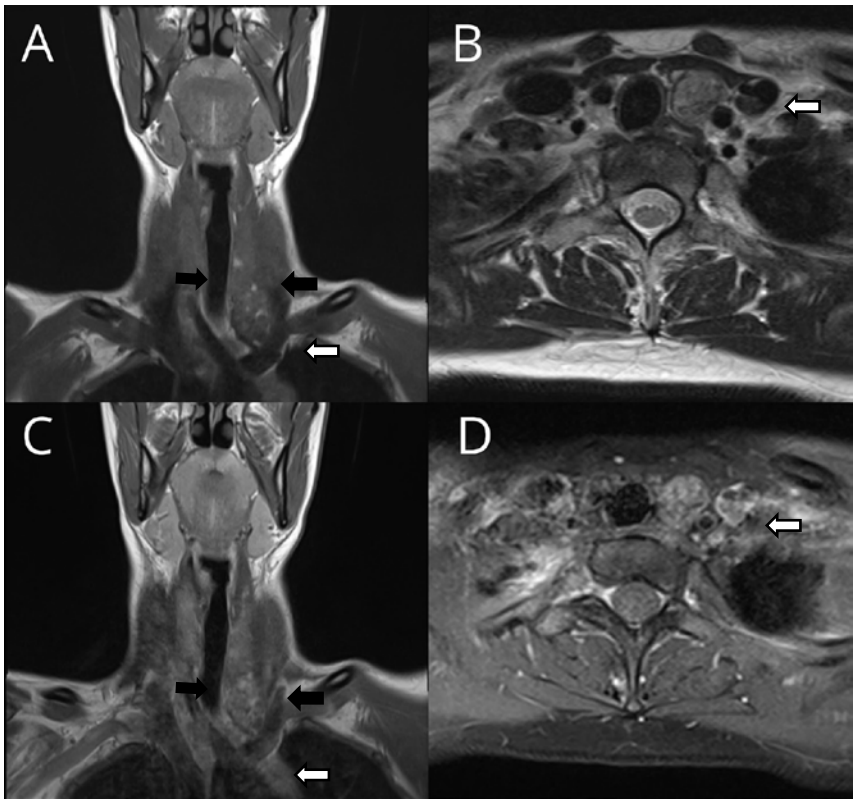

Fig. 2 – Magnetic resonance imaging (MRI) examination: T1 weighted image in the coronal plane (A) and T2 weighted image in the axial plane (B) showing a well-circumscribed paratracheal mediastinal soft tissue mass on the left (white arrows) with mild compressive effect on the trachea; T1 weighted image in the coronal plane (C) and T1 weighted image with fat saturation in the axial plane (D) after gadolinium contrast administration showing an inhomogeneous enhancement of the soft tissue mass (white arrows). Black arrows in A and C indicate normal right and left thyroid gland lobes.

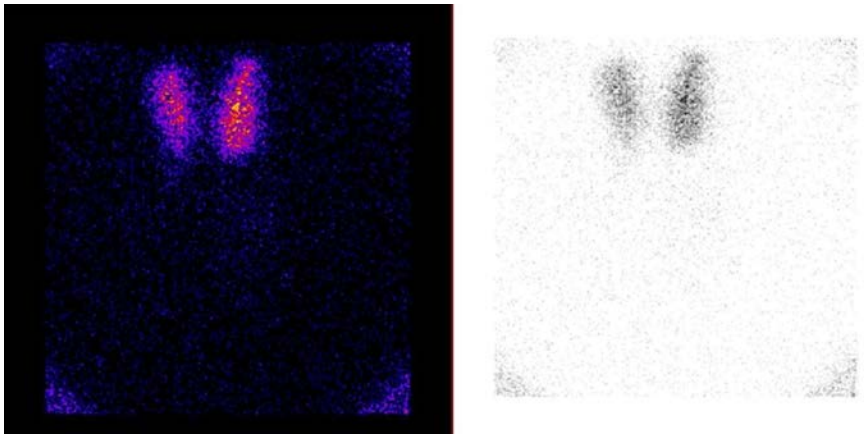

Fig. 3 – Thyroid gland radioiodine scintigraphy with ^{131}I performed with a gamma camera (Symbia E, Siemens) fitted with a high-energy, parallel-hole collimator, 24 hours after oral administration of 1.8 MBq of the radionuclide.

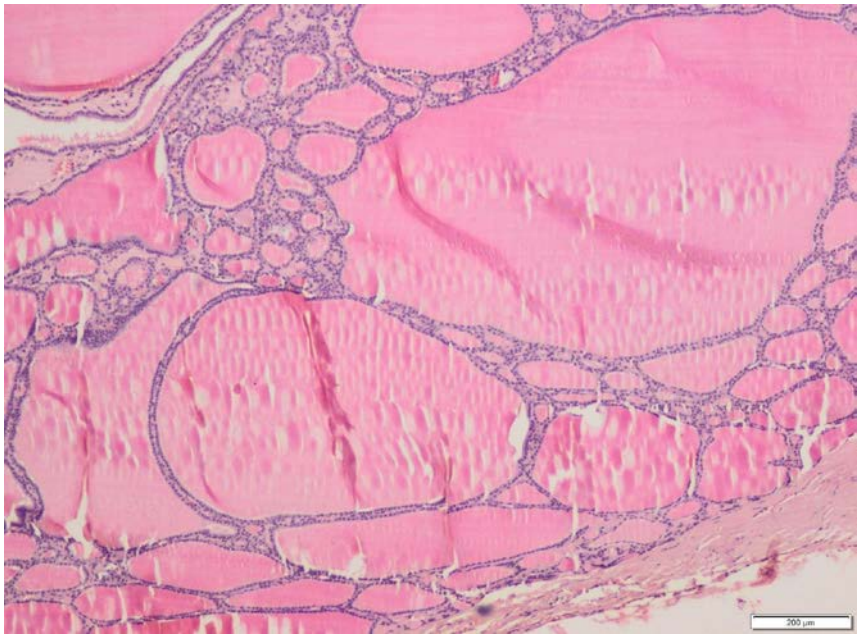

Fig. 4 – Pathohistological finding: Colloid goiter (hematoxylin eosin, × 40).

Discussion

ETT is a rare developmental anomaly. Some studies suggest the genetic base of this anomaly, due to mutation of regulatory genes and transcriptional factors that determine the development of the thyroid gland. Several mutations in genes playing a role during thyroid morphogenesis such as NKX2-1, PAX8, FOXE1, NKX2-5 and TSHR, have been reported, but the molecular mechanisms are not yet fully understood [1, 5, 6]. ETT can appear at any time, but most commonly in childhood, adolescence or in menopause. The female to male ratio is about 4:1 [5]. The presence of a normal thyroid gland in patients with ETT is not necessary. All pathological processes that can develop in the normal gland can also develop in the ETT. Clinical symptoms are typically related to the size and location as well as thyroid function. However, it is mostly an asymptomatic and incidental finding. An increase in the size of the ETT typically correlates with physiological conditions with increased demands for thyroid hormones that is seen during puberty and pregnancy [1, 2].

Mediastinal ETT is an extremely rare entity. To our knowledge only a few cases were reported [5]. It represents about 1% of mediastinal tumors; because of that, it is necessary to be included in the differential diagnosis of mediastinal masses with lymphomas, thymic tumors and dermoid cysts. Hodgkin lymphoma, large B cell lymphoma and lymphoblastic lymphoma are the most common mediastinal lymphomas, while thymic and neuroendocrine carcinomas are rare but highly malignant [7, 8]. Substernal thyroid goiter needs to be differentiated from the ETT. CT and MRI both have a very important role in the diagnosis of ETT, especially when it is distant from the descending pathway of the thyroid [5, 7, 9, 10]. Other imaging modalities such as single-photon emission computed tomography (SPECT CT) with ^{131}I SPECT CT and endoscopic bronchial ultrasound guided biopsy are useful especially in cases of mediastinal ETT [11–14]. Mediastinal ETT can coexist with the orthotopic thyroid gland and in most cases patients are euthyroid, as in our case. If there is no significant mass effect on the surrounding structures and thyroid function is normal, the patients should be followed. In other cases, treatment is surgery. Even in elderly patients, surgical treatment is suggested because of its low risk [7–9]. Both benign and malignant alterations can occur in ETT of any location. Malignancy may occur within ETT with a variety of cell types (papillary, follicular, medullary thyroid cancer, and also Hurtle cell tumor). There are few cases of teratoma and B cell lymphoma in mediastinal ETT [1, 5, 6].

Rarely, a patient with normal TSH can have differences in radionuclide thyroid imaging using technetium pertechnetate vs. iodine scan [15]. This false negative iodine scan could be explained by the presence of the non-organifying thyroid tissue in the ETT (follicular cells which have access to the iodine pump, but without organification).

In our case, the first scintigraphy, at the time of diagnosis, was performed with technetium-99m pertechnetate and the uptake by the ETT was present. The second scintigraphy performed a year later with more sensitive ^{131}I did not show any uptake of the radionuclide. Having in mind the MRI finding, heterogeneous morphology of the tissue, and possible alteration, surgical removal was performed and the pathologist confirmed that it was a benign lesion.

Conclusion

Although mediastinal ETT is rare, it is necessary to be kept it in mind in cases of mediastinal tumor masses. Beside scintigraphy and ultrasound, both CT and MRI have important role in the diagnostic algorithm of ETT. Benign and malignant alterations can occur in ETT of any location. Treatment of mediastinal ETT is either follow-up or surgery, depending on size, location, growth and morphologic changes.

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Laryngeal schwannoma – A case report with short literature review

Švanom larinksa – prikaz slučaja sa kratkim pregledom literature

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Abstract

Introduction. Laryngeal schwannomas are rare benign neurogenic tumors. They are usually localized in the supraglottic portion of the larynx. We reported a rare case of laryngeal schwannoma with a focus on clinical presentation, diagnosis and management. **Case report.** A 61-year old male patient with a 3-year history of hoarseness underwent telescopic examination which revealed an oval submucosal tumefaction of the left ventricular fold extending over the left vocal fold toward the right ventricular fold. Multislice computed tomography showed a 22 x 15 mm well defined, oval heterodense mass in the region of the left ventricular fold, extending toward the left vocal fold and the posterior commissure of the larynx, with signs of initial compression lesion of the thyroid cartilage. Biopsy and histopathology revealed a primary benign encapsulated mesenchymal tumor, while immunohistochemistry analysis confirmed schwannoma diagnosis. The patient underwent tracheotomy and left hemilaryngectomy, with complete removal of the tumor. There were no signs of recurrence at the six-month follow-up. **Conclusion.** Schwannomas are rare among benign tumors of the larynx and might grow for years before being diagnosed. Biopsy with histopathology analysis is used to confirm the diagnosis of laryngeal schwannoma, although extreme care should be taken during biopsy. Treatment consists of complete surgical excision; the surgical approach depends on the size and localization of the tumor, as well as on the presence of a peduncle.

Key words:
diagnosis; laryngeal neoplasms; multidetector computed tomography; neurilemmoma; otorhinolaryngologic surgical procedures.

Apstrakt

Uvod. Švanomi larinksa su retki benigni neurogeni tumori. Najčešće su lokalizovani u supraglotičnom delu larinksa. Prikazan je redak slučaj laringealnog švanoma sa fokusom na kliničkoj slici, dijagnozi i lečenju. **Prikaz bolesnika.** Muškarac, star 61 godinu, zbog promuklosti predhodne tri godine javio se na teleskopski pregled. Tom prilikom mu je otkriven ovalni submukozni izraštaj na levom ventrikularnom naboru koji se pružao preko leve glasnice ka desnom ventrikularnom naboru. Multislajsna kompjuterizovana tomografija larinksa i vrata sa kontrastom pokazala je ovalnu, heterodenznu, jasno ograničenu promenu veličine oko 22 x 15 mm u regiji levog ventrikularnog nabora koja se širila ka levoj glasnici i zadnjoj komisuri larinksa, sa znacima početne uzure tireoidne hrskavice. Biopsija i histopatološki nalaz otkrili su primarni benigni inkapsulirani mezenhimni tumor, dok je imunohistohemijska analiza potvrdila dijagnozu švanoma. Bolesniku je urađena hirurška traheotomija i levostrana hemilaringektomija, sa potpunim uklanjanjem tumora. U toku šestomesečnog postoperativnog praćenja nije uočeno prisustvo recidiva bolesti. **Zaključak.** Među benignim tumorima larinksa švanomi su retki, a mogu rasti godinama pre postavljanja dijagnoze. Biopsija sa histopatološkom analizom potvrđuje dijagnozu laringealnog švanoma, mada je neophodan poseban oprez tokom biopsije. Lečenje podrazumeva kompletnu hiruršku eksciziju, a sam hirurški pristup zavisi od veličine i lokalizacije tumora, kao i od prisustva peteljke.

Ključne reči:
dijagnoza; larinks, neoplazme; tomografija, kompjuterizovana, multidetektorska; švanom; hirurgija, otorinolaringološka, procedure.

Introduction

Schwannoma is a benign slow-growing encapsulated neurogenic tumor, originating from Schwann cells that surround somatic and autonomic peripheral and cranial nerves, except the olfactory and optic nerves which lack Schwann cell sheath [1–3]. Between 25% and 45% of all schwannomas are located in the head and neck area, predominantly in the

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parapharyngeal space [1]. The only known risk factors for schwannoma occurrence are genetics and exposure to radiation [4]. Laryngeal schwannomas are extremely rare and account for 0.1%–1.5% of all benign laryngeal tumors [5].

We presented a rare case of laryngeal schwannoma in a 61-year-old male patient.

Case report

A 61-year-old male was admitted to our clinic due to hoarseness over the past three years, which had been gradually worsening. He was a nonsmoker and consumed alcohol occasionally. Telescopic examination revealed an oval submucosal mass of the left ventricular fold extending over the left vocal fold toward the right ventricular fold (Figure 1). Laboratory and biochemical blood tests were within normal limits. We performed microlaryngoscopy and biopsy of the left ventricular fold mass. The histopathology (HP) finding showed atypical squamous cell hyperplasia with moderately edematous stroma and mild subepithelial inflammatory infiltrate. At the follow-up, we found that the left ventricular fold mass was increased in size. Multislice computed tomography showed a 22 x 15 mm well defined, oval heterodense mass in the region of the left ventricular fold, extending toward the left vocal fold and the posterior commissure of the larynx, with signs of initial lesion of the thyroid cartilage due to compression (Figure 2). After the repeated biopsy, HP finding revealed a primary encapsulated mesenchymal tumor with benign characteristics (Figure 3). Immunohistochemistry analysis showed diffuse and intensive S-100 and vimentin protein positivity, without expression of alpha-smooth muscle actin, desmin, epithelial membrane antigen, with a low proliferation index of around 1%, confirming schwannoma diagnosis. Operative treatment consisted of tracheotomy and left hemilaryngectomy, with complete removal of the encapsulated tumor. Postoperative HP report confirmed diagnosis of laryngeal schwannoma. The patient was decannulated 14 days after surgery and discharged from the hospital 16 days after surgery. There were no signs of recurrence present during the follow-up after six months.

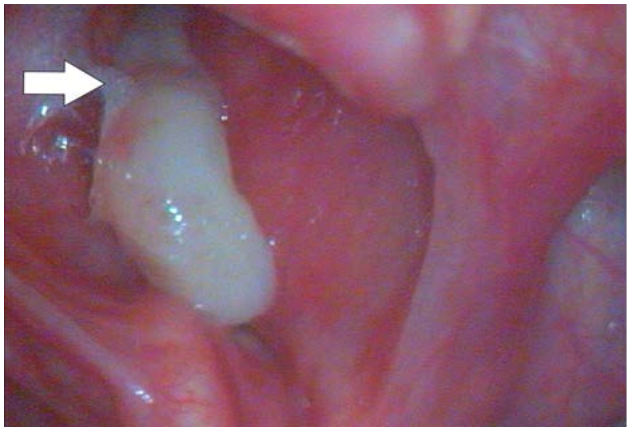

Fig. 1 – Telescopic examination showing oval submucosal mass at the left ventricular fold extending over the left vocal fold and toward the right ventricular fold (arrow showing the tumor peduncle at the left ventricular fold).

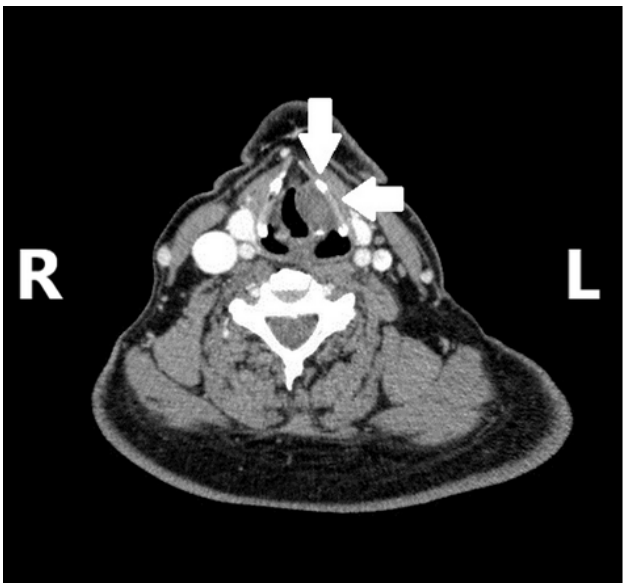

Fig. 2 – Multislice computed tomography showing an oval, well defined, heterodense mass in the region of the left ventricular fold, extending toward the left vocal fold and posterior commissure of the larynx, with signs of initial destruction of the thyroid cartilage.

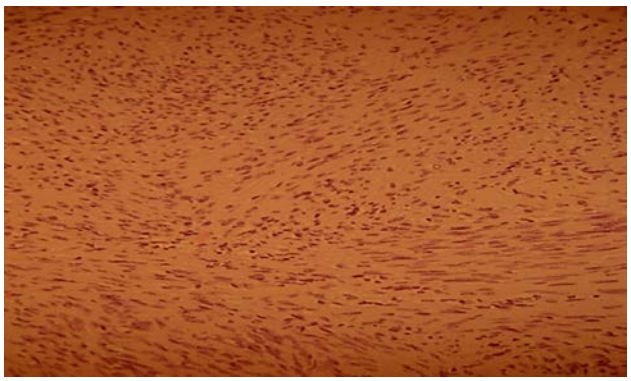

Fig. 3 – Elements of a biphasic tumor, made of irregularly shaped, loosely arranged cells with hyperchromatic nuclei, immersed in the myxoid stroma, as well as bundles of elongated cells in parallel arrays, with nuclei arranged in palisade form – schwannoma (hematoxylin and eosin staining, x200).

Discussion

Laryngeal schwannomas are most common in females in the fourth and fifth decade of life, although they can occur at any age [6]. On the contrary, our patient was a male in his seventh decade. Most commonly, the nerve of origin is the inner branch of the superior laryngeal nerve or small nerve fibers that innervate the laryngeal submucosa. In 80% of cases, laryngeal schwannomas are located at the aryepiglottic fold, while 20% occur at the vocal or ventricular fold, as in our case [2].

Clinical manifestations of laryngeal schwannomas depend on their size and localization. The most common symptoms include hoarseness (71.2%), dysphagia (24.6%), dyspnea (23.3%) and globus sensation (16.4%) [7]. Acute respiratory failure might occur, mainly in large pedunculated

tumors, which can cause complete airway obstruction [6]. A case of asphyxial death caused by laryngeal schwannoma has also been described [8]. Our patient complained of hoarseness for the past three years that had been gradually worsening. Typically, laryngeal schwannomas do not cause symptoms such as palpable cervical mass and weight loss [1], which were not present in our case either. There is only one described case of laryngeal schwannoma presenting as a painless neck mass [9].

Differential diagnoses of schwannoma include laryngeal cyst, laryngoceles, mucoceles, chondromas, adenomas, lipomas and neurofibromas. It is important to differentiate schwannoma from neurofibroma, because the latter have a higher incidence of malignant transformation (around 10% of cases) and a higher recurrence rate [1]. The presence of neurofibroma could also indicate possible neurofibromatosis [3].

Diagnosis of laryngeal schwannoma is based on anamnesis, direct or indirect laryngoscopy, imaging methods such as computed tomography and/or magnetic resonance imaging and biopsy with HP analysis [1]. Ultrasonography of the larynx is rarely used, due to the difficulties in visualizing posterior structures of the tumor and the larynx. As in our case, computed tomography and magnetic resonance imaging show typical characteristics of a benign lesion: circular or oval mass with well-defined borders, mostly isodense compared to muscle and compressing without infiltrating surrounding tissue [7]. The gold standard in diagnosing schwannoma is the HP analysis [2]. In our case histological diagnosis was based on Enger and Weiss' three histological criteria: 1) capsule presence, 2) presence of a stromal Antoni A (compacted, bipolar cells with nuclei arranged in palisade form) and/or Antoni B (loosely arranged spindle cells within a myxoid matrix) pattern and 3) positive S-100 staining [5]. However, it must be noted that incisional biopsy does not always result in correct diagnosis [6]. Results of our first biopsy showed atypical squamous cell hyperplasia instead of a schwannoma. We believe this is due to schwannoma being an encapsulated tumor and the possibility of taking superficial tumor tissue during the biopsy. This can also be explained by the lack of changes in mucosa serving as a guiding point for biopsy [2].

The treatment of choice for laryngeal schwannoma is complete surgical excision [1]. The surgical approach primarily depends on the presence of a peduncle. Pedunculated tumors are removed endoscopically regardless of their size, while nonpedunculated tumors are treated based on their size and localization [2]. Small nonpedunculated tumors can be removed endoscopically, while large nonpedunculated tumors demand an open surgical approach [1, 2]. The least invasive approach should be used, while also providing the best visualization and complete tumor removal [2]. Considering the size of the tumor and its extension toward the left vocal fold and the posterior commissure of the larynx, we opted for a left hemilaryngectomy with complete removal of the tumor. Temporary tracheostomy was conducted in order to prevent asphyxia due to possible postoperative laryngeal swelling.

The prognosis of laryngeal schwannoma is favorable. Wong et al. [1] report that 27/32 (84.38%) of patients had no signs of the disease on follow-up, while relapse occurred in 5/32 (15.62%) of patients, two of which had incompletely excised tumor. Complications caused by laryngeal schwannoma rarely occur [3, 8], although there is a risk of postoperative recurrent laryngeal nerve paralysis [1]. However, it should be noted that open surgical approaches, such as lateral thyrotomy and lateral pharyngotomy, are more frequently associated with this risk, compared to an endoscopic approach [1, 2].

There is still no consensus on how long the patient should be monitored after the treatment. Tulli et al. [7] report that the follow-up differs widely, from 2 months to 17 years, with laryngeal schwannoma recurrence usually being observed within 3 months after tumor excision. Therefore, controls are recommended every 3 months during the first year after excision, and then at least once a year for the next two years. We did not notice any signs of recurrence six months after surgery. We advised the patient to check in regularly, at least once a year.

Conclusion

Laryngeal schwannomas are very rare and can be overlooked for years due to their mild clinical manifestations. The gold diagnostic standard is histopathology analysis. Because of the tumor encapsulation, special care must be taken while performing a biopsy in order to avoid false-negative results. By the time the diagnosis is made, laryngeal schwannomas could significantly increase in size causing destruction of surrounding structures and consequently demand an open surgical approach. Hence, otolaryngologists should always consider schwannomas in cases of an oval or circular submucosal mass in the larynx.

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CASE REPORT
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Various aspects of two treatment approaches to patients with problems of hypodontia of upper lateral incisors

Različiti aspekti dva terapijska pristupa kod pacijenata sa hipodoncijom gornjih lateralnih sekutića

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Abstract

Introduction. The treatment of hypodontia of the upper lateral incisors could be orthodontic or multidisciplinary with combined orthodontic, surgical and prosthetic involvement. Both options have their *pros* and *cons*. They could be quite challenging to manage, particularly in the cases of unilateral missing of the upper lateral incisor in adult patients. We presented two cases with these different treatment approaches in young adult patients with unilateral missing of upper lateral incisors. **Case report**. The first case presents a combined orthodontic, surgical and prosthetic treatment of the missing upper right lateral incisor in an adult patient. Our clinical examination of a 22-year-old girl showed her missing tooth 12 with almost completely closed space, midline deviation, reduced overjet and overbite, Class III molar relationship on the right side and Class I molar relationship on the left side with V-shape maxillary arch and crossbite tendency in the frontal region. Based on the skeletal Class III relationship and intraoral findings, it was decided to open the space for tooth 12 and to establish the overjet, overbite and Class I occlusion as well. A surgical implant insertion followed the orthodontic preparation, with crown positioning after surgical healing. The second case described the orthodontic treatment of unilateral hypodontia in a young adult patient. Clinical and radiographic examinations of a 24-year-old female revealed hypodontia of tooth 12 with microdontic conical tooth 22 with severe crowding in the lower arch, Class I molar relationship on the right side and half-Class II relationship on the left side. The treatment decision was to extract atypical tooth 22, teeth 35 and 44 and to move the upper teeth forward to close the space. After the orthodontic treatment, upper canines were mesially moved to replace those missing lateral incisors. **Conclusion.** Both treatments successfully resolved malocclusion and obtained solid aesthetic and functional results. The treatment plan and decision to open or close the space in a case of hypodontia should be made individually for each patient according to their age, malocclusion, canines' shape and size and patient preferences.

Key words:
anodontia; dental implants; incisor; malocclusion; orthodontics; orthodontics, corrective.

Apstrakt

Uvod. Terapija hipodoncije gornjih lateralnih sekutića može biti ortodontska ili kombinovana, uključujući ortodontsku, hirurško i protetsku terapiju. Oba terapijska pristupa imaju svoje prednosti i nedostatke i mogu biti veoma zahtevni za terapeuta, naročito kod odraslih pacijenata sa jednostranim nedostatkom gornjeg lateralnog sekutića. Prikazana su dva različita terapijska pristupa kod mladih odraslih osoba sa jednostranim nedostatkom gornjeg lateralnog sekutića. **Prikaz bolesnika.** Prvi slučaj predstavlja kombinovanu ortodontsko-hirurško-protetsku terapiju kod mlade odrasle osobe ženskog pola, starosti 22 godine, kod koje je kliničkim pregledom ustanovljen nedostatak zuba 12 sa potpuno zatvorenim prostorom, pomerenom sredinom gornjeg zubnog niza, smanjenim incizalnim razmakom i preklopom, interkuspidacijom molara III klase sa desne strane i I klase sa leve strane, V oblika gornjeg niza, uz postojanje tendencije ka obrnutom preklopu sekutića. Na osnovu skeletnog odnosa III klase i intraoralnog nalaza, odlučeno je da se otvori

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prostor za zub 12 i koriguje incizalni razmak i preklop uz uspostavljanje okluzije I klase. Nakon ortodontske pripreme, postavljen je implant u zoni zuba 12 sa krunicom nakon završene faze hirurškog zarastanja. U drugom slučaju opisan je ortodontski tretman kod mlade odrasle ženske osobe, starosti 24 godine, sa jednostranom hipodoncijom lateralnog sekutića. Kliničkim i radiografskim pregledom ustanovljen je nedostatak zuba 12, sa mikrodontnim koničnim zubom 22, izraženom teskobom u donjem zubnom nizu, interkuspidacijom molara I klase sa desne strane i polu-II klase sa leve strane. Ortodontskom terapijom, uz ekstrakciju zuba 22, 35 i 44, korigovana je postojeća malokluzija, uz mezijalno pomeranje gornjih očnjaka na mesto lateralnih sekutića. **Zaključak.** Primenom oba terapijska pristupa, postignuti su zadovoljavajući estetski i funkcionalni rezultati i uspešno je korigovana malokluzija. Odluka o terapijskom pristupu treba da bude individualna, zasnovana na uzrastu, malokluziji, obliku i veličini očnjaka kao i željama samog pacijenta.

Ključne reči:
bezubost; implanti, stomatološki; sekutići; malokluzija; ortodoncija; ortodoncija, korektivna.

Introduction

A restorative treatment in hipodontia cases can be significantly facilitated by an orthodontic treatment. Orthodontic management of these patients includes many procedures – from space management, uprighting and aligning teeth to retention and stability of the whole treatment [1–6].

There are various terms for the reduction in the number of teeth which are used in bibliography, such as teeth absence, aplasia of teeth, congenitally missing teeth, agenesis of teeth, oligodontia. The most commonly used term is hypodontia, which in general sense refers to the absence of a smaller number of teeth. The phenomenon of 1 to 2 absent teeth has been found in 80% of cases, while agenesis of 4 or more teeth can occur in 10%. Severe oligodontia can appear in 1% or fewer cases [7–15]. In the period between 1936 and 2002, according to a meta-analysis by Polder et al. [16], missing teeth were more prevalent in Europe and Australia than in North America. The absence of teeth is more frequent in permanent teeth. In the upper jaw, the missing tooth is the last tooth of any given type, and it usually affects the upper lateral incisors. The absence of a primary tooth means that the same permanent one will be missing as well.

During the early stages of tooth formation, disturbances can result in hypodontia, which could be a part of a syndrome (Down syndrome, Rieger and Book syndrome [17–19]), could occur in patients with clefts [20–22] or could be an isolated occurrence [23–25]. The last-mentioned case can be either familial or sporadic. Studies have shown that the concordance rate for monozygotic twins is significantly higher than for dizygotic ones [26–28]. The polygenetic inheritance pattern is found in the cases of missing teeth [29]. Some patients do not have any hereditary history while others do have due to a combination of genetic and environmental factors [30]. Males and females are not equally affected by the absence of some teeth. Namely, in females there is an association between missing teeth and microdontia, while on the other hand, hyperdontia occurs more frequently in males and can be connected with macrodontia [31–32]. The problems of missing teeth can be more or less accompanied with a lesser or greater degree of impacted canines or with the transposition of canine and first premolar and taurodontism [33].

The treatment of patients with hypodontia must be seriously planned and sometimes within an interdisciplinary team. The orthodontic treatment plan may involve two different therapeutic approaches concerning space opening or closing, each one having its own separate criteria. All decisions with all their benefits could show some negative aspects [34–36].

The first option of opening the space, according to some authors, is the ideal functional and occlusal choice since it allows the perfect position of the canines. The negative side of this approach can be the prolonged treatment time and also the higher cost of the total treatment because of the use of implants along with crowns. Also, younger patients have to wait until the age of 18 before the implant procedure can be done [37–41].

On the other hand, the second choice is not as easy as it might appear. Closing the extra free spaces can be slow because of a reduction of the alveolar bone. Furthermore, the negative aspect of this option could lie in the difference in appearance of the canines compared to the adjacent teeth as they are usually pointed, darker, and wider. Many patients tend to choose this option due to the significantly lower cost of the total treatment [42–51].

There are two choices of retention in these cases: a removable or bonded retainer. Which type of retainers will be selected depends on many considerations, such as the age of the patients, i.e. in younger patients the removable retainer is preferred, while in the others, the lingual bonded retainer is a good option [52–54].

We present two different treatment approaches in cases of uni/bilateral incisor hypodontia: the first case was treated with a multidisciplinary approach, while the second one was treated only orthodontically.

Case report

Case 1

A female patient, Caucasian, 22-year-old, was presented due to chief complaints: disturbed aesthetic of the smile mainly due to certain midline asymmetry, limited occlusal contact in the anterior area and sporadic facial pain though her medical history was negative and there was no abnormal lifestyle detected. The patient had regular dentistry controls, orthodontic treatment with removable appliances

from age 8 to 13. The dentist referred the patient for an orthodontic evaluation.

The extraoral examination showed a dolichofacial pattern, slight mandibular asymmetry with a left shift and evidence of slight lateral black corridors during the smile. Her intraoral examination revealed a permanent dentition up to the second molars, absence of the right maxillary second incisor and completely closed space between teeth 11 and 13. The upper midline was deviated on the right side with V shape of the maxillary arch. There was some reduced overjet and overbite (edge to edge relationship) with a crossbite tendency in the front region. Class III molar and Class I canine relationship was present on the right side, Class I molar and Class II canine relationship on the left side and mild crowding in the anterior area of the lower arch (Figure 1).

The patient was 22 years old at the time of her first visit. Total treatment time, including interceptive and corrective phase, was 31 months.

Pretreatment records included lateral x-ray, panoramic x-ray both at initial stage and at the end of active treatment. An upper dentascan was performed at the end of the treatment to evaluate the bone condition for the implant substitution (Figure 2). Dental casts, cast analysis as well as the photographic documentation and aesthetic analysis were performed.

Treatment challenges were: recovery of the upper anterior aesthetic of the smile due to psychological impacts of previous orthodontic and prosthetic treatments, space reopening for tooth 12 that was completely closed, correction of occlusal relationship in a skeletal class III in an adult patient.

Objectives of the treatment were as following: correction of the occlusal relationships, both on the transversal and sagittal plane; acquisition of the adequate interradicular and interdental space for an implant substitution of tooth 12; upper arch expansion with a dentoalveolar expander type QH with the aim of coordinating the shape of the upper and lower arches, while the secondary objective was to gain elongation of the upper perimeter due to the lateral expansion (Figures 3a and 3b).

Application in both arches of a fixed appliance (MBT)

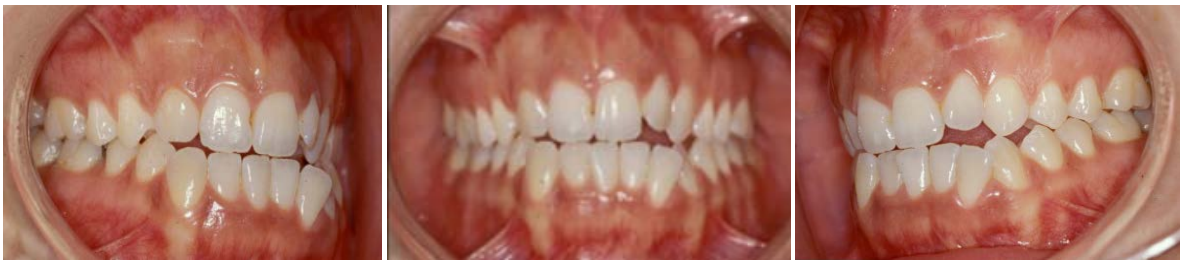

Fig. 1 – Intraoral features at the beginning of the whole treatment.

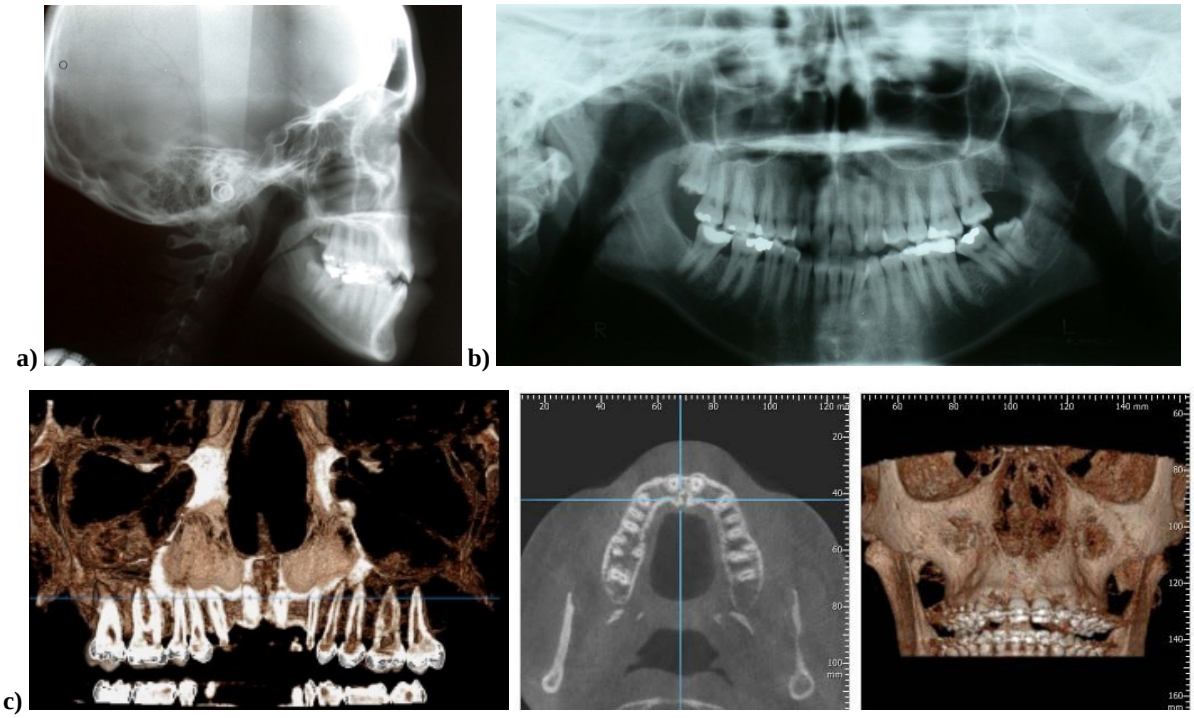

Fig. 2 – Pretreatment records: a) lateral x-ray; b) panoramic x-ray; c) upper Dentascan.

with conventional arch sequence was performed. Class III and intercuspation intraoral elastics were included. During the active phase, temporary prosthetic substitution of the lateral incisor was used with a single tooth bonded on the orthodontic arch (Figure 3c).

No changes of the treatment plan were made during the orthodontic therapy. The implant positioning required the choice of a small design of the implant due to the narrowed basal bone in the anterior area of the upper arch (skeletal class III malocclusion). Total orthodontic treatment time lasted 31 months with controls every 4th week (Figures 4 and 5).

The patient showed an optimal cooperation with a limited number of emergencies. The treatment plan was peculiar because of the adult age of the patient, the skeletal class III malocclusion with initial dysfunction signs and the complete closure of the 12 space requiring the extensive movement of the adjacent teeth to reach the correction of the malocclusion and implant positioning.

The presence of severe alteration on the sagittal and transversal plane of the upper arch in the adult patient needed a phase of correction with dentoalveolar expander and then a corrective treatment with braces and extensive usage of an intraoral elastic. A short period of retention was necessary to wait for the healing of the surgical phases.

The case illustrates a solution for a monolateral agenesis in an adult patient with corrective implant-prosthetic orthodontic treatment. The patient was satisfied with the aesthetic restoration of the smile (Figure 6).

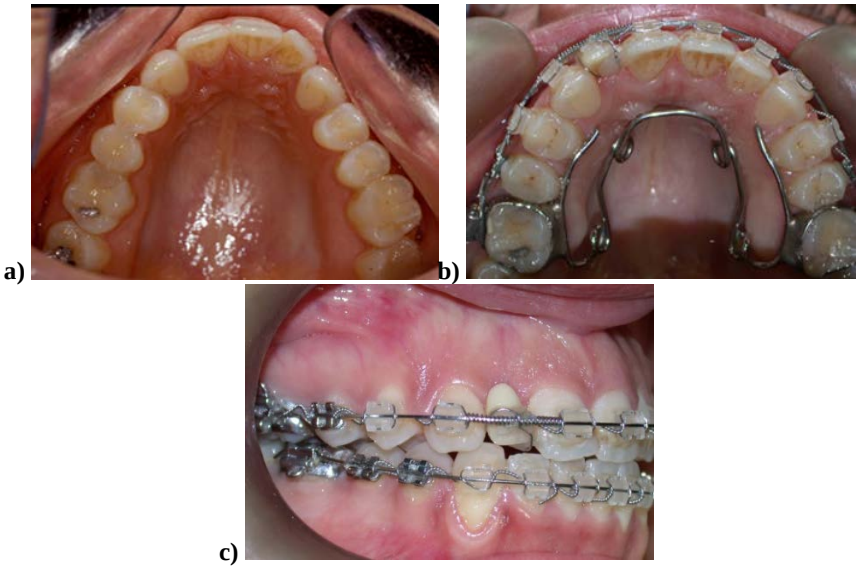

Fig. 3 – The upper arch: a) before the treatment; b) after the treatment with expander; c) temporary prosthetic substitution of the missing lateral incisor 12.

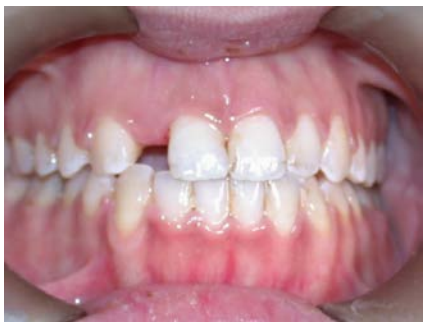

Fig. 4 – Patient at the end of orthodontic treatment – time for the implant replacement of the upper lateral incisor 12.

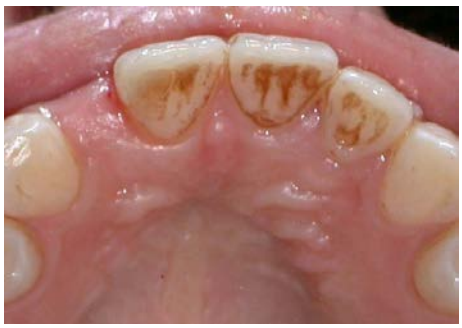

Fig. 5 – The end of orthodontic treatment (similarly to Fig. 4).

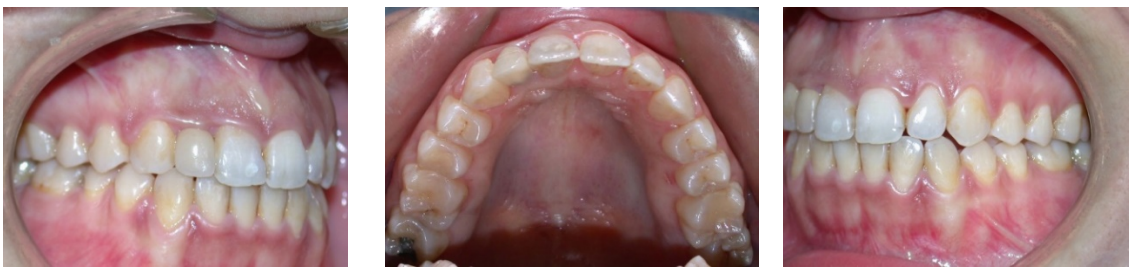

Fig. 6 – The results of the complete treatment.

Case 2

A Caucasian female 24-year-old patient was presented due to crucial complaints: asymmetric smile with atypical left lateral incisor and crowding in the lower arch. Her medical history was negative. The patient was referred for orthodontic treatment by her dentist.

Extraoral examinations showed slightly increased central third of the face in the frontal and lateral views, with a straight profile. Intraoral examinations revealed absent tooth 12, atypical tooth 22, severe crowding in the lower arch and both upper and lower dental arch midlines symmetrically deviated to the right in comparison to the midline of the face. Intercuspidation of the first molars on the right side was Class I, while on the left side her first molars were in the half-Class II. Intercuspidation of the canines on both sides were half-Class II. There was a crossbite in the contact area of teeth 13 and 43 (Figure 7a, 1–5).

A radiographic examination of the panoramic view showed the absence of teeth 12 and atypical 22 (Figure 7b). Our cephalometric analysis showed bimaxillary retrognathism with skeletal Class I by angle classification, with protruding lower central incisors. The dental cast analysis showed severe crowding in the mandibular arch with ectopic tooth 43 and Bolton discrepancy of the upper and lower dentition, with mandibular teeth too wide in comparison with the maxillary teeth.

Objectives of the treatment were to resolve the lower arch crowding and to place the ectopic 43 within dental arch, to correct both midlines to facial midline, to establish a symmetry of the smile line and Class I molar and canine relationship on both sides. Due to the lip competence and balanced profile, canines' shapes and sizes, it was decided to extract atypical tooth 22, and to move posterior teeth mesially to close the space, so that upper canines would replace upper lateral incisors. To resolve the crowding in the mandibular arch, it was agreed to extract teeth 44 and 35 on the left side because of the periapical lesion on the tooth 35 root.

This treatment started first in the upper arch with upper fixed appliance (Roth prescription). After the upper arch alignment and leveling, the lower arch was also included in the treatment. Because of the deep curve of Spee, leveling in the lower arch included a reverse curve of Spee arches. When the lower arch was leveled, space closing in both arches was done on stainless-steel wires. Having closed the space at the end, the patient was satisfied with the treatment outcome and her fixed appliance was removed (Figure 8). During the retention period our patient was instructed to wear removable plastic orthodontic retainers.

Total treatment time lasted 2 years and 5 months.

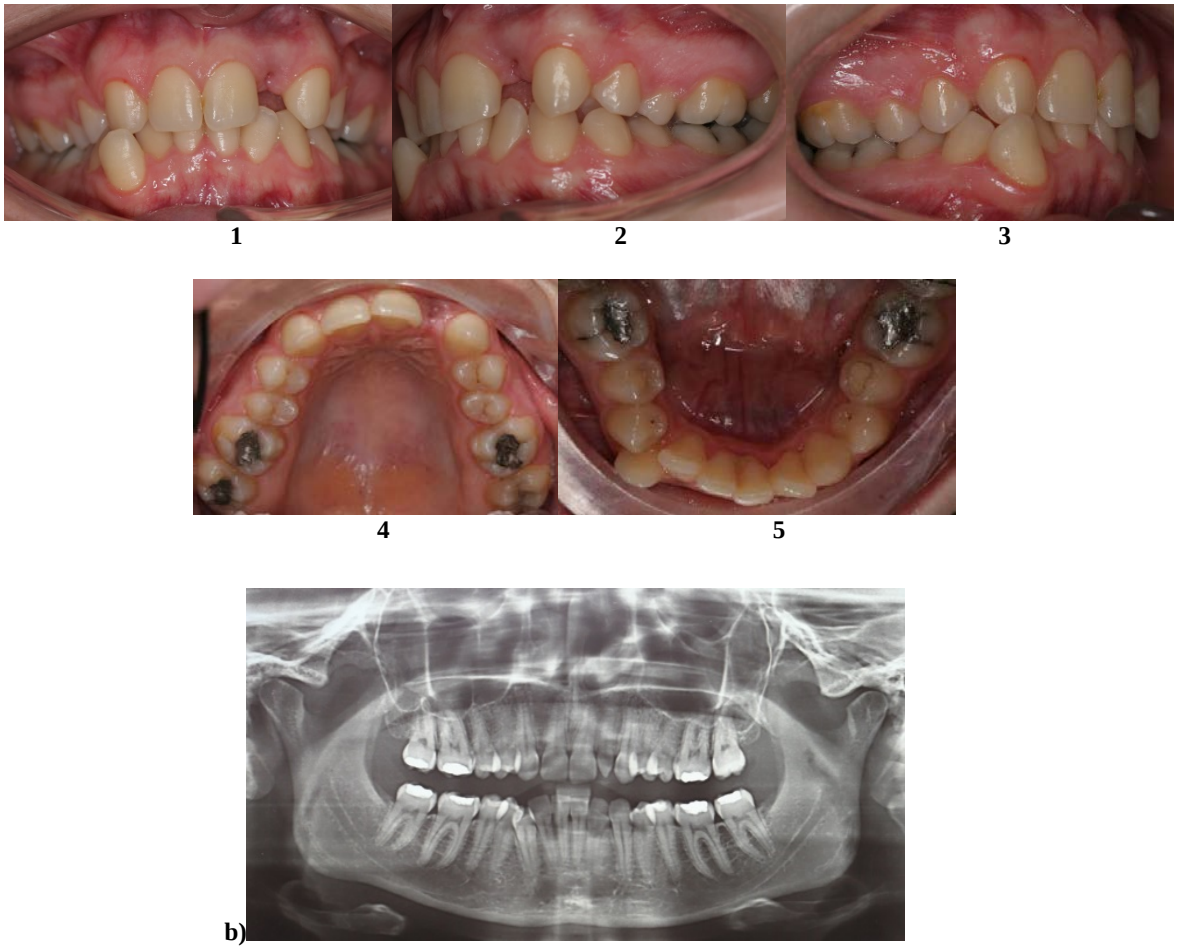

Fig. 7 –a, 1-5) intraoral photos of the second patient before the treatment; b) pretreatment panoramic view.

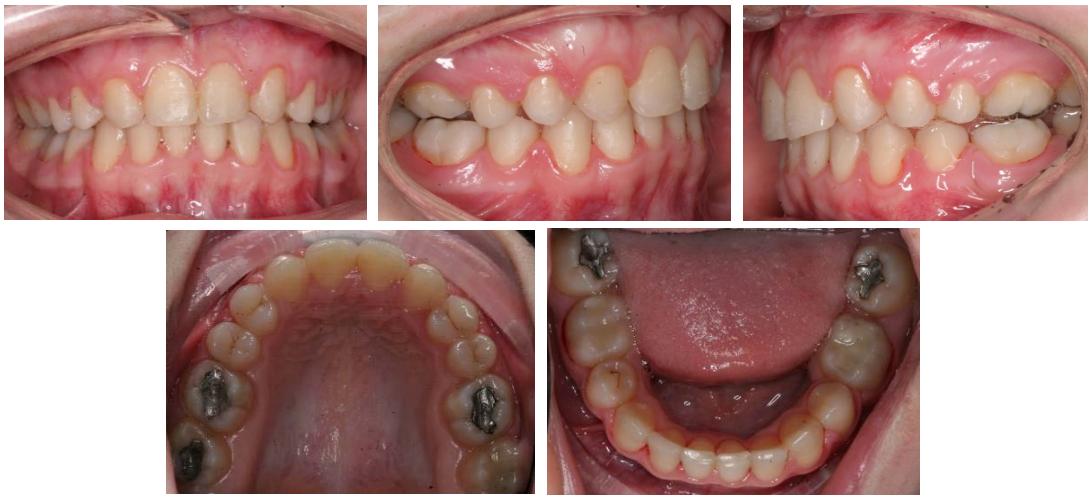

Fig. 8 – Final results of all goals and plans at the treatment completion.

Discussion

In cases with hypodontia of uni/bilateral lateral incisors, it is quite demanding to make an appropriate treatment plan to improve facial aesthetics, smile aesthetics and to rehabilitate oral functions.

In the first patient with skeletal Class III tendency toward anterior crossbite, our multidisciplinary treatment approach was presented. This decision was based on the collapsed and V-shaped upper arch, as well as on the narrowing especially in the anterior part. The upper arch expansion provided better arch shape and space for the missing tooth 12. As the space for the upper right lateral incisor was completely closed, it was decided to reopen it for the single tooth implant. Extensive protrusion of the upper incisors was avoided owing to the narrow basal bone in this area. Intermaxillary elastics were used during the orthodontic treatment to create the overbite, overjet and to resolve the malocclusion. In this case space opening provided not only implant preparation, but it was performed to correct the malocclusion.

The second patient had skeletal Class I with a balanced profile and lip competence. The shape and size of the canines' tooth crown, atypical tooth 22, severe crowding in the lower arch directed the treatment in the way of space closing and canine substitution of the lateral incisors and extractions of tooth 22, as well as of teeth 35 and 44. Following the orthodontic management, a restorative treatment was planned to reshape the crowns of the canines to achieve optimal aesthetic results. In this particular case, this female patient did not want any restorative treatment and was satisfied with the orthodontic treatment only.

Patients with unilateral missing upper lateral incisors could be quite challenging to treat. Unilateral hypodontia is usually accompanied with other intra-arch and inter-arch irregularities which makes the whole issue even more complicated.

Each treatment approach, with opening or closing the spaces, has its own advantages and disadvantages, so neither solution could prevail in clinical practice. According to Kokich and Kinzer [55] , there are three possible approaches: canine substitution, closing the space and creating the space for the implant or prosthetic replacement of the missing tooth. Comparing orthodontic and prosthetic approach, Kiliaridis et al. [56] and Bukvić et al. [57] have given the advantage to orthodontic treatment. Contrary to this, Rafałowicz and Wagner [58], concluded that an implant with porcelain-fused-to-metal crown, was the most effective treatment. How to reach the right decision and what to do within the problem of "space" depends on many factors such as: patient's malocclusion, profile, smile line, as well as on the color, shape and size of the adjacent teeth – the canines. The age of the patient influences the choice of the treatment, too. The period of early adolescence is the best time for it since the eruption of permanent canines can be controlled and at the same time the decision of closing or opening the space can be made.

Conclusion

The best solution for providing optimal results in the missing upper lateral incisor cases is the adequate individual decision plan, careful treatment management for achieving fine occlusion as well as satisfactory aesthetics and masticatory function. Some cases could be solved only by orthodontic management without any restorative treatment (as in our second case), while the first one needed the interdisciplinary team approach for achieving satisfactory results.

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CASE REPORT

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Treatment of velopharingeal insufficiency with turn over mucoperiosteal palatal flap in a patient with DiGeorge syndrome

Lečenje velofaringealne insuficiencije primenom prevrnutog mukoperiostnog režnja sa nepca kod bolesnice sa DiGeorge-vim sindromom

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Abstract

Introduction. DiGeorge syndrome (velocardiofacial syndrome) occurs in about 1 in 4,000 people. It is characterized by incomplete gene penetration, due to which there is significant variability in the clinical picture in different patients. The paper describes the successful application of an inverted mucoperiosteal flap of the palate in a nine-year-old girl with DiGeorge syndrome who had pronounced velopharyngeal insufficiency (VPI). **Case report.** The girl was the first-born child from a normal pregnancy and a normal birth with a normal early psychomotor development. During breastfeeding, milk was occasionally returned to the nose, and later deviations from normal speech (incomprehensible and nasal speech) were noticed. At the age of 4, detailed genetic testing was performed and a microdeletion of 22q11 chromosome was found. Also, a submucosal cleft palate was established, and magnetic resonance angiography of the head and neck revealed an abnormal position of the left internal carotid artery (ICA) that extended submucosally to the central axis of the posterior wall of the pharynx. Then, the submucosal cleft palate was surgically resolved in other clinical center, but without speech improvement. Pharyngoplasty was not performed due to the risk of serious postoperative complications. It was explained to the parents that speech recovery will not be satisfactory without surgical treatment of VPI. At the age of 9, the girl was admitted to the Clinic for Plastic and Reconstructive Surgery and Burns of the Military Medical Academy in Belgrade for surgical treatment of VPI. Taking into account the potential risks of certain surgical methods, it was decided to perform intravelar veloplasty according to Furlow. Since it was intraoperatively found that the soft palate is too short and that this procedure cannot provide its sufficient length, the mucoperiosteal flap was lifted from the palate to the palatal aponeuroses on the posterior edges of the palatine bones leaving their oral surfaces exposed. The mucoperiosteal flap raised in this way could not also provide the required length of the soft palate only by retroposition. However, its length is 160% of the soft palate axis length, which was enough to turn over the front of the flap towards the pharynx of the soft palate to reach its posterior wall. The raised mucoperiosteal palatal flap has no muscles, so its motility was achieved by the fact that along the edges of the existing short and mobile palate, the flap was fixed to the existing palate and uvula. This provided the anatomical preconditions for speech recovery, shown during the one-year postoperative follow-up of the child. Exposed palatine bones and short palate was covered by mucosal tissue, without cystic formations. **Conclusion.** The mucoperiosteal palatal flap can be easily, successfully and maximally safely applied in the resolution of VPI in patients with DiGeorge syndrome where there is an aberrant submucosal position of the ICA. This flap could be a 'flap of choice' for such patients with atopic position of the ISA, too.

Key words:
digeorge syndrome; velopharingeal insufficiency; cleft palate; speech disorders; rehabilitation of speech and language disorders.

Apstrakt

Uvod. DiGeorge-ov sindrom (velokardiofacijalni sindrom) javlja se kod oko 1 od 4 000 ljudi. Karakteriše ga nepotpuna penetracija gena, zbog čega postoji značajna varijabilnost u kliničkoj slici kod različitih pacijenata. U radu je opisana uspešna primena prevrnutog mukoperiostnog režnja nepca kod devetogodišnje devojčice sa DiGeorge-ovim sindro

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mom kod koje je postojala izražena velofaringealna insuficijencija (VFI). **Prikaz bolesnika.** Devojčica je prvorođeno dete iz uredne trudnoće i urednog porođaja sa urednim ranim psihomotornim razvojem. Tokom dojenja povremeno je dolazilo do vraćanja mleka na nos, a kasnije je primećeno odstupanje od normalnog govora (nerazumljiv i nazalni govor). U dobi od 4 godine urađeno je detaljno genetičko testiranje i nađena je mikrodelecija 22q11 hromozoma. Ustanovljen je i submukozni rascep nepca, a magnetno rezonantnom angiografijom glave i vrata otkrivena je abnormalna pozicija leve unutrašnje karotidne arterije koja se širila submukozno skoro do centralne osovine zadnjeg zida ždrela. Prvo je hirurški rešen submukozni rascep nepca, ali bez poboljšanja govora. Faringoplastika je bila kontraindikovana zbog rizika od ozbiljnih postoperativnih komplikacija. Roditeljima je objašnjeno da bez hirurškog tretmana VFI, oporavak govora neće biti zadovoljavajući. Sa nepunih devet godina devojčica je primljena u Kliniku za plastičnu i rekonstruktivnu hirurgiju i opekotine Vojnomedicinske akademije u Beogradu na hirurško lečenje VFI. Uzimajući u obzir potencijalne rizike od pojednih hirurških metoda, odlučeno je da se uradi intravelarna veloplastika po Furlow-u. Intraoperativno je utvrđeno da je meko nepce suviše kratko i da ovaj zahvat ne može da obezbedi dovoljno produženje mekog nepca. Zbog toga je odignut mukoperiostni režanj sa nepca do palatalnih aponeuroza na zadnjim ivicama palatalnih kostiju ostavljajući njihove ogoljene oralne površine. Ovako podignut mukoperiostni režanj nije mogao samo retropozicijom da obezbedi potrebnu dužinu mekog nepca. Budući da je ustanovljeno da dužina ovako ispreparisanog režnja iznosi 160% dužine osovine mekog nepca, bilo je dovoljno da se prevrtanjem prednjeg dela režnja ka farinksu mekog nepca, dosegne njegov zadnji zid. Ovaj režanj nema mišiće, pa mu je pokretljivost postignuta tako što je po obodima postojećeg kratkog i pokretnog nepca, režanj fiksiran za postojeće meko nepce i uvulu. Time su bili obezbeđeni anatomski preduslovi za značajan oporavak govora, što je i pokazano tokom jednogodišnjeg postoperativnog praćenja deteta. Ogoljena palatalna kost je bila prekrivena granulacijom, a s vremenom i mukozom, kao na mekom nepcu, bez pojave cističnih formacija. **Zaključak**. Mukoperiostni palatalni režanj se može jednostavno, uspešno i maksimalno bezbedno primenjivati u rešavanju VFI kod pacijenata sa DiGeorge-ovim sindromom gde postoji aberantna submukozna pozicija unutrašnje karotidne arterije, koja doseže skoro do srednje osovine zadnjeg zida farinksa. Opisani režanj i njegova primena pokazuju da on može biti metod izbora u rešavanju VFI kod pacijenata sa DiGeorge-ovim sindromom i aberantnom submukoznom pozicijom unutrašnje karotidne arterije.

Ključne reči:
digeorge sindrom; velofaringealna insuficiencija; nepce, rascep; govor, poremećaji; rehabilitacija poremećaja govora i jezika.

Introduction

American physician Angelo DiGeorge was first described the syndrome in 1968, which in 1981 using genetics was defined as a deletion of a small segment of chromosome 22. This syndrome, known as 22q11.2 deletion syndrome, is inherited autosomally dominantly, with prevalence of 1 in 4,000 people [1, 2]. Diagnosis of DiGeorge syndrome is based on the symptoms and confirmed by genetic testing [3, 4]. Signs and symptoms are as follows: congenital heart disease (40% of individuals), particularly conotruncal malformations [interrupted aortic arch (50%)], persistent *truncus arteriosus* (34%), tetralogy of Fallot, ventricular septal defect, cyanosis, palatal abnormalities (50%), particularly velopharyngeal incompetence, submucosal cleft palate, and cleft palate, characteristic facial features (present in the majority of Caucasian individuals) including hypertelorism, learning difficulties (90%), hypocalcemia (50% – due to hypoparathyroidism), significant feeding problems (30%), renal anomalies (37%), hearing loss, laryngo-trachea-esophageal anomalies, growth hormone deficiency, autoimmune disorders, immune disorders due to reduced T cell numbers, seizures (with or without hypocalcemia), skeletal abnormalities, and psychiatric disorders [1–5]. Current research demonstrates a unique profile of speech and language impairments is associated with 22q11.2DS. The most common problems are hypernasality, language delay, and speech errors, so they are often perform lower on speech and language evaluations in comparison to their nonverbal IQ scores. Hypernasality occurs if air comes out through the nose during the formation of oral speech sounds, which results in reduced intelligibility. This phenomenon is seen in velopharyngeal insufficiency (VPI), due to the altered structure of the soft palate vellum. Hearing loss can also contribute to increased hypernasality, because children with hearing impairment may have difficulty self-controlling their oral speech [6–8].

Case report

A girl, born on May 11, 2009, was the first-born child from a normal pregnancy and a normal birth with a normal early psychomotor development. During breastfeeding, milk was occasionally returned to the nose, and later deviations from normal speech (incomprehensible and nasal speech) were noticed. In April 2011, she was sent to an otorhinolaryngologist-phoniatrician by an audiologist because of incomprehensible and nasal speech. During the first examination, it was revealed nasal speech by type of open nasalization and bad articulation. During the phoniatric examination, VPI was noted with suspected submucosal cleft palate. Suspected DiGeorge syndrome was diagnosed in 2013 in the Clinical Center of Vojvodina, Novi Sad, Serbia. The diagnosis was confirmed by detailed genetic testing in the Institute for Molecular Genetics and Genetic Engineering in Belgrade, in May/June 2013. In 300 (100%) analyzed metaphasis there were found microdeletion of 22q11 chromosome (velocardiofacial syndrome; DiGeorge syndrome). The diagnosis of the submucosal cleft was confirmed at the age of 4 by the use of

flexible nanofiber with optics, at the other Clinical Center. The examination also confirmed the pulsation of a larger blood vessel (carotid artery) on the posterior wall of the pharynx. Magnetic resonance imaging (MRI) angiography of the head and neck was then performed and an abnormal position of the left internal carotid artery (ICA) extending submucosally to the posterior wall of the pharynx was diagnosed (reaching medially from 10 mm to 7 mm from the median axis of the posterior pharyngeal wall) (Figure 1). The right ICA was in the normal position submuscularly. The parents were explained which surgical method should be applied for correction of nasal speech while avoiding possible complications. It was also explained to the mother that the child should be included in speech therapy that would have limited possibilities. In April 2013, speech therapy status was characterized as age-appropriate language skills, but spontaneous speech, although fluent, had significantly impaired articulation and phonation, rhythm and tempo of speech were neat, speech melody was significantly disturbed with monotone voice. Assessment of articulation and discrimination of voices revealed: incorrect pronunciation of all voices and vocals and consonants by distortion type except nasal /M/, /N/, /Nj/. The substitution of sonic consonants with silent pairs or nasals was present – pronouns: voices /P/ and /B/ substituted by voice /M/; voice /G/ substituted by voice /K/; nasally produced to be perceived in most cases as nasal /N/; voice /D/ substituted by voice /T/; fricatives: /Z/ substituted by voice /S/; /Ž/ substituted by voice /Š/; nasally produced voices /V/, /H/, without voice /R/ with a properly spoken voice /J/; affricates: voice /Đ/ substituted by voice /Ć/; voice /Dž/ substituted by voice /Č/; distorted voice /C/ for nasalization of oral voices; laterals: voice replacement /L/ and /LJ/ by voice /N/; speech intelligibility was significantly impaired.

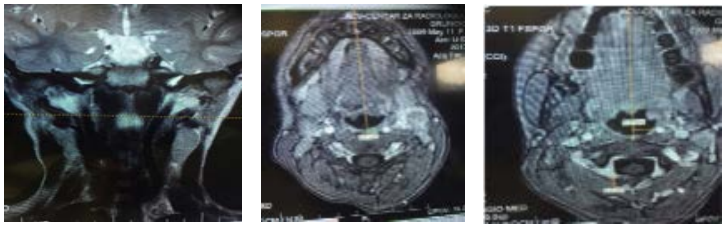


Fig. 1 – Magnetic resonance imaging (MRI) angiography of the head and neck showing an abnormal position of the left internal carotid artery (ICA) extending submucosally to the posterior wall of the pharynx.

Having successfully mapped the anatomical variation of the ICA, the possibilities of nasal speech correction by surgery and possible complications applying certain methods of correction of VPI were explained to the parents. In May 2013, the patient was hospitalized in other Clinical Centre and subjected to the surgical treatment of submucosal cleft palate. The postoperative course was neat. Nevertheless, postoperatively VPI with poor speech was persisted. The pediatric examination of the patient's neurological development did however show areflexia, as well as a neat trophic of the upper and lower extremities. A genetic examination further determined clinodactyly of the small toes, overlapping toes as low growth. Cardiological examination revealed the aberrant left ventricular horde and mitral valve dysmorphia that were consistent with the described syndrome. Kidney ultrasonography showed a fibrotic band of left kidney with ruptured cysts. Pharyngoplasty was dismissed because of the risk of serious postoperative complications. The mother was explained that the child should be included in speech therapy with limited possibilities. Furthermore, the basic principles of rehabilitation of VPI were also explained, as well as that the rehabilitation would take a long time. The parents were also explained that without surgical treatment of VPI, rehabilitation could not give satisfactory results. In December 2018 (3 months before surgery), speech therapy status was estimated as follows: spontaneous speech – fluent with partially impaired articulation and phonation. Assessing articulation and discrimination of votes revealed distorted pronunciation of a certain number of votes: pronunciation of vocals /I/ and /U/ in certain positions with nasal voices; the substitution of sonic consonants with silent pairs or nasals was present. The pronunciation significantly improved with occasional substitution of pleisus /B/ nasal /M/; fricatives: /Z/ in most situations substituted by voice /S/, /Ž/ substituted by voice /Š/; affricates: voice /Đ/ substituted by voice /Ć/ in certain combinations, voice /Dž/ substituted by voice /Č/; laterals: replacement of voice /L/ voice /N/ in most speech discourses. Rhythm and tempo of speech were as follows: during the insistence on the proper articulation of voices, occasionally pausing and repetition of syllables could be observed, which could be described as mild dysfunction. The speech melody was partially disturbed. Speech intelligibility was also partially impaired. During spontaneous speech, the girl had worse performance in speech production compared to speech quality during speech therapy. Articulation finding was: incorrect pronunciation of all voices of the mother tongue due to open nasalization, less intensity than before surgery; vocals: slightly distorted vocals /I/ and /U/ especially pronounced in the combination of syllables with nasals; plays: substitution of voice /B/ by voice /M/, voice /D/ by voice /N/ during continuous speech occasionally; fricatives: nasal colored voices / W /, / S /, / Z /; affricates: nasal colored affricates: /Č/, /J/, /Ć/, /Đ/; laterals: nasal voice pronunciation /L/ nasally: proper pronunciation of the voices /M/, /N/, /NJ/. The performance of speech was slightly impaired.

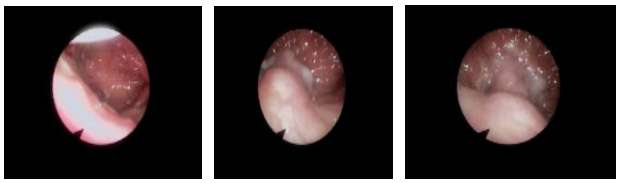
Surgical methods for correction VPI as pharyngoplasty and sphincteroplasty groups are contraindicated in patients with DiGeorge syndrome, due to the risk for severe peri- and postoperative complications. Operation with risk also includes augmentation methods to increase Passavant's thickening. The applying of bucal flaps is more harder and complicated procedure than palatoplasties in treatment of VPI. Surgical methods from the palatoplasty groups, Furlow "Z" intravelar veloplasty, and "Dorrance like flap", include relatively less risk. After consular consideration, it was decided to perform intravelar veloplasty according to Furlow. Intraoperatively, measurement showed that the soft palate is too short and that intra-velar veloplasty "Z" plastic according to Furlow, by elongation, would not provide sufficient length of the soft palate. A harvested mucoperiosteal flap was removed from the hard palate. The "dorrance-like" flap raised to the palatine aponeuroses at the posterior edges of the palatine bones, leaving the oral surface of the bones exposed. The flap raised in this way, just by its repositioning by "sliding" could not provide the necessary retroposition of the soft palate. Comparing the length of the soft palate and the mucoperiosteal palatal flap, the length of raised mucoperiosteal flap of the palate was 160% of the length of the soft palate (Figure 2a). We turned over raised mucoperiosteal palatal flap (Figure 2b) over the patient's short palate and reached the length of the flap for contact with Passavant fold. This mucoperiosteal palatal flap lacks muscle and active mobility. Its motility is achieved by fixing the mucoperiosteal flap to the mobile, short soft palate and uvula with the individual stitches (Figures 2c and 2d). In that way, we provided safely and effectively anatomical prerequisites for speech recovery and rehabilitation (Figure 3). This described surgery method, the application of the "turn over" palatal mucoperiosteal flap in the VPI treatment, caused subjective and objective improvement in the patient. After six month postoperatively, a reduction of hypernasality, and slightly better and clearer speech of the last palate consonants were recorded. The speech recording one year after the surgery indicated that the speech recovery of the patient progressed neatly.

Discussion

Because our patient with DiGeorge syndrome had anatomic variation of the topographic position of the left ICA, we made a more detailed analysis of the possible surgical methods for VPI treatment [8], and choose the most optimal technique due to possible intraoperative complications (ACI injury, dramatic bleeding, and the need for ligation of ACI in a very small and inaccessible operating place for intervention), as well as postoperative complications (scarring and possible partial Philip puncture of the ACI). It was estimated that augmentation methods such as pharyngoplasty and sphincteroplasty were contraindicated in this patient. Surgical methods (palatoplasty) for the correction of VPI related to elongation or retroposition of the soft palate (Furlow) could be applied. However, intraoperatively, a scarred and short soft palate is encountered, which did not allow sufficient mobilization and elongation of the soft palate, leaving only the palatal mucoperiosteal flap. Exposed palatine bones and short palate was covered by mucosal tissue, without cystic formations. We did not find in the available literature that anybody applied such palatal mucoperiosteal "turn over" flap for treatment of incomprehensible speech or for any other kind of reconstruction.

a) b) c) d)

Fig. 2 – a) The ratio of lengths of raised mucoperiosteal flap of the palate and the soft palate; b) Turned over the raised mucoperiosteal palatal flap; c) The soft palate with fixed mucoperiosteal palatal flap; d) The soft palate two months after turnovered mucoperiosteal palatal flap applaying.



a) b) c)

Fig. 3 – Nasopharyngoscopy findings after operation:
a) Passavant fold formed;
b) Contact of newly formed uvula with Passavant;
c) Contact of newly formed uvula during ingestion.

Conclusion

New anatomical structure, obtained by the presented surgical method, caused subjective and objective improvements in the clinical picture of the patient with DiGeorge syndrome. Six months after operation, a reduction of hypernasality, slightly better and clearer speech of the last palate consonants were recorded. The finding was even better after the next six months of follow-up. Because of that, we consider that the mucoperiosteal palatal flap can be easily, successfully and maximally safely applied in the resolution of VPI in patients with DiGeorge syndrome where there is an aberrant submucosal position of the ICA. We also recommend this "turn over" mucoperiosteal palatal flap as the flap of choice for treatment patients with atopic position of the ICA.

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Tabele

Sve tabele pripremaju se sa proredom 1,5 na posebnom listu. Obeležavaju se arapskim brojevima, redosledom pojavljivanja, u levom uglu (**Tabela 1**), a svakoj se daje kratak naslov. Objašnjenja se daju u fus-noti, ne u zaglavlju. Svaka tabela mora da se pomene u tekstu. Ako se koriste tuđi podaci, obavezno ih navesti kao i svaki drugi podatak iz literature.

Ilustracije

Slikama se zovu svi oblici grafičkih priloga i predaju se kao dopunske datoteke u sistemu **aseestant**. Slova, brojevi i simboli treba da su jasni i ujednačeni, a dovoljne veličine da prilikom umanjivanja budu čitljivi. Slike treba da budu jasne i obeležene brojevima, onim redom kojim se navode u tekstu (**Sl. 1; Sl. 2** itd.). Ukoliko je slika već negde objavljena, obavezno citirati izvor.

Legende za ilustracije pisati na posebnom listu, koristeći arapske brojeve. Ukoliko se koriste simboli, strelice, brojevi ili slova za objašnjavanje pojedinog dela ilustracije, svaki pojedinačno treba objasniti u legendi. Za fotomikrografije navesti metod bojenja i podatak o uvećanju.

Skraćenice i akronimi

Skraćenice i akronimi u rukopisu treba da budu korišćeni na sledeći način: definisati skraćenice i akronime pri njihovom prvom pojavljivanju u tekstu i koristiti ih konzistentno kroz čitav tekst, tabele i slike; koristiti ih samo za termine koji se pominju više od tri puta u tekstu; da bi se olakšalo čitaocu, skraćenice i aktinome treba štedljivo koristiti.

Abecedni popis svih skraćenica i akronima sa objašnjenjima treba dostaviti pri predaji rukopisa.

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