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June Almeida (October 5, 1930 – December 1, 2007), a Scottish virologist was a pioneer in the development of virus visualization techniques. Using an electron microscope, in 1964, she indentified the first human coronavirus – the same type of virus as SARS and SARS-CoV-2, the virus causing COVID-19.

June Almeida (5. oktobar 1930–1. decembar 2007), škotski virusolog, bila je pionir u razvoju tehnika za vizualizaciju virusa. Koristeći elektronski mikroskop ona je 1964. godine identifikovala prvi humani koronavirus – isti tip virusa kao što su SARS i SARS-KoV-2, izazivač COVID-19.

ORIGINAL ARTICLES

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Myeloid-derived suppressor cells in secondary sepsis: Is there an association with lethal outcome?

Supresorske ćelije mijeloidnog porekla u sekundarnoj sepsi: postoji li povezanost sa smrtnim ishodom?

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Abstract

Background/Aim. Role of myeloid-derived suppressor cells (MDSCs) in human host response to sepsis still needs to be clarified. The aim of our study was to determine whether frequency and/or absolute numbers of the MDSCs were associated with outcome in critically ill patients with secondary sepsis and/or septic shock. **Methods.** Total of 40 critically ill patients with secondary sepsis were enrolled in a prospective study. We detected and enumerated both main subsets of MDSCs: granulocytic (G)-MDSCs and monocytic (M)-MDSCs on the Day 1 (the day of hospital admission) and the Day 5 after the. The primary end-point was hospital mortality. **Results.** Increased frequencies and absolute numbers of subpopulations corresponding to MDSCs were associated with poor outcome. As far as relative kinetics was concerned, in both survivors and non-survivors, sepsis duration from 1th to 5th day was accompanied by an increase in MDSCs values of both investigated subpopulations. In contrast to findings of stepwise multivariate logistic regression analysis of the variables on the Day 1, on the Day 5 it was determined that the Sequential Organ Failure Assessment (SOFA) score (OR 2.350; $p < 0.05$) and G-MDSCs frequencies (OR 3.575; $p < 0.05$) were independent predictors of lethal outcome. **Conclusion.** These findings suggest harmful role of MDSCs in secondary sepsis.

Key words:
myeloid cells; myeloid-derived suppressor cells; mortality; prognosis; sepsis; treatment outcome.

Apstrakt

Uvod/Cilj. Uloga supresorskih ćelija mijeloidnog porekla (MDSCs) u imunskom odgovoru bolesnika sa sepsom tek treba da bude razjašnjena kod ljudi. Cilj istraživanja je bio da se utvrdi da li kod kritično obolelih sa sekundarnom sepsom i/ili septičkim šokom postoji udruženost učestalosti i/ili apsolutnih brojeva MDSCs sa ishodom bolesti. **Metode.** U prospektivnu studiju je bilo uključeno ukupno 40 kritično obolelih pacijenata sa sekundarnom sepsom. Detektovane su i kvantifikovane obe glavne podvrste MDSCs: granulocitna (G)-MDSCs i monocitna (M)-MDSCs, po prijemu na bolničko lečenje (prvi dan) i petog dana posle prijema. Primarni ishod je bio bolnički mortalitet. **Rezultati.** Veća učestalost i apsolutni brojevi subpopulacija koje odgovaraju MDSCs bili su udruženi sa lošim ishodom. Što se relativne kinetike tiče, i kod preživelih i kod umrlih, trajanje sepse od prvog do petog dana bilo je praćeno povećanjem vrednosti MDSCs u obe ispitivane subpopulacije. Multivarijantna logistička regresiona analiza je pokazala da su, za razliku od prvog dana, petog dana *the Sequential Organ Failure Assessment* (SOFA) skor (OR 2.350; $p < 0,05$) i frekvenca G-MDSCs (OR 3.575; $p < 0,05$) bili nezavisni prediktori letalnog ishoda. **Zaključak.** Ovi nalazi ukazuju na štetnu ulogu MDSCs u sekundarnoj sepsi.

Ključne reči:
kostna srž, ćelije; kostna srž, ćelije, supresorske; mortalitet; prognoza; sepsa; lečenje, ishod.

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Introduction

Since myeloid-derived suppressor cells (MDSCs) have been first described, almost 30 years ago in the context of cancer, their roles and importance are expanding, lately rather rapidly [1]. MDSCs are heterogeneous population of cells of myeloid origin encompassing myeloid progenitor cells, immature macrophages, immature granulocytes and immature dendritic cells. One of the main features of MDSCs is potent suppression of T-cell function. In the state of activation, these cells increasingly produce arginase 1, reactive nitrogen-species and reactive oxygen species (ROS) [2, 3]. Apart from acting as regulators of adaptive immune response, MDSCs also exert their influence over cytokine production by macrophages, so innate immune response is also affected. Two main subsets of MDSCs have been identified: monocytic (M)-MDSCs and granulocytic (G)-MDSCs.

Special interest was focused on role of MDSCs in immuno-inflammatory cascade in sepsis and/or trauma [4–6]. Sepsis remains a leading cause of mortality, multiple organ dysfunction syndrome (MODS) and prolonged stay in intensive care units (ICUs) despite enormous efforts from both clinicians and researchers. More than 250,000 deaths annually in the United States can be attributed to sepsis. Incidence of sepsis is rising for the most part of the world because of ageing population. In elderly, immune function is not efficient as it used to be, this important entity is known as immunosenescence. The Third International Consensus Definitions for Sepsis and Septic Shock (Sepsis 3) taskforce was well aware of how complex and intricate host response to infection is and how important it would be to know when protective and adaptive response becomes deleterious and maladaptive [7, 8]. It has been proposed that "persistent inflammation-immunosuppression catabolism syndrome (PICS)" is the predominant phenotype that has replaced late occurring multiorgan dysfunction syndrome (MODS) in surgical ICU (SICU) patients who fail to recover [9–12]. Beneficial or detrimental role of MDSCs in host response to infection is still controversial [13–17].

It is obvious that role of MDSCs in sepsis still needs to be clarified. The primary aim of the study regarding MDSCs in critically ill septic patients was to determine whether frequencies and/or absolute numbers of MDSCs were associated with outcome. The measure of outcome was hospital mortality.

Methods

Patients

Total of 40 critically ill patients with secondary sepsis due to peritonitis, pancreatitis and severe trauma, admitted to SICU, were enrolled in a prospective study conducted in a tertiary university hospital (Military Medical Academy, Belgrade, Serbia). Approval in concordance with the Declaration of Helsinki was obtained from local ethics committee and informed consent from a patient or first-degree relative. The study was conducted in accordance with the approved guidelines. Sepsis patients were enrolled if they had fulfilled current Sepsis-3 diagnostic criteria for sepsis (formerly severe sepsis) and/or septic shock (acute change in total Sequential Organ Failure Assessment (SOFA) score ≥ 2 points and vasopressors required to maintain mean arterial pressure (MAP) ≥ 65 mmHg and serum lactate level > 2 mmol/L despite adequate volume resuscitation) [8]. The study lasted 2 years and 1 month. The diagnostic criteria encompass any of the following variables thought to be a result of the infection: sepsis-induced hypotension, lactate levels greater than 2 mmol/L, urine output less than 0.5 mL/kg/h for more than two hours despite adequate fluid resuscitation, acute lung injury with PaO_2/FiO_2 less than 250, creatinine greater than 2.0 mg/dL (176.8 μmol/L), bilirubin greater than 2.0 mg/dL (34.2 μmol/L), platelet count less than 100,000 and coagulopathy – international normalised ratio (INR) greater than 1.5. Critically ill surgical patients with severe trauma [Injury Severity Score – ISS (determined using Abbreviated Injury Scale – AIS) > 25 points] were enrolled after they developed secondary sepsis. Regarding mechanism of injury, most frequently it was motor vehicle accident both as occupants and pedestrians. Also, fall from height and fall from standing height were present. Polytraumatized patients had predominant orthopedic, thoracic and head trauma. The exclusion criteria were as follows: secondary sepsis and/or septic shock with an underlying cause other than severe peritonitis, pancreatitis or trauma and malignant disease of any origin. A total of 25 patients were excluded out of 65 patients initially considered for enrolment.

Blood samples for MDSCs analysis were collected on admission to the SICU (Day 1) and on the Day 5 after admission. Also, samples of blood were simultaneously drawn for a blood culture. SOFA score, the Simplified Acute Physiology Score (SAPS) II and the Acute Physiology and Chronic Health Evaluation (APACHE) II score were calculated and recorded within the first 24 h after admission to the SICU (Day 1) [18–20]. SOFA score was recorded daily during SICU stay to assess severity of organ dysfunction in secondary sepsis.

The use of antibiotics, circulatory volume replacement, vasoactive support and source controlled were performed according to guidelines [21]. Various modes of mechanical ventilation and surgical procedures were performed if and when necessary in all patients. Outcome measure was hospital mortality; patients were followed until hospital discharge (survivors) or hospital death (non-survivors).

Sampling and analysis

Fresh peripheral blood samples were analyzed, frequency and absolute number of MDSCs were determined. Both main subsets of MDSCs were detected, G-MDSCs and M-MDSCs.

Three mL of venous blood were collected from the sepsis patients and 100 μL were dispensed in test tubes for staining with below listed monoclonal antibodies. After incubation for 30 min, erythrocytes were removed using the lysing buffer (EDTA, NH_4Cl, $KHCO_3$) for 20 min. The remaining nucleated cells were washed out twice in the Roswell Park Memorial Institute (RPMI) 1640 culture medium

with 5% of normal human serum, centrifuged and resuspended. Separation of peripheral blood mononuclear cells (PBMC) for the comparative analysis was performed using Lymphocyte Separation Medium (LSM) 1077. The separation process was performed by centrifugation at 1.200 × g for 20 min. The interphase layer between the plasma and the separation solution was extracted with a Pasteur pipette and washed twice in culture medium. The cell counting was done manually, in an improved Neubauer chamber, and automatically, using the Beckman Coulter ACT differ blood counter. Finally, the suspension with 1×10^6 cells/100 μL was aliquoted in 12 × 75 mm test tubes for further immunostaining.

The following antihuman monoclonal antibodies were used in different combinations for multicolor analysis of the fresh peripheral blood samples: CD15-PECy7 (Biolegend, USA), CD45-PEDyLight 594 and PECy5 (EXBIO, Czech Republic), HLA-DR-FITC (Miltenyi Biotec, Germany), CD14-PEDyLight 594 (EXBIO, Czech Republic), CD16-PECy7 (Biolegend, USA), CD11b-PE (Miltenyi Biotec, Germany), CD10-PECy5 (BD Biosciences, USA), CD3-PEDyLight 594 (EXBIO, Czech Republic), CD19-PEDyLight 594 (EXBIO, Czech Republic) and CD56-PEDyLight 594 (EXBIO, Czech Republic). The flow cytometry was performed using Beckman Coulter FC 500 flow cytometer with CXP analysis software. Given the fact that this was pilot study and we had not performed the suppressive assay yet, the acronyms M-MDSCs and G-MDSCs, refer to the phenotypically corresponding cells.

Statistical analysis

Complete statistical analysis of data was done with the statistical software package, SPSS Statistics 18. Most of the variables were presented as frequency of certain categories, while statistical significance of differences was tested with χ^2 test. In case of continuous data, variables were presented as mean value ± standard deviation (SD), median, minimal and maximal values. Kolmogorov-Smirnov test was used for evaluation of distribution of continual data. Statistical significance between groups was tested by Wilcoxon or Mann-Whitney test. Spearman's Rank Correlation analysis was used to establish the relation between parameters. Receiver operating characteristic (ROC) curves were constructed and analyzed to determine the sensitivity and specificity of variables for prediction of lethal outcome. Calculations of odds ratios (OR) and their 95% confidence intervals (CI) were done to determine the strength of the association between risk factors and outcomes. For that purpose, the most promising independent variables as single or combined risk factors were incorporated into binary logistic regression analyses. All the analyses were estimated at $p < 0.05$ level of statistical significance.

Results

Forty patients (average age was 59.3 years; range: 27–86 years; 12 females, 28 males) with secondary sepsis and/or septic shock due to pancreatitis (16 patients – 40%), peritonitis (14 patients – 35%) and trauma (10 patients – 25%) as the underlying cause, were enrolled. Of the 40 patients, 20 (50%) patients developed Gram-positive bacteriemia – GPB, 8 (20%) patients developed Gram-negative bacteriemia – GNB, and 10 (25%) patients had polymicrobial bacteriemia – POLY. In 2 (5%) patients no pathogen was isolated from blood culture. ISS (determined using AIS) was calculated and recorded in all polytrauma patients (mean ± SD): 35.24 ± 4.67. The demographic and clinical data of the patients are shown in Table 1.

Table 1

Demographic and clinical data

Parameter	Values
Patients, n	40
Age (years), median (range)	59.3 (27–86)
Sex, n (%)	
male	28 (70)
female	12 (30)
Simplified Acute Physiology II (SAPS II) score, mean ± SD	57.05 ± 9.37
Acute Physiology and Chronic Health Evaluation II (APACHE II) score, mean ± SD	21.65 ± 3.360
Sequential (Sepsis) Organ Failure Assessment (SOFA) score, mean ± SD	6.850 ± 2.832
[1]Severe sepsis due to, n (%)	
pancreatitis	16 (40)
peritonitis	14 (35)
trauma	10 (25)
Blood cultures, n (%)	
Gram-positive	20 (50)
Gram-negative	8 (20)
polymicrobial	10 (25)
sterile	2 (5)
Overall hospital mortality, n (%)	20 (50)

[1] – Reason for intensive care unit (ICU) admission.
SD – standard deviation.

Detection of MDSC subsets

Both main subsets of MDSCs were detected in sepsis patients. The cells were first gated on CD45 positive events to exclude the detritus in both, the sepsis patients and the healthy controls (Figures 1A and 2A, respectively). In the next step, on HLA-DR vs. CD11b dot plot, the HLA-DR$^{-/low}$CD11b+ events were selected (Figures 1B and 2B) and further analyzed for the lineage markers (CD3, CD19 and CD56, not shown) as well as for the CD10 (not shown), CD15 (Figures 1C and 2C), CD14 (Figures 1D and 2D) and CD16 (not shown) expression. The classification of granulocytic and monocytic subsets was based on the CD15 and CD14 expression, respectively. The G-MDSC were separated from mature granulocyte population on the basis of CD10 negativity, as well as lower and inhomogeneous expression of virtually all positive markers (CD11b, CD15 and CD16). The MDSC frequency was expressed as a percentage of these cells out of all CD45 positive events.

In order to investigate whether the assumed MDSCs had altered buoyancy, we have analyzed leukocytes from fresh lysed peripheral blood samples in paralel with peripheral blood mononuclear cells obtained on density gradient centrifugation from the same patient's samples. We have found that the cells of the same phenotype retain in the mononuclear layer on density gradient (not shown). Well known immunoparalysis, decrease of HLA-DR expression on monocytes in sepsis patients, was also observed (Figures 1B and 2B).

Detection of MDSCs in healthy control represents referent value from healthy donors blood pool.

The G-MDSCs and M-MDSCs frequencies and absolute numbers are higher in nonsurvivors

Of the 40 sepsis patients there were 20 survivors (discharged from hospital) and 20 non-survivors. In both groups of patients, survivors and non-survivors, sepsis duration from 1th to 5th day was accompanied with an increase in MDSCs values of both investigated subpopulations (Figures 3 A, B, C, D).

Baseline characteristics of patients on the Day 1 and the Day 5 according to outcome are shown on Table 2.

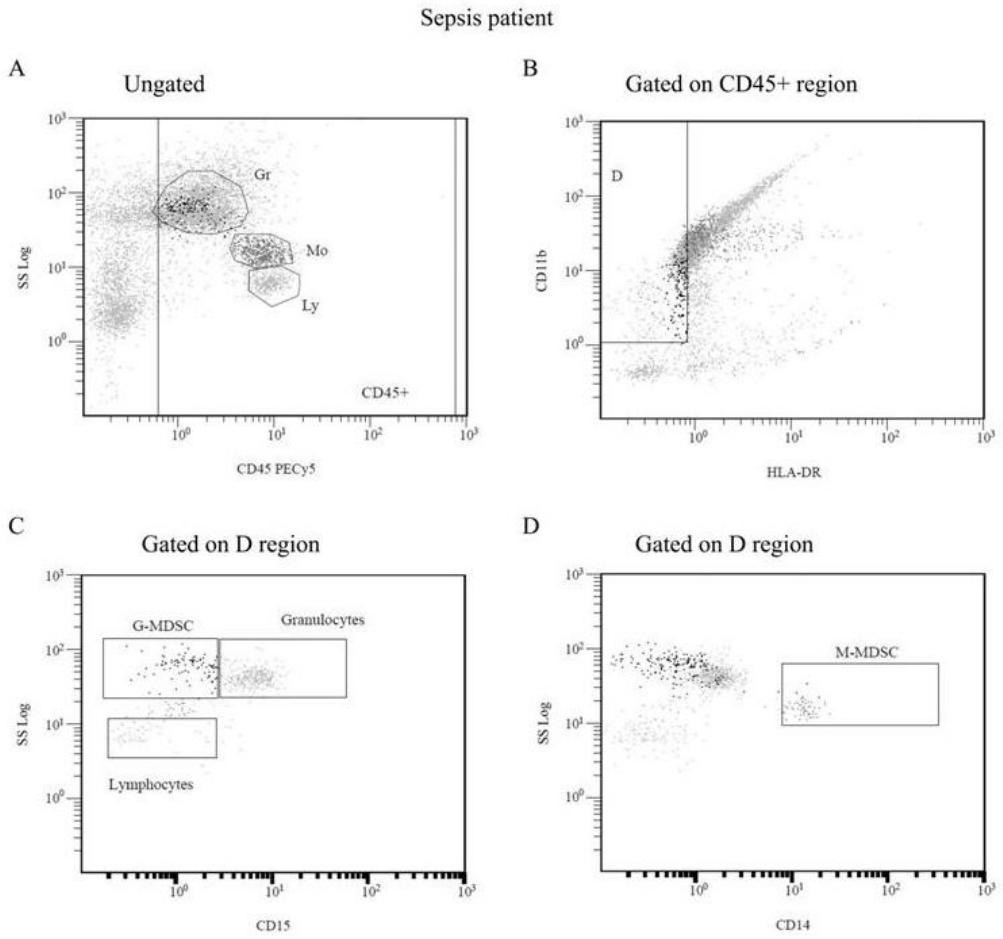

Fig. 1 – Detection of myeloid-derived suppressor cells (MDSCs) in the sepsis patients. Representative two-parameter dot plots showing identification of granulocytic (G)-MDSC in lysed peripheral blood samples. (A) The main leukocyte populations were selected based on CD45 expression. Monocytes are colored slightly darker grey for further tracking. (B) Darker grey monocytes showing low HLA-DR expression. The HLA-DR$^{-/low}$CD11b^{+} events were selected and assessed for the (C) CD15 expression, and (D) CD14 expression. The G-MDSCs are black colored for easier tracking.

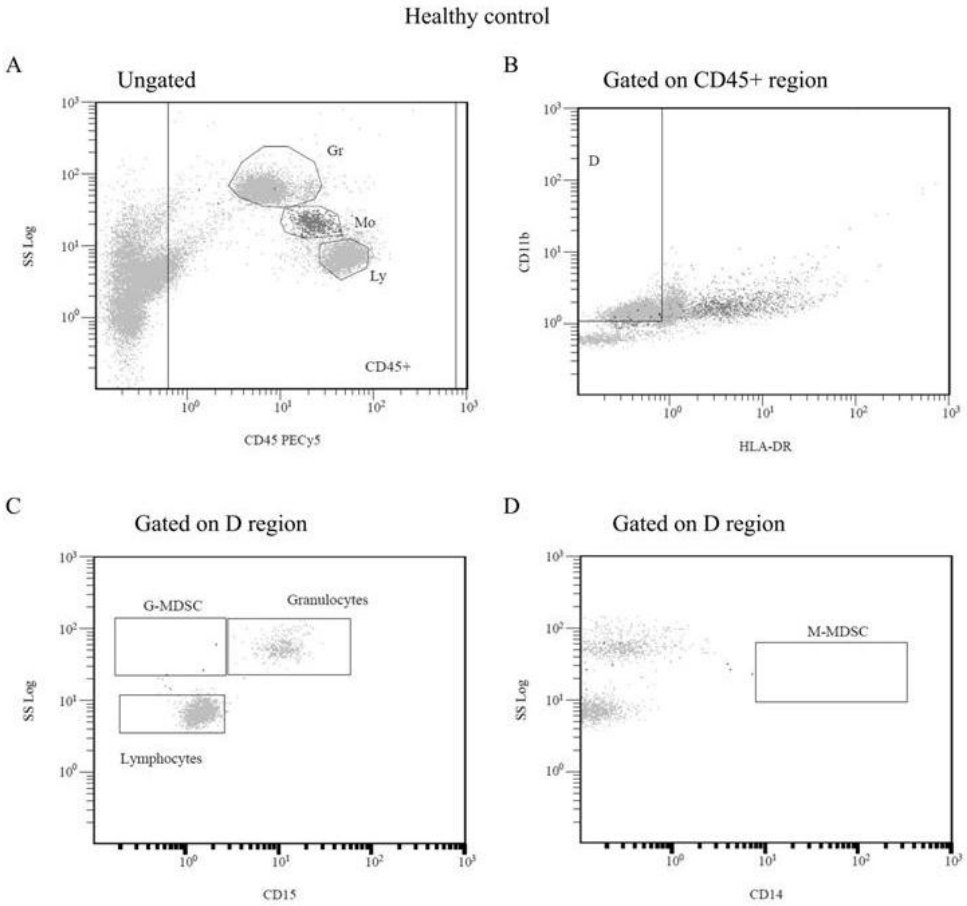

Fig. 2 – Detection of myeloid-derived suppressor cells (MDSCs) in the healthy control. Representative two-parameter dot plots showing identification of granulocytic (G)-MDSC in lysed peripheral blood samples. (A) The main leukocyte populations were selected based on CD45 expression. Monocytes are colored slightly darker grey for further tracking. (B) Darker grey monocytes showing higher HLA-DR expression than in sepsis patients. Assesment of the HLA-DR$^{-/low}$CD11b^{+} events showing „empty" (C) the G-MDSC region, as well as (D) the monocytic (M)-MDSC region in a healthy donor.

Table 2

Baseline characteristics of the patient population according to outcome on the Day 1and the Day 5

Parameters	Survivors (n = 20) mean ± SD; M; (min–max)	Non-survivors (n = 20) mean ± SD; M; (min–max)
SAPS II score 1st day	47.20 ± 11.07; 46.50; (22–65)	56.90 ± 15.52; 55.00; (23–85)
APACHE II score 1st day	14.50 ± 5.37; 15.00; (5–22)	20.80 ± 5.57; 21.00; (11–30)
SOFA score		
1st day	4.50 ± 2.87; 5.00; (0–9)	8.60 ± 3.50; 8.50; (1–14)
5th day	3.10 ± 2.53; 3.00; (0–9)	9.00 ± 4.52; 10.00; (3–14)
G-MDSCs frequencies (%)		
1st day	0.56 ± 0.61; 0.30; (0.02–1.99)	1.99 ± 2.72; 0.48; (0.02–9.35)
5th day	0.83 ± 0.82; 0.48; (0.03–2.95)	2.36 ± 2.44; 1.39; (0.37–9.00)
G-MDSCs absolute numbers		
1st day	114.28 ± 182.99; 37.14; (2.35–644.76)	180.42 ± 280.09; 55.29; (5.20–991.10)
5th day	152.17 ± 175.42; 72.24; (2.05–525.10)	268.27 ± 272.00; 178.35; (31.45–864.24)
M-MDSCs frequencies (%)		
1st day	0.44 ± 0.69; 0.25; (0.02–2.56)	0.59 ± 0.78; 0.19; (0.04–2.18)
5th day	0.55 ± 0.55; 0.53; (0.01–1.85)	0.93 ± 0.82; 0.87; (0.12–2.49)
M-MDSCs absolute numbers		
1st day	48.28 ± 45.89; 38.18; (1.67–157.95)	103.46 ± 165.89; 10.77; (1.77–533.92)
5th day	118.99 ± 158.91; 39.01; (0.68–519.85)	145.05 ± 202.22; 75.66; (3.51–689.73)

SD – standard deviation; M – median; min – minimum; max – maximum.
SAPS – Simplified Acute Physiology Score; APACHE – Acute Physiology and Chronic Health Evaluation;
SOFA – Sequential Organ Failure Assessment; G – granulocytic; MDSCs – myeloid-derived suppressor cells; M – monocytic.

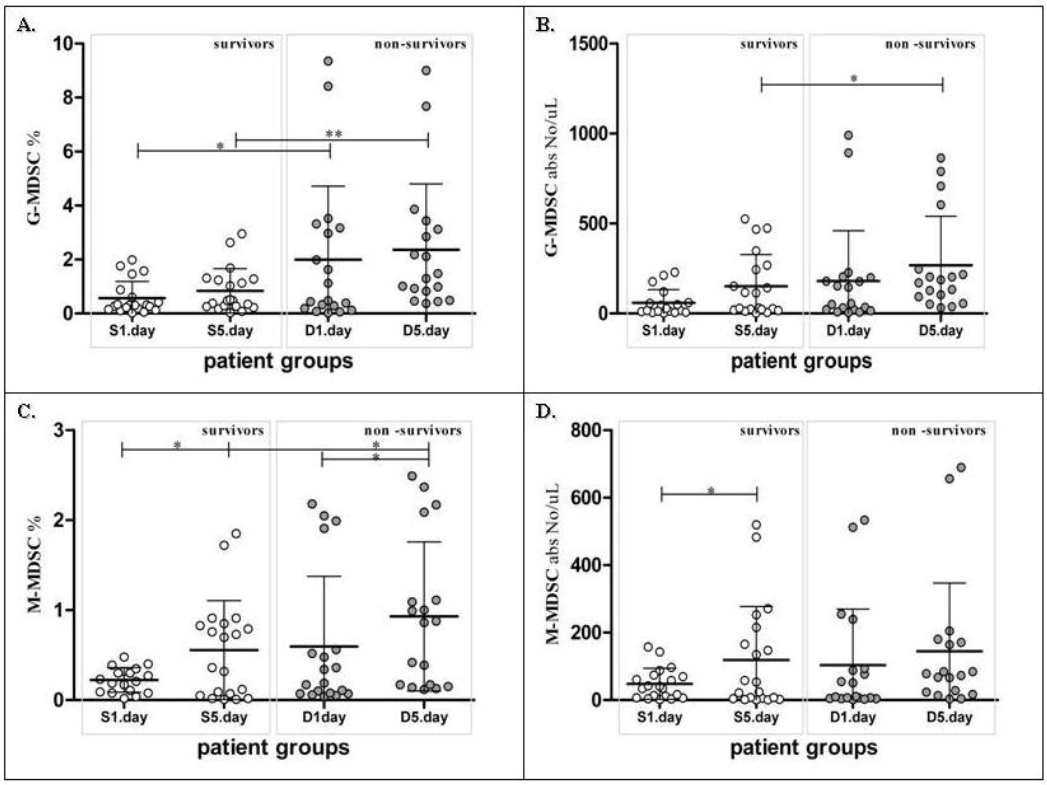

Fig. 3 – Comparison of myeloid-derived suppressor cells (MDSCs) values between survivors and non-survivors, 1th and 5th day: A) Relative number of granulocytic (G)-MDSC (%); B) Absolute number of G-MDSC (N/μL); C) Relative number of monocytic (M)-MDSC (%); D) Absolute number of M-MDSC (N/μL). S1, S5 – survivors on the Day 1 and the Day 5; D1, D5 – non-survivors on the Day 1 and the Day 5. Relative and absolute numbers given as mean ± standard deviation (Mann Whitney test, *$p < 0.05$, **$p < 0.01$).

The frequency of G-MDSCs was significantly higher in non-survivors, both on the Day 1 ($p < 0.05$) and the Day 5 ($p < 0.01$) of follow-up (Figure 3A). Absolute number of G-MDSCs was higher in non-survivors on the Day 1 (there was a trend which did not reach statistical significance) and on the Day 5 (statistically significant increase, $p < 0.05$) (Figure 3B).

Frequency of M-MDSCs was significantly higher on the Day 5, compared to the Day 1 ($p < 0.05$) in both survivors and non-survivors, but on the Day 5, frequency of M-MDSCs was also significantly higher in non-survivors compared to survivors ($p < 0.05$) (Figure 3C).

Regarding absolute number of M-MDSCs, although there was trend of higher values on the Day 5 in both survivors and non-survivors, only difference between the Day 1 and the Day 5 in survivors group reached statistical significance ($p < 0.05$) (Figure 3D).

Univariate logistic regression analyses were performed in order to determine whether associations of each individual variable with lethal outcome existed. Standardized regression coefficient (β) and OR with 95% CI were calculated for each variable. Forward stepwise multivariate logistic regression model was performed in order to determine the independent predictors of lethal outcome, without the effect of possible confounders. In Table 3 univariate OR of variables for predicting lethal outcome in patient population, on the Day 1 and the Day 5 are shown.

Univariate logistic regression analyses of investigated variables regarding lethal outcome on the Day 1 revealed that all three severity scores (SAPS II, SOFA, APACHE II) along with G-MDSCs frequencies had statistically significant power for predicting lethal outcome. When stepwise multivariate logistic regression analyses of the same variables on the Day 1 were performed, it was demonstrated that none of the investigated variables was independent predictor of lethal outcome.

Univariate logistic regression analyses of investigated variables regarding lethal outcome on the Day 5 revealed that SOFA score along with G-MDSCs frequencies had statistically significant power for predicting lethal outcome. In contrast to findings of stepwise multivariate logistic regression analyses of variables on the Day 1, on the Day 5 it was determined that SOFA score and G-MDSCs frequencies were independent predictors of lethal outcome which is shown in Table 4.

ROC curves were constructed to assess predictive values of investigated variables regarding lethal outcome. On the Day 1 neither frequencies nor absolute numbers of G-MDSCs and M-MDSCs were significant in discriminating between survivors and non-survivors.

Table 3

Univariate odds ratios (ORs) of variables for predicting lethal outcome in the patient population on the Day 1 and the Day 5

Variables	Standard β value	Odds ratio	95% confidence interval		*p*-value
			lower bound	upper bound	
SAPS II score 1st day	0.059	1.061	1.001	1.124	0.045*
SOFA score					
1st day	0.411	1.508	1.147	1.982	0.003**
5th day	0.40	1.504	1.167	1.938	0.002**
APACHE II score 1st day	0.216	1.241	1.068	1.443	0.005**
G-MDSCs frequencies					
1st day	0.671	1.956	0.958	3.997	0.040*
5th day	0.821	2.272	1.075	4.800	0.032*
G-MDSCs absolute numbers					
1st day	0.001	1.001	0.998	1.004	0.387
5th day	0.002	1.002	0.999	1.006	0.135
M-MDSCs frequencies					
1st day	0.292	1.339	0.552	3.252	0.519
5th day	0.807	2.242	0.820	6.131	0.116
M-MDSCs absolute numbers					
1st day	0.005	1.005	0.998	1.012	0.199
5th day	0.001	1.001	0.997	1.004	0.651

Significant differences are marked by *($p < 0.05$) or **($p < 0.01$).
For abbreviations see under Table 2.

Table 4

Independent predictors of lethal outcome by multivariate logistic regression analysis on the Day 5

Variables	Standard β value	Odds ratio	95% confidence interval		*p*-value
			lower bound	upper bound	
SOFA score	0.854	2.350	0.929	5.941	0.042*
G-MDSCs frequencies	1.274	3.575	1.098	11.639	0.030*

Significant differences are marked by *($p < 0.05$) or **($p < 0.01$).
For abbreviations see under Table 2.

Table 5

Clinical accuracy of variables in predicting lethal outcome in the patient population on the Day 5

Variables	AUC ROC	*p*-value	95% confidence interval		Cut-off value	Sensitivity (%)	Specificity (%)	Youden index
			lower bound	upper bound				
SOFA score	0.861	0.000**	0.748	0.975	6.50	67.0	90.0	0.56
G-MDSCs frequencies	0.758	0.007**	0.607	0.909	0.36	100.0	40.0	0.40
G-MDSCs absolute numbers	0.692	0.040*	0.519	0.864	30.75	100.0	50.0	0.50
M-MDSCs frequencies	0.699	0.037*	0.530	0.867	0.86	56.0	80.0	0.35

Significant differences are marked by *($p < 0.05$) or **($p < 0.01$).
AUC ROC – area under curve; ROC – receiver operating characteristic; for other abbreviations see under Table 2.

Table 6

Spearman's rho correlations between variables and lethal outcome in the patient population on the Day 5

Variables	G-MDSCs frequencies	G-MDSCs absolute numbers	M-MDSCs frequencies	M-MDSCs absolute numbers
Lethal outcome	0.447; $p = 0.005$	0.332; $p = 0.042$	0.344; $p = 0.035$	0.168; $p = 0.313$
G-MDSCs frequencies		0.818; $p = 0.000$	0.484; $p = 0.002$	0.389; $p = 0.016$
G-MDSCs absolute numbers			0.663; $p = 0.000$	0.749; $p = 0.000$
M-MDSCs frequencies				0.899; $p = 0.000$

G – granulocytic; MDSCs – myeloid-derived suppressor cells; M – monocytic.

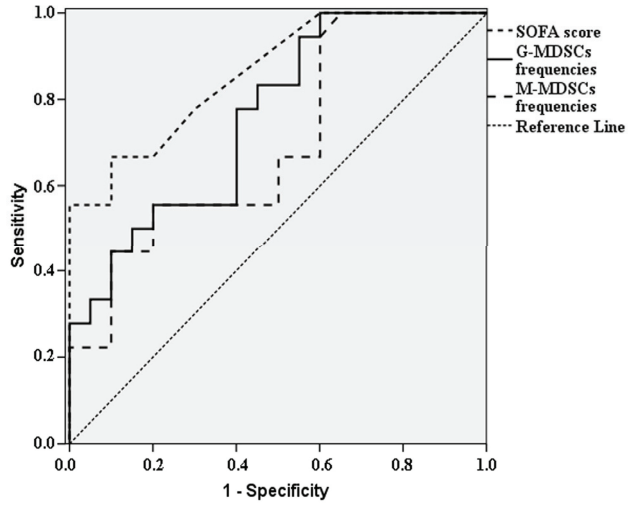

Fig. 4 – Receiver operating characteristic (ROC) curves for SOFA score, G-MDSCs and M-MDSCs frequencies in patient population on the Day 5 and the lethal outcome. For abbreviation see under Table 2.

In contrast to the Day 1, on the Day 5 all investigated variables were good predictors of lethal outcome apart from M-MDSCs absolute numbers [area under curve (AUC) 0.597; $p = 0.306$]. Frequencies and absolute numbers higher than cut-off values were predictors of lethal outcome. In Table 5 and Figure 4, clinical accuracy of variables in predicting lethal outcome in patient population on the Day 5 is shown.

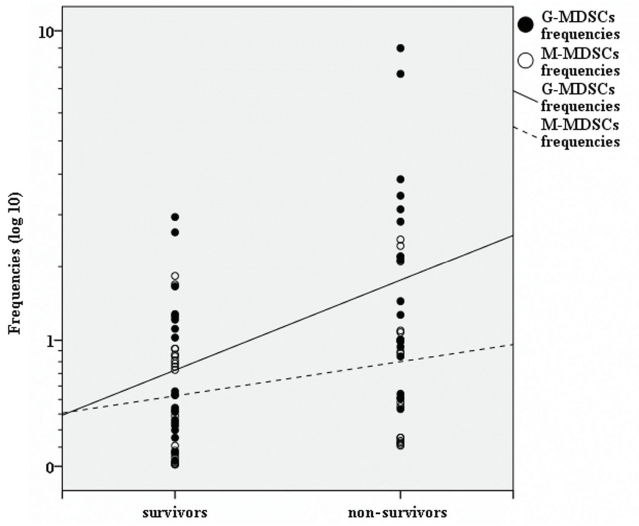

Fig. 5 – Scattergram on $\log_{10}$ scales of G-MDSCs and M-MDSCs frequencies versus lethal outcome in the patient population on the Day 5. G – granulocytic; MDSCs – myeloid-derived suppressor cells; M – monocytic.

The Spearman's rho test of correlation between frequencies and absolute numbers of G-MDSCs and M-MDSCs, on one hand, and lethal outcome, on the other hand, was performed to assess strength of association. On the Day 1 neither frequencies nor absolute numbers of G-MDSCs and M-MDSCs correlated significantly with lethal outcome. In contrast to the Day 1, on the Day 5, apart from M-MDSCs absolute numbers, there were significantly positive correlations between investigated variables and lethal outcome (Table 6). The strongest correlation was between G-MDSCs frequencies and lethal outcome (Figure 5).

There was no statistically significant association of gender, age, cause of secondary sepsis or nature of blood culture with outcome.

Discussion

The role of MDSCs has been extensively studied in a cancer field, but investigations regarding their function in sepsis are still sparse with previously contradictory results. While some studies demonstrated their deleterious effects [5], the others showed that the MDSCs expansion and activation could actually protect the sepsis host [6, 22]. In the present study, which included 40 patients with sepsis and/or septic shock secondary to pancreatitis, peritonitis and trauma, we detected and enumerated MDSCs on the Day 1 (the day of SICU admission and fulfillment of current sepsis and/or septic shock criteria) and on the Day 5 after the admission . These two specific time points were chosen because animal studies have shown dynamic change in MDSCs function during sepsis. In one study, although both MDSCs harvested at the Day 3 and the Day 10 were able to inhibit T cell proliferation, only MDSCs harvested at the Day 10 were also able to decrease peritoneal release of cytokines, enhance bacterial clearance and improve rate of survival [16]. In another study, authors demonstrated, on animal sepsis model, that early (Day 3) MDSCs adoptive transfer from septic into naive mice led to increased proinflammatory cytokine profile, decreased peritoneal bacterial growth with high early mortality rate. Contrary to that, transfer of late (Day 12) MDSCs effect was completely opposite [17]. To the best of our knowledge, this has not been investigated in humans yet. So, the Day 1 corresponds to early MDSCs in animal sepsis model. The Day 5 was chosen bearing in mind that survival of critically ill patients with secondary sepsis and/or septic shock on the day 10 or 12 is rather uncertain. Previously, we have emphasized that sample handling is of great importance during flow cytometric detection of MDSCs in the study with melanoma patients and indicated several reasons why we decided to analyze fresh, lysed peripheral blood samples [23]. However, Sagiv et al. [24], showed remarkable ability of mature neutrophils to change their density from 'normal' high, to low-density neutrophils and *vice versa* in the peripheral blood of tumor-bearing mice and human lung cancer patients. If this could be the truth for MDSCs as well, then analysis of fresh lysed samples might have the advantage in preserving the possible "high-density'' MDSCs. Altered buoyancy of our targeted cells is, however, in accordance with many studies that showed immunosuppressive capacity of a low density granulocyte-like cells [25–28].

As mentioned, it is still not definitely clarified whether MDSCs are friends or foes in sepsis and what determines whether they carry benefit or harm to the sepsis patients. De-

lano et al. [5] showed, on experimental animal sepsis model, that the Gr-1$^+$CD11b$^+$ MDSCs accumulate in bone marrow and peripheral lymphoid organs in mice during polymicrobial sepsis, and contribute to the T cell suppression seen after sepsis, as well as to the polarization from a Th1 towards Th2 immune response. Based on Delano et al. [5] findings, it was expected to connote the MDSC population as detrimental to the septic host. Surprisingly, blockages of the MDSCs expansion by using gemcitabine or anti-Gr-1 antibodies, with an aim to improve survival in septic mice, have led to unexpected, significantly worsened outcomes. This worsening in septic mice survival is partially explained by nonselective action of gemcitabine and anti-Gr-1 antibodies, but still, the beneficial effect of blocking MDSCs has not been reached [4]. The aforementioned evidences could lead towards opinion that the MDSCs accumulation is beneficial to the septic host. But, as emphasized by Cuenca et al. [4] and Delano et al. [5], the function and role of MDSCs in sepsis cannot be simplified to this point. There is still complex and intertwined relationship between impact of MDSCs on sepsis severity and survival, on one hand, and kinetics of their accumulation in sepsis, on the other hand. In that regard, we found that non-survivors had significantly higher frequencies of G-MDSCs both on the Day 1, and the Day 5, but on the fifth day difference was more pronounced, statistically highly significantly. On the Day 5, G-MDSCs frequencies were independent predictors of lethal outcome, determined by stepwise multivariate logistic regression analysis; this was confirmed by ROC curve analysis, which revealed good discriminative power regarding outcome, and by the Spearman's rho test showing the strongest positive correlation between G-MDSCs frequencies and lethal outcome in comparison with other investigated variables. Similarly, in the animal model of sepsis, Cuenca et al. [4] found no changes in either splenocyte or peripheral lymph node CD11b$^+$GR-1$^+$ numbers in the first twenty-four hours after sepsis. They found first expansion of the CD11b$^+$GR-1$^+$ cells in the spleen and peripheral lymph nodes only after 3–5 days, with continuous increase in their concentrations for the next 10–14 days. All of these results, regarding the increase in MDSCs, are consistent with the theory that the host immune response to sepsis is characterized by an initial hyperinflammatory phase which evolves over several days into a more protracted immunosuppressive phase [12]. In support of this notion that MDSCs contribute to the secondary, immunosuppressive phase of sepsis, are also the findings of Brudecki et al. [17]. These investigators clearly showed that GR-1$^+$ CD11b$^+$ cells from late sepsis are endowed with immunosuppressive capabilities. Namely, they showed that adoptive transfer of GR-1$^+$ CD11b$^+$ cells from the bone marrow of the day 12 septic mice into naive mice, immediately after induction of sepsis by cecal ligation and puncture, significantly improved early sepsis survival. In addition, IL-10 and TGF-β levels were significantly higher in mice that received GR-1$^+$ CD11b$^+$ cells from the day 12 septic mice than in mice which received saline or cells from the day 3 septic mice. Dramatic expansion of the GR-1$^+$ CD11b$^+$ cells in late sepsis was also documented in this study [17]. Predictive value of many components of immune response in sepsis, regarding disease severity and outcome, has been investigated; future large sample studies are required to explore MDSCs in this regard [29, 30].

As already mentioned, MDSCs are heterogeneous group of immature myeloid cells, poor phagocytes, which can prevent overactivation of the immune system by producing IL-10 or TGF-β [31]. But, protracted presence of these cells can lead to persistent inflammation (*via* NO, myeloperoxidase and ROS) and induce immunosuppression (by T-cell proliferation, anti-inflammatory mediators elaboration or defective presentation of antigens) [32].

A year ago, two very important studies regarding MDSCs in patients with sepsis and/or septic shock were published, emphasizing and reiterating the importance and novelty of this subject. Mathias et al. [33] focused their attention on patients with PICS, the predominant clinical phenotype in the ICU population, for which current interventions are ineffective. They noted that pivotal for the immune response in chronic sepsis (as well as in cancer) was the expansion of MDSCs, aimed at preserving innate immunity. Their hypothesis was that after sepsis in humans, MDSCs would be persistently increased, functionally immunosuppressive and associated with adverse clinical outcome. They enrolled 74 patients with sepsis and/or septic shock and 18 healthy controls. Blood was obtained at set intervals out to 28 days, MDSCs were phenotyped. They also performed functional and genome-wide expression analyses. This study design allowed them to assess role of MDSCs after sepsis. They found circulating MDSCs to be persistently increased, functionally immunosuppressive and associated with adverse long-term outcome consistent with PICS. These results are similar to our findings that higher values of MDSCs are associated with adverse outcome.

Uhel et al. [34] performed peripheral blood transcriptomic analysis in 29 patients with sepsis and 15 healthy donors, and in a second cohort of 94 patients with sepsis, 11 severity-matched ICU patients and 67 healthy donors, they performed functional analysis in order to clarify phenotype, suppressive activity, origin and clinical impact of MDSCs in patients with sepsis. Their results showed that MDSCs were major players in sepsis-induced immunosuppression. They concluded that CD14^{pos}HLA-DR$^{low/neg}$ M-MDSCs and CD15^{pos} G-MDSCs strongly contributed to T-cell dysfunction in patients with sepsis. Our findings generally go in the same direction. In both studies, authors stated that role of MDSCs in host response to sepsis was still not well-defined and needed to be clarified in large trials [35]. Multicentre large trials of this sort are difficult to conduct due to complexity of the design. Nevertheless, in the future, in our opinion, effort will be made because these important cells are potential target for future immunomodulating therapies. In this regard, it should be noted that MDSCs are phenotypically plastic which allows them a diverse functionality in response to their environmental conditions [36–38].

Main limitation of our study is sample size. Significant number of critically ill patients with secondary sepsis due to diffuse peritonitis had to be excluded because of malignant disease. Larger trial is essential for possible confirmation of our findings.

Conclusion

The role of MDSCs in different clinical settings, especially in sepsis, where the proinflammatory and antiinflammatory responses are simultaneously initiated, is not completely elucidated yet. In this study, we demonstrate that subpopulations corresponding to MDSCs can be phenotypically identified in the whole blood samples of sepsis patients and that their increased frequencies and absolute numbers are associated with poor outcome. As far as relative kinetics is concerned, we found that, in both survivors and nonsurvivors, sepsis duration from 1th to 5th day was accompanied by an increase in MDSCs values of both investigated subpopulations. These findings suggest that there is harmful role of MDSCs in sepsis and that larger trials are warranted in future research of these intriguing cells.

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Nasal polyposis: a semiquantitative morphometric histopathological study

Nazalna polipoza: semikvantitativna morfometrijska patohistološka studija

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Abstract

Background/Aim. Nasal polyps are inflammatory hypertrophic proliferations of the sinonasal mucosa composed of both epithelial and stromal elements. The aim of this study was to determine histopathological hallmarks of nasal polyposis via semiquantitative morphometric study. **Methods.** The study comprised 77 patients with chronic rhinosinusitis and nasal polyposis (CRSwNP) that underwent functional endoscopic sinonasal surgery performed by the same surgeon. The control group consisted of 9 different nasal mucosal samples that were taken from patients without CRSwNP that underwent functional and esthetic surgery. Morphometric analysis included gradation of tissue edema within polyps, thickening of epithelial basal membrane, degree of inflammation, presence/absence of metaplasia within epithelium, degree of fibrosis within polyps, and percentage of inflammatory cells within inflammatory infiltrate (lymphocytes, macrophages, plasma cells, neutrophils and eosinophils). **Results.** As expected, samples from the study group showed significantly higher degree of inflammation than samples from the control group ($\chi^2 = 35.89$, with $p < 0.01$). Degree of fibrosis in nasal polyposis was in positive correlation with duration of symptoms ($r = 0.25$, $p < 0.05$) and with percentage of macrophages in inflammatory infiltrate ($r = 0.26$, $p < 0.05$). Patients with nasal polyposis had significantly lower number of lymphocytes ($r = -7.66$, $p < 0.01$), but significantly higher number of eosinophils ($r = 3.84$, $p < 0.01$), macrophages ($r = 3.34$, $p < 0.01$) and plasma cells ($r = 3.14$, $p < 0.01$) than controls ($p < 0.01$). **Conclusion.** Tissue samples from patients with nasal polyposis show significant changes that reflect in various degrees of inflammation, fibrosis and basement membrane thickening which may contribute to more difficult surgical management and perioperative complications such as bleeding.

Key words:
nasal polyps; otorhinolaryngologic surgical procedures; postoperative complications; histology.

Apstrakt

Uvod/Cilj. Nazalni polipi predstavljaju inflamatorne izrasline hipertrofične respiratorne sluznice i sačinjeni su od epitelnih i stromalnih elemenata. Cilj ove studije bio je da odredimo patohistološka obeležja nazalnih polipa kroz semikvantitativnu morfometrijsku studiju. **Metode.** Izvršena je semikvantitvna morfometrijska analiza uzoraka nazalne sliznice uzetih od 77 bolesnika sa hroničnim rinosinuzitisom i nazalnim polipima. Kontrolnu grupu sačinjavali su uzorci nazalne sluznice, uzeti od 9 pacijenta bez nazalne polipoze koji su bili podvrgnuti funkcionalnoj i estetskoj hirurgiji. Kod svih bolesnika je učinjena funkcionalna endoskopska sinonazalna hirurgija od strane istog hirurga. Morfometrijska analiza je uključivala gradaciju edema tkiva sa polipima, debljinu bazalne membrane, stepen inflamacije, prisustvo/odsustvo metaplazije u epitelu, stepen fibroze, kao i procenat zapaljenskih ćelija sa zapaljenskim infiltratom (limfocite, makrofage, plazma ćelije, neutrofile i eozinofile). **Rezultati.** Kao što je i očekivano, uzorci iz ispitivane grupe su imali značajno veći stepen inflamacije u odnosu na kontrolnu grupu ($\chi^2 = 35.89$, $p < 0.01$). Stepen fibroze kod polipa

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nosa je bio u pozitivnoj korelaciji sa trajanjem dužine simtoma ($r = 0.25$, $p < 0.05$) i sa procentom makrofaga u zapaljenskom infiltrate ($r = 0.26$, $p < 0.05$). Bolesnici sa nazalnom polipozom imali su značajno veći broj limfocita ($r = -7.66$, $p < 0.01$), ali i značajno veći broj eozinofila ($r = 3.84$, $p < 0.01$), makrofaga ($r = 3.34$, $p < 0.01$) i plazma ćelija ($r = 3.14$, $p < 0.01$) nego kontrolna grupa ($p < 0.01$). **Zaključak.** Uzorci tkiva kod bolesnika sa nazalnom polipozom pokazuju značajne promene koje se ogledaju u različitom stepenu inflamacije, fibroze i zadebljanja bazalne membrane što može značajno otežavati hirurški zahvat, kao i uticati na veći stepen perioperativnih komplikcija kao što je krvarenje.

Ključne reči:
nos, polipi; hirurgija, otorinolaringološka, procedure; postoperativne komplikacije; histologija.

Introduction

Nasal inflammatory polyps are nonneoplastic proliferations of the sinonasal mucosa composed of both epithelial and stromal elements. The pathogenesis of these lesions is still uncertain; however, mucosal edema and inflammation, cytokine secretion, and collagen synthesis stimulated by eosinophils have all been implicated [1–3]; polyps are frequently associated with salicylates intolerance, asthma and cystic fibrosis [1–9]. Symptoms at presentation include nasal obstruction, rhinorrhea, headache, impaired sense of smell and postnasal discharge [1–6]. Nasal polyposis (NP) is slightly more prevalent in men, with an incidence in the fifth decade of life, and affects between 1% and 4% of the population [5].

Patients who have failed medical management may benefit from surgical intervention in the form of transnasal ethmoidectomy or, more recently, functional endoscopic nasal surgery. Even after appropriate surgical therapy, a significant number of patients with chronic rhinosinusitis (CRS) with NP (CRSwNP) experience recurrences [9], with disease-free interval significantly shorter in patients with eosinophilic-type polyposis. NP often present as multiple bilateral masses arising from the lateral nasal wall. Inflammatory polyps can measure up to several centimeters in diameter, with usually a broad stalk and have a myxoid or gelatinous appearance with a smooth surface. Histologically, they are lined with respiratory epithelium with a variably thickened basement membrane. The epithelium often exhibits some degree of squamous metaplasia. The stroma is abundant and highly edematous or myxoid and contains a mixed inflammatory infiltrate composed of eosinophils, lymphocytes, and plasma cells. Sometimes Charcot-Leyden crystals associated with abundant eosinophils may be seen. These crystals are a result of eosinophil degeneration and are formed at the surface of nasal mucosa and within mucus. In cases associated with infection, neutrophils may be present in large numbers. The stroma contains a variable number of fibroblasts and blood vessels [1].

The aim of this study was to determine histopathological hallmarks of nasal polyposis via semiquantitative morphometric study.

Methods

We conducted a study during period of January 1st, 2016 until December 31st, 2016. The study comprised 77 patients with CRSwNP that underwent endoscopic sinus surgery performed by the same surgeon. Patients had no history of cystic fibrosis, antrochoanal polyp or primary ciliary dyskinesia. Nasal steroid treatment was given to patients pre and postoperatively. Nasal polyps were sent for histopathological examination. Representative tissue samples were processed routinely, were formalin-fixed and paraffin embedded. Tissue sections that were 5 μm thick were made and stained with hematoxillin & eosin. The control group consisted of 9 different nasal mucosal samples that were taken from patients without CRSwNP that underwent functional and esthetic surgery. The samples of mucosa were taken from inferior nasal concha. After the histopathological diagnosis of nasal polyposis was established, semiquantitative morphometric analysis was performed. It included gradation of tissue edema within polyps according to the degree of lamina propria expansion (0 - no edema, 1-slight edema/slight lamina propria expansion, 2 - moderate edema/moderate lamina propria expansion, 3 - severe edema/marked lamina propria expansion), thickening of epithelial basal membrane (0 - no thickening, 1 - slight thickening, 2 - moderate thickening, 3 - severe thickening), degree of inflammation (0 - no inflammation, 1 - slight inflammation with inflammatory infiltrate comprising less than 30% of the sample/per 100 x magnification, 2 - moderate inflammation, with inflammatory infiltrate comprising between 30% and 60% of the sample/per 100 x magnification, 3 - severe inflammation, with inflammatory infiltrate comprising more than 60% of the sample/per 100 x magnification), presence/absence and type of metaplasia within epithelium (goblet cell metaplasia and squamous metaplasia), degree of fibrosis within stroma (0 - no fibrosis, 1 - slight fibrosis that comprises less than 30% of stromal surface, 2 - moderate fibrosis that comprises up to 50% of stromal surface, 3 - severe fibrosis that comprises more than 50% of the stromal surface), and percentage of inflammatory cells within inflammatory infiltrate (lymphocytes, macrophages, plasma cells, neutrophils and eosinophils). We also evaluated gender and age in both the control and the study group, duration of symptoms, prior history of allergies and polyposis laterality. We did not evaluate the percentage of eosinophils within nasal mucus. Analysis was performed using a Cell F imaging analysis programme and was performed by one pathologist.

Data were analyzed by the χ^2-test, Pearson's correlation coefficient and *t*-test with p values ≤ 0.05 that were considered significant. All analyses were done in the software package Statistical Package for Social Sciences 18 (SPSS 18).

Results

Our study included 77 patients, 46 (59.7%) male and 31 (40.3%) female. Control group consisted of 9 patients, 6 (66.7%) male and 3 (33.3%) female. Average age (Table 1) in the study group was 45.40 ± 14.92 years (age ranged from 13 to 71 years). Duration of symptoms ranged from 1 to 31 months, the average being 12.10 ± 6.81 months. Majority of patients (89.6%) had bilateral NP. We found no gender differences in our patients in comparison with any of examined morphological data. Samples from the study group showed significantly higher degree of inflammation than samples from the control group ($\chi^2 = 35.89$, $p < 0.01$). Slight inflammation was found in 35 patients, moderate in 33 patients and severe in 9 patients from the study group (Figure 1). Fibrosis (Figure 2) was slight in 13 patients, moderate in 34 and severe in 17 patients within the study group, whilst 17 showed no morphological signs of fibrosis. Degree of fibrosis in NP was in positive correlation with duration of symptoms ($r = 0.25$, $p < 0.05$) and with percentage of macrophages in inflammatory infiltrate ($r = 0.26$, $p < 0.05$). There was no such correlation between degree of tissue edema and age/duration of symptoms. There were no patients with 50% or more macrophages in the inflammatory infiltrate. Patients with NP had significantly lower number of lymphocytes ($r = -7.66$, $p < 0.01$), but significantly higher number of eosinophils ($r = 3.84$, $p < 0.01$), macrophages ($r = 3.34$, $p < 0.01$) and plasma cells ($r = 3.14$, $p < 0.01$) than the controls ($p < 0.01$).

Table 1
Clinical characteristics of the study group with nasal polyposis and the control group

Characteristics	Study group	Control group
Gender, n (%)		
male	46 (59.7)	6 (66.7)
female	31 (40.3)	3 (33.3)
Total	77 (100)	9 (100)
Age (years), min–max.	13–71	18–55
Duration of symptoms (months), min–max . (mean ± SD)	1–31 (12 ± 6.81)	-

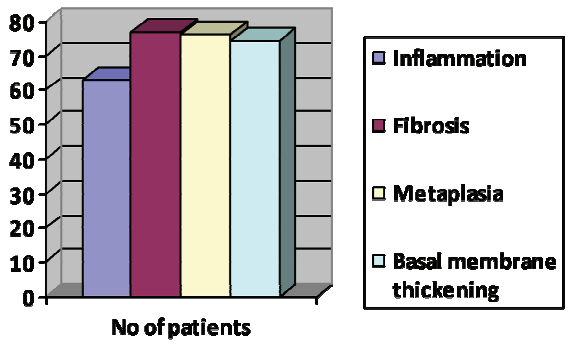

Fig. 1 – Histopathological hallmarks of the study group with nasal polyposis.

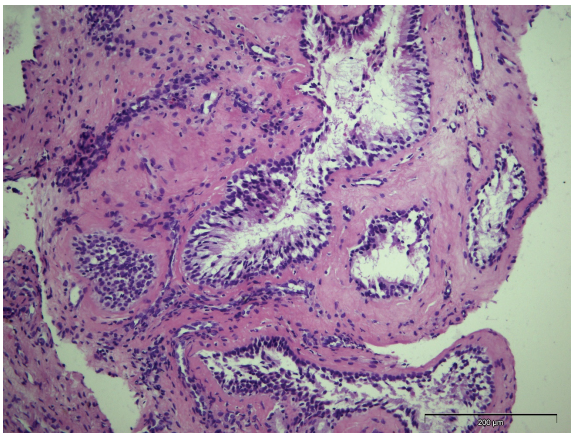

Fig. 2 – Nasal polyp with mild stromal fibrosis (hematoxillin & eosin, original magnification ×200).

Epithelial metaplasia was found in a great majority of patients: isolated goblet cell metaplasia in 70.1% (Figure 3) and combined goblet cell and focal squamous metaplasia in 26%. Only 1 (1.3%) patient showed no adaptive epithelial changes. We also found no correlation of basal membrane thickening (Figure 4) with age of patients and duration of symptoms.

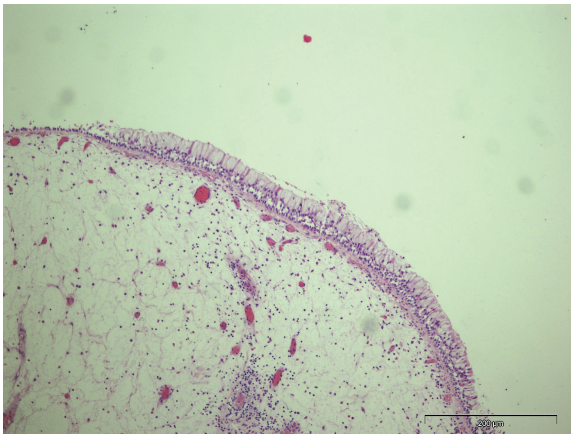

Fig. 3 – Nasal polyp with goblet cell metaplasia (hematoxillin & eosin, original magnification ×200).

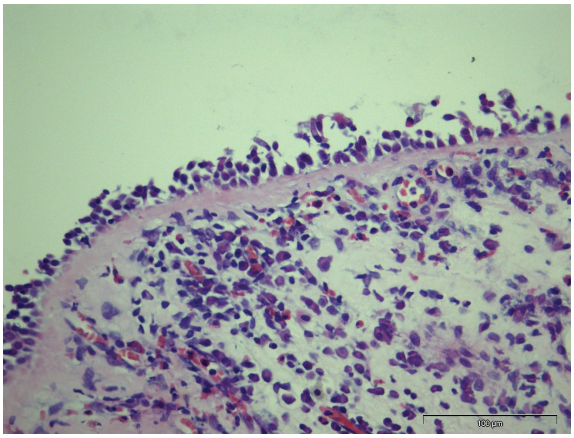

Fig. 4 – Basal membrane thickening within nasal polyp (hematoxillin & eosin, original magnification ×400).

Discussion

Rhinosinusitis can be defined as an inflammation with two or more of the following symptoms: nasal congestion/blockade, nasal discharge, facial pain, reduction/loss of smell; there are also complementary endoscopic signs and computed tomography changes. If rhinosinusitis persists for more than 12 weeks it is classified as chronic, with or without NP. NP consists of mucosal edema, inflammatory infiltrates, hyperplastic / hypertrophic sero-mucous glands often with some degree of epithelial metaplasia. A vast variety of inflammatory cells can be found in NP such as eosinophils, neutrophils, mast cells, plasma cells, lymphocytes, monocytes and fibroblasts. CRSwNP is also characterized with increased fibrosis and collagen deposition and with thickened epithelial basement membrane. Recent studies often discuss and explain different immunological pathways of tissue damage and edema, also different inflammatory pathways and different responses to treatment between CRSwNP and CRS without NP [10–12]. It is well known that inflammatory reactions can stimulate epithelial proliferation. Inflammatory cells produce various growth factors that stimulate epithelial proliferation. Recent studies report that NP with recurrent disease displayed higher scores for proliferation markers [12], but not significantly higher than that in non-recurring NP; preoperative steroid treatment might have resulted in inhibition of inflammatory response [12]. The presence of eosinophils greatly increases the risk of recurrent disease [13, 14]. Nakayama et al. [13] report eosinophilic inflammation in 59.6% of patients with NP. Patients with mucosal eosinophilia had higher recurrence rate than patients without mucosal eosinophilia, whereas patients with NP did not have higher polyp recurrence rate than patients without NP [13]. Vlaminck et al. [14] found tissue eosinophils in 78% of CRS with NP in comparison to 42% patients with CRS without NP. Eosinophilic mucin was observed in 52% of patients with CRSwNP and in 20% of patients CRS without NP. CRSwNP patients showed a recurrence rate of 48%; those with additional eosinophilic mucin showed 56% of recurrences [14]. In our study, after the follow-up period, there were no reccurences.

Recently macrophages invaded the spotlight in NP. Banks et al. [15] found that NP patients had significantly increased numbers of macrophages compared to control patients or patients without polyposis, regardless of atopic status. Our results concur with this report: we found significantly higher number of eosinophils, macrophages and plasma cells in patients with NP compared to the control ones, regardless of symptom duration, patients age and atopic status. We also found significant positive correlation between degree of fibrosis within NP and duration of symptoms and correlation between percentage of macrophages and degree of fibrosis. There was no such correlation between degree of tissue edema and age/duration of symptoms. We found that higher degree of tissue fibrosis may aggravate the operating process during endoscopic nasal surgery. We also found that younger patients with NP had significantly higher degree of neutrophils in inflammatory infitrates, regardless of symptom duration. These findings were not reported in previously published histopathological studies.

Conclusion

Tissue samples from patients with nasal polyposis show significant changes reflecting in various degrees of inflammation, fibrosis and basement membrane thickening which may contribute to more difficult surgical management and perioperative complications such as bleeding.

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The correlation between metabolic syndrome quantification scores and numerous laboratory parameters related to this syndrome

Korelacija između kvantifikacionih skorova metaboličkog sindroma i brojnih laboratorijskih parametara udruženih sa njim

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Abstract

Background/Aim. Metabolic syndrome (MS) is characterized by basic cluster risk factors – waist circumference (WC), glucoregulation disorders, hypertension, hypertriglyceridemia, low HDL-cholesterol followed by associated factors such as insulin resistance (IR), C-reactive protein (CRP), uric acid, plasminogen activator inhibitor-1 (PAI-1), fibrinogen, hyperhomocysteinemia (HHcy), nonalcoholic fatty liver disease (NAFLD) and microalbuminuira. The aim of this study was to analyze basic and associated factors of MS in patients with and without MS as well as correlation of siMS score, siMS risk score with basic and confounding factors of MS. **Methods**. The study included 148 overweight [body mass index (BMI) 25–30 kg/m^2 and obese patients (BMI > 30 kg/m^2)], age 30–75 years, classified into two groups: I – with MS (68 patients); II – without MS (80 patients). For quantification of MS, siMS score was used as a method, and siMS risk score was used as atherosclerotic complications risk indicator. **Results.** Patients with MS had statistically higher values of WC, hypertension, triglycerides ($p < 0.001$), glycemia ($p = 0.006$), as well as values of associated factors of MS [homeostatic model assessment (HOMA-IR)] ($p = 0.002$), CRP ($p = 0.01$), uric acid ($p < 0.001$), alanin transaminase (ALT) ($p = 0.007$) i gammaglutamyl transferase (GGT) ($p = 0.001$) and lower values of HDL-cholesterol ($p < 0.001$) compared to patients without MS. siMS score has shown correlation with associated factors of MS (log HOMA IR, logCRP, uric acid, ($p < 0.001$), fibrinogen ($p = 0.005$), liver enzymes logALT ($p = 0.001$) and log GGT ($p < 0.001$) and renal parametars (creatinine ($p = 0.013$) and serum protein ($p = 0.006$). siMS risk score correlated significantly with homocysteine, platelets, uric acid, blood urea nitrogen, albumins and proteins. **Conclusion.** In our study we found that patients with MS had higher values of associated factors of MS (HOMA-IR, CRP, uric acid, ALT, GGT), which was confirmed by correlation with siMS score. siMS score further indicated that IR, CRP, fibrinogen, uric acid and NAFLD are associated factors of MS. siMS risk score is another score that indicated that obesity and hyperprotein diet aggravates HHCy with age, increasing the risk for renal dysfunction and promoting atherosclerotic complications.

Key words:
biomarkers; homocysteine; metabolic syndrome; risk assessment; risk factors.

Apstrakt

Uvod/Cilj. Metabolički sindrom (MS) karakterišu osnovni faktori rizika [obim struka (OS), poremećaji glikoregulacije, hipertenzija, hipertrigliceridemija, nizak HDL-holesterol] kao i pridruženi faktori rizika – insulinska rezistencija (IR), C-reaktivni protein (CRP), mokraćna kiselina, inhibitor aktivacije plazminogena-1 (PAI-1), fibrinogen, hiperhomocisteinemija (HHci), nealkoholna masna bolest jetre (NAMBJ) i mikroalbuminurija. Cilj rada bio je da se analiziraju osnovni

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i pridruženi faktori rizika od MS kod bolesnika sa i bez MS i ustanovi korelacija siMS skora i siMS skora rizika sa osnovnim i pridruženim faktorima rizika od MS. **Metode.** Studijom su bila obuhvaćena 148 bolesnika sa prekomernom telesnom težinom [*body mass index* ((BMI) 25–30 kg/m^2) i gojazni (BMI > 30 kg/m^2), starosti 30–75 godina, podeljeni u dve grupe: I – sa MS (68 bolesnika) i II – bez MS (80 bolesnika). Korišćeni su siMS skor, kao metod za kvantifikaciju MS, i siMS skor rizika, kao indikator aterosklerotskih komplikacija. **Rezultati.** Bolesnici sa MS imali su statistički značajno više vrednosti OS, hipertenzije, triglicerida (p < 0,001), glikemije (p = 0,006), kao i pridruženih faktora rizika od MS [HOMA IR (p = 0,002) CRP (p = 0,01) mokraćne kiseline (p < 0,001), alanin aminotranferaze (ALT) (p = 0,007) i gama-glutamil transferaze (GGT) (p = 0,001)] i niže vrednosti HDL-holesterol, (p < 0,001) u odnosu na bolesnika bez MS. Skor siMS pokazao je korelaciju sa pridruženim faktorima MS [log HOMA IR, logCRP, mokraćnom kiselinom (p < 0,001) i fibrinogenom (p = 0,005), parametrima jetrene funkcije: logALT (p = 0,001), log GGT, (p < 0,001) i bubrežne funkcije: kreatininom (p = 0,013) i serumskim proteinima (p = 0,006)]. Skor siMS rizika je statistički značajno korelirao sa vrednostima homocisteina, trombocita, mokraćne kiseline, uree, albumina i proteina. **Zaključak.** Statistički značajno više vrednosti pridruženih faktora rizika od MS (HOMA-R, CRP, mokraćne kiseline, ALT, GGT) kod bolesnika sa MS potvrđene su i korelacijom sa siMS skorom. Skor siMS ukazuje na to da su insulinska rezisencija, CRP, fibrinogen, mokraćna kiselina, NAMBJ pridruženi faktori rizika od MS. Skor siMS rizika ukazuje na to da gojaznost i hiperproteinski unos povećavaju HHCi sa starenjem, te da povećavaju rizik od bubrežnih poremećaja i aterosklerotskih komplikacija.

Ključne reči:
biomarkeri; homocistein; metabolički sindrom; rizik, procena; faktori rizika.

Introduction

Hyperhomocysteinemia (HHcy) was found in some age-related clinical entities such as osteoporosis, hypothyroidism, cardiovascular diseases (CVD), cancer, end-renal stage disease and neurodegenerative diseases. Homocysteine (Hcy) is increased by several mechanisms as methionine enriched diets, defects in the methionine metabolism and B6, B12 and folate deficits [1].

Plasma Hcy directly correlates with age, waist circumference (WC), fasting glucose, triglyceride, uric acid, fibrinogen levels, insulin resistance, and inversely with creatinine clearance, and HDL-cholesterol [2].

Animal studies suggested HHcy as additional component of the metabolic syndrome (MS). Studies were based on theory that insulin might affect Hcy metabolism, in which hyperinsulinism caused increased levels of Hcy [3, 4]. Further studies have shown that MS and HHcy are established independent risk factors for CVD, and HHcy might be co-founding factor of MS [5, 6].

In our previous studies correlation of siMS score with Hcy indicated that Hcy is a co-founding factors of MS. [7] siMS score defined by Soldatović et al. [8] presents summary score of all MS factors [abdominal obesity, glycemia, systolic and diastolic blood pressure, triglycerides and high density lipoprotein (HDL)-cholesterol]. siMS score correlates with values of uric acid, microalbuminuria, fibrinogen, as well as with an inflammation parameter, C- reactive protein (CRP) [7]. Next clinical entity, nonalcoholic fatty liver disease (NAFLD) is also considered as a sign of MS. NAFLD is a chronic liver disease, which includes a spectrum of hepatic pathology from simple steatosis, steatohepatitis, to cirrhosis. Incresed Hcy may be associated with hepatic fat accumulation, both caused by hyperinsulinism [9]. Hcy induces endothelial cell injury and impairs vasodilatation by increased inactivation of nitric oxide and decreased generation of nitric oxide [10]. Hcy promote oxidative stress in vascular cells and tissues by reactive oxygen species (ROS), who have been shown to cause endothelial injury and the development of atherosclerosis [11]. Correlation between Hcy, hypertension and hyperlipoproteinemia indicated that Hcy could be promoting factor for atherosclerosis [12].

The aim of this study was to analyze and correlate MS cluster factors [WC, glycoregulation disorders, hypertension, hypertrigliceridemia, low HDL-cholesterol] and associated factors of MS [insulin resistance, CRP, uric acid, plasminogen activator inhibitor-1 (PAI-1), fibrinogen, HHcy, NAFLD and microalbuminuria] in patients with MS and without MS. siMS score and siMS risk score correlation with basic cluster MS factors and associated factors were also examined.

Methods

The study included 148 overweight [body mass index (BMI) 25–30 kg/m^2] and obese (BMI > 30 kg/m^2) patients, aged 30–75 years, classified into two groups: I – with MS (68 patients), and II – without MS (80 patients). Measured anthropometric parameters were body weight (BW), body height (BH), BMI, and WC. BMI was calculated as BW in kilograms divided by the square of BH in meters. Blood pressure (BP) was measured in seating position using sphygmomanometer. Oral glucose tolerance test (OGTT) with 75 g glucose was used for estimation of glycoregulation early disorders. Values of glycemia and insulin were measured during OGTT in 0, 30 and 120 min. Lipid status was determined by total cholesterol, HDL-cholesterol, low density lipoprotein (LDL)-cholesterol, triglycerides by spectrophotometer methods and apolipoprotein (Apo) A1, Apo B, Apo E and lipoprotein a [Lp(a)] by immunochemical methods. The Adult Treatment Panel (ATP) III classification was applied for diagnosing MS. A diagnosis of MS was confirmed if three out of five parameters were found as follows: WC > 102 cm for males and > 88 cm for females, BP > 135/85 mm/Hg, fasting blood glucose > 6.1 mmol/L, increased triglycerides (> 1.7 mmol/L), decreased HDL-C (< 1.03 mmol/L for males and HDL-C < 1.29 mmol/L for females). Patients who consumed more than 2 units of alcohol per day (for females), or 3 units per

day (for males), or more than 14 units per week (females) and 21 units per week (for males) were excluded from the study [one unit of alcohol (10 g) is equivalent to one glass of whiskey – 3 cL, or one glass of brandy – 3 cL, or one glass of wine – 20 cL, or one glass of beer – 25 cL) [13].

In this study we analyzed cluster factors of MS and associated factors such as insulin resistance, Hcy, CRP, PAI-1, fibrinogen, uric acid, liver and renal function parameters. Insulin was measured using radioimmunoassay method. Insulin resistance and insulin sensitivity was determined by Homeostatic Model Assessment Insulin Resistance (HOMA IR): HOMA-IR = insulinemia (mU/L) × glycaemia (mmol/L)/ 22.5 (cut off value is 3.2 μmol/mU/mL). Hcy as an independent marker of atherosclerosis was determined on Abbott´s Architect analyzer, using CMIA technology. Levels of CRP, as an inflammation marker, were determined by immunometric method. PAI-1, as an thrombogenic marker, was determined by plasminogen substrate essay. Liver function parameters determined were aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma-glutamyltransferase (GGT), albumin, total proteins. Renal function parameters, determined by immune-nephelometric method, were: urea, creatinine, creatinine clearance, microalbuminuria from 24-hour urine. Soldatovic et al. [8] established a new siMS score for MS quantification, simple for clinical use and scientific research.

The formula for siMS score using MS reference values is calculated as follows:

$$siMS\ score = \frac{2 \times Waist}{Height} + \frac{Gly}{5.6} + \frac{Tg}{1.7} + \frac{TA\ systolic}{130} - \frac{HDL}{1.03\ or\ 1.3\ (male\ or\ female)}$$

Age and positive family anamnesis were added to siMS score; siMS risk score, useful for cardio/cerebrovascular events risk evaluation, was thus obtained [14].

$$siMS\ risk\ score = siMS\ score \times \left(\frac{Age}{45\ or\ 50\ (males\ or\ females)}\right) \times \left(\frac{Family\ history\ of\ cardio\ or\ cerebro-vasular\ event}{(event = 1.2, else = 1)}\right)$$

Complete internist-cardiology examination: ECG, BP and other methods necessary or possible to determine the cardiac status were carried out.

Ethics

The Ethics Committee of the Faculty of Medicine, University of Belgrade approved the present study. All patients have given their consent.

Statistics

Data are presented as count (%) or mean ± standard deviation, depending on data type. Student's *t*-test and Mann-Whitney *U* test were used to assess significant differences between groups. Pearson's correlation was used to explore the significant relationship between Hcy and other parameters. All *p* values less than 0.05 were considered significant. All data were analyzed using SPSS 20.0 (IBM corp.) statistical software.

Results

Average age of 68 patients with MS was 46.69 ± 15.04 years, while average age of patients without MS was 47.73 ± 16.66 years ($p > 0.5$). MS was found in 45.95% of 80 patients. The gender distribution was as follows: in the MS group, there were 20.3% of male and 79.7% of female patients, while in the group of MS free patients, there were 5.6% of male and 94.4% of female patients.

Anthropometric parameters (BW, BMI, WC, systolic BP, diastolic BP, mean BP ($p < 0.001$) were statistically much higher in patients with MS than in patients without MS. Higher fasting glycemia ($p = 0.006$) and significantly higher values of triglycerides ($p < 0.001$) as well as lower HDL-cholesterol ($p < 0.001$) were also found in patients with MS (Table 1). The distribution of patients regarding to each criterion, showed that the increased WC had 88.0% of patients, 48.2% of patients had hypertension, 21.2% of patients had hyperglycemia, 45.9% of patients had increased triglyceride values and 38.1% of patients had decreased HDL-cholesterol.

Table 1

Anthropometrical and biochemical parameters in patients with metabolic syndrome (MS) and without MS

Parameter	With MS	Without MS	*p*-value
Age (years), mean ± SD	46.7 ± 15.0	47.7 ± 16.7	0.695
Gender (male), n (%)	21 (30.9)	15 (18.8)	0.086
Alcohol consumption, n (%)	13 (20.3)	4 (5.6)	0.009
BW (kg), mean ± SD	97.3 ± 20.1	82.7 ± 17.1	< 0.001
BMI (kg/m^2), mean ± SD	33.2 ± 6.1	29.5 ± 6.1	< 0.001
WC (cm), mean ± SD	105.8 ± 14.4	92.7 ± 14.2	< 0.001
sBP (mmHg), mean ± SD	135.8 ± 12.1	118.7 ± 11.2	< 0.001
d BP (mmHg), mean ± SD	88.1 ± 8.8	77.8 ± 8.8	< 0.001
BP mean (mmHg), mean ± SD	104.0 ± 8.9	91.4 ± 9-2	< 0.001
Cholesterol (mmol/L), mean ± SD	5.9 ± 1.2	5.8 ± 1.2	0.669
HDL-C (mmol/L), mean ± SD	1.22 ± 0.3	1.45 ± 0.3	< 0.001
LDL-C (mmol/L), mean ± SD	3.65 ± 1.2	3.7 ± 1.1	0.627
Triglycerides (mmol/L), mean ± SD	2.1 ± 0.9	1.3 ± 0.5	< 0.001
Glycemia (mmol/L), mean ± SD	5.4 ± 1.4	4.9 ± 0.8	0.006

BW – body weight; BMI – body mass index; WC – waist circumference; sBP – systolic blood pressure; dBP – diastolic blood pressure; HDL-C – high density lipoprotein cholesterol; LDL-C – low density lipoprotein cholesterol; SD – standard deviation.

Table 2
Metabolic syndrome (MS) associated parameters in patients with metabolic syndrome (MS) and without MS

Parameter	With MS	Without MS	*p*-value
Homocysteine (μmol/L)	13.3 ± 3.6	12.9 ± 4.2	0.119
Insulin fasting (mlU/L)	31.2 ± 31.5	20.9 ± 16.8	0.007
Insulin at 120 minute (mlU/L)	61.6 ± 42.3	45.8 ± 45.4	0.014
Mean value insulin (mlU/L)	63.0 ± 52.8	52.8 ± 36.8	0.066
HOMA IR (μmol/mU/mL)	7.7 ± 8–6	4.5 ± 3.7	0–002
CRP (mg/dL)	5.5 ± 6.8	3.7 ± 5.4	0.00
Uric acid (μmol/L)	359.2 ± 85–6	307.3 ± 77.9	< 0.01
Fibrinogen (g/L)	268-5 ± 62.8	253.4 ± 63.1	0.153
Thrombocytes (10^9/L)	3.8 ± 0–8	3.7 ± 0–7	0.517
PAI-1 (U/mL)	5.98 ± 1.84	5.77 ± 1.78	0.776
AST (U/L)	25.2 ± 15.2	22.9 ± 7.6	0.227
ALT (U/L)	29.7 ± 20.3	23.0 ± 11.8	0.007
GGT (U/L)	28.7 ± 14.8	19.5 ± 11.9	< 0.01
Urea (mmol/L)	4.9 ± 1.3	4.7 ± 1.1	0.467
Creatinine (μmol/L)	76.9 ± 15.8	72.9 ± 15.1	0.115
Creatinine clearance (mL/min)	121.7 ± 57.3	111.4 ± 33.4	0.224
Microalbuminuria (mg/24 h)	81.9 ± 80.8	55.9 ± 57.4	0.225
ApoB (g/L)	1.64 ± 0.38	1.61 ± 0.27	0.603
ApoA1 (g/L)	1.12 ± 0.28	0.96 ± 0.26	0-004
Apo A2 (g/L)	357.9 ± 65.5	351.0 ± 66.6	0.674
Apo E (g/L)	47.0 ± 12.3	43.3 ± 14.5	0.255
Lp (a) (g/L)	0.166 ± 0.185	0.236 ± 0.290	0.223

The results are expressed as mean value ± standard deviation.
HOMA IR – Homeostatic Model Assessment of Insulin Resistance; CRP – C-reactive protein; PAI-1 – plasminogen activator inhibitor-1; AST – aspartate aminotransferase; ALT – alanine aminotrasferase; GGT – gamma glutamyl transferase; Apo – apolipoprotein; Lp – lipoprotein.

Insulin values (p = 0.007), insulin at 120 min during OGTT (p = 0.014), mean value insulin in OGTT (p = 0.066) and HOMA-IR (p = 0.002) were significantly higher in patients with MS. Higher Apo B (p = 0.01), CRP (p = 0.01), uric acid (p < 0.001) and liver enzymes ALT (p = 0.007) and GGT (p < 0.001) were also found in patients with MS. Thrombocytes, fibrinogen, PAI-1, urea, creatinine, creatinine clearance, microalbuminuria values were higher in patients with MS then in those without MS but without any significance (p > 0.5) (Table 2).

In order to determine the effect of MS on liver enzymes, a dual factorial analysis of variance was used, in which MS and alcohol consumption were independent variable, and ALT and GGT were dependent variables. Based on this analysis, it was found that MS correlates with ALT and GGT independently of alcohol consumption (p = 0.011; p < 0.001).

Presence of MS risk factors in patients with MS was as follows: 6.9% patients were with no MS factors, 17.2% had one MS factor, 31% had two MS factors, 26.9% had three MS factors, 15.2% had four MS factors and 2.8% had all five MS factors.

Hcy correlated significantly (Pearson's correlation) with values of thrombocites (p=0.046), urea (p = 0.002), creatinine (p = 0.006), creatinine clearance (p = 0.047) and siMS risk score (p = 0.015).

The siMS score confirmed significant correlation with log CRP, uric acid, log HOMA IR, log GGT (p < 0.001), log ALT (p = 0.001), thrombocytes (p = 0.01), fibrinogen (p = 0.005), proteins (p = 0.006), creatinine (p = 0.013). This risk score showed a statistically significant correlation with values of urea (p < 0.01), albumin (p = 0.003), total proteins (p = 0.057), thrombocytes (p = 0.046), uric acid (p = 0.038), and Hcy (0.015) (Table 3).

Table 3
Pearson' correlation analysis of siMS score and siMS risk score, and various parameters of metabolic syndrome (MS)

Parameter	siMS score	siMS risk score
Homocysteine	0.120 (0.177)	0.215 (0.015)
Log HOMA IR	0.457 (< 0.001)	0.130 (0.181)
Log CRP	0.333 (< 0.001)	-0.125 (0.189)
Uric acid	0.336 (< 0.001)	0.183 (0.038)
Fibrinogen	0.250 (0.005)	-0.099 (0.272)
Thrombocytes	0.281 (0.001)	-0.176 (0.046)
Log ALT	0.281 (0.001)	0.105 (0.237)
Log GGT	0.369 (< 0.001)	0.114 (0.211)
Total proteins	0.241 (0.006)	-0.168 (0.057)
Albumin	0.037 (0.681)	-0.265 (0.003)
Urea	0.040 (0.649)	0.388 (< 0.001)
Creatinine	0.218 (0.013)	-0.115 (0.191)

***Results are presented as correlation coefficient rho and *p*-value (in brackets).**
HOMA – Homeostatic Model Assessment; CRP – C-reactive protein; GGT – gamma glutamyl transferase.

Figure 1 shows the correlation between siMS score and log HOMA IR, log CRP, fibrinogen, log ALT, log GGT, and Hcy.

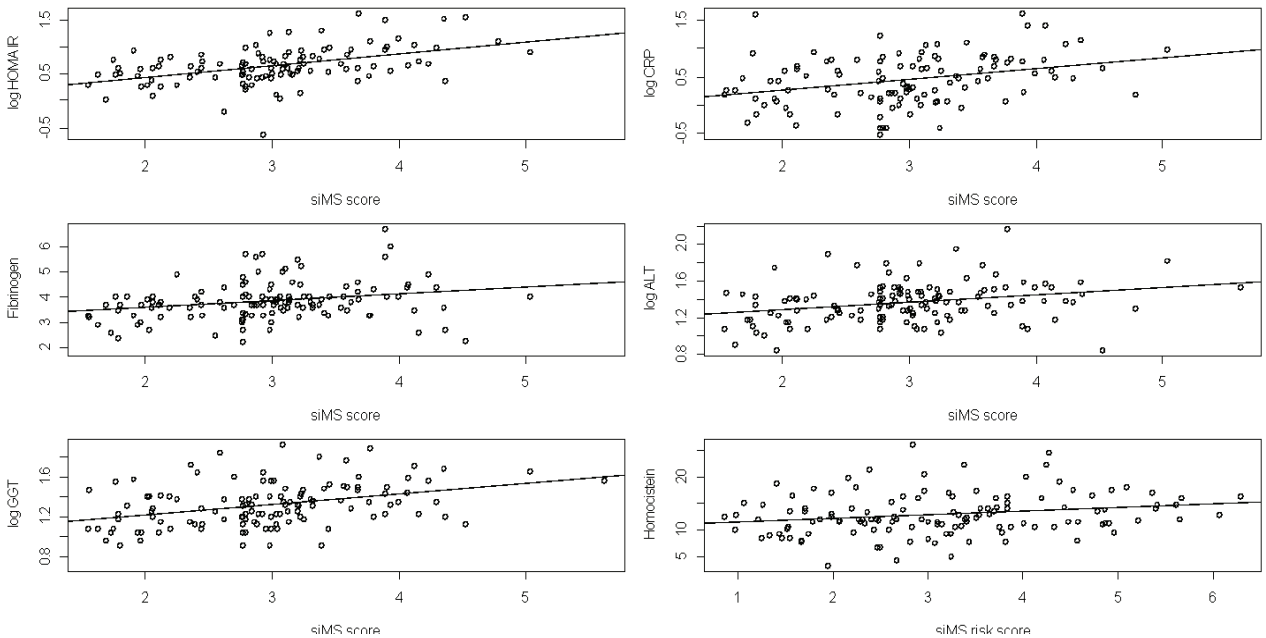

Fig. 1 – Correlation between siMS score and log Homeostatic Model Assessment Insulin Resistance (HOMA IR), log C-reactive protein, fibrinogen, log alanine aminotransferase (ALT), log gamma glutamyl transferase (GGT) and homocystein.

Discussion

Chronic diseases as diabetes, osteoporosis, hypothyroidism, as well as renal dysfunction and diet are considered to be associated with moderately elevated Hcy concentrations [15]. Hcy is amino acid formed in metabolism cycle of methionine to cysteine. HHcy is recognized as an independent risk factor for atherosclerosis [14]. Connection of Hcy and insulin resistance is explained by disruption of insulin signaling by Hcy interfering phosphorylation of insulin receptors. The result of this impaired insulin receptor signal cascade is lowered GLUT4 translocation to the plasma membrane and therefore reduced glucose uptake [16]. Catena et al. [2] showed that plasma Hcy was directly correlated with age, a factor of MS and insulin resistance, while inversely correlated with creatinine clearance and HDL-cholesterol, vitamin B12, and folate levels. A correlation of Hcy with hypertension and hyperlipoproteinemia in our previous studies indicates that Hcy can be an important indicator of risk for atherosclerotic complications and their progression [12]. Sheu et al. [17] found in their studies higher Hcy values in hypertensive patients than in normotensive ones, and significant correlation of plasma Hcy with insulin values in OGTT was also found. The latest results of our studies showed a positive correlation of Hcy with a long term glycoregulation parameter HbA1C, HOMA-IR, Apo B, and negative correlation with Apo E. The siMS score significantly correlated with Hcy, uric acid, microalbuminuria, a thrombosis factor – fibrinogen, an inflammation factor – CRP, and confirmed that these are metabolic syndrome assiciated factors [7]. Our study in patients with coronary heart disease showed correlation between Hcy and systolic BP, triglycerides and uric acid, which confirms association of Hcy with insulin resistance and MS as well as the further risk of atherosclerosis complications [18].

Patients with MS covered by the present study were characterized by statistically important much higher values of anthropometric parameters (BW, BMI, WC), BP, triglycerides, insulinemia in OGTT at 0 min and 120 min, mean value of insulin levels, HOMA-IR, CRP, uric acid, Apo B as well as liver function parameters ALT and GGT as markers of NAFLD, and statistically lower HDL-cholesterol. Summarized above mentioned, these results showed that patients with MS had higher values of basic cluster factors of MS (WC, hypertension, hyperlipoproteinemia type IV) as well as values of associated factors of MS such as hyperinsulinemia, insulin resistance, CRP, uric acid and NAFLD.

Abdominal obesity and insulin resistance have a significant role in MS development [19]. Recent studies have shown that patients with MS have significantly higher levels of high sensitive CRP, compared to the control group, which is a marker of chronic inflammation in patients with MS, whose values increased linearly with the increase number of factors for MS [20]. Obesity is characterized by elevated levels of inflammatory factors such as CRP and prothrombogenic factors such as fibrinogen, which occur before other MS disorders and are useful in the assessment of cardiovascular risk [21].

Results obtained by Dimitrijević-Srećković et al. [22] indicate the existence of NAFLD even in the youngest obese population: children (7.3%), adolescents (18.9%), and youth 20 to 30 years old (29%). NAFLD is a liver sign of MS, while youth with NAFLD manifested, besides increased ALT and GGT values, abdominal obesity, hyperinsulinism in OGTT, pronounced insulin resistance, increased triglyc-

erides, CRP and uric acid. The study of Čolak et al. [23] have also shown elevated liver enzymes in obese students with increased risk for CVD. Other studies of obese and adolescent population indicate the association of NAFLD with insulin resistance [24].

In the present study, siMS score showed a correlation with MS associated factors (log CRP, uric acid, log HOMA-IR, fibrinogen, thrombocytes), liver parameters (log ALT, log GGT) and hyperproteinemia, retention of nitrogen substances and increased risk for kidney damage. The correlation of siMS score with liver function parameters indicates that fatty liver is a MS associated factor. Hcy is an intermediate in methionine metabolism, which takes place mainly in the liver [25]. Impaired remethylation of Hcy to methionine leads to increased levels of Hcy promoting the liver damage from NAFLD to non alcoholic steatohepatitis [26].

Correlation of siMS score with renal function parameters, creatinine and total proteins, as shown in the present study, indicates that even initial renal function disorders can represent MS associated factors. Higher values of microalbuminuria in patients with MS, compared to patients without MS, indicate the initial stage of kidney damage in obese patients. Our previous study has shown the appearance of microalbuminuria in obese children, adolescents and young people, which is normalized after weight reduction [27]. Correlation of homocysteine with platelets, renal function parameters (urea, creatinine, creatinine clearance) and siMS risk score was also confirmed. Hyperprotein diet based on meat and dairy products, most frequent in MS obese people nutrition, can contribute to increased glomerular filtration with increased creatinine clearance and provoke HHcy and renal damage. Berstad et al. [28] have shown that higher intake of animal saturated fatty acids correlates positively with higher Hcy levels. Microalbuminuria as associated factor of MS is a strong indicator of CVD and renal dysfunction. It is suggested that HHcy enhances oxidative stress, inducing endothelial and mesangial cell dysfunction, resulting in microalbuminuria [29]. High animal-protein diet correlates positively with high Hcy levels, whereas high plant-protein diet inversely correlates with total Hcy levels [30]. A correlation of siMS score with liver and renal function parameters indicates that disorders of these systems could appear in obese MS patients as associated MS factors. HHcy can be caused by increased intake of proteins from dairy and meat products abounding in saturated fats of animal origin and reduced intake of vegetables rich in folic acid, which all contributes to progression of atherosclerotic complications, fatty liver and renal damage.

In the present study, siMS risk score correlated with Hcy, platelets, uric acid and renal function parameters (urea, total albumins and proteins). Our results also indicate that HHcy increases with age and represents a vascular complications risk indicator. Correlation with uric acid indicates that hyperproteinic intake could contribute considerably to vascular complications and values of total proteins and albumins.

Mediterranean diet, rich in dietary fibers and complex carbohydrates in fruits, vegetables and cereals, monounsaturated fats in olive oil, omega-3 polyunsaturated fats in fish and reduction of saturated fats and proteins of animal origin proved favorable effects on body mass reduction, glycoregulation, hypertension, lipid status, insulin resistance, inflammatory and thrombotic factors, and HHcy [30]. Han et al. [31] highlight the importance of increasing folic acid and vitamin B supplementation, diet which consists of daily fruit and vegetable intake, healthy lifestyle based on regular exercise and refraining from tobacco smoking and alcohol consumption for prevention of HHcy.

Conclusion

Patients with MS had statisticaly significant higher values of MS associated factors (HOMA-IR, CRP, uric acid, ALT, GGT) which correlated well with siMS score. siMS score correlation with fibrinogen, creatinine and proteins indicated that thrombosis factors, so as renal function parametars could be associated with MS. Used as a method of MS quantification, siMS score confirmed that MS patients have an increased risk for glycoregulation disorders – prediabetes and diabetes type 2 (abdominal obesity followed by hyperinsulinism and insulin resistance), hyperlipoproteinemia type IV (elevated triglycerides and low HDL-cholesterol), and hypertension. siMS score further indicated that insulin resistance, IR, CRP, fibrinogen, uric acid and NAFLD are associated factors of MS. siMS risk score is another score that indicated that obesity and hyperprotein diet aggravates HHCy with age, increasing the risk for renal dysfunction and promoting atherosclerotic complications.

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Efficacy and safety of pegylated-interferon alpha therapy in patients with chronic hepatitis B in recource-limited settings: A Serbian single-center experience

Efikasnost i bezbednost pegilovanog interferona alfa-2a u terapiji hroničnog virusnog hepatitisa B u uslovima ograničenih resursa: iskustvo jednog centra u Srbiji

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Abstract

Background/Aim. In Serbia, pegylated interferon (PEG-IFN) alpha-2a has been registered since 2013 for the treatment of patients with chronic hepatitis B (CHB). Numerous advantages, new experiences during the past five years and lack of any published data in our specific population, have initiated this study, with the aim to examine efficacy and safety of PEG-IFN in patients in a Serbian referral center. **Methods.** This prospective study included 36 patients with CHB who were treated in the Hepatology Department of the Clinic for Infectious and Tropical Diseases, Clinical Center of Serbia in Belgrade, during 2012–2017. Patients had a standard 48-week treatment protocol with PEG-IFN, with measurements of liver enzymes, serology and viraemia before, during, at the end of the treatment and follow-up 6 months afterwards. Treatment outcome was determined using serology (clearance of HBeAg), biochemical [normalization of alanine aminotransferase (ALT)] and virological response [hepatitis B virus (HBV) DNA < 2,000 IU/mL]. **Results.** Virological success in patients with HBeAg positive CHB was achieved in 50% of patients, HBeAg clearance in 62.5%, and normalization of ALT in 37.5% of patients. In patients with HBeAg negative CHB, 38% of the patients achieved virologic success, biochemical success was obtained in 47.6% of the patients and only one (4.7%) patient had HBsAg clearance. **Conclusion.** PEG-IFN is important for treatment of patients with CHB in well-defined situations, and in our population success rates are similar to other published studies. Although safety and tolerability are satisfactory, there is a possibility of more serious side-effects so it is necessary to monitor patients regularly during the treatment.

Key words:
biochemistry; hepatitis b; antigens; hepatitis b, chronic; peginterferon alfa-2; treatment outcome; virology.

Apstrakt

Uvod/Cilj. Pegilovani interferon (PEG-IFN) alfa-2a je registrovan u Srbiji od 2013. godine za lečenje bolesnika sa hroničnim hepatitisom B (HHB). S obzirom na njegove mnogobrojne prednosti u odnosu na dotadašnju terapiju, nova iskustva u periodu od proteklih pet godina i nedostatak publikovanih rezultata u našoj populaciji, cilj rada bio je da se prvi put među bolesnicima sa HHB u Srbiji ispita efikasnost i bezbednost primene PEG-IFN u tercijarnoj zdravstvenoj ustanovi. **Metode.** U prospektivnoj studiji u petogodišnjem periodu od 2012. do 2017. godine analizirano je ukupno 36 bolesnika sa HHB, lečenih standardnim protokolom PEG-IFN tokom 48 nedelja, u Hepatološkom odeljenju Klinike za infektivne i tropske bolesti Kliničkog centra Srbije u Beogradu. Svim bolesnicima su merene bazalne vrednosti transaminaza, serologije i viremije, uključujući praćenje tih parametara tokom terapije, na kraju terapije i u periodu praćenja. Za procenu uspeha terapije analiziran je serološki odgovor (gubitak HBeAg), biohemijski odgovor [normalizacija alanin aminotransferaze (ALT)] i virusološki odgovor na terapiju [supresija DNK hepatitis B virusa (HBV) < 2000 IU/mL. **Rezultati.** Virusološki uspeh terapije kod bolesnika sa HBeAg pozitivnim HHB postignut je kod 50% bolesnika, gubitak HBeAg kod 62,5%, a biohemijski odgovor kod 37,5% bolesnika. Kod HBeAg negativnog HHB, virusološki uspeh terapije postignut je kod 38% bolesnika, biohemijski odgovor kod 47,6%, a samo jedan (4,7%) bolesnik imao je i gubitak HBsAg. **Zaključak.** Primena PEG-IFN u

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lečenju HBV infekcije važna je u dobro selektovanoj grupi bolesnika, a u našoj populaciji lečenih bolesnika procenat uspešnosti terapije sličan je onom od drugih autora. Bezbednost i podnošljivost terapije je dobra, ali se mogu očekivati i ozbiljniji neželjeni događaji zbog čega je neophodno redovno praćenje bolesnika tokom lečenja.

Ključne reči:
biohemija; hepatitis b; antigeni; hepatitis b, hronični; interferon alfa-2a, pegilovani; lečenje, ishod; virologija.

Introduction

Hepatitis B viral infection (HBV) remains a significant challenge in hepatology, in spite of decades of successful immunization worldwide. In Serbia, compulsory immunization against HBV was introduced in 2000 for all newborns, followed by additional campaigns including adolescents and healhcare workers. However, the number of patients with chronic hepatitis B (CHB) infection seeking medical treatment is still significant, although there are only estimates and no published data concerning prevalence in the Serbian population.

Treatment of CHB is primarily oriented towards prevention of disease progression to end-stage liver disease including cirrhosis and hepatocellular carcinoma [1, 2]. Ideally, the ultimate treatment goal is the eradication of viral DNA, which still remains elusive. Current recommendations include two groups of drugs: oral nucleoside/nucleotide analogues (lamivudine, adefovir, entecavir, emtricitabine, tenofovir) and pegylated interferons (PEG-IFN alpha 2a and 2b) [1]. However, both treatment options have certain disadvantages. Although PEG-IFN is a less potent antiviral, it has an additional immunomodulatory effect which may account for durability of sustained virological suppression. The significant advantage of PEG-IFN lies in the absence of drug resistance, clearly defined treatment duration and higher anti HBe seroconversion rates. Other disadvantages, beside less potent antiviral effect, include tolerance issues with more side effects compared to oral analogues [1, 2].

In Serbia, the only treatment options were lamivudine and interferon alpha-2a, until 2012. But as the efficacy and tolerability of interferon are very poor, in everyday practice the single reliable treatment option for patients with CHB was lamivudine. After 2012, two more treatment options became available – second oral analogue tenofovir disoproxyl fumarate (TDF) and, in 2013, PEG-IFN alpha-2a. However, our treatment experience with PEG-IFN during past five years is limited, due to strict selection criteria for its application: elevated alanine aminotransferase (ALT) > 5 times upper limit, basal HBV DNA < 10^7 UI/mL and intermediate necroinflammatory activity in liver histology. Very few patients with CHB fulfilled these criteria, especially due to the fact that in our surroundings virology testing [HBV DNA polymerase chain reaction (PCR)] was often unavailable due to limited resources and funding, not only for treatment of naive patients but also patients who had commenced treatment. Although PEG-IFN was officially registered for the treatment of CHB in 2013, our study included an additional number of 20 patients treated with the donated PEG-IFN in 2012 through a compassionate treatment programme. This treatment group was selected mostly based on clinical judgment, and not always according to the mentioned criteria, especially due to the unavailability of HBV DNA PCR testing.

The aim of study was to present the results of our 5-years treatment experience with PEG-IFN in patients with CHB and to examine its efficacy and possible predictors of sustained virological response concerning tolerability issues and side effects in our study population, as well.

Methods

This study was performed in order to examine the efficacy and safety of PEG-IFN in patients with CHB, who were treated in the Hepatology Department of the Clinic for Infectious and Tropical Diseases, Clinical Center of Serbia in Belgrade during 2012–2017. In total 36 patients consented to participate in this study and fulfilled inclusion criteria: diagnosis of CHB, HBV DNA > 20,000 IU/mL for hepatitis B extractable antigen (HBeAg) positive patients and for HBeAg negative patients, HBV DNA > 2,000 UI/mL, elevated ALT > 5 x upper limit. Exclusion criteria were: hepatitis C and/or HIV coinfections, liver decompensation, active substance abuse, alcohol consumption.

All patients were treated with PEG-IFN alpha-2a 180 μg subcutaneously, once a week for 48 weeks. Pretreatment patient data were collected from patients records, including demographic data (sex, age), previous treatment options, pretreatment levels of ALT and HBV DNA, the presence of HBeAg, and liver histology reports including METAVIR scores. Liver enzymes and full blood counts were analyzed every 4 weeks during treatment, and then during the follow-up period in 12th and 24th weeks after the treatment was finished. Serology, i.e. HBeAg and anti HBe antibodies were analysed every 12 weeks. In 16 patients basal levels of hepatitis B surface antigen (qHBsAg) were available, then retested in 10 patients during duration of the treatment every 12 weeks, at the end of the treatment and during 6-months follow-up period. Level of HBV DNA was determined in all patients in the same time intervals as previously mentioned.

Treatment success was considered as primary and secondary. Primary treatment success was defined in HBeAg positive patients as end-treatment HBeAg seroconversion and viral suppression of HBV DNA < 2,000 IU/mL. In HBeAg negative patients primary treatment success was defined as end-treatment favourable virological response – HBV DNA < 2,000 IU/mL. Secondary treatment success in HBeAg positive patients included HBeAg seroconversion, sustained suppression of HBV DNA < 2,000 IU/mL and clearance of HBsAg 24 weeks after the end of the treatment. In HBeAg negative patients end-treatment success was con-

sidered as sustained viral suppression of HBV DNA < 2,000 IU/mL and HBsAg elimination 24 weeks after treatment. All possible predictors of treatment success were analysed including pretreatment blood tests (liver enzymes, blood count), serology (HBeAg) as well as levels of qHBsAg and HBV DNA before, during and after the treatment.

Biochemical analysis of blood samples was performed using Siemens Dimension Xpand® biochemistry analyzer in the Center for Medical Biochemistry, Hepatology Department of the Clinic for Infectious and Tropical Diseases, Clinical Center of Serbia. Hepatitis B serology (HBsAg, HBeAg, anti-HBeAb) was performed using commercial ELISA tests (Abbot Laboratories, North Chicago, IL, USA) at the Virology Laboratory, Microbiology Department, Clinical Center of Serbia.

HBV DNA was analysed in the same departments using CombasAmpliPrep/CobasTaqMan HBV essay (CAP/CTM version 2.0, Roche Diagnostics Indianapolis, IN, USA with detection levels of 10–107 UI/mL). Quantitative HBsAg was performed in the Biochemistry Laboratory of the Clinical Center "Zvezdara" using Architect HBsAgQT assay (Abbott Diagnostic Germany) with sensitivity levels of 0.05–250 UI/mL. Safety and tolerability of the treatment were assessed during clinical examinations and check-ups, based on the occurrence of side effects and completion of the full treatment protocol. All patients who received at least one dose of PEG IFN were included in safety and tolerability examination.

Statistical analysis was performed using SPSS®, version 11.5 and included both descriptive and analytical methods. Patients were categorised according to the presence of HBeAg into two groups – HBeAg positive and HBeAg negative. Both parametric (Student *t*-test) and nonparametric tests (χ^2, Fisher test) were used, depending on the normality of variables. Linear regression and Spearman's correlation rank were also computed for the analysis of association. Values at the $p \leq 0.05$ level were considered statistically significant, the confidence interval (CI) was 95% and all performed tests were 2-tailed.

All participants provided their informed consent and the study protocol was performed according to the Helsinki declaration, including Ethics Committee permission and institutional approval.

Results

Baseline (pretreatment) patients characteristics

Studied patients (n = 36) were mostly male (86.5%, X2 = 19.7, $p < 0.0001$), with an average age of 37.9 ± 12 years (interval ranging from 18–60 years). Patients were categorised according to the presence of HBeAg into two study groups, with a predominance of HBsAg negative form of CHB (72.2%), but there was no statistically significant difference in age or sex distribution between study groups (Table 1). Most of the patients were treatment naive (83.3%). However, six patients were previously treated with lamivudine, but there was no statistically significant difference in distribution between the two study groups (Table 1). Although most of the patients had a lower degree of fibrosis, four patients had cirrhosis (11.4%), but without differences between study groups ($p = 0.718$). Elevated activity of ALT > 2 x upper limit was registered in most of the patients (72.4%), but there was no significant difference in gradations of enzyme activity between two study groups ($p = 0.308$). Average viraemia was 7.4 log (5.2–8.2) IU/mL, without statistically significant differences between groups (Table 1).

In 44.4% of the patients (16/36) baseline level of qHBsAg was performed, averaging 8,400 UI/mL (345–42,390 UI/mL); however, there was no statistically significant correlation with baseline viraemia ($\rho = -0.082$, $p = 0.589$).

Table 1
Baseline (pretreatment) demographic, clinical and laboratory characteristics of patients with chronic hepatitis B

Variable	Patients			*p*
	total (n = 36)	HBeAg+ (n = 10)	HBeAg- (n = 26)	
Sex, n (%)				
male	31 (86.1)	9 (90)	22 (84.6)	0.676
female	5 (13.9)	1 (10)	4 (15.4)	
Age (years), mean ± SD	38±12	32.1 ± 8.7	40.2 ± 12.6	0.072
Previous treatment, n (%)				
lamivudine	6 (16.7)	3 (30)	3(11.5)	0.317
treatment-naive	30 (83.3)	7 (70)	23 (88.5)	
[1]Liver histology				
F0	4 (11.4)	1 (11.1)	3 (11.5)	0.718
F1	8 (22.9)	1 (11.5)	7 (26.9)	
F2	13 (37.1)	5 (55.6)	8 (30.8)	
F3	6 (17.1)	1 (11.1)	5 (19.2)	
F4	4 (11.4)	1 (11.1)	3 (11.5)	0.972
Elevated ALT, n (%)				
< 2x upper limit	10 (27.8)	1 (10)	9 (34.6)	0.308
2x–5x upper limit	16 (44.4)	6 (60)	10 (38.5)	
> 5x upper limit	10 (27.8)	3 (30)	7 (26.9)	
HBV DNA ($\log_{10}$ IU/mL), mean ± SD	7.4 ± 0.9	7.9 ± 0.4	7 ± 1	0.068

HBeAg – hepatitis B extractable antigen; [1]METAVIR score; HBV – hepatitis B virus; ALT – alanine aminotransferase (upper limit of ALT > 37 IU/L); SD – standard deviation.

Efficacy of PEG- IFN alpha-2a in patients with HBeAg positive CHB

Full treatment protocol of 48 weeks was completed in 8 patients (80%), and in two patients due to the rise in HBV DNA, it was stopped in 12th week. HBeAg clearance after 24 weeks and at the end of the treatment was observed in 50% of patients (4/8), and after follow-up period (6 months after the end of the treatment) in 62.5% of the patients (5/8). However, HBeAg seroconversion with anti-HBeAb was present in only 25% (2/8) of the patients. The only statistically significant baseline factor that influenced HBeAg clearance was the presence of HBcIgM ($p = 0.008$).

Biochemical response, e.g. ALT normalization was achieved at the end of the treatment and after follow-up period in 37.5% (3/8) of the patients (Table 2).

Table 2
Efficacy of pegylated interferon (PEG-IFN) in patients with HBeAg+ chronic hepatitis B (n = 8)

Parameters	End of the treatment (48 weeks)	Follow-up (72 weeks)
Serology response, n (%)		
clearance HBeAg	4 (50)	5 (62.5)
clearance HBsAg	0	1 (12.5)
Virological response, n (%)		
< 2000 IU/mL	1 (12.5)	2 (25)
undetecteble viraemia	3 (37.5)	2 (25)
Biochemical response, n (%)		
ALT normalization	3 (37.5)	3 (37.5)

HBeAg – hepatitis B extractable antigen; HBsAg – hepatitis B surface antigen; ALT – alanine aminotransferase.

In 50% of the patients (4/8) virological suppression was achieved with end-treatment HBV DNA < 2,000 IU/mL, including three patients with undetectable viraemia (Table 2). After the follow-up, virological response of HBV DNA < 2,000 IU/mL was sustained in 50% (4/8) of the patients. The success rates remained unchanged, although one of the patients who had achieved end-treatment success had relapsed (HBV DNK 16,400 UI/mL, ALT > 2x), as another patient who had end-treatment failure had a HBV reduction < 2,000 IU/mL with reduction of ALT > 1.5x upper limit during follow-up period. Undetectable viraemia was sustained in 25% of the patients (2/8), among whom one had HBsAg elimination.

Complete treatment success, eg. secondary success was confirmed in only one (1.25%) patient in this group. This patient had HBeAg clearance after follow-up period of 24 weeks, undetectable viraemia and negative HBsAg. After a year of follow-up period, this patient had achieved seroconversion and anti-HBsAb.

The in-depth statistical analysis did not identify possible predictors of sustained virological response, such as sex, age, previous lamivudine treatment, baseline values of ALT, HBV DNA < 2,000 IU/mL after 12 weeks of the treatment, baseline qHBsAg ($p > 0.05$) (Table 3).

Efficacy of PEG IFN alpha-2a in patients with HBeAg negative CHB

Treatment protocol of 48 weeks was completed in 21 patients with HBeAg negative CHB. In five patients it was stopped before the completion due to early virological failure (in two patients) and serious side-effects (three patients).

Favourable end-treatment biochemical response was achieved in 23.8% (5/21) of the patients, with an additional number of patients who achieved ALT normalization during follow-up period [47.6% (10/21)] (Table 4).

Table 4
Efficacy of pegylated interferon (PEG-IFN) in patients with HBeAg+ chronic hepatitis B (n = 21)

Parameter	End of the treatment (48 weeks)	Follow-up (6 months)
Biochemical response, n (%)		
ALT normalization	5 (23.8)	10 (47.6)
Serology response, n (%)		
clearance of HBsAg	1 (4.7)	1 (4.7)
Virological response, n (%)		
< 2,000 IU/mL	15 (71.42)	7 (33.33)
undetectable viraemia	2 (9.52)	1 (4.76)

HBeAg – hepatitis B extractable antigen; HBsAg – hepatitis B surface antigen; ALT – alanine aminotransferase.

Table 3
Predictors of virological success after follow-up period in patients with HBeAg+ chronic hepatitis B (n = 4)

Parameter	HBeAg+		p^{α}
	HBV DNA < 2,000 IU/mL	HBV DNA > 2,000 IU/mL	
Male, n (%)	4 (100)	3 (75)	0.356
Age (years), mean ± SD	37 ± 11	28 ± 5	0.201
Severe fibrosis$^{\beta}$, n (%)	1 (25)	1 (25)	1.000
Cirrhosis, n (%)	1 (25)	0	0.708
Lamivudine-experienced, n (%)	1 (25)	1	1.000
Baseline HBV DNA (log10 IU/mL), mean ± SD	7.2 ± 1.8	7.6 ± 0.5	0.666
Baseline ALT (U/L), mean ± SD	148 ± 62	177 ± 119	0.624
> 5x upper limit, n (%)	1 (25)	3 (75)	0.437
Baseline HBsAg ($\log_{10}$ IU/mL), mean	4.31	4.62	0.157
HBV DNA ($\log_{10}$ IU/mL , 12th week), mean ± SD	4.08 ± 2.9	5.9 ± 2.1	0.709
HBV DNA decline >2 log 12th week, n (%)	3 (75)	4 (100)	0.748
HBsAg < 150 IU/mL ,12th week, n (%)	0	0	1.000

$^{\alpha}$logistic regression, significance level $p < 0.05$; $^{\beta}$patients with METAVIR > F3; HBV – hepatitis B virus; ALT – alanine aminotransferase (upper limit of ALT > 37 IU/L); SD – standard deviation; HBeAg – hepatitis B extractable antigen; HBsAg – hepatitis B surface antigen.

Table 5

Predictors of virological success after follow-up period in patients with HBeAg- chronic hepatitis B

Parameter	HBeAg-		p^{α}
	HBV DNA < 2,000 U/mL (n = 8)	HBV DNA > 2,000 IU/mL (n = 13)	
Male, n (%)	8 (100)	11 (84.6)	0.266
Age (years), mean ± SD	34.6 ± 15.9	43.7 ± 11	0.136
Severe fibrosis$^{\beta}$, n (%)	3 (37.5)	5 (38.4)	0.764
Cirrhosis, n (%)	2 (25)	1 (7.7)	0.769
Lamivudine-experienced, n (%)	1 (12.5)	2 (15.4)	0.865
Baseline HBV DNA (log_{10} IU/mL), mean ± SD	6.05 ± 1.32	5.85 ± 1.1	0.346
Baseline ALT (U/L), mean ± SD	151 ± 120	148 ± 199	0.947
> 5x upper limit, n (%)	1 (12.5)	5 (38.4)	0.213
Baseline HBsAg (log_{10} IU/mL), mean ± SD	3.5 ± 0.4	3.4 ± 0.09	0.208
HBV DNA (log_{10} IU/mL ,12th week), mean ± SD	2.92 ± 1.45	3.66 ± 1.58	0.639
HBV DNA decline > 2 log 12th week, n (%)	5 (62.5)	9 (69.2)	0.765
HBsAg < 150 IU/mL 12th week, n (%)	0	1 – (7.7)	1.000

$^{\alpha}$logistic regression, significance level $p < 0.05$; $^{\beta}$patients with METAVIR > F3; HBeAg – hepatitis B extractable antigen; HBsAg – hepatitis B surface antigen; HBV hepatitis B virus; ALT – alanine aminotransferase (upper limit ALT > 37 IU/L); SD – standard deviation.

End-treatment HBsAg clearance was achieved in one patient, but with a recurrence of HBsAg during follow-up period. At the end of follow-up, one patient had HBsAg clearance but without seroconversion to anti-HBsAb.

End-treatment virological response, eg. HBV DNA < ,000 IU/mL was achieved in 81% of the patients (17/21), of whom 9.5% (2/21) had undetectable viraemia (Table 4). After a relapse in ten patients during follow-up period, a sustained virological response was maintained in 38% of the patients (8/21), with a patient from this group who had achieved undetectable viraemia and normalization of ALT but without clearance of HBsAg (Table 4). In-depth statistical analysis of possible treatment outcome predictors, did not show a significant correlation between sustained virological response and following variables: age, sex, previous lamivudine treatment, baseline ALT, HBV DNA < 2,000 IU/mL after 12 weeks of the treatment, baseline HBsAg, reduction of HBsAg < 150 IU/mL after 12 weeks of the reatment ($p > 0.05$) (Table 5).

Kinetics of qHBsAg during treatment with PEG IFN

In 16/36 patients we were able to determine baseline qHBsAg in addition to viraemia, and in 10/16 kinetics of qHBsAg was followed during and after the treatment (two patients from this group had HBeAg positive CHB). During treatment, a decline in qHBsAg was observed in 6/10 (60%) of the patients after 12 weeks of the treatment. A further decline of qHBsAg after 24 weeks of the treatment was sustained in 7/10 patients, although we observed levels of HBsAg lower than 20,000 UI/mL in 9/10 patients. However, a continuous decline of qHBsAg even during the follow-up was observed in only one patient, who had achieved a sustained virological response. High baseline and treatment levels of qHBsAg were observed in patients with HBeAg positive CHB (2/10), which remained unchanged for the duration of treatment and follow-up period.

Safety

On-treatment stopping occurred in 7 (19.44%) patients, mostly [in 4 (11.1%) of patients] due to early virological failure after 12 weeks of the treatment. Due to serious side effects, the treatment was stopped in three patients (8.33%): *de novo* diagnosed ovarian cancer, a severe form of depression and debilitating myalgias and arthralgias in a patient who had an early virological response.

In five (17.24%) of the patients there were occasional dosage reductions of PEG-IFN due to the expected side effects – thrombocytopenia in 4 (13.79%) of the patients, neutropenia in one patient (6.89%). During the follow-up period, oral analogues were introduced in five patients (17.24%) due to the rise in liver enzymes over 10x upper limit and risk of hepatic decompensation.

Discussion

The quality of treatment for CHB has been significantly improved in Serbia during past years, especially with the introduction of tenofovir disoproxil fumarate and PEG-INF alfa-2a starting from 2012.

As the treatment with PEG-IFN is covered by state health insurance in Serbia, criteria of the National Health Fund for the administration are low viraemia HBV DNA < 107 copies/mL and elevation of liver enzymes > 2x upper limit [3]. These criteria are mostly fulfilled by younger patients in the immunoeliminatory phase of CHB, e.g. with chronic HBeAg positive hepatitis. However, among Serbian patients, the most predominant are those with HBeAg negative form of CHB, characterized by high viraemia and fluctuating levels of ALT. Unfortunately, due to common shortages and unavailability of HBV DNA PCR testing, as clinicians, we are often faced with a delayed and incorrect diagnosis of CHB in our patients. In this study, we were able to include an additional number of patients who did not fulfil

the National Health Fund criteria, and who were selected based on clinical judgement and treated with donated medication. This enabled us to include patients in the immunoreactive phase of CHB, who were previously not able to receive treatment with PEG-IFN, and reach a total of 36 patients which is a significant number for a single centre experience.

A large portion of patients (80.55%) completed full treatment protocol for the duration of 48 weeks. In seven (19.44%) of the patients treatment was stopped because of lack of early virological response as well as side effects.

Our results showed that in patients with HBeAg positive CHB, treatment with PEG-IFN resulted in HBeAg seroconversion in 62.5% of the patients, stable immunological control in 50% of the patients, with a complete success of the treatment in one patient (12.5%). Previously published results in different European centres have shown PEG-IFN treatment success rates for these patients ranging from 20%–30% [4, 5]. An important aim of treating HBeAg positive patients is the elimination of HBeAg, which was achieved in 62.5% of the patients, among whom a half (4/8) had achieved it during first 24 weeks of the treatment. This particular effect of PEG IFN during the first six months of the treatment has been previously observed by other authors, but the overall treatment success rates are higher after 48 weeks compared to shorter administration [5]. Combined treatment success in this group of patients, eg. HBeAg elimination with HBV DNA < 2,000 IU/mL ,was achieved in 23% of the patients in a study performed by Sonnevald et al. [4], similar to our results of 25%.

However, although we had only one occurrence of HBsAg elimination during follow-up in this group (12.5%), this is still significantly higher than in most published authors who report this in extremely low percentage of treated patients ranging from 3%–7%. One of the limitations for a possible interpretation of this particular result in our population is a small patient sample size. Biochemical response, e.g. ALT normalization in this group was achieved in five (62.5%) of the patients, but after follow-up this percentage was lower, reaching 37.5%. These rates were lower compared to most authors who reported biochemical response rates above 41% in patients with HBeAg positive CHB [4–6]. Possible differences in success rates may be due to different reference limits in numerous studies, as in our study we considered a level of 37 IU/mL normal, and every value measured above this limit at least twice during a three months period was considered elevated.

Virologic success rates of PEG-IFN in patients with HBeAg negative CHB have been reported around 44% [7, 8]. However, in patients with genotype D, these rates are lower, reaching 20% [2]. Although we were unable to perform genotype testing in our study, previously published genotype prevalence studies by Serbian authors report a predominance of genotype D which may explain our success rates of 38% [9, 10].

Elimination of HBsAg is a very rare treatment outcome in this group of patients, published results ranging from 3% after follow-up period of 24 weeks, up to 12% after 5 years [11, 12]. In our study, only one (4%) patient achieved HBsAg elimination, which is similar to results of foreign authors. Biochemical response, e.g. ALT normalization in this group was achieved in five (23.8%) of the patients, and the success rates were even higher after follow-up period (47.6%), similarly to other published results of 51% of patients with HBeAg negative CHB [1].

One of the most important advantages of achieving successful PEG-IFN treatment is its prolonged effect and sustainability of virological suppression even for years after successful completion as well as the rising percentage of HBsAg elimination during follow-up period [12, 13]. Marcellin et al. [12] conducted a 5 year follow-up study of efficacy of PEG-IFN in patients with HBeAg negative CHB and reported a rise in sustained virological response of 28%, as well as HBsAg clearance in 12% of patients with favourable predictors (HBV DNA < 2,000 IU/mL after a one year of follow-up) [12]. In all of our eight (38%) patients with virological response, we were able to confirm that after 1–3 years after the end of the treatment, all of them had sustained virological suppression. These results are in concordance with foreign authors who had much larger patient samples showing that a prolonged stability of achieved HBeAg seroconversion is maintained in more than 80% of patients and virological response sustained in more than 60% of the treated patients [13]. However, in our patients, we did not observe a rise in HBsAg clearance, which has been reported by numerous authors, as none of our patients after 3 years of treatment had achieved HBsAg clearance [11, 13, 14].

Tolerability of PEG-IFN is often a limiting factor, but years of treatment experience in chronic hepatitis C (CHC) patients has improved our possibilities of timely detection and intervention in case of any side-effects. The incidence of side-effects is significantly lower and milder compared to our patients with CHC, as patients with CHB are mostly younger with fewer comorbidities [1, 2]. After the treatment initiation, we observed flu-like symptoms in four patients, as well as neutropenia and thrombocytopenia in five patients, prompting for dosage reduction. We observed a case of drug-induced thyroiditis, which was completely resolved and the patient was treated with PEG-IFN in full, as well as two serious side effects prompting for the secession of the treatment – a patient with an ovarian carcinoma discovered during 4th month of the treatment and a case of severe depression during 32th week of the treatment. There are published data concerning the late occurrence of psychiatric side-effects during PEG IFN treatment [15]. There were no death outcomes or occurrences of hepatocellular carcinoma during the follow-up period.

Current guidelines for treatment of HBeAg positive form of CHB of the European Association of the Study of the Liver (EASL) state certain predictors of successful PEG-IFN treatment including low viraemia, higher activity of liver enzymes, female sex, and genotype A [1, 2]. However, in HBeAg negative patients, there are no clear predictors of successful treatment outcome. In both of our patient groups, we found no such statistically significant predictors, which may be due to the sample size. Yeh et al. [16] have pointed to the importance of previous treatment options as a possible

predictor of successful PEG-IFN treatment. They have shown that patients previously treated with oral analogues have lower PEG-IFN success rates compared to treatment-naive and interferon experienced patients. In our study group, 7 (25%) of the patients have been previously treated with lamivudine, but we found no difference in PEG-IFN success rates compared to the treatment-naive patients.

Current protocols have implemented viraemia kinetic and qHBsAg as major predictors of successful PEG-IFN treatment [2]. Rijckborst et al. [17] have pointed to the importance of HBV kinetics after 12 weeks of the treatment and implementation of the rule of stopping treatment in patients with HBeAg negative form of CHB. The EASL guidelines also underline the rule of stopping in 12th and 24th week of the treatment [2]. These guidelines were followed and we stopped PEG-IFN treatment after 12 weeks of the treatment in 11.11% (4/36) of the patients, of whom three did not fulfill the National Health Fund treatment criteria for PEG-IFN administration (two patients had ALT levels < 2x upper limit and a high viraemia > 108 IU/mL). Current guidelines also state that besides HBV DNA levels, qHBsAg should be measured in order to decide to stop or continue treatment in both forms of CHB. However, as qHBsAg detection was not available at the time, as clinicians we are in doubt if the treatment was stopped prematurely in these patients, especially as it was based solely on HBV DNA levels.

Our treatment experience concerning the role of qHBsAg is very limited and can not be used for a more significant conclusion, as pretreatment qHBsAg was available in only 16 patients, and in only 10 during the treatment and follow-up period. On this small sample, we did not observe any correlation between the decline of viraemia and levels of HBsAg. There are multiple publications pointing out to the importance of qHBsAg at the end of the treatment and during follow-up period, as its continuous decline is correlated with sustainability of virological response, and may predict a possible relapse if there is no decline in levels of HBsAg during follow-up [2, 12, 14].On the other hand, there are published results showing that patients with HBeAg negative CHB and genotype D may benefit from prolonged PEG-IFN treatment (96 weeks instead of 48 weeks) including higher success rates (up to 29%) and HBsAg clearance up to 6% [18]. As this option for prolonged PEG-IFN treatment was not available in our patients who are also mostly HBeAg negative, and considering the local prevalence of genotype D, we suspect that this approach may prove beneficial to patients in Serbia.

This study has some limitations, such as sample size and limited availability of qHBsAg. However, we believe that some of our experiences may prove beneficial to other clinicians who are using PEG-IFN for treatment of CHB patients.

Conclusion

These results are the first published data concerning the efficacy and safety of PEG-IFN in Serbian patients with CHB, as this drug was mostly described and observed in the treatment of patients with CHC. Our modest results showed that although PEG-IFN is important for treatment of patients with CHB in well-defined situations, such as relatively low viraemia, elevated liver enzymes and in younger patients, other treatment predictors are also necessary, especially qHBsAg.

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The level of endogenous testosterone and its correlation with lipid profile in men older than 40 years with acute myocardial infarction

Nivo endogenog testosterona i njegova korelacija sa lipidnim profilom kod muškaraca sa akutnim infarktom miokarda, starijih od 40 godina

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Abstract

Background/Aim. The influence of lipid profile on acute myocardial infarct (AMI) is well known. On the other hand, the role of testosterone (T), as one of the possible predictive factors of AMI in men and its influence on lipid profile in men is still controversial. The aim of the study was to determine levels of T in AMI and six months after AMI in the same group of patients, and to compare with T levels in healthy men. Also we correlated T levels with lipid profile in patients with AMI and 6 months after AMI. **Methods.** The study was designed as prospective study. Patients were divided into III groups: Group I included 35 men, aged 55 ± 3 years, with AMI. Group II included the same 35 patients, analyzed 6 months after AMI. The group III consisted of 20 healthy men aged 57 ± 2.12 years (control group). Blood samples of the group I (AMI) were taken in the first 12 hours from the AMI beginning and also 6 months after AMI (group II). Following analyses were performed: levels of total cholesterol, triglycerides, low density lipoprotein (LDL) cholesterol, high density lipoprotein (HDL) cholesterol, lipoprotein(a) [Lp(a)], apolipoprotein A1 (Apo A1), apolipoprotein B (Apo B) and T. **Results.** Levels of T in patients with AMI (16.86 ± 7.18 nmol/L) as well as 6 months after AMI (18.12 ± 7.96 nmol/L) were statistically significantly lower than those in healthy persons of the same age (27.11 ± 10.48 nmol/L) ($p < 0.001$). In the group I, statistically significant, positive correlation was found between levels of T and HDL cholesterol ($r = 0.403$, $p < 0.05$), and levels of T and Apo A1 ($r = 0.747$, $p < 0.01$). In the group II, statistically significant, positive correlation was also found between levels of T and HDL cholesterol ($r = 0.388$, $p < 0.05$) and T and Apo A1($r = 0.354$, $p < 0.05$). **Conclusion.** This study showed that men, over 40 years of age, with AMI had statistically significantly lower concentrations of endogenous T compared to healthy male population of the same age. Levels of T in the same patients after 6 months from AMI maintained statistically significantly lower values in comparison to those in healthy men.

Key words:
lipids; lipoproteins; myocardial infarction; testosterone.

Apstrakt

Uvod/Cilj. Uticaj lipidnog profila na akutni infarkt miokarda (AIM) dobro je poznat. Nasuprot tome, uloga nivoa endogenog testosterona (T), kao jednog od mogućih prediktivnih faktora AIM i njegovo dejstvo na lipidni profil kod muškaraca sa AIM i dalje su kontroverzni. Cilj studije je bio da se odredi nivo endogenog T u AIM, kao i 6 meseci nakon AIM kod iste grupe ispitanika i da se uporedi sa nivoima T kod zdravih ispitanika. Pored toga, određena je i korelacija nivoa T sa parametrima lipidnog profila u AIM kao i šest meseci nakon AIM. **Metode**. Sprovedena je prospektivna studija u koju su bili uključeni muškarci podeljeni u tri grupe: grupu I činilo je 35 muškaraca, starosne dobi 55 ± 3 godine sa AIM; grupu II sačinjavalo je istih 35 muškaraca koji su analizirani 6 meseci nakon AIM, dok se grupa III sastojala od 20 zdravih ispitanika starosne dobi 57 ± 2,12 godina. Uzorci krvi kod ispitanika grupe I uzimani su u periodu od 12h od nastanka AIM. Kod istih ispitanika krv za analize uzeta je šest mesci nakon preležanog AIM (grupa II). U krvi su određivani nivoi: ukupnog holesterola, triglicerida, lipoproteina male gustine (LDL) holesterola, lipoproteina velike gustine (HDL) holesterola, apolioporoteina A1 (Apo A1), apolipoproteina B (Apo B), lipoproteina(a) [Lp(a)] i nivo endogenog T. **Rezultati.** Nivo T kod ispitanika sa AIM (grupa I) (16,86 ± 7,18 nmol/L), kao i kod istih ispitanika

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šest meseci nakon AIM (grupa II) (18,12 ± 7,96 nmol/L) bio je statistički značajno niži u odnosu na zdravu kontrolnu grupu iste starosti (27,11 ± 10,48 nmol/L) ($p < 0,001$). U grupi I dobijena je statistčki značajna, pozitivna korelacija između nivoa T i HDL holesterola ($r = 0.403$, $p < 0,05$) i visoko statistički značajna, pozitivna korelacija između nivoa T i Apo A1($r = 0,747$, $p < 0,01$). U grupi II, takođe je nađena statistički značajna pozitivna korelacija između nivoa T i HDL holeserola ($r = 0,388$, $p < 0,05$), kao i nivoa T i Apo A1 ($r = 0,354$, $p < 0,05$). **Zaključak.** Ova studija je pokazala da su nivoi T kod mušaraca starijih od 40 godina sa AIM visoko stistički značajno niži u odnosu na nivoe T kod zdravih muškaraca iste životne dobi. Nivoi T kod ispitanika sa AIM zadržavaju statistički značajno niže vrednosti i šest meseci nakon AIM u poređenju sa zdravom kontrolnom grupom.

Ključne reči:
lipidi; lipoproteini; infarkt miokarda; testosteron.

Introduction

Androgens as well as estrogens show influence on many risk factors related to cardiovascular diseases (CVD) [1]. The basic risk factors for CVD are: hypercholesterolemia, low lovel of high density lipoprotein (HDL) cholesterol, high level of low density lipoprotein (LDL) cholesterol, hypertension and cigarette consumption. Epidemiological studies have shown that each of these factors is of high importance depending on the degree of exposure. The common feature of these factors is their ability to damage the arterial endothelium. Hypertension produces an increased mechanical stress on blood vessels. Cigarette smoking causes transient but intensified release of free radicals into the arterial system, and oxidized cholesterol can act as endothelial toxin [2]. Besides conventional risk factors including: diabetes mellitus, positive family history and age, the additional factors such as abdominal obesity, alcohol consumption and physical inactivity could be added; those risk factors represented the focus of research in many studies, e.g. INTERHART, a global risk factors study for acute myocardial infarction (AMI) [3]. Major studies concerning risk factors for CVD (INTERHART, AMORIS, MONICA/CORA), focused special attention to the role of apolipoproteins as informative indicators for CVD and AMI, primarily apolipoprotein B (Apo B) and apolipoprotein A1 (Apo A1) [4].

The influence of androgens on lipid status and interpretation of results obtained is extremely controversial. The fact that androgens usually reduce levels of HDL cholesterol has been used throughout history to characterize these steroids as harmful to blood vessel health [5]. But, along with these findings, it has been noticed that reduction of lipoprotein(a) [Lp(a)] level and plasma triglycerides could lead to a reduction of CVD risk [1].

Although risk factors for CVD do not appear to be isolated, and cholesterol and triglycerides metabolism is highly interconnected, the fact that triglycerides concentrations vary day by day in an individual to a much greater extent compared to cholesterol concentrations, cholesterol level was marked as stronger predictor for CVD [6].

In puberty boys, the increase in testosterone concentrations was followed by a decrease in HDL cholesterol concentrations, probably as a result of hepatic lipase induction, a sex hormone sensitive enzyme of the lipoprotein metabolism. This decline in HDL cholesterol levels represents the basic difference and a higher risk for early development of CVD in men compared to women. Unlike the puberty period in men, in the later years there is a positive correlation between concentrations of testosterone and HDL cholesterol due to the influence of testosterone on the hepatic synthesis of Apo A1 [7].

The effect of androgen on levels of LDL cholesterol in plasma, which represents a classic metabolic risk factor in men, is difficult to be interpreted and analyzed. In some men who abused anabolic–androgenic steroids (AAS), extremely high values of LDL cholesterol were found, indicating an elevated risk for CVD. In contrast, an increase in LDL cholesteril did not occur in patients who used androgens for the purpose of contraception or substitution therapy, whereas in one of studies, decline in LDL cholesterol levels was observed in patients who abused AA [6].

Although LDL cholesterol is known as the primary lipid risk factor for CVD, there are several limiting factors for using only it as a main risk factor. Recent data suggest that apolipoproteins are important indicators and predictors for CVD primarily Apo A1, which represents anti-atherogenic high density lipoprotein. Several studies, including two major AMORIS [7] and INTERHART [8], as well as MONICA/KORA STUDY [9], showed strong direct relationship between high levels of the Apo B/Apo A1 ratio and the increased risk of fatal AMI. Apo B is found in very low density lipoproteins (VLDL), medium density lipoproteins (IDL), as well as in large boyant LDL and sd-LDL, with one molecule of Apo B in each of these atherogenic particles. Therefore, the total number of Apo B reflects the total number of atherogenic particles. Apo B also plays role in the "capture" of these lipoproteins in the walls of blood vessels. Apo B synthesized in the liver also stabilizes and allows the transport of cholesterol and triglycerides in VLDL, IDL to large boyant LDL and plasma sd-LDL. Apo B serves as a ligand for Apo B and Apo E receptors and thus facilitates cholesterol intake in peripheral tissues and liver. Apo A1 is the main protein of HDL particles and is the major initiator of reverse cholesterol transport. The balance between Apo B and Apo A1, as well as the Apo B/ Apo A1 ratio increase the risk of CVD, the higher ratio the higher is risk.

In addition to the standard lipid profile parameters as well as the above mentioned apolipoproteins, Lp(a) which originates from LDL modification, may also be one of the predictors of CVD and AMI. Due to its structural similarity to plasminogen, Lp(a) impairs plasma synthesis and the fibrinolysis process [10]. Lp(a) also plays a role in macrophage binding through high affinity receptors, which leads to the

formation of foam cells and discharge of cholesterol into atheromatous plaques [11]. The correlation between Lp(a) and risk for CVD and AMI was first suggested in some cross-sectional and prospective studies, while in some studies contradictory results were obtained. In a prospective PROCAM study which included 788 men aged 35–65, with follow-up period of 10 years, the risk of acute coronary events was 2.7 times higher in patients with Lp(a) levels > 20 mg/dL [12].

The aim of the study was to determine levels of testosterone in men older than 40 years in AMI and six months after AMI, and to compare with testosterone levels in healthy men of the same age. Another aim was to examine correlation of testosterone levels with lipid profile prameters in men over 40 years of age in AMI and six months after AMI.

Methods

The study was designed as prospective clinical study. Clinical examination and recruitment of participants were conducted at the Clinic for Endocrinology, Diabetes and Metabolic Diseases of the Clinical Center of Serbia and the Clinic for Cardiology of the Emergency Center (Coronary Unit) in Belgrade. Laboratory analyses were performed in the Center for Medical Biochemistry of the Clinical Center of Serbia in Belgrade.

Patients were divided into three groups: group I included 35 men aged 40–80 years with AMI; group II included the same 35 men who were analyzed six months after the AMI; group III (control group) consists of 20 healthy men aged 40–80 years.

All groups were homogeneous concerning body mass index (BMI) and age. Total ischemic time in the group I was shorter than 12 hours. All participants were taken blood samples early in the morning for the following analyses of lipid profile: total cholesterol, triglycerides, low LDL cholesteril, HDL cholesterol, Lp(a), Apo A1 and Apo B. Hormone analysis included levels of testosterone.

Hormone and lipid parameters were determined immediately in AMI event (in patients of the group I) as well as six months after discharge from the hospital (the group II).

All patients were informed concerning the methodology of the study and all of them voluntarily filled out the informed consent. The study was approved by the Ethics Committee of the Clinical Center of Serbia.

Biochemical analyses were performed by chromatography methods, and tetsosteron levels by radioimmunoassay (RIA).

Results were reported as mean ± standard deviation and presented in tables. Differences between groups were assessed by Student's *t* test. Correlations between parameters were analyzed with Spearman's correlation test. Differences were considered statistically significant at $p < 0.05$. SPSS 20.0 software was used for the statistical analyses.

Results

The group of patients with AMI and the control group were homogenous in BMI and age, and no statistically significant differences were find between them for BMI (28.40 ± 2.84 kg/m^2 vs. 26.45 ± 2.01 kg/m^2, respectively) and age (55 ± 3 years vs. 57 ± 2.12 years, respectively).

Testosterone levels in patients of the group I (16.86 ± 7.18 nmol/L) were statistically significantly lower than those in the control group (27.11 ± 10.48 nmol/L) ($p < 0.001$). Also, highly statistically significant difference was obtained by comparing testosterone levels in patients 6 months after AMI (the group II) (18.12 ± 7.96 nmol/L) with those in the control group (27.11 ± 10.48 nmol/L) ($p < 0.001$). No statistically significant difference was found between testosterone levels in patients with AIM and 6 months after AMI (Table 1).

Statistically significantly higher levels of cholesterol, LDL cholesterol and Apo B were obtained in AMI patients (the group 1) compared to those 6 months after AMI in the group 2 ($p < 0.05$) (Table 2). All these three values were slightly increased in the group I, comparing with referent range for cholesterol (3.1–5.1 mmol/L), LDL cholesterol (1.55–3.4 mmol/L) and Apo B (0.66–1.33 g/L).

Correlations of levels of testosterone and parameters of the lipid profile in patient with AMI and six months after AMI are given in Table 3.

In the group I, statistically significant, positive correlation was found between levels of testosterone and HDL cholesterol ($p < 0.05$), as well as testosterone and Apo B ($p < 0.01$).

In the group II, statistically significant positive correlation was also found between levels of testosterone and HDL cholesterol ($p < 0.05$) as well as testosterone and Apo A1 ($p < 0.05$).

Table 1
Testosterone levels in patients with AMI (the group I), 6 months after AMI (the group II) and in the control group (the group III)

Groups of patients	Testosterone levels (nmol/L)			
	min	max	mean	SD
I (n = 35)	1.52	48.62	16.86	7.18
II (n = 35)	6.88	46.12	18.12	7.96
III (n = 20)	15.80	49.04	27.11	10.48

AMI – acute myocardial infarction; SD – standard deviation.

Table 2

Lipid parameters in patients with AMI (the group I) and 6 months after AMI (the group II)

Parameters	Group I	Group II	
	mean ± SD	mean ± SD	*p*
Total cholesterol, mmol/L	5.75 ± 1.33	4.70 ± 1.29	< 0.05
HDL cholesterol, mmol/L	1.13 ± 0.31	1.05 ± 0.29	> 0.05
LDL cholesterol, mmol/L	3.70 ± 1.11	2.79 ± 1.23	< 0.05
Triglycerides (mmol/L)	1.89 ±1.52	1.98 ± 1.13	> 0.05
Apolipoprotein A1 (Apo A1), g/L	2.39 ± 0.69	2.02 ± 0.46	> 0.05
Apolipoprotein B (Apo B), g/L	1.62 ± 0.73	1.11 ± 0.41	< 0.05
Apo B/Apo A1	0.66 ± 0.23	0.59 ± 0.23	> 0.05
Lipoprotein(a), g/L	0.47 ± 0.21	0.38 ± 0.26	> 0.05

AMI – acute myocardial infarction; HDL – high density lipoprotein; LDL – low density lipoprotein; SD – standard deviation.

Table 3

Correlation of levels of testosterone with levels of lipid parameters in patients with AMI (group I) and 6 months after AMI (group II)

Lipid parameters	Group I		Group II	
	r	*p*	r	*p*
Total cholesterol	0.318	> 0.05	0.184	> 0.05
HDL cholesterol	0.403	< 0.05	0.388	< 0.05
LDL cholesterol	0.268	> 0.05	0.255	> 0.05
Triglycerides	-0.052	> 0.05	-0.065	> 0.05
Apolipoprotein A1 (Apo A1)	0.747	< 0.01	0.354	< 0.05
Apolipoprotein B (Apo B)	0.298	> 0.05	0.133	> 0.05
Apo B /Apo A1	-0.118	> 0.05	-0.079	> 0.05
Lipoprotein(a)	0.281	> 0.05	0.328	> 0.05

AMI – acute myocardial infarction; HDL – high density lipoprotein; LDL – low density lipoprotein; r – coefficient of correlation.

Discussion

Men, unlike women, do not experience a sudden decrease in concentration and production of sex hormones in middle age, but a gradual decline of endogenous testosterone has been present since 30 years of every man's life. Research of the role of testosterone in maintenance of male health, as a new field of endocrinology, occurred in 1998 at the first world congress "Aging Male". During the past 20 years, decrease in testosterone levels has gone from "Andropause" to "late onset hypogonadism – LOH" and, ultimately, the involutive hypoandrogenism as the most acceptable definition of changes in concentrations of endogenous testosterone in the aging process in men. The problem with the name and nomenclature is only part of the controversy associated with testosterone and its role in the development of various pathological processes in men.

Many large-scale studies with a large number of participants tried and partly managed to give an answer on the role of sex hormones and their impact on the cardiovascular system in women, but in men this mostly was not the case. For this reason, and especially because of the diametrically different results obtained in animal models and in some smaller studies, over the past 10 years, increasing attention has been paid to the role of sex hormones in the prevention, treatment and occurrence of CVD and AMI in men [13].

Testosterone in AMI

In the last few years, several studies, trials, and case studies reported an increased risk of developing AMI in men who received testosterone [14, 15]. Thus, in a study of Layton et al. [15], 2,898 patients with coronary events demonstrated an increased risk of AMI, cardiovascular insult and unstable angina pectoris immediately after testosterone injection [15]. What has always provoked controversy concerning levels of testosterone is the question what are the appropriate, "normal" values of testosterone levels depending on the age. Avoiding supraphysiological doses and maintaining a physiological balance potentially unwanted effects of testosterone are omitted. For this reason, in recent years one of the largest studies, a retrospective cohort study of Li et al. [16] compared occurance of AMI in 200,000 participants receiving testosterone therapy with that in 200,000 hypogonadic patients who did not receive testosterone therapy over a one-year period, and no association between testosterone therapy and AMI was found. In favor of the positive effect of substitution therapy with testosterone in hypogonadal males, a large cohort study of Cheetham et al. [17], conducted on 8,808 individuals, reported smaller risk of developing AMI in the follow-up period of 3.4 years.

What differentiated our study from recent trials was that we monitored levels of testosterone in patients with AMI and

six months after AMI, as well as in age and BMI comparable group of healthy men. The obtained results showed highly significantly lower levels of testosterone not only in the ischemic period of 12 hours from the onset of AMI but six months after the acute phase as well, compared to the healthy control group.

Lipids as risk factors for CVD and AMI

Lipid status with all its components (cholesterol, HDL cholesterol, LDL cholesterol, Lp(a), triglycerides, Apo A1, Apo B) was completely processed and statistically analyzed in order to determine its correlations with concentrations of endogenous testosterone in patient with AMI and 6 months later.

Statistically significantly higher values were demonstrated for LDL cholesterol and cholesterol in subjects with AMI compared to values found after six months in the same subjects.

HDL cholesterol with its anti-atherogenic effects marks one of frequent controversies associated with levels of endogenous testosterone and its influence on HDL cholesterol level [18]. The evident decline in HDL cholesterol in puberty is associated with testosterone jumping (negative correlation) due to the induction of sex hormone sensitive enzyme of lipoprotein metabolism, hepatic lipase, is one of the main causes of the early onset of CVD in men compared to women [7]. Contrary to this, in many studies a positive correlation between levels of endogenous testosterone and HDL cholesterol has been demonstrated in older man [19], which we also confirmed in our study. In our study statistically significant positive correlation was observed between levels of testosterone and HDL cholesterol in AMI patients (the group I), as well in the group II, six months after AMI. This positive correlation can be explained by hepatic effect of testosterone on the production of Apo A1. The Massachusetts male aging study (MMAS) showed a positive and highly statistically significant correlation of HDL cholesterol levels and levels of endogenous testosterone in males over 40 years of age with or without CVD, thus definitely confirming the fact that there is difference of endogenous testosterone effect on HDL cholesterol and risk factors in older men compared to men immediately after puberty [20]. Similar results were obtained in the San Antonio Hearth Study, where a positive correlation between levels of endogenous testosterone and HDL cholesterol in 178 men with normal glycemic values was demonstrated. It was concluded that the less atherogenic lipid profile (lower triglyceride values and higher HDL cholesterol values) was present in men with a higher concentration of endogenous testosterone vs. women in whom the increased concentration of androgens was in a strong correlation with high levels of triglycerides and low HDL values [21].

There were no statistical significant correlation between testosterone levels with the levels of triglycerides. A negative correlation was obtained, which, although not statistically significant, corresponded in many ways to the results of large studies. Tromso Study also dealt with the effect of endogenous testosterone on levels of triglycerides during the day in 1,274 men who did not have a verified CVD and who participated in the population study. Analyzing triglyceride levels taken during the day, their linear increase has been demonstrated in subjects with endogenous testosterone levels below 50th percentile. On the contrary, in men with values of endogenous testosterone above 50th percentile there were no statistically significant changes in triglyceride levels during the day. Also highly statistically negative correlation was found between levels of triglycerides and endogenous testosterone and it was highly statistically positive related to HDL cholesterol. Men with poor lipid profile (HDL cholesterol < 0.9 mmol/L and triglicerides > 1.8 mmol/L) had significantly lower testosterone levels compared to men with normal lipid profile [22]. The conclusion of this large study was that low level of endogenous testosterone correlates with the linear rise in triglycerides during the day, and that it is independently associated to a poor lipid profile indicating that low levels of testosterone affect the poor triglyceride metabolism.

LDL cholesterol represents one of the risk factors for the development of CVD and, unlike HDL cholesterol, shows positive correlation with that risk [23]. In addition, the role of Lp(a) as an important risk factor for the development of CVD has been highlighted over the past years. Due to the structural similarity with plasminogen, as well as its binding properties with high affinity macrophages and the formation of foam cells, Lp(a) directly affects the development of CVD [24]. In our study, we did not find statistically significant correlation between levels of testosterone and levels of LDL cholesterol and Lp(a) in patients with AMI as well as six month later in the same patients.

In our study we found statistically significant, positive correlation between testosterone levels and levels of Apo A1 in both AMI groups (the group I and the group II). The role of Apo A1, Apo B, as well as their ratio (quotient) in development of CVD and AMI is known from major studies such as AMORIS [7], INTERHART [8] and MONICA/CORA [9].

The AMORIS study showed that high levels of Apo B highly correlated with an increased risk of developing CVD and AMI, while the level of Apo A1 had a protective role in both men and women. In that prospective study, more than 175,000 men and women of the Swedish population were monitored during 98 months, nearly 2,000 of them died due to AMI. Apo B was labeled as a stronger marker for CVD than LDL cholesterol, and especially for subjects with normal/lower LDL cholesterol values [7]. A single variable representing the strongest indicator for the occurrence of fatal myocardial infarction was the Apo B/Apo A1 ratio. This ratio was an indicator of the risk of fatal myocardial infarction, independently of lipid phenotype, especially when other lipid levels were normal or low [25]. This ratio was a stronger risk factor for CVD compared to all other ratios: triglicerides/HDL cholesterol, LDL cholesterol/HDLcholesterol or non-HDL cholesterol/HDL cholesterol [26].

The impressive INTRHARTH study, based on 30,000 patients from 52 countries all over the world, also showed that the Apo B/Apo A1 ratio was the strongest risk factor among the other conventional risk factors for AMI [8].

Several other studies have confirmed that the Apo B/Apo A1 ratio is in a strong correlation with increased ca-

rotid artery intima-media thickness and that this ratio progressively increases in patients with metabolic syndrome. This ratio was in a positive correlation with the CVD risk, described as the first or myocardial reinfarction [27].

The MONICA/Cora Study included 1,414 men and 1,436 women aged 35–64 years without a previous history of myocardial infarction. The period of follow-up was in average 13 years, during which 114 men and 31 women had a coronary event, of which 71 were fatal and 74 were not. The strongest correlation was demonstrated for high Apo B levels as well as Apo B/Apo A1 ratio and risk for myocardial infarction [4]. The results of that are completely coherent with those obtained in the INTERHART study, based on 15,000 AMI patients compared to 15,000 healthy controls. Both studies have shown that Apo B/Apo A1 ratio is the most important among all risks factors besides: smoking, hypertension, abdominal obesity, diabetes, alcohol, psycho-social stress, vitamin intake, and physical inactivity. The results were independent concerning gender, age and ethnicity. The Apo B/Apo A1 ratio remained the strongest risk factor after the multivariate analyses were performe [8].

In the last few years, numerous studies estimated the role as well as the significance of the Apo B/Apo A1 ratio concerning CVD and AMI incidence. The study published in 2015 explored the predictive value of Apo B/Apo A1 ratio and non-HDL cholesterol values and their effects on CVD incidence [28]. The study was conducted on 826 patients, of whom 532 had CVD, 165 of them unipolar, 175 bipolar disorders, and 192 multipolar CVD vs. 294 healthy subjects. After a follow-up period of 3 years, it has been confirmed that there is a positive correlation among high values of the Apo B/Apo A1 ratio and non-HDL cholesterol with the most serious, multiply forms of coronary heart disorders and the increased risk of developing adverse events such as: angina pectoris, AMI, heart insufficiency, stroke and death caused by CVD.

Statistically significantly higher Apo B values we found in the patient with AMI compared to the same subjects six months after myocardial infarction.

Negative, but not statistically significant correlation of endogenous testosterone levels and the Apo B/Apo A1 ratio we found in both AMI groups (groups I and II).

Analyses of studies conducted so far as well as the results of our study suggest that natural endogenous testosterone has a positive or neutral effect on the development of CVD and AMI. The antiatherogenic mechanism of testosterone is unknown, in general, but several solutions have been offered so far. Some data emphasize the modulating effect of endogenous testosterone on the risk factors for CVD: diabetes [29], insulin resistance [30], obesity [31], hypercholesterolemia [32, 33], and hypertriglyceridemia [32]. It has been assumed that increase of triglycerides levels is modified by changes in hepatic triglyceride lipase [34]. On the contrary, endogenous testosterone can have a direct effect on HDL cholesterol by increasing the hepatic production of Apo A1 as the main protein component of nascent high density lipoprotein [28].

Conclusion

This study showed that men over 40 years of age with AMI have highly statistically significantly lower concentrations of endogenous testosterone compared to healthy male population of the same age. Statistically significantly lower concentrations of testosterone are maintained even six months after AIM. In our study, highly statistically significant, positive correlation was found between levels of endogenous testosterone and levels of HDL cholesterol and Apo A1 in men with AMI as well in the same patients six months after AMI. Long-term, well designed prospective clinical trials are required to verify potential testosterone role, its interaction with parameters of the lipid profile and possible predictive value in men with AMI.

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A randomized trial of surgery alone versus surgery plus compression in the treatment of venous leg ulcers in patients with primary venous insufficiency

Randomizovano ispitivanje efikasnosti hirurškog tretmana naspram kombinacije hirurškog i kompresivnog tretmana u lečenju venskih ulceracija kod bolesnika sa primarnom venskom insuficijencijom

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Abstract

Background/Aim. Venous leg ulcers (VLU) are a significant health problem worldwide. It is well known that VLU are difficult to treat and that they have high tendency for recurrence. Compression therapy is the preferred treatment modality but there is growing evidence that correction of underlying venous disorder in early stages of the disease in addition to compression treatment may improve ulcer healing and reduce recurrence rate. **Methods.** An open, prospective, randomized, single-center study, with a 6-months follow-up was performed to determine the efficacy of two different treatment modalities (surgery alone versus surgery plus compression) in the treatment of VLU in patients with primary venous insufficiency. Patients with secondary venous insufficiency and/or thrombosis were excluded from the study. Overall, 71 patients were randomized (37 men, 34 women; mean age 60 years) into two groups: the group A – 34 patients who underwent surgical intervention (stripping) and postoperatively were treated with simple wound dressing only, and the group B – 37 patients who underwent surgical intervention (stripping) and wore a heelless open-toed elastic class III compression device knitted in tubular form –Tubulcus® (Laboratoires Innothera, Arcueil, France). All patients in group B were instructed to wear compression device continuously during the day and night. The study was performed at the Clinic for Cardiovascular and Transplant Surgery, Clinical Centre Niš (Serbia) with primary endpoint of the study being complete ulcer healing at 180 days. **Results.** The healing rate was 29.41% (10/34) in the group A, and 56.76% (21/37) in the group B ($p < 0.01$). Mean healing time in the group A was 141 ± 15 days, and in the group B it was 98 ± 12 days (Log-rank life table analysis: $p < 0.001$). **Conclusion.** This study suggests that for VLU in patients with primary venous insufficiency, surgery plus compression therapy provides higher healing rate and faster healing time compared to surgery only.

Key words:
varicose ulcer; vascular surgical procedures; stockings, compression; treatment outcome.

Apstrakt

Uvod/Cilj: Venske ulceracije nogu (VUN) predstavljaju široko rasprostranjen zdravstveni problem. Poznato je da se VUN teško leče i da postoji visoka stopa recidiva. Kompresivna terapija predstavlja terapiju izbora, ali postoji sve veći broj dokaza da korigovanje osnovnog venskog oboljenja u ranom stadijumu bolesti, uz kompresivnu terapiju, može poboljšati zarastanje ulceracija i smanjiti stopu recidiva. **Metode.** U cilju utvrđivanja efikasnosti dva različita načina lečenja (samo hirurgija *vs* hirurgija plus kompresivna terapija) venskih ulceracija, kod pacijenata sa primarnom venskom insuficijencijom, sprovedena je otvorena, prospektivna, randomizirana studija, sa šestomesečnim praćenjem. Bolesnici sa sekundarnom venskom insuficijencijom i/ili venskom trombozom su bili isključeni iz studije. Studijom je obuhvaćeno ukupno 71 bolesnika (37 muškaraca i 34 žene), podeljenih u dve grupe: grupu A – 34 bolesnika kod kojih je urađena operacija (*stripping*) i koji su postoperativno tretirani samo običnim previjanjem rane, i grupu B – 37 bolesnika kod kojih je urađena operacija (*stripping*) i koji su postoperativno nosili elastičnu čarapu III klase kompresije, sa otvorenim prstima, satkanu u tubularnoj formi – Tubulcus® (Laboratories Innothera, Arcueil, France). Svim bolesnicima iz

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grupe B je objašnjeno da kompresivnu čarapu nose konstantno tokom dana i noći. Bolesnici su tretirani ambulantno, na Klinici za kardiovaskularnu i transplantacionu hirurgiju, Klinički Centar Niš (Srbija), sa primarnim ciljem da do zarastanja ulceracije dođe unutar 180 dana. **Rezultati.** Stopa zarastanja ulceracija je bila 29,41% (10/34) u grupi A i 56,76% (21/37) u grupi B ($p < 0.01$). Srednje vreme zarastanja ulceracija je bilo 141 ± 15 dana u grupi A, a u grupi B, 98 ± 12 dana (Log-rank analiza: $p < 0{,}001$). **Zaključak.** Rezultati studije su pokazali da lečenje venskih ulceracija kod bolesnika sa primarnom venskom insuficijencijom, hirurškom metodom u kombinaciji sa kompresivnom terapijom daje veću uspešnost i kraće vreme zarastanja, u poređenju sa samo hirurškom metodom.

Ključne reči:
venska ulceracija; hirurgija, vaskularna, procedure; čarape, kompresivne; lečenje, ishod.

Introduction

Venous leg ulcers (VLU) are a significant health problem worldwide. The treatment costs are very high and many patients due to this condition experience early retirement. It is well known that VLU are difficult to treat and that they have high tendency for recurrence. Compression therapy is the preferred treatment modality and has been used in different forms (compression hosiery, elastic or inelastic bandages usually applied as either two or multilayer bandaging systems) [1–4]. Healing rates of VLU obtained with compression treatment vary widely from 40%–95% [5–7]. Despite the widespread use of compression therapy, recurrence rates of VLU remain high, between 25%–70% [8–10]. During the last couple of years published data suggest that correction of underlying venous disorder in early stages of the disease in addition to compression treatment may improve ulcer healing and reduce recurrence rate [11–12].

Methods

An open, prospective, randomized, single-center study, with a 6-months follow-up was performed to determine the efficacy of two different treatment modalities (surgery alone versus surgery plus compression) in the treatment of VLU in patients with superficial venous reflux. Patients with secondary venous insufficiency and/or thrombosis were excluded from the study.

Population

Patients aged at least 18 years with VLU and primary venous insufficiency present on ultrasound examination were screened for inclusion in the trial. Significant arterial disease, pregnancy, rheumatoid disease, malignancy, restricted range of ankle motion and diabetes mellitus were exclusion criteria from the study.

Before randomization, all patients were examined by Color Duplex Scan investigation (CDS). In order to establish significant arterial diseases ankle brachial pressure index (ABPI) measurements were performed. For ultrasound investigation a Siemens Sonoline Sienna device with a 7 MHz probe was used. Exclusion of venous thrombosis was determined by assessing venous compressibility and establishing flow characteristics. The flow direction was determined during a Valsalva maneuver in the 20–30° reverse Trendelenburg position. The reflux was induced using a rapid cuff deflation in the standing position. The presence of reflux was confirmed if the reflux time was > 0.5 seconds.

Sample size

With a power of 80% and a confidence level of 95%, assuming VLU healing rates of at least 20% in the group A and 40% in the group B, a total of 70 patients were needed for this study. One hundred and eleven patients were examined for potential participation in this study. Of these, seventy-one were accepted and randomized.

Randomization

Randomization was computer generated and, in total, 71 patients were randomized (37 men, 34 women; mean age 60 years) into two groups: the group A – 34 patients who underwent surgical intervention (*stripping*) and postoperatively were treated with simple wound dressing only, and the group B – 37 patients who underwent surgical intervention (*stripping*) and wore a heelless open-toed elastic class III compression device knitted in tubular form-Tubulcus® (Laboratoires Innothera, Arcueil, France). All patients in the group B were instructed to wear compression device continuously during the day and at night. This study was performed at the Clinic for Cardiovascular and Transplant Surgery, Clinical Centre Niš (Serbia) with the primary endpoint of the investigation being complete VLU healing at 180 days.

The relevant authorities approved the study protocol and all patients who were included in the study gave their written consent.

Study protocol

All patients included in the study were treated and monitored by the same clinical team comprising of three doctors and three medical nurses. Patients were treated and monitored on the ambulatory basis at the Clinic for Cardiovascular and Transplant Surgery, Clinical Centre of Niš (Serbia) (3 visits per week for the 6 months period). The surgical procedure was performed on all patients and included crossectomy with stripping of the great saphenous vein. The patients were operated in local anesthesia and received 2 g of cephalosporine intraoperatively.

Treatment regimen

The local treatment regimen for ulcers was the same for all patients included in the study. The dressings were changed in regard to amount of wound exudation (from 1 to 7 days). The patients did not receive any additional local or systemic therapy. No medication including antibiotics or venous-active drugs were used. Simple mechanical debridement was performed at each patient's visit using sterile gauze to remove dead tissue and slough. After this, ulcers were covered by sterile gauzes and one layer of creep bandage was applied to affected leg. The patients in the group B received tubular compression deviceknee-high (Tubulcus®). This elastic stocking is classified as a compression device Class 3 that exerts the interface pressure of 35–40 mmHg. The interface pressure achieved with this device is graduated and the highest pressure is exerted at the ankle in the region of medial malleoli, diminishing upwards towards the knee. Tubulcus® elastic stocking comes in 5 different sizes (S, M, L, XL and XXL) and the size for each patient was determined according to the circumference of the affected leg. Two measures of the affected leg were taken: one at the ankle and the second at the largest part of the calf. One pair of elastic stockings were changed after the 6 months period and the circumference of the affected leg was remeasured in order to provide elastic stockings of adequate size. The Tubulcus® elastic stockings were placed on the affected leg over the local dressing using the special positioner. After slipping over the Tubulcus® device over the positioner, the stocking was placed to the desired position. The positioner was then removed by pulling it down using special handle [3]. The patients in the group B were instructed to wear Tubulcus® elastic stockings all the time during the day and at night.

Outcomes

Primary endpoint of our study was complete ulcer healing at 180 days. The ulcer closure was defined as the point at which complete epithelialization of the affected leg occurred.

Statistical analyses

After the 6 months follow up, data were collected and statistically analyzed. Our primary analysis compared time to ulcer healing on an intention-to-treat basis using the Kaplan-Meier survival analysis with log rank comparisons. In order to determine whether covariates (age, gender, body mass index – BMI, ulcer size, duration of venous ulceration) significantly influenced the ulcer healing rate, Cox regression analysis with backward method was used.

The χ^2 test was used to compare categorical parameters between the groups. Differences in median values between the two groups were analyzed with the Mann-Whitney *U* test.

The Fisher exact tests and Mantel-Haenszel χ^2-test were used to compare the frequencies. In order to compare means between the examined groups, an independent samples *t*-test was used. Single variable logistic regression was performed to calculate odds ratios (ORs) with 95% confidence intervals (CIs). The age was considered as a continuous, and other monitored factors as a categorical variables. Calculated *p* values were represented by the estimated regression coefficient divided by its standard error.

Statistical package SPSS 16.0 was used for the analyses (SPSS Inc., Chicago, Ill), with *p* values less than 0.05 considered as significant.

Results

One hundred and eleven patients were examined for possible inclusion onto this study and 71 were recruited and randomized.

The study excluded 40 patients: patients with diabetes mellitus (seven patients), heart insufficiency (one patient), pregnancy (one patients), malignant disease (two patients), patients with significant arterial disease (six patients), and patients who had secondary venous insufficiency and/or thrombosis (twenty-three patients).

Overall, 71 patients (37 men, 34 women; mean age 60 years) completed the study. The two study groups were comparable in terms of age, gender, general medical history, previous episodes of ulceration, size and duration of the ulcer (Table 1).

Table 1
Characteristics of the treatment and control groups (median, range)

Parameter	Group A (n = 34)	Group B (n = 37)	*p*
Male : female ratio	16 : 18	21:16	0.480
Age (years)	60 (33–80)	61 (40–77)	0.853
BMI, kg/m^2	28 (23–34)	29 (22–35)	0.903
Size of the ulcer (cm^2)	52.7 (11–134)	46.6 (8–142)	0.698
Duration of the ulcer (years)	5.1 (0.7–12)	4.4 (0.7–11)	0.484

Group A – patients treated by surgery only; Group B – patients treated by surgery plus compression.
BMI – body mass index.

The clinical, etiologic, anatomic and pathophysiologic (CEAP) classification was presented as follows: clinical – all patients included in the study had an active VLU (C6); etiologic – all patients had primary CVI; anatomic – superficial vein reflux was present in all 71 patients included in the study; pathophysiologic – reflux was the pathophysiology established in all 71 patients included in the study.

Ulcer characteristics

The median size of the ulcer in the group A was 52.7 cm^2 (range, 11.0–154.0 cm^2) and in the group B it was 46.6 cm^2 (range, 8.0–142.0 cm^2), (Table 1). The ulcer median duration time in the group A was 5.1 years (range, 7 months-12 years) and in the group B it was 4.4 years (range, 7 months-11 years) (Table 1).

Time to healing and healing rate

The healing rate was 29.41% (10/34) in the group A, and 56.76% (21/37) in the group B ($p < 0.01$). Mean healing time in the group A was 141 ± 15 days, and in the group B it was 98 ± 12 days (Log-rank life table analysis: $p < 0.001$), (Figure 1).

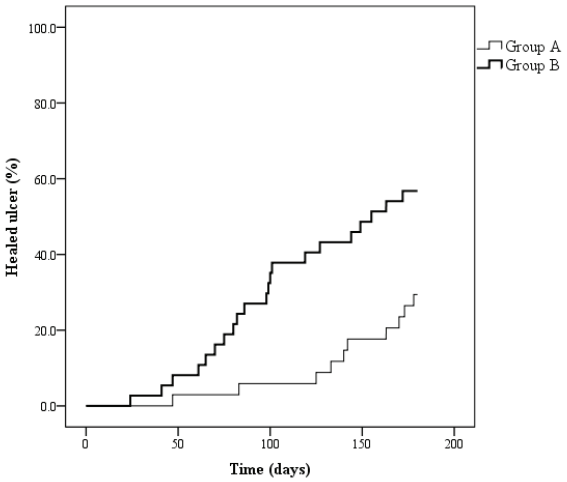

Fig. 1 – Cumulative healing rate of venous leg ulcers in the combined treatment group (surgery plus compression – the Group B) and the control group (surgery only – the Group A).

Age, gender, ulceration size, duration of the ulcer, body mass index are not independent parameters of success or failure of compression treatment (Table 2).

Table 2
Covariates entered in Cox regression model with enter method

Variables not in the equation	OR	95% CI	*p*
Age	0.988	0.951–1.026	0.517
Sex	1.504	0.717–3.155	0.280
Ulceration surface	1.006	0.997–1.015	0.202
Time since ulcer onset	0.981	0.895–1.076	0.687
Body mass index	1.003	0.913–1.102	0.948

CI – confidence interval; OR – odds ratio.

Discussion

Venous leg ulcers develop as a result of ambulatory venous hypertension. There are two main reasons for this: venous reflux and obstruction. As a result of ambulatory venous hypertension, inflammation process and leukocytes activation are triggered which leads to skin changes, at first and, in time, skin brake appears usually below the knee in the region of medial malleoli [13].

The aim of compression treatment in patients with VLU is ulcer healing, prevention of ulcer recurrences and reduction of pain and edema [14]. Regrettably, a large number of venous leg ulcers remain refractory to compression therapy and it is evident that healed venous ulcers have a high tendency for recurrence.

Compression therapy is the preferred treatment modality and has been used in different forms (compression hosiery, elastic or inelastic bandages usually applied as either two or multilayer bandaging systems) [1–4]. However, there is growing evidence supporting conclusion that surgical correction of underlying venous disorder in addition to compression may improve ulcer healing and reduce the rate of ulcer recurrences [11–12, 15]. There are many published studies comparing the efficacy of different compression systems, efficacy of surgery treatment in addition to compression compared to compression alone, but, there are no studies that compare contemporary surgical treatment alone to compression systems.

Our study clearly demonstrated the superiority of compression therapy plus surgery in the treatment of active venous ulcers compared to surgical treatment only.

This study could not verify risk factors for VLU healing rate and healing time based on patient's basic characteristics (age, sex, BMI, previous operations, medical history.

Interestingly, a recently published study by Gohel et al. [15] showed that treating venous ulcers early with endovenous ablation could significantly improve healing times and delay the recurrence of ulcers. In this study the patients were treated with compression as an addition to surgery which is in concordance with our finding that compression plus surgery achieves better results compared to surgery alone.

Our study also clearly demonstrated that surgical correction of venous reflux may resolve ulcer healing in small ulcers of short duration only. Large ulcers of long duration may be successfully treated with compression only. The inflammation lasted a long time and pathological skin changes were more profound compared to patients with small ulcers of short duration. These results show that patients with venous ulcers should be surgically treated as soon as venous ulcers develop in order to accelerate ulcer healing and provide a longer length of time free from ulcers (ulcer-free time). Surgical correction of underlying venous disorder, whenever is possible, is mandatory to abolish ambulatory venous hypertension and prevent continuous inflammation.

Among other risk factors, Nelson et al. [10] identified previous ulcers as a risk factor for VLU healing and recurrence rates. We could not confirm this finding in our study. This is probably because most of the patients included in our study had never been treated with compression previously. High percentage of our patients had an active venous ulcer for decades and previous ulcers were recognized only during the initial phase of venous ulcer development when they experienced spontaneous wound closure without compression treatment.

The ESCHAR study [11, 12] reported that patients who were surgically treated in addition to compression had a lower ulcer recurrence rate at 4 years compared to patients who were treated with compression only. However, healing rate and healing time was the same in both examined groups.

One of our previously published studies [7] found that high-compression systems healed more ulcers than compression systems with low or moderate compression. This trial is in concordance with these findings and it supports the premise that compression systems are mandatory in the treatment of venous leg ulcers.

Conclusion

The results obtained in this study suggest that compression therapy plus surgery provide statistically significant higher healing rate and faster healing time compared to surgery alone.

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IL-32 expression associated with lymph vessel invasion in intestinal type of gastric cancer

Udruženost ekspresije IL-32 sa invazijom limfnih sudova u intestinalnom tipu karcinoma želuca

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Abstract

Background/Aim. Gastric cancer (GC) is fourth most frequent malignant tumor worldwide, frequently diagnosed at advanced stages with poor prognosis. The aim of study was to determine expression of interleukin (IL)-32, pro-inflammatory and angiogenic mediators in the tumor, peritumor and healthy tissue, in patients with intestinal gastric cancer and the relationship with the disease severity. **Methods.** The tissue samples of intestinal type of the tumor of 60 patients with GC were analyzed. Expression of IL-32, vascular endothelial growth factor (VEGF), IL-17 and CD31 were measured by immunohistochemistry. **Results.** IL-32, VEGF and IL-17 expression as well as microvascular density (MVD) were diminished in adjacent tumor tissues compared with the tumor ones. Further, more intense expression of IL-32 and VEGF and enhanced MVD were noticed in patients with severe (TNM stages III and IV) and more progressive GC (lymph vessel invasion). **Conclusion.** Higher expression of IL-32, VEGF and intense MVD in the tumor tissue of GC patients with detectable lymph vessel invasion may be considered as a sign of the tumor's malignant progression. This indicates a protumorogenic and proangiogenic role of IL-32 in biology of intestinal type of gastric cancer.

Key words:
stomach neoplasms; il 32 protein, human; anti-allergic agents; severity of illness index; vascular endothelial growth factors; immunohistochemistry.

Apstrakt

Uvod/Cilj. Karcinom želuca (KŽ) četvrti je najčešći maligni tumor širom sveta, često dijagnostikovan u naprednim stadijumima sa lošom prognozom. Cilj studije bio je da se utvrdi ekspresija IL-32, pro-inflamatornih i angiogenih medijatora u tumoru, peritumoru i zdravom tkivu kod bolesnika sa intestinalnim tipom KŽ, kao i povezanost sa težinom bolesti. **Metode.** U studiji su analizirani uzorci tkiva intestinalnog tipa tumora od 60 bolesnika sa KŽ. Ekspresija interleukina (IL)-32, vaskularnog endotelnog faktora rasta (engl. *vascular endothelial growth f*actor – VEGF), IL-17 i CD31 merena je imunohistohemijskom metodom. **Rezultati.** Ekspresija IL-32, VEGF-a i IL-17, kao i mikrovaskularna gustina (engl. *microvascular density* – MVD) bili su smanjeni u peritumorskom tkivu u poređenju sa tumorskim tkivom. Intenzivnija ekspresija IL-32 i VEGF-a i pojačana MVD bili su registrovani kod bolesnika sa težim (TNM stadijumi III i IV) i progresivnijim karcinomom želuca (prisutna invazija limfnih sudova). **Zaključak.** Veća ekspresija IL-32, VEGF-a i intenzivnija MVD u tumorskom tkivu bolesnika sa KŽ i prisutnom invazijom limfnih sudova može se smatrati znakom progresije maligne bolesti. Ovaj rezultat ukazuje na protumorogenu i proangiogenu ulogu IL-32 u biologiji intestinalnog tipa KŽ.

Ključne reči:
želudac, neoplazme; il 32 protein, humani; zapaljenje, medijatori; bolest, indeks težine; faktori rasta endotela krvnih sudova; imunohistohemija.

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Introduction

Gastric cancer (GC) is the fourth most frequent malignant tumor and the second cause of cancer-related death worldwide [1]. Lauren [2] classified gastric cancer in two major forms: intestinal and diffuse type. *Helicobacter pylori* and chronic inflammation are two primary causes of intestinal gastric cancer [3, 4]. It is believed that persistent inflammation induces mucosal atrophy and hypochlorhydria, thus increases the risk for development of intestinal metaplasia, dysplasia and finally intestinal type of GC [4, 5]. Late diagnosis and mild or absent symptoms and clinical signs contribute to delayed therapy and high mortality [6].

Interleukin (IL)-32 is cytokine known to its involvement in the pathogenesis of diverse allergic, infectious, cancerous, and inflammatory diseases [7, 8]. Moreover, this pleiotropic cytokine has important role in various biological functions such as cell differentiation, stimulation of proinflammatory cytokines and cell death [8–10]. It plays important role in immunomodulation as well in tumor biology [11]. But, its precise role in this processes is still unknown. IL-32 stimulates production of pro-inflammatory cytokines including IL-8 and tumor necrosis factor (TNF)-α, prostaglandin E2 and also stimulates macrophages to produce pro-inflammatory factors [12, 13]. In line with this, IL-32 and IL-8 are significantly expressed in patients with estrogen receptor (ER)-positive tumors with detected lymph nodes. It is believed that IL-32 promotes angiogenesis and invasiveness *via* stimulation of pro-inflammatory cytokines IL-8 and TNF-α and thus contributes to tumor metastasis [14]. The other study showed that IL-32 induces development of distant and lymph node metastasis in patients with colorectal cancer (CRC) and thus can be considered as the marker of CRC metastasis [15]. In opposite, previous study reported an immunosuppressive role of IL-32, by inducing production of anti-inflammatory cytokine, IL-10 and immunosuppressive indoleamine 2,3-dioxygenase (IDO) [16]. It has been shown that IL-32 expressed in various cancers suppresses cancer cell growth by induction of apoptosis in cancer cells. Moreover, antitumorigenic function of natural killer (NK) cells is stimulated by IL-12 and IL-18, which further induce IL-32 production that stimulates TNF-α synthesis thus enhancing NK-mediated apoptosis [11, 17, 18]. The data about expression of this cytokine in tumor and peritumor tissue in intestinal type of GC. The aim of this study was to evaluate differences in expression of IL-32 and proangiogenic and proinflammatory molecules, VEGF and IL-17 as well as microvascular density (MVD) in the tumor, peritumor and healthy tissue in intestinal form of GC.

Methods

Ethic approvals

The study was conducted at the Center for Abdominal Surgery and the Center for Pathology, Clinical Center of Kragujevac and the Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Serbia. All patients gave their informed consent and research project was approved by relevant Ethics Committees of the Clinical Center of Kragujevac, Kragujevac, Serbia, and the Faculty of Medical Sciences, University of Kragujevac, Serbia. All research procedures were made according to the Principle of Good Clinical Practice and the Declaration of Helsinki.

Patients

The study included totally 60 patients with intestinal form of GC. The cancer was diagnosed on the basis of gastroscopic and histopathological criteria. The study did not include patients with no well-defined pathology, no adequate clinical document available or with previously diagnosed GC who were treated with radiation and chemotherapy. Data about age, gender, nuclear grade, well/moderate/poor differentiation and clinical stage by TNM (tumor, nodes, and metastasis) were recorded and analyzed in the study.

Immunohistochemical staining of VEGF, IL-32, IL-17 and CD31

The tissue samples of stomach from patients were fixed in 10% buffered formalin, routinely processed, and embedded in paraffin. Four-μm-thick sections from paraffin blocks were used for immunohistochemistry (IHC). IHC steps were carried out at room temperature. After deparaffinization and rehydration with graded ethanol, the sections were placed into a pressure cooker in 10 Mm sodium citrate buffer (pH 6.0) at full power for 20 min, followed by treatment with 3% hydrogen peroxide solution for 10 min. The primary mono-/poli-clonal antibodies against VEGF (ab16883, Abcam, Cambridge, UK, at a 1:200 dilution), IL-32 (ab37158, Abcam, Cambridge, UK, at 10 μg/mL), IL-17 (ab79056, Abcam, Cambridge, UK, at a 1:100 dilution) and CD31 (ab79056, Abcam, Cambridge, UK, at a 1:200 dilution) were incubated for 60 min with the tissue sections in a humid chamber, respectively and exposed to EnVision reagent (DakoCytomation, Glostrup, Denmark) for 30 min. The slides were then sequentially incubated with the chromogen reagent for 5 min, counterstained with Meyer's hematoxylin, and mounted. Negative control staining was performed by using mouse IgG1 isotype antibody. An Olympus microscope (BX50 model) equipped with a digital camera was used to prepare microphotographs with magnifications of ×200 or ×400.

Immunohistochemistry scoring

All tissue specimens were investigated by two independent pathologists. They used semi-quantitative modified scoring system based on the percentage of tumor tissue stained with IL-32 and intensity of staining [7, 19]. The IHC score was calculated by adding the percentage of positively stained cells to the staining intensity. The percentage of positive cells ranged between 0 and 3: 0 – if less than 10% of tumor cells were stained; 1 – if 10–25% of tumor cells were stained; 2 – if 25–50% were positive; and 3 – if > 50% were

positive. The staining intensity was scored as: 0 – negative immunoreaction; 1 – weak intensity; 2 – moderate intensity; and 3 – strong intensity. The sum of the two parameters varied between 0 and 6.

VEGF scoring was based on the presence, intensity and percent of positive cells, as previously described [19, 20]. Brown or brown-yellow staining signals found in the cell membrane or cytoplasm were considered to indicate VEGF immunopositivity. The negative controls were unstained. The number of positive cells in 500 tumor cells was counted within 3 randomly selected high power fields (×400). Four grades were defined according to the percentage of positively stained cells: 0 – no immunopositive cells; 1 – < 25% immunopositive cells; 2 – 25–50% immunopositive cells; 3 – > 50% immunopositive cells. Four grades were defined according to color-staining intensity: 0 – no color; 1 – weak, pale yellow; 2 – medium, brown; 3 – strong, dark brown.

Single endothelial cells or clusters of endothelial cells positive for CD-31 were considered as a microvessel, by two pathologists. At first, slides were examined at an original magnification of ×40. Three „hot spots" (areas with the highest MVD) from each slide were identified and these are photographed by a digital camera at an original magnification of ×200. The area of this histological field was 0.704 μm. MVD (microvessel/HPF – high-power field) and number of microvessels were evaluated according to MVD of the specimen that was estimated as a mean of MVD in three histological fields.

Expression of IL-17 was localized in the cytoplasm of mononuclear cells. Light-microscopic analysis was performed by manually counting positively stained cells in 3 separate areas of intratumor regions under ×400 high power magnifications [21].

Statistical analysis

The data were analyzed using commercially available SPSS 20.0 software. The results were reported as mean and standard error (SE). In determining statistically significant difference between the means of two groups it was used the Student's *t*-test for independent samples if the data had normal distribution or Mann-Whitney *U*-test for data without normal distribution. The Spearman's correlation evaluated the possible relationship between the expression of IL-32 and presence of lymphatic vessels invasion in GC. Strength of correlation was defined as negative or positive: weak (-0.3 to -0.1 or 0.1 to 0.3), moderate (-0.5 to -0.3 or 0.3 to 0.5) or strong (-1.0 to -0.5 or 1.0 to 0.5). *P*-value of 0.05 was considered as statistically significant.

Results

Sixty adult patients, between 54 and 92 years of age, with diagnosed and histologically confirmed intestinal form of GC were enrolled in this study. There was significant difference in gender distribution: 47 men (78.33%) and 13 women (21.67%). Clinical and pathologic characteristics of these patients are presented in Table 1. We have assessed expression of IL-32, CD31, VEGF and IL-17 in the tumor, peritumor and healthy tissue. Patients with GC were classified into two groups based on TNM stage of the disease: I + II and III + IV. Further, patients were divided according to the invasion of lymph vessels (+ and −). We analyzed values of previously defined markers of interest between defined groups.

Table 1
Baseline characteristics of patients with intestinal type of gastric cancer (GC)

Characteristics	Values
Gender (male/female), n	47/13
Age (years), mean (range)	75 (54–92)
TNMcClassification, (I and II/III and IV),	27/33
Nuclear grade (I/II/III), n	5/41/14
Histological differentiation rate (well/moderate/poor), n	11/31/18
Lymph vessel invasion (absent/present), n	10/50
Necsrosis (absent/present), n	21/39

TNM – tumor, nodes, metastasis; n – number of patients

IL-32 expression associated with lymph vessel invasion

We assessed expression of IL-32 cytokine in the tumor, peritumor and healthy tissue of GC patients. Imunohistochemistry data are illustrated in Figure 1C. The results obtained from this experiment showed that IL-32 was significantly more expressed in the tumor tissue in comparison to its expression in the peritumor tissue ($p = 0.001$; Figure 1a). Patients with GC were divided into two categories on the basis of TNM stage of the disease: I + II and III + IV. There was no significant difference in IL-32 expression between defined groups (data not shown). Further, expression of IL-32 was analyzed in patients divided into two groups, based on the invasion of lymphatic vessels (+ and -). Expression of IL-32 was significantly increased in patients with detected lymph vessel invasion ($p = 0.041$; Figure 1b). The relationship between IL-32 expression in the tumor tissue and the invasion of lymphatic vessels revealed a moderate positive correlation between IL-32 expression and presence of lymphatic vessels invasion ($r = 0.364$; $p = 0.040$).

Micro-vascular density associated with TNM system and lymph vessel invasion

We analyzed MVD in the tumor, peritumor and healthy tissue of GC patients. As the expression of molecule CD31 (PECAM-1) indicates the angiogenesis and the presence of blood vessels, immunohistochemistry was carried out in the tumor, peritumor and healthy tissue of all 60 patients with intestinal form of gastric cancer. Our results showed that MVD was significantly higher in the tumor tissue in comparison to the peritumor one of GC ($p = 0.001$; Figure 2a). Next, patients were divided into two categories on the basis of TNM stage of the disease: I + II and III + IV.

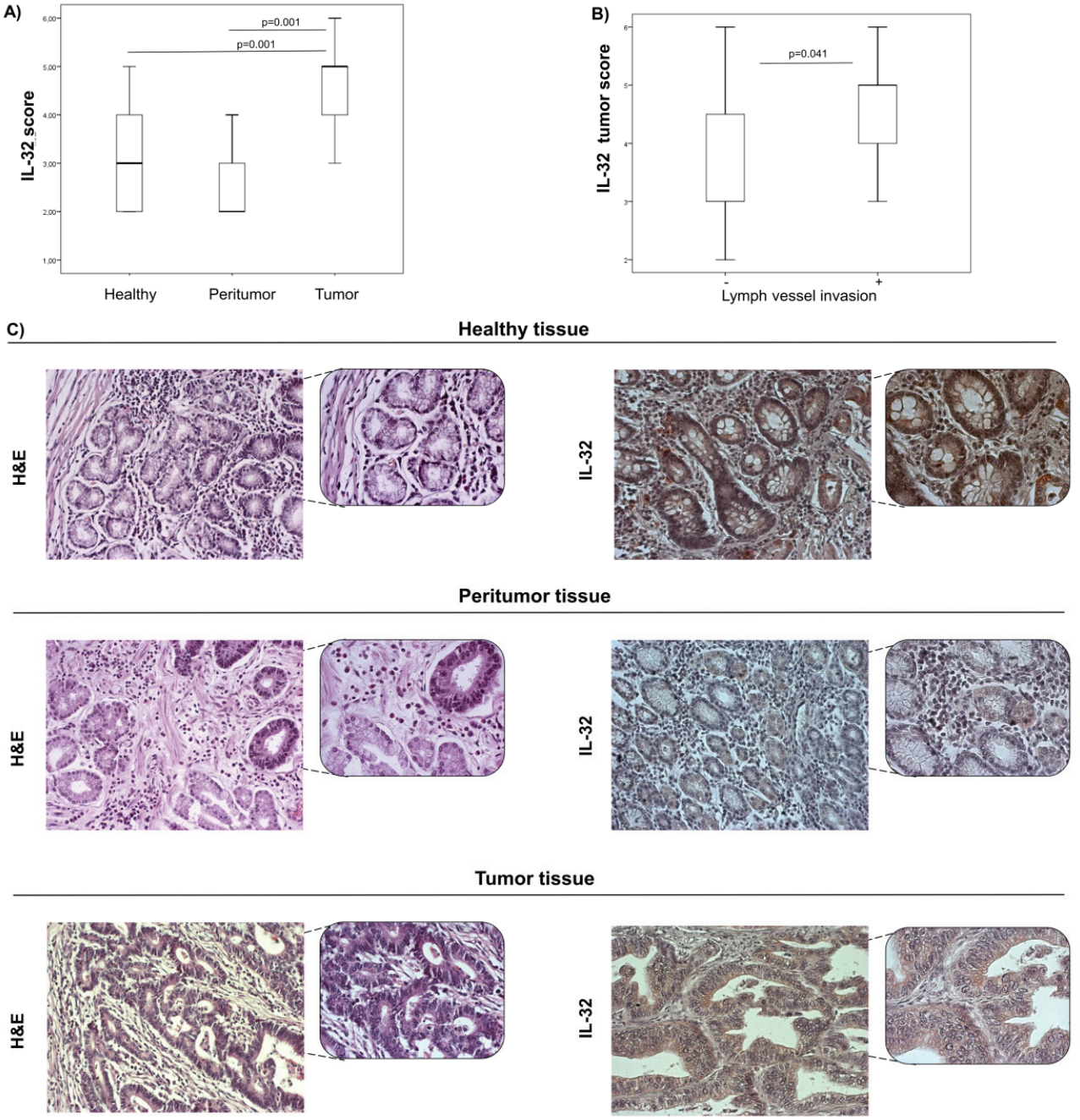

Fig. 1 – IL-32 expression in the tumor, peritumor and healthy tissue of patients with intestinal gastric carcinoma (GC).

A) Significantly higher IL-32 expression in the tumor tissue in comparison to its expression in the peritumor tissue ($p < 0.001$); B) Patients with detected lymph vessel invasion had significantly higher expression of IL-32 compared to patients without lymph vessel invasion ($p = 0.041$) (p values were assessed by the Mann-Whitney Rank Sum test); C) Hemotoxilyne-eosin (H&E) staining of representative tumor and peritumor tissues and representative IL-32 staining in the tumor, peritumor and healthy tissue of intestinal GC patients (×200 and ×400 magnification).

Patients with TNM stages III + IV revealed significantly higher MVD in the tumor tissue in comparison to patients with TNM stages I + II; ($p = 0.018$; Figure 2b).

Further, we divided patients on the basis of invasion of lymph vessels (+ and -), and analyzed MVD in the tumor tissue. MVD was significantly increased in the tumor tissue of patients with detectable lymphatic vessels invasion ($p = 0.012$; Figure 2c).

VEGF expression associated with TNM system and lymph vessel invasion

Focus of our further research was based on analyzing different proangiogenic soluble factors. Initially, we investigated expression of VEGF, one of the main proangiogenic molecules.

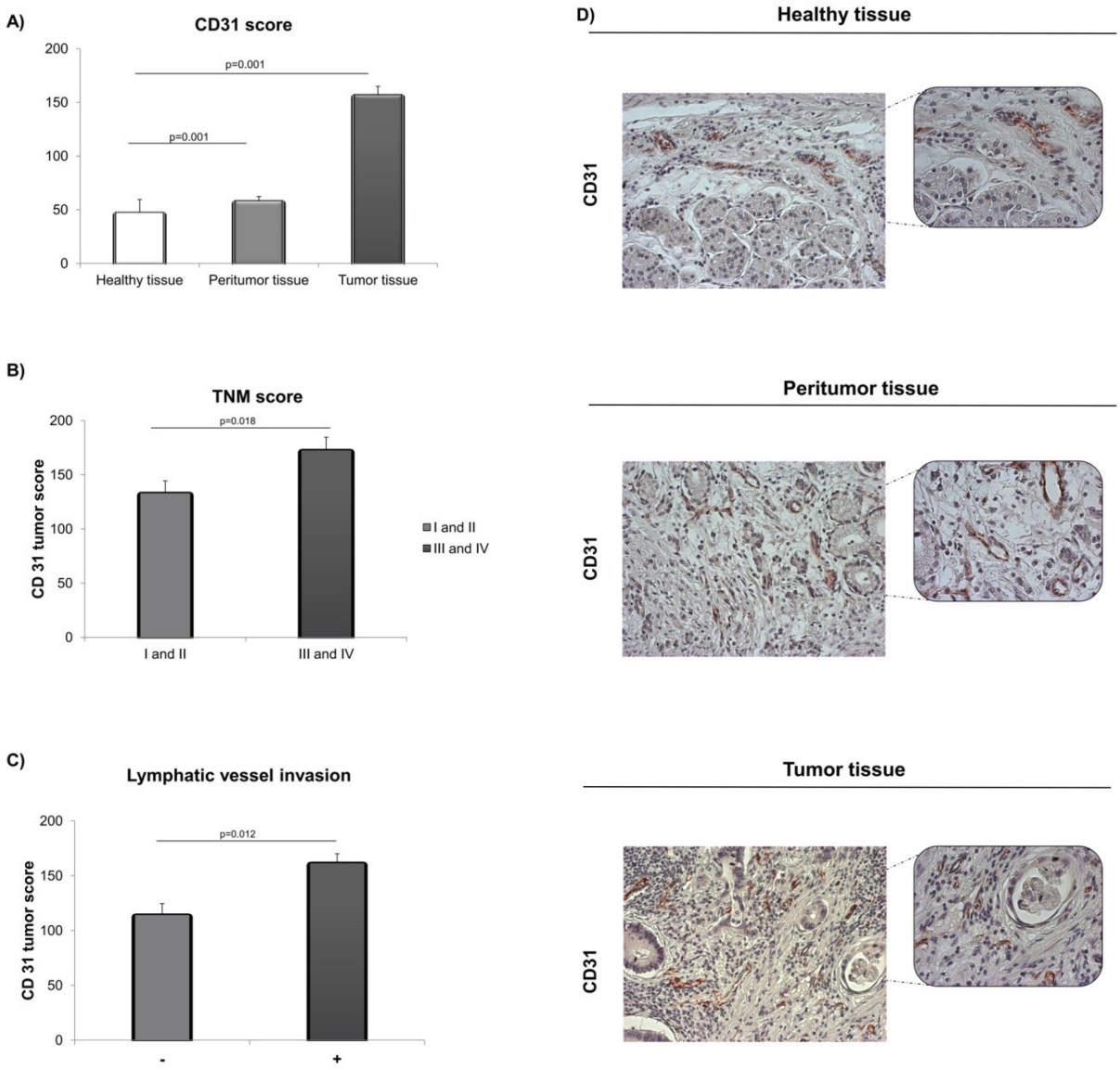

Fig. 2 – Microvessel density (MVD) in the tumor, peritumor and healthy tissues of patients with intestinal gastric carcinoma (GC).

A) CD31 expression was significantly higher in the tumor tissue in comparison to its expression in the peritumor tissue ($p = 0.001$); B) Patients with higher TNM stage (stage III + IV) had significantly higher expression of CD31 compared to patients with lower TNM stage (stage I + II) ($p = 0.018$); C) Patients with detected lymph vessel invasion had significantly higher expression of CD31 compared to patients without lymph vessel invasion ($p = 0.012$) (p values were assessed by the Mann-Whitney Rank Sum test); D) Representative CD31 staining in the tumor, peritumor and healthy tissues of patients with intestinal GC (×200 and ×400 magnification).

Results obtained from the experiment discovered that VEGF was significantly more expressed in the tumor tissue in comparison to the peritumor one of patients with GC ($p = 0.001$; Figure 3a).

Further, patients were divided into two groups based on TNM stages of the disease: I + II and III + IV. Patients with TNM stages III + IV had significantly higher expression of VEGF in tumor tissue compared to patients with TNM stages I + II ($p = 0.018$; Figure 3b). Next distribution of patients was created according to the existence of lymphatic invasion and analyzed them for expression of VEGF. Expression of VEGF was significantly higher in the tumor tissue with lymphatic invasion ($p = 0.002$; Figure 3c).

IL-17 expression associated with tumor necrosis

Analyses of the expression of IL-17 revealed that tumor tissue had significantly higher expression of IL-17 in comparison to the peritumor tissue ($p = 0.001$; Figure 4a). According to presence of necrotic fields in the tumor tissue, patients were divided into two groups (+ and -) and analyzed to the expression of IL-17. Results showed that IL-17 was significantly higher expressed in the tumor tissue with detectable necrotic fields ($p = 0.001$; Figure 4b).

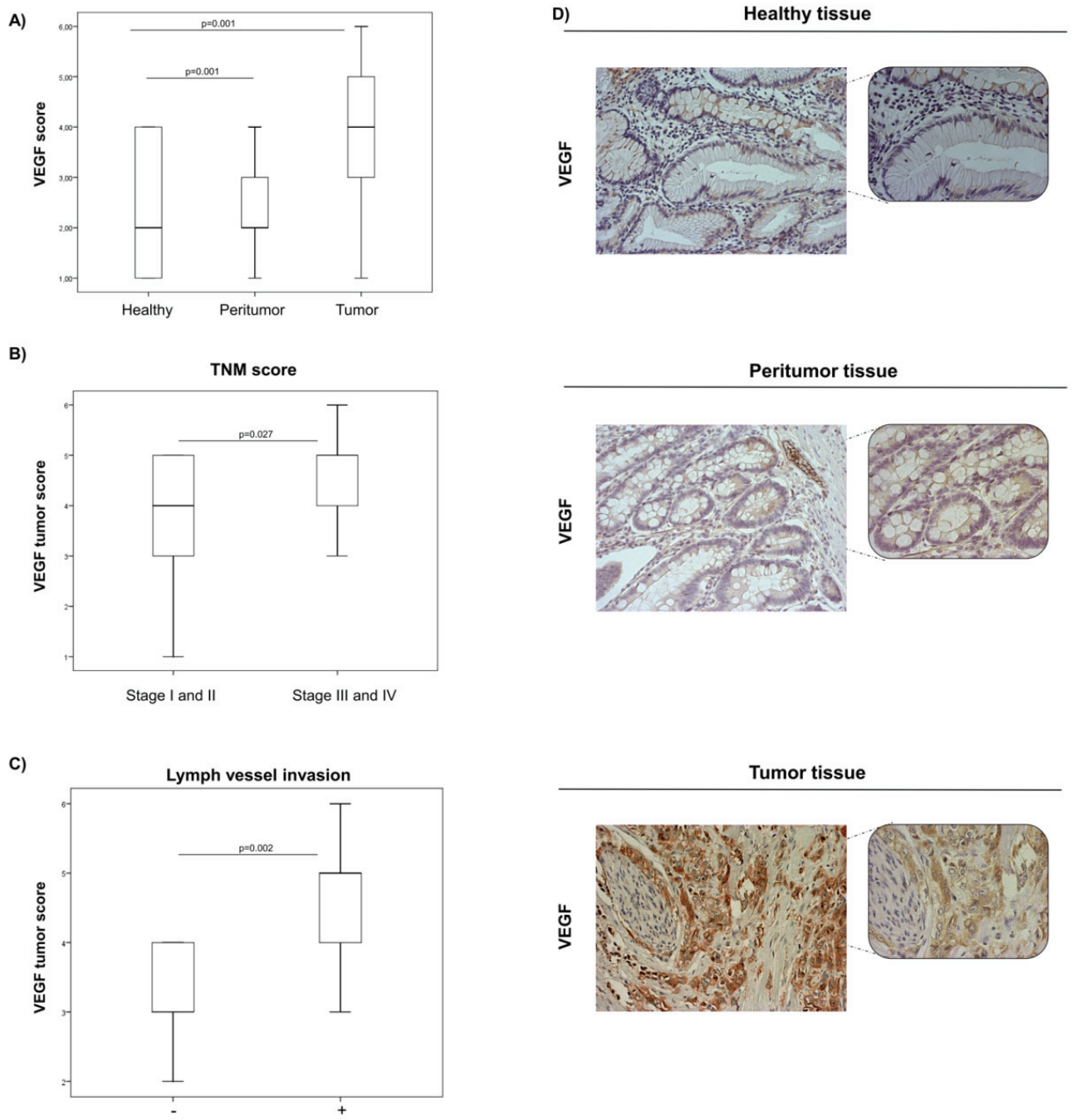

Fig. 3 – Immunohistochemical analysis of VEGF in the tumor, peritumor and healthy tissues of patients with intestinal gastric carcinoma (GC).

A) Significantly higher VEGF expression in the tumor tissue in comparison to its expression in the peritumor tissue ($p = 0.001$); B) Significantly higher expression of VEGF in the tumor tissue of patients with TNM stages III + IV compared to patients with TNM stages I + II ($p = 0.018$); C) Expression of VEGF was significantly higher in the tumor tissue of patients with detected lymphatic invasion in comparison to patients with no detected lymphatic invasion ($p = 0.002$). *P* values were assessed by the Mann–Whitney Rank Sum test; D) Representative VEGF staining in the tumor, peritumor and healthy tissues of patients with intestinal GC (×200 and ×400 magnification).

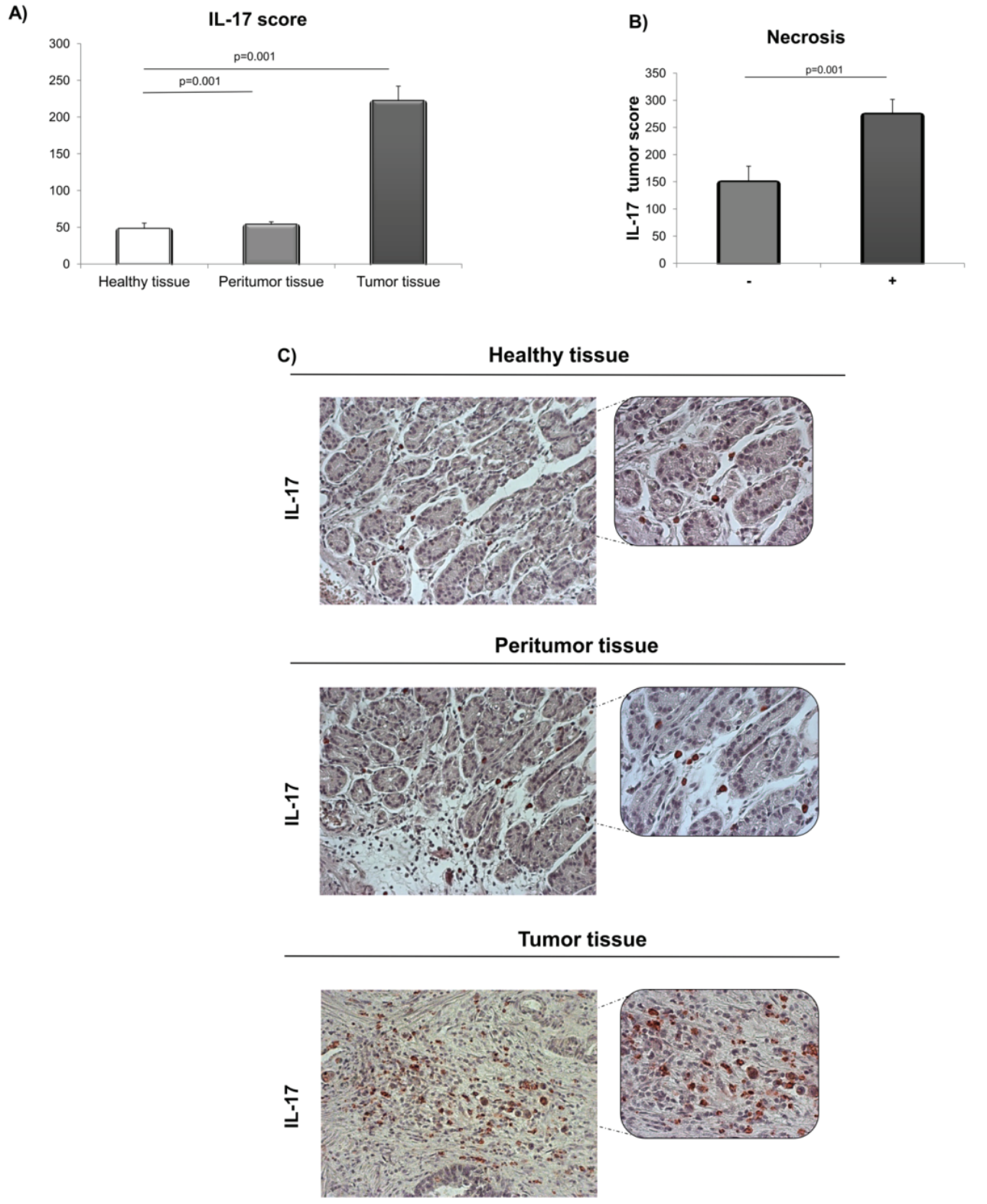

Fig. 4 – IL-17 expression in the tumor, peritumor and healthy tissues of patients with intestinal gastric cancer (GC).

A) Significantly higher expression of IL-17 in the tumor tissue in comparison to the peritumor tissue ($p = 0.001$); B) Significantly higher IL-17 expression in the tumor tissue of patients with detectable necrotic fields compared to patients without detectable necrosis ($p = 0.001$); C) Representative IL-17 staining in the tumor, peritumor and healthy tissues of patients with intestinal GC (×200 and ×400 magnification).

Discussion

Gastric cancer is the fourth most common cancer throughout the world behind lung, breast and colorectal cancers and the second major cause of cancer-related death [22, 23]. Around 90% of all GCs are adenocarcinomas, created from the glands of stomach mucosa [24]. According to Lauren's classification, there are two major histological types of GC: intestinal and diffuse type [2]. Intestinal type of GC consists of tubular or glandular metaplastic cell formations [25]. It is more frequent in elder males, with a lower TNM stage and a low risk of lymph node metastasis [26].

IL-32 is cytokine known to its important biological functions. Due to its proinflammatory function, IL-32 induces production of different chemokines and proinflammatory cytokines, including IL-1β, TNF-α, IL-6, IL-8, and macrophage inflammatory protein-2 (MIP-2) and activation of the p38 mitogen-activated protein kinase (MAPK), nuclear factor κB (NF-κB), and activator protein-1 (AP-1) signaling pathways [27]. IL-32 plays role in genesis and progression of GC. In the present study, we analyzed expression pattern of IL-32 in the tumor and peritumor tissue. We found significantly higher expression in the tumor tissue in comparison to the peritumor one. Moreover, IL-32 expression in the tumor tissue was significantly higher in patients with more progressive GC (lymph vessel invasion). These results are in line with previous studies claiming that IL-32 is higher in sera of GC patients [28, 29] and that IL-32 is linked to development of *Helicobacter pylori*-associated GC [30]. We obtained a positive correlation between IL-32 expression in the tumor tissue and disease severity (lymph vessel invasion), indicating its protumorogenic role. Moreover, IL-32 facilitates angiogenesis trough induction of production of matrix metalloproteinase and VEGF thus facilitating invasion and migration of tumor cells [31]. According to these data, further step was focused on analyses of MVD, proangiogenic and proinflammatory soluble molecules in the tumor and peritumor tissue of GC patients. CD31 is one of the most useful markers for detection of MVD. Platelet/endothelial cell adhesion molecule-1 (PECAM-1 or CD31) has pleiotropic effects such as transendothelial migration of leukocytes and inflammation as well as endothelial cell biology [32]. Moreover, CD31 plays important role in the tumor biology in few ways. It is one of the most abundant junctions set deep between endothelial cells thus supporting the integrity of endothelial membrane and regulating leukocyte migration and vascular permeability [33, 34]. We found increased MVD in the tumor tissue in comparison to the peritumor tissue. Moreover, MVD was significantly more explicit in patients with severe TNM stages III and IV and more progressive disease (lymph vessel invasion). MVD may be one of the important prognostic factors for GC patients and MVD value and lymph node metastasis represent independent prognostic factors [35].

Analysis of VEGF expression revealed its higher expression in the tumor tissue in comparison to the peritumor tissue of patients with GC, as well as more intense expression in patients with severe TNM stages III and IV and more progressive disease (lymph vessel invasion). In line with this finding, tumors with lymph node metastasis were associated with high VEGF-A, VEGF-B and VEGF-C, mRNA in lung adenocarcinoma [36]. The VEGF expression positively correlates with GC progression (TNM stage, tumor size, positive lymph nodes and lymphovascular invasion) [37].

As it is known that IL-32 promotes angiogenesis and inflammation, our further investigations were focused on analyses of proangiogenic and proinflammatory cytokine IL-17, in the tumor and peritumor tissue of GC patients. The tumor tissue had significantly higher expression of IL-17 in comparison to the peritumor tissue. Interestingly, we found increased IL-17 in the tumor tissue with detectable necrotic fields. Only a few studies evaluated IL-17 in GC, mainly describing IL-17 as promoter of cancer progression [38].

The selective process of metastasis requires active cross-talk between tumor cells and peritumor tissue, which is mediated by direct tumor cell-stromal cell contact or paracrine cytokine and growth factor signaling [39]. The peritumor environment should be fully taken into account in assessing the process of the tumor progression. Therefore, our goal was to evaluate the peritumor expression of IL-32, VEGF, IL-17 and MVD. We found lower expression of IL-32, VEGF and IL-17 as well as decreased MVD in adjacent tumor tissues compared with tumor tissues. Most studies have focused on the intratumor environment, and potential roles of angiogenesis and immunomodulation in the peritumor environment remain unclear. To our knowledge, this is the first study investigating peritumor IL-32 in any localization. In line with our findings, analysis of tumor and peritumor tissues of eyelids revealed that VEGF and MVD are highly expressed in tumors [40]. Interestingly, recent study revealed significantly higher peritumor expression of VEGF in hepatocellular carcinoma [41], opposite to our results. In the other study, peritumor expression of IL-17 corresponded with a significantly lower overall survival and maight be present as independent prognostic factor in patients with intrahepatic cholangiocarcinoma [42].

Conclusion

In summary, increased local expression of IL-32, in GC patients with detectable lymph vessel invasion may be considered as a sign of the tumor's malignant progression and, consequently, of a poor prognosis for patients. Increased IL-32, as well as VEGF and MVD in severe and advanced gastric cancers, may indicate a protumorogenic and proangiogenic role of IL-32 in intestinal type of gastric cancer. These observations point at possible facilitating role of IL-32 in biology of intestinal form of gastric cancer and its potential use as therapeutic target.

Declaration of interest

The authors declare that they have no conflict of interests.

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Effect of long-term strenuous training on the plasma phospholipid fatty acid composition in handball players

Efekat dugotrajnog napornog vežbanja na masnokiselinski profil fosfolipida plazme kod rukometaša

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Abstract

Background/Aim. Consensus on the exercise effect on the fatty acid metabolism has not been reached, and probably depends on the type of sports (aerobic, anaerobic or mixed). The aim of this study was to investigate effect of long-term handball training on the body composition, lipid profile and the plasma phospholipid fatty acid composition in female and male younger players. **Methods.** Seventeen female and 15 male active handball players, aged 16–20 years, who competed at the national/international level, were enrolled in the study. A control group was established from healthy, sedentary individuals (13 females and 19 males, aged 17–21 years), comparable to the athletes in terms of age, sex and body mass index. **Results**. In both groups of handball players a higher percentage of palmitoleic acid and alpha linolenic acid (18:3, n-3), were found and lower percentage of oleic acid and docosahexaenoic acid (22:6, n-3), when compared with corresponding control group. On the other hand, the lower level of stearic acid and estimated activity of plasma elongase was detected in female players than in sedentary women. Furthermore, higher proportion of linoleic acid (18:2, n-6), n-6 polyunsaturated fatty acids (PUFA) and total PUFA was found only in female players in comparison to the control group. **Conclusion.** The observed differences between handball players and sedentary individuals showed that handball training influenced lipid and fatty acid metabolism. Follow-up of these changes could indicate potential need for supplementation or nutritional intervention in young handball players.

Key words:
body composition; lipid metabolism; fatty acids; sports; sex factors.

Apstrakt

Uvod/Cilj. Konsenzus o uticaju treniranja na metabolizam masnih kiselina nije postignut, a taj uticaj verovatno zavisi od tipa sporta – aerobno, anaerobno ili mešovito vežbanje. Cilj ove studije bio je da se ispita efekat dugotrajnog, aktivnog treniranja rukometa na telesnu kompoziciju, profil lipida i masnih kiselina fosfolipida plazme kod mlađih kategorija rukometaša oba pola. **Metode.** U studiju je bilo uključeno 17 devojaka i 15 mladića, starosne dobi od 16 do 20 godina koji treniraju rukomet i takmiče se na nacionalnom i internacionalnom nivou. Kontrolnu grupu činilo je 13 devojaka i 19 mladića starosti od 17 do 21 godine, koji su bili uporedivi sa sportistima po godinama, polu i indeksu telesne mase. **Rezultati.** Procenat palmitoleinske i alfa-linolenske kiseline (18:3, n-3) bio značajno viši, dok je procenat oleinske i dokozaheksaenske kiseline (22:6, n-3) bio značajno niži u fosfolipidima plazme kod obe grupe sportista u odnosu na kontrolnu grupu. Sa druge strane, niži nivo stearinske kiseline i procenjene aktivnosti elongaze, ali i visok nivo linolne kiseline (18:2, n-6), ukupnih n-6 masnih kiselina, kao i ukupnih polinezasićenih masnih kiselina, utvrđen je kod rukometašica u odnosu na ispitanice iz kontrolne grupe, dok u grupi muškaraca nisu utvrđene takve razlike. **Zaključak.** Utvrđene razlike između rukometaša i rukometašica, sa jedne strane, i kontrolne grupe, sa druge strane, ukazale su na to da treniranje rukometa utiče na metabolizam lipida i masnih kiselina. Praćenje tih promena moglo bi ukazati na moguću potrebu za suplementacijom kod mladih rukometaša i rukometašica.

Ključne reči:
telo, sastojci; lipidi, metabolizam; masne kiseline; sport; pol, faktori.

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Introduction

Beneficial effects of regular physical activity on health are well established [1]. However, long-term strenuous training could have the opposite effect by production of proinflammatory cytokines and promotion of low grade inflammation. Previous studies have shown that sports with high degree of stressful physical exertion (e.g. soccer and volleyball), are accompanied by unfavorable plasma lipid and lipoprotein profiles, while sports with low levels of stressful exercise, such as swimming, appear to have a beneficial effect on plasma lipids [2].

Beside alternations in the levels of triacylglycerol (TG), total cholesterol, high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol in the circulation, chronic exercise leads to significant changes in the fatty acid (FA) composition of blood and tissue phospholipids [3, 4]. As elements of all natural membranes, FA are required for several basic functions, playing pivotal roles in regulation of intracellular signaling pathways, gene expression and production of important lipid mediators [5]. Although FA composition in biological membranes depends on the dietary intake, many other factors, including physical activity, may influence their metabolism [6–8]. Alterations in the FA profiles of plasma and erythrocytes phospholipids were found in elite water polo, football, basketball players and boxers when compared with sedentary subjects [9–11]. In addition, changes were not similar in different groups of athletes, suggesting that FA composition may depend on type of sport [9].

Handball is a globally popular team sport with almost 20 million players in the world. Due to its fast-paced game involving a lot of running, jumping, turning and slamming, it is a great workout for the whole body. Thus it is related to boosting the body's agility and flexibility, building up muscle tone and strength and improving cardiovascular function and oxygen supply [12, 13]. However, prolonged intense exercise promotes reliance on lipids as a primary fuel source, that is also connected with increased rate of harmful lipid peroxidation when compared to moderate or no physical activity [14]. Although the effect of exercise training on the FA composition of total lipids and different lipid classes have been studied [15–17], consensus on the effect of exercise on FA metabolism has not been reached, and probably depends on the type of sports (aerobic, anaerobic or mixed). Considering all these facts, the aim of this study was to investigate whether handball training modifies body composition, lipids profile and plasma phospholipid FAs composition in young female and male players.

Methods

Subjects

Seventeen female and 15 male active handball players aged 16–20 years, who competed at the national/international level, were recruited from elite sport clubs in Belgrade and Kragujevac, Serbia. The study was conducted during the period of preparatory training prior to the next competition season. A control group was established from healthy, sedentary individuals (13 females and 19 males, aged 17–21 years), comparable to the athletes in terms of age, sex and body mass index (BMI). All subjects were apparently healthy at the recruitment and during the study, and none of them was taking any drugs, or dietary supplements that might have influenced the lipid profile results. General data, such as age, duration of regular daily training, period of time of weekly training, dietary habits and use of supplements were obtained from the subjects through standardized questionnaires under supervision of a trained nutritionist. Female study participants reported regular menstrual cycles (26–32 days) and those who were taking oral contraceptives were excluded. All of them were included in the study in the early follicular phase of the menstrual cycle. The study protocols were approved by the Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac, Serbia in accordance with the Declaration of Helsinki and principles of Good Clinical Practice. All subjects gave written informed consent to participate in the study.

Anthropometric measurements

Standing height was measured in participants without shoes and socks, to the nearest 0.1 cm by a wall mounted stadiometer (Perspective Enterprises, Kalamazoo, MI). For measuring body weight (to the nearest 0.1 kg), BMI, percentage of body fat, fat mass, fat free mass and total body water, Tanita body composition analyzer (TBF-300, Tanita Corp., Tokyo, Japan) was used.

Analytical methods

Blood samples were taken in the morning after a 12 hrs fast, and 18 hrs after the end of the last training bout. Glucose, cholesterol and triglyceride concentrations were measured in the serum using automated enzymatic methods (Roche Diagnostics, Mannheim, Germany), on Cobas c111 analyzer (Roche, Basel, Switzerland).

Total lipid extract was prepared as described previously [10]. One-dimensional thin-layer chromatography in a neutral solvent system (petrol ether: diethyl ether: acetic acid 87:12:1 v/v) on Silica Gel GF plates (C. Merck, Darmstadt, Germany) was performed to isolate phospholipid fractions. Phospholipids were subjected to trans-esterification and obtained FA methyl esters were analyzed by the gas chromatograph Shimadzu 2014 (SHIMADZU, Kyoto, Japan) fitted with a capillary column (Rtx 2330, RESTEK, USA) as described previously [18]. The individual FA methyl esters were identified from the retention times of authentic standard mixtures (Sigma Chemical Co., St. Louis, MO, USA) and/or polyunsaturated FA (PUFA-2) standard mixture (Supelco, Inc., Bellefonte, Pennsylvania, USA). The results were expressed as the relative percentage of total identified FAs. Product-to-precursor ratios were used to estimate activities of certain enzymes involved in FA biosynthesis: 18:0/16:0 for elongase activity, 18:1/18:0 ratio for delta-9-desaturase (Δ9-desaturase) activity, 20:3/18:2 ratio for delta-6-

desaturase (Δ6-desaturase) and elongase activity, 20:4/20:3 ratio for delta-5-desaturase (Δ5-desaturase) activity.

Statistical analysis

Statistical analysis was performed using the statistical package SPSS 20.0 for Windows. The results are presented as means ± standard deviation. Normality was tested using the Shapiro-Wilk test before statistical analysis. For all variables which showed normal distribution, statistical comparisons of means were performed using the unpaired Student's *t*-test. For those which showed non-normal distribution [Δ6-desaturase, alpha linolenic acid (ALA) and eicosapentaenoic acid (EPA)], the Mann-Whitney *U*-test was performed. Differences were considered significant at *p*-values of < 0.05.

Results

The anthropometric characteristics and basic biochemical parameters of the study subjects are presented in Table 1. All anthropometric parameters, including height, weight, BMI and body fat were similar in both female groups. Although the level of all biochemical parameters was within reference ranges, concentrations of glucose and triglycerides in the serum were higher and lower, respectively in female players than in control women, as shown by the Student's *t*-test.

On the other hand, sportsmen had higher height, weight, fat free mass, and total body water, as well as lower body fat mass than control men. In addition, we found no difference in studied biochemical parameters between male athletes and control subjects.

FA composition of plasma phospholipids of the study participants are presented in Table 2. Among saturated FA (SFA), only percentage of stearic acid (18:0) was significantly lower in female handball players than in the control group. The percentage of oleic acid (18:1, n-9) was lower, and that of palmitoleic acid (16:1, n-7) was higher in both groups of athletes when compared to controls. In addition, female players had higher proportion of linoleic acid (LA, 18:2, n-6), n-6 PUFA, total PUFA than sedentary women, while higher ALA (18:3, n-3) and lower percentage of docosahexaenoic acid (DHA, 22:6, n-3) were observed in both groups of players in comparison to the control groups. The Student's *t*-test was used for all comparisons except ALA, which was analyzed by the Mann-Whitney *U*-test.

As shown in Table 3, the estimated activity of plasma elongase was lower in female handball players than in sedentary subjects, whereas estimated activities of desaturases were similar among the examined groups.

Discussion

It has been well established that long-term intense physical training modulates lipid profile of many tissues, not only concentration and distribution of lipid classes but also their FA composition [4]. We have previously shown that FA profiles in plasma and erythrocyte phospholipids differ between sportsmen and sedentary subjects [10, 11], as well as that type of regular training may affect metabolism of FA in elite athletes [9]. Here we examined the effects of handball training on plasma phospholipid FA profile in young players.

Different anthropometric parameters (Table 1) including body fat (both % and kg), fat free mass (kg) and total body water (kg) between male players and controls were expected due to intense trainings and in line with our previous results [9, 10]. Because of different body constitution, these changes in female athletes were not significant. Namely, women generally have higher % of body fat than men, due to sexual hormones, and this % markedly varies among women, including handballers. Thus, the standard deviation is higher and there was no statistically significant difference in body composition between athletes and the control group. Moreover, Bayios et al. [19] have published that Greek female handball players were shorter and had higher levels of body fat than basketball and volleyball players, and that their body composition was even close to general female population in Greece. They concluded that hours of training and sport-specific physiological demands during the game could explain the observed differences.

Table 1

The anthropometric characteristics of male and female handball players

Parameter	Male handball player	Control	Female handball player	Control
Age (years)	18.47 ± 1.06	19.05 ± 0.85	16.89 ± 1.00	17.91 ± 1.38
Height (cm)	192.73 ± 6.32***	182.44 ± 6.56	172.11 ± 7.64	171.18 ± 4.40
Weight (kg)	90.66 ± 14.96***	78.13 ± 10.04	64.36 ± 8.71	63.93 ± 8.72
BMI (kg/m^2)	24.37 ± 3.80	23.41 ± 2.12	21.78 ± 2.30	21.78 ± 2.07
Body fat (%)	9.66 ± 2.20***	14.70 ± 2.69	20.69 ± 4.94	24.09 ± 5.11
Fat mass (kg)	8.06 ± 2.99***	13.42 ± 4.55	14.34 ± 4.05	15.65 ± 4.99
Fat free mass (kg)	81.25 ± 9.38***	65.84 ± 6.62	51.00 ± 6.98	48.30 ± 5.53
Total body water (kg)	59.58 ± 6.87***	48.21 ± 4.85	37.43 ± 5.13	35.36 ± 4.05
Glucose (mmol/L)	4.67 ± 0.34	4.33 ± 0.35	4.40 ± 0.28*	4.17 ± 0.33
Triglycerides (mmol/L)	0.96 ± 0.30	0.99 ± 0.34	0.46 ± 0.13**	0.81 ± 0.30
Cholesterol (mmol/L)	4.07 ± 0.42	4.16 ± 0.94	3.95 ± 0.49	4.40 ± 0.66

Data are presented as a mean ± standard deviation.
BMI – body mass index.
***p* < 0.05, ****p* < 0.01, *****p* < 0.001 compared to the control group.**

Table 2

Plasma phospholipid fatty acid composition in male and female handball players

Fatty acid (%)	Male handball player	Control	Female handball player	Control
SFA				
16:0	26.39 ± 2.24	25.84 ± 1.59	27.95 ± 1.53	27.50 ± 1.44
18:0	15.26 ± 1.25	15.78 ± 1.44	13.91 ± 1.13*	15.43 ± 1.40
Total SFA	41.65 ± 1.42	41.63 ± 2.33	41.87 ± 1.29	42.93 ± 1.49
MUFA				
16:1, n-7	0.53 ± 0.17**	0.34 ± 0.12	0.46 ± 0.09**	0.39 ± 0.09
18:1, n-9	8.87 ± 1.13*	9.82 ± 1.10	8.51 ± 0.33*	8.85 ±1.07
18:1, n-7	1.56 ± 0.22	1.42 ± 0.24	1.45 ± 0.17	1.41± 0.16
Total MUFA	10.93 ± 1.22	11.58 ± 1.23	10.49 ± 0.84	10.65 ± 1.22
n-6 PUFA				
18:2, n-6	26.46 ± 2.68	26.10 ± 2.03	29.88 ± 2.24*	27.87 ± 2.58
20:3, n-6	3.31 ± 0.58	2.90 ± 0.59	2.74 ± 0.74	2.71 ± 0.68
20:4, n-6	13.16 ± 2.46	12.78 ± 1.94	10.90 ± 1.52	11.08 ± 1.59
22:4, n-6	0.70 ± 0.18	0.62 ± 0.14	0.50 ± 0.12	0.55 ± 0.15
Total n-6 PUFA	43.64 ± 2.14	42.38 ± 2.78	44.02 ± 1.34*	42.21 ±1.75
n-3 PUFA				
18:3, n-3	0.37 ± 0.16***	0.13 ± 0.04	0.26 ± 0.10**	0.12 ± 0.04
20:5, n-3	0.38 ± 0.08	0.34 ± 0.13	0.25 ± 0.09	0.20 ± 0.07
22:5, n-3	0.65 ± 0.15	0.73 ± 0.14	0.51 ± 0.12	0.54 ± 0.15
22:6, n-3	2.36 ± 0.52**	3.23 ± 0.97	2.60 ± 0.59*	3.19 ± 0.56
Total n-3 PUFA	3.69 ± 0.69	4.19 ± 1.31	3.56 ± 0.80	4.02 ± 0.68
Total PUFA	47.32 ± 1.42	46.56 ± 3.02	47.57 ± 1.40**	45.40 ± 2.60
n-6/n-3 ratio	12.17 ± 1.98	10.52 ±2.72	12.72 ± 5.34	10.85 ± 2.00

Data are presented as a mean ± standard deviation.
SFA – saturated fatty acids (16:0 – palmitic acid; 18:0 – stearic acid); MUFA – monounsaturated fatty acids (16:1, n-7 – palmitoleic acid; 18:1, n-9 – oleic acid; 18:1, n-7 – vaccenic acid); PUFA – polyunsaturated fatty acids (18:2, n-6 – linoleic acid; 20:3, n-6, – dihomo gamma-linolenic acid; 20:4, n-6:4 – arachidonic acid; 22:4, n-6 – adrenic acid; 18:3, n-3 – alpha-linolenic acid; 20:5, n-3 – eicosapentaenoic acid; 22:5, n-3 –docosapentaenoic acid; 22:6, n-3 – docosahexaenoic acid).
***p* < 0.05, ****p* < 0.01, *****p* < 0.001 compared to the control group.**

Table 3

The estimated plasma desaturase and elongase activities in male and female handball players

Enzyme	Male handball	Control	Female handball	Control
Elongase (18:0/16:0)	0.59 ± 0.10	0.61 ± 0.06	0.50 ± 0.06*	0.56 ± 0.07
Δ9- desaturase (18:1/18:0)	0.59 ± 0.11	0.63 ± 0.08	0.62 ± 0.10	0.58 ± 0.13
Δ6- desaturase and elongase (20:3, n-6/18:2, n-6)	0.13 ± 0.03	0.11 ± 0.03	0.09 ± 0.03	0.10 ± 0.03
Δ5- desaturase (20:4, n-6/20:3, n-6)	4.10 ± 1.01	4.55 ± 1.03	4.29 ± 1.26	4.30 ± 1.18

Data are presented as a mean ± standard deviation.
***p* < 0.05, compared to the corresponding control group.**

Furthermore, reduced plasma TG levels, which are used as energy sources during exercise, were found, but only in female athletes, the finding in accordance with the literature data [20]. Reduced plasma TG levels, which are used as energy source during exercise, were found in only female athletes.

Even though glucose levels in both examined groups were within referent values, we detected higher level of glucose in female athletes when compared to the control group. Plasma glucose concentration can increase in response to intermittent sport activity due to an increase in circulating catecholamines [21, 22]. Catecholamine-stimulated glycogenolysis results in an elevated plasma glucose level even exceeding resting values [21], which returns to the basal level after a few hrs recovery period [23]. Since glucose was determined 18 hrs after the last bout of exercise, we think that this difference can be a natural difference between two groups, unrelated to sport, especially as we did not find the same in males. Nevertheless, it should be checked comparing glucose levels in other handball and control groups.

Our results on FA composition of plasma phospholipids (Table 2) showed lower level of stearic acid and estimated elongase activity in female players than in the sedentary women. This is contrary to our previous study where female football players had higher level of stearic acid than controls, suggesting the effects of type of exercise on the elongase activity [9]. Increased SFA in plasma and/or erythrocytes is posi-

tively associated with the development of diabetes [24] and coronary heart disease [25], but this effect can be attributed to palmitic acid rather than stearic acid, which even exerts cardioprotective effects [26]. The lower level of stearic acid might be explained by the effect of handball training on elongase included in synthesis of stearic acid. Since we have not observed differences in the levels of stearic acid in male players nor in the estimated elongase activity, we can assume that the effect of exercise on the FA profile in plasma phospholipids is gender dependent. Still, further research is required to elucidate the relationship between exercise and modulation of activities of enzymes included in FA synthesis.

Unlike SFA, the impact of handball on monounsaturated fatty acids (MUFA) plasma phospholipids is similar in both groups of athletes. Namely, we found a significantly higher level of palmitoleic acid and lower level of oleic acid in both handball groups than in the control groups. These results are in line with our previous results on female athletes [9], but in male football and basketball players no differences were found [6, 10, 27]. Regarding beneficial cardioprotective effect of oleic acid [28], our results indicate the importance of increased dietary intake of olive oil as the best source of oleic acid. Furthermore, level of linoleic acid, and thus n-6 PUFA and total PUFA in plasma phospholipids was significantly higher in female players than in sedentary women. However, proportions of LA considerably vary between groups of athletes. For instance, LA and n-6 PUFA were decreased in female football players [9], increased in male basketball players [10], and similar to controls in male players in our study and in the study by Andersson et al. [6]. This is important since LA is precursor of the other n-6 PUFAs, including arachidonic acid which is a strong proinflammatory mediator [29].

Furthermore, handball players had higher levels of ALA than control groups. As precursor of n-3 PUFA family, ALA can reduce systemic inflammation by decreasing synthesis of inflammatory cytokines and stimulating synthesis of antiinflammatory eicosanoids [29]. Higher level of ALA, which we observed, could be of special importance in handball players, since strenuous exercise promotes synthesis of proinflammatory cytokines, and elite athletes often have altered immune response [30]. However, lower level of DHA, found in both athletes groups, suggest possibly decreased conversion of ALA to long chain n-3 PUFA – EPA and DHA, that could be a reason for elevated ALA proportion. Considering strong antiinflammatory properties of EPA and DHA and their importance not only for sport performances, but also for health, in general, our results indicate the need for nutritional intervention and/or n-3 PUFA supplementation in handball players.

Conclusion

The observed differences between handball players and sedentary individuals as well as between female and male players can be attributed to handball training and gender differences, although the mechanism underlying these changes requires further investigations. Since millions of people train handball, investigation and follow-up of lipid and FA profiles in handball players would indicate potential need for supplementation early in their career to avoid far-reaching consequences for their health.

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Conflict of interests

The authors declare that they have no competing interests.

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Inhalatory and intravenous colistin in treating ventilator-associated pneumonia due to *Acinetobacter* species: should we combine them?

Inhalatorni i intravenozni kolistin u lečenju ventilatorom udružene pneumonije izazvane *Acinetobacter* species: da li ih treba kombinovati?

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Abstract

Background/Aim. *Acinetobacter* is one of the most common causes of nosocomial infections, especially ventilator-associated pneumonia (VAP). Considering the increased presence of multidrug-resistant microorganisms and the lack of novel antibiotics, colistin merged as the last-resort antibiotic for life threatening nosocomial infections. Intravenous use of antibiotics is accepted as a gold standard for the treatment of pneumonia, but additional administration of inhaled antibiotics in the treatment of VAP has shown to be advantageous in some clinical trials. The aim of this study was to investigate the effect of inhalatory colistin as an adjunct to intravenous colistin on the survival of patients with VAP caused by *Acinetobacter* species. **Methods.** We conducted a retrospective study to evaluate the efficacy of combination of inhalatory and intravenous colistin vs. intravenous colistin alone in 69 patients in the Intensive Care Units (ICU) with VAP caused by *Acinetobacter baumannii*. The patients were treated in the ICU at the Institute for Pulmonary Diseases of Vojvodina, Sremska Kamenica (Serbia) in the period from January, 2013 to March, 2018. Baseline demographic data, severity of the disease, comorbidities, colistin regimen and length of the treatment were collected. The primary outcome was 28-day mortality. **Results.** Twenty seven of total 69 (39.1%) patients received combined intravenous and inhalatory colistin. Forty two (60.9%) patients received only intravenous colistin. Compared to the combined use of the drug (intravenous and inhalatory colistin), patients receiving intravenous colistin alone had a significantly increased risk of death during 28 days [25.9% vs. 61.9%, respectively; odds ratio (OR) 4.464, 95% confidence interval (CI) 1.539–2.925; $p = 0.006$]. Length of colistin use (> 7 days) was also associated with reduced survival (OR 0.22; 95% CI 0.080–0.606; $p = 0.003$). After adjusting for baseline severity of the illness (APACHE score) and length of colistin treatment, patients receiving only intravenous colistin had greater 28-day mortality rate compared to patients receiving both intravenous and inhalatory colistin (OR 6.305; 95% CI 1.795–22.153; $p = 0.004$). **Conclusion.** Our results suggest that adding inhalatory to intravenous colistin might be beneficial in the treatment of VAP caused by *Acinetobacter* species.

Key words:
pneumonia, ventilator-associated; acinetobacter; colistin; administration, inhalation; infusions, intravenous; treatment outcome.

Apstrakt

Uvod/Cilj. *Acinetobacter* je jedan od najčećih uzročnika nozokomijalnih infekcija, posebno pneumonije udružene sa upotrebom ventilatora (VAP). Uzimajući u obzir da je sve veći broj multirezistentnih mikroorganizama, uz nedostatak novih antibiotika, kolistin je našao svoje mesto u lečenju životno ugrožavajućih nozokomijalnih infekcija. Intravenska primena antibiotika je zlatni standard u lečenju pneumonija, ali dodatak inhalatorne, njihovoj sistemskoj primeni u lečenju VAP, pokazala je svoje prednosti u nekim istraživanjima. Cilj naše studije bio je da se ispita efekat inhalatorne primene kolistina, kao dodatka intravenskom načinu primene, na preživljavanje bolesnika sa VAP čiji je uzročnik *Acinetobacter*. **Metode.** Sprovedena je retrospektivna studija kako bi se procenila efikasnost kombinovane inhalatorne i intravenske primene kolistina u odnosu na samo intravensku primenu leka, kod 69 bolesnika sa VAP izazvanim *Acinetobacter* spp. Bolesnici su lečeni u periodu od januara 2013. do marta 2018. godine u Jedinici intenzivnog lečenja Instituta za plućne bolesti Vojvodine u Sremskoj Kamenici (Srbija). Prikupljeni su osnovni demografski podaci, podaci o težini bolesti, komorbiditetima, režimu kolistina i dužini lečenja. Primarni cilj studije bio je 28-dnevni

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mortalitet. **Rezultati.** Dvadeset sedam od ukupno 69 (39,1%) bolesnika primalo je kominaciju intravenskog i inhalatoronog kolistina. Kod 42 bolesnika dat je samo intravenski kolistin (60,9%). U poređenju sa bolesnicima kod kojih je primenjena kombinacija intravenskog i inhalatornog kolistina, bolesnici kod kojih je primenjen samo intravenozni kolistin imali su statistički značajno veći rizik od 28-dnevnog mortaliteta [25,9% vs. 61,9%, *odds ratio* (OR) 4,464; 95% *confidence interval* (CI) 1,539–2,925; p = 0,006]. Dužina lečenja kolistinom (preko 7 dana) takođe je bila povezana sa smanjenim preživljavanjem (OR 0,22; 95% CI 0,080–0,606; p = 0,003). Nakon prilagođavanja uzorka prema težini bolesti (APACHE skor) i dužini lečenja kolistinom, bolesnici koji su primali samo intravenozni kolistin imali su veći 28-dnevni mortalitet u poređenju sa bolesnicima lečenih kombinovanom primenom kolistina: intravenozno i inhalatorno (OR 6,305; 95% CI 1,795–22,153; p = 0,004). **Zaključak.** Rezultati naše studije su pokazali da bi inhalatorna primena kolistina, kao dodatak intravenoznoj primeni leka, mogla da poboljša ishod lečenja VAP uzrokovane *Acinetobacter* spp.

Ključne reči:
pneumonija, respiratorom uzrokovana; acinetobacter; kolistin; inhalaciona primena; infuzije, intravenske; lečenje ishod.

Introduction

According to the Cochrane database review, ventilator associated pneumonia (VAP) occurs in 10% of mechanically ventilated patients [1]. Earlier studies reported that depending on the underlying conditions and the pathogenicity of the infecting organisms, the mortality rates varied from 10% to 70% [2–4]. As stated in guidelines of the Infectious Diseases Society of America (IDSA) and the American Thoracic Society (ATS), the empirical treatment of VAP is based on the risk assessment of multidrug resistant infection. Inadequate initial therapy is associated with higher mortality and prolonged length of stay in an intensive care unit (ICU LOS) [5]. Early application of adequate antibiotic therapy is of crucial importance in the treatment of VAP. Postponement of antibiotic application as well as inadequate antibiotic therapy, even when later changed according to microbiological cultures, lead to higher mortality [6]. The choice of therapy should be based on the initial microbiological map, minding the side effects, as well as the previous antibiotic therapy in the last two weeks [5, 7].

Due to its high virulence and increased antimicrobial resistance, *Acinetobacter* is one of the most common causes of nosocomial infections, especially VAP. Imipenem was recommended as the first line treatment of pneumonia caused by *Acinetobacter baumannii*, until its resistance occurred to most antibiotics including aminoglycosides, carbapenems and fluoroquinolones [8–10].

In the 1950s, antibiotics polymyxin B and E (also known as colistin) were introduced for the treatment of infections caused by Gram-negative bacilli, but even though they were highly effective, they fell out of favor in human medicine due to nephrotoxicity [11, 12]. Considering the increased presence of multidrug-resistant microorganisms (*Acinetobacter baumannii, Klebsiella pneumoniae, Pseudomonas aeruginosa*), and the lack of novel antibiotics, polymyxins emerged as the last-resort antibiotics for life threatening nosocomial infections in the 21st century [13, 14].

Intravenous use of antibiotics is accepted as a gold standard for the treatment of pneumonia, but additional administration of inhaled antibiotics with their systemic use in the treatment of VAP has shown to be advantageous in some clinical studies [15–18].

Even though the idea to enhance the antibiotic concentration in the lungs by inhalation is rational, there is not enough published reports to elucidate the benefits of such a route of administration [19–21]. The studies related to this subject are scarce and have conflicting results. Despite the emerging colistin use, the recommendations for dosing regimens vary and the beneficial effects of inhalatory treatment remains insufficiently investigated [22, 23].

The aim of this study was to investigate the effect of inhalatory colistin as an adjunct to intravenous colistin on the survival of patients with VAP caused by *Acinetobacter* species.

Methods

A retrospective analysis was conducted in the period from January 2013 to March 2018. All ethical procedures were done in accordance with requirements of the Institute for Pulmonary Diseases of Vojvodina (IPDV), Sremska Kamenica, Serbia.The study included a total of 69 patients who were treated in the ICU of the IPDV. Those 69 patients received colistin for the treatment of VAP caused by *Acinetobacter*. Colistin was administered in two ways, only intravenously or in combination, both inhalatory and intravenously. The experimental group consisted of 27 patients who received both intravenous and inhalatory colistin, while the control group consisted of 42 patients who received only intravenous colistin.

The criteria for diagnosing VAP were based on recommendations for hospital-acquired pneumonia (HAP) and VAP from 2016 [5]. The patients were mechanically ventilated for a minimum of 48 hours, with a new infiltration on the chest X-ray or a progression of already existing infiltration with two of the following three criteria: fever over 38.5 °C or hypothermia below 35.5 °C, leukocytosis > 10,000/μL or leukopenia < 4,000/μL and purulent endotracheal aspiration. Non-invasive sampling and semi-quantitative determination were performed to determine the microbiological cause. The significant non-invasive quantitative sampling value was ≥ 10^5 colony forming unit (CFU)/mL. If the sampling was invasive with the quantitative determination of the causative agent, the threshold for the diagnosis of VAP was ≥10^4 CFU/mL for bronchoalveolar lavage [5].

Baseline demographic data and severity of illness [the Acute physiology and chronic health evaluation (APACHE) II [24], and the Sequential organ failure assessment (SOFA) scores] [25], presence of acute respiratory distress syndrome (ARDS) [26], septic shock [27] and acute renal failure (defined by the Kidney Disease: Improving Global Outcomes – KDIGO) [28], comorbidities, colistin regimen (intravenous vs. intravenous and inhalatory) and length of treatment were recorded. The primary outcome was 28-day mortality.

For statistical analysis, continuous variables were presented as mean and standard deviations (SD), while categorical variables were expressed as whole numbers and percentages. The influence of different colistin protocols on 28-day mortality was investigated using binary logistic regression analysis. All predictors that were statistically significant in the univariate analysis were entered into the multivariate model. The final model included APACHE score, length of treatment and colistin regimen. Statistical significance for all variables was set on p value 0.05. All statistical tests were performed using SPSS version 21.

Results

A total of 69 patients, 48 (69.6%) men, median age 56.64 ± 14.22 years, were included in the study. Mean APACHE score was 20.8 (± 5.8) and mean SOFA score was 6.8 (± 2.8). At admission, 55.1% of the patients were diagnosed with ARDS, 33.3% with septic shock and 36.2% with acute kidney injury. Almost 25% of patients, who developed VAP, had chronic respiratory diseases, primarily chronic obstructive pulmonary disease (COPD). Among other comorbidities, cardiovascular diseases, immune deficiency and diabetes were most common. The ICU mortality was 53.6% (37/69), 28-days mortality was 47.8% (33/69) and median ICU LOS was 19.59 (± 12.5) days. The differences in baseline characteristics between the patients who received intravenous and those who received combined intravenous and inhalatory colistin are presented in Table 1. There was no difference in length of hospital stay (35 ± 17 days in combined regimen group vs. 27 ± 19 days in intravenous regimen group; $p = 0.07$).

In Table 2 the univariate analysis of the factors associated with 28-days mortality is presented. In our study, 27 (39.1%) of total 69 patients received combined intravenous and inhalatory colistin. Forty two (60.9%) patients received only intravenous colistin. Compared to the combined use of the drug, patients receiving intravenous colistin alone had a significantly increased risk of death during 28 days (OR 4.464; 95% CI 1.539–2.925; $p = 0.006$). Length of colistin use was also associated with the increased risk of death (OR 0.22; 95% CI 0.080–0.606; $p = 0.003$ for patients receiving colistin for more than 7 days). In the multivariate analysis when adjusted for baseline severity of illness and length of colistin treatment, patients receiving only intravenous colistin had greater 28-day mortality rate compared to the patients receiving both intravenous and inhalatory colistin (OR 6.305; 95% CI 1.795–22.153; $p = 0.004$) (Table 3).

Table 1

Baseline characteristics of patients

Characteristcs	Values
Total number, n (%)	48 (69.6)
Gender, n (%)	
male	48 (69.6)
female	21(30.4)
Severity of illness, mean (± SD)	
APACHE	20.8 (± 5.8)
SOFA	6.8 (± 2.8)
ARDS, n (%)	
no	31 (44.9)
yes	38 (55.1)
Sepsis, n (%)	
no	23 (33.3)
yes	46 (66.7)
Septic shock, n (%)	
no	46 (66.7)
yes	23 (33.3)
Acute kidney failure, n (%)	
no	44 (63.8)
yes	25 (36.2)
Chronic comorbidities, n (%)	
COPD	
no	52 (75.4)
yes	17(24.6)
diabetes	
no	57 (82.6)
yes	12 (17.4)
malignancy	
no	63 (91.3)
yes	6 (8.7)
chronic kidney insufficiency	
no	67 (97.1)
yes	2 (2.9)
hepatic insufficiency	
no	66 (95.7)
yes	3 (4.3)
cardiovascular comorbidities	
no	55 (79.7)
yes	14 (20.3)
neurological comorbidities	
no	62 (89.9)
yes	7 (10.1)
immune compromise	
no	52 (75.4)
yes	17 (24.6)
gastric ulcer	
no	65 (94.2)
yes	4 (5.8)
Need for CRRT, n (%)	
before colistin use	
no	52 (75.4)
yes	17 (24.6)
after colistin use	
no	41 (59.4)
yes	28 (53.6)

APACHE – Acute physiology and chronic health evaluation;
ARDS – Acute respiratory distress syndrome;
SOFA – Sequential organ failure assessment;
COPD – Chronic obstructive pulmonary disease;
CRRT – Continuous renal replacement therapy;
SD – standard deviation.

Table 2
Impact of predictive factors on 28-day mortality by univariate analysis

Predictive factors	*p*	OR	95% CI	
			lower limit	upper limit
Gender				
male	0.308			
female		1.00[a]		
		1.174	0.609	4.828
Age	0.211	1.022	0.988	1058
*APACHE	0.023	1.114	1.015	1.233
SOFA	0.287	1.098	0.925	1.303
WBC ($\times 10^{-9}$)	0.639	0.988	0.942	1.037
ARDS				
no	0.570	1.00[a]		
yes		0.759	0.293	1.965
Sepsis				
no	0.308	1.00[a]		
yes		1.697	0.613	4.696
Septic shock				
no	0.051	1.00[a]		
yes		2.917	1.028	8.273
Acute kidney insufficiency				
no	0.601	1.00[a]		
yes		1.300	0.486	3.477
COPD				
no	0.299	1.00[a]		
yes		1.801	0.594	5.466
Diabetes mellitus				
no	0.159	1.00[a]		
yes		2.560	0.691	9.481
Malignancy				
no	0.103	1.00[a]		
yes		6.250	0.690	56.621
Hepatic insufficiency				
no	0.514	1,00[a]		
yes		2.258	0.195	26.132
Cardiovascular comorbidities				
no	0.437	1.00[a]		
yes		1.600	0.490	5.288
Neurological comorbidities				
no	0.605	1.00[a]		
yes		1.517	0.313	7.351
Immune compromise				
no	0.528	1.00[a]		
yes		0.528	0.231	1.965
Gastric ulcer				
no	0.929	1.00[a]		
yes		1.097	0.146	8.264
CRRT before colistin				
no	0.627	1.00[a]		
yes		1.312	0.438	3.933
CRRT after colistin				
no	0.079	1.00[a]		
yes		2.415	0.902	6.462
Febrile				
no	0.204	1.00[a]		
yes		0.528	0.197	1.415
Creatinine clearance	0.75	1.004	0.981	1.027
*Intravenous and inhalatory colistin				
no	0.006	4.464	1.539	2.925
yes		1.00[a]		
Bolus dose of colistin	0.527	0.942	0.782	1.134
Dose of colistin	0.686	2.362	0.037	151.692
Dosing interval of colistin	0.257	1.080	0.946	1.233

Table 2 (*continued*)

Predictive factors	*p*	OR	95% CI	
			lower limit	upper limit
*Length of colistin treatment				
≤ 7days	0.003	1.00[a]	0.080	0.606
> 7 days		0.220		
Ventilator days	0.402	1.018	0.976	1.063
ICU days	0.461	0.985	0.946	1.025

APACHE – Acute physiology and chronic health evaluation; SOFA – Sequential organ failure assessment; ARDS – Acute respiratory distress syndrome; COPD – Chronic obstructive pulmonary disease; CRRT – Continuous renal replacement therapy; WBC – white blood cells; ICU – intensive care unit; OR – odds ratio; CI – confidence interval. [a] – reference category; *statistically significant.

Table 3
Impact of predictive factors on 28-daily mortality by multivariate analysis

Predictive factors	*p*	OR	95% CI	
			lower limit	upper limit
APACHE	0.008	1.171	1.042	1.317
Intravenous and inhalatory colistin				
no	0.004	6.305	1.795	22.153
yes		1.00[a]		
Length of colistin treatment				
≤ 7days	0.019	1.00[a]	0.069	0.733
> 7days		0.225		

APACHE – Acute physiology and chronic health evaluation; [a] – reference category; OR – odds ratio; CI – confidence interval.

Considering the adverse effects of colistin use, need for continuous renal replacement therapy (CRRT) before and after colistin use was recorded. There was no difference in frequency of renal failure requiring continuous renal replacement therapy between the two groups of patients (17/42, 40.5% vs. 11/27, 40.7%; $p = 0.98$).

Discussion

The results of this study indicated that intravenous treatment with colistin was associated with 6-fold increase in 28-days mortality compared to combined intravenous and inhalation colistin regimen (61.9% vs. 25.9%, respectively; OR 6.305; 95% CI 1.795–22.153). The combined treatment resulted in prolonged length of hospital stay in relation to the intravenous only regimen, that was not statistically significant difference (35 vs. 27 days, respectively; $p = 0.07$).

Literature search revealed a small quantity of published studies that investigated the relation of the inhalatory colistin addition to the intravenously administered drug and their correlation with the 28-day mortality rate. Nevertheless, results from previous studies examining effects of the inhalatory colistin addition to the intravenous monotherapy treatment are conflicting [21, 29, 30]. These discrepancies among published studies were explained in the conclusion of the study by Tumbarello et al. [30] where it was stated that their investigation was conducted on a substantially larger population (being the largest study so far with 208 patients) and significant improvement of clinical cure rates were observed [31, 32]. These findings are in direct correlation with our investigation elucidating the substantial decrease in risk of ICU mortality and 28-day mortality when a combined treatment was carried out. Moreover, Tumbarello et al. [30] emphasized that an important role in further investigation should be to optimize the colistin use in order to enhance the efficacy without increasing the adverse renal effects. Additionally, it was stressed out that randomized controlled trials are needed for further clarification of benefits and risks of the combined treatment. Earlier review studies indicated that major adverse effect of colistin use could be nephrotoxicity, but results were inconclusive and could not allow for a more significant conclusion concerning the correlation of nephrotoxicity and colistin use [33]. These concerns have also been raised in recent publications for both intravenous and inhalatory route of the drug administration, where no increase in nephrotoxicity was reported with inhaled colistin as adjunctive therapy to the intravenous one, which is also in accordance with our findings [21, 34–36]. The overall conclusion of these studies was that the inhaled colistin seems to be beneficial in the VAP therapy and can be considered as safe, even though limitations and drawbacks were observed, mainly as inconsistent and limited data. A more detailed investigation of colistin nephrotoxicity and neurotoxicity was recently reported in the study of Abdellatif et al. [37], where renal safety was underlined as one of several benefits of aerosolized colistin regimen vs. intravenous.

It should be noted that the significant benefits of the colistin inhalotory enrollment in the combined therapy was

recognized in the latest hospital-associated pneumonia (HAP) and VAP guidelines of IDSA and ATS suggesting both inhaled and systemic antibiotics for patients with VAP, but with very low quality evidence [5]. Therefore, the results of our study could contribute to stronger evidence, essential for future guidelines as well as to the ongoing investigation of this therapeutic approach. Two studies out of nine, that were cited in the mentioned guidelines, directly concentrated their research on the beneficial effects of the inhaled colistin combined with intravenous colistin monotherapy [36, 38]. Korbila et al. [36] concluded that the application of the inhaled colistin was an independent predictor of cure of VAP, but no difference in all-cause in-hospital mortality and all-cause ICU mortality was detected. Three years later, Doshi et al. [38] published their results, obtained from three tertiary-care academic medical centers, stating that the addition of aerosolized colistin to intravenous colistin may improve clinical cure and mortality for patients with multidrug resistant gram-negative (MDR-GN) pneumonia. These findings are in accordance with our results elucidating the hypothesis of our research.

As previously mentioned, results obtained in our study showed that patients receiving only intravenous colistin had greater ICU mortality compared to the group of patients who received combined intravenous and inhalatory colistin (24/42, 57.1% vs. 13/27, 48.1%, respectively; $p = 0.465$). These results are in correlation with other studies comparing these two regimens of colistin administration, where collected data showed ICU mortality of 35.9–52.9% vs. 24–43.3%, respectively [21, 30, 36, 38].

The present study has some limitations that are very similar to the limitations stated in almost all previous investigations published on this subject. The limitations of our study are retrospective single-center nature, slight variations in the administration of the inhalatory colistin as well as dosing variations.

Conclusion

Our study demonstrated that adjunct of inhalatory colistin to intravenous colistin may significantly decrease 28-day and ICU mortality in the treatment of VAP caused by *Acinetobacter*. Therefore, we suggest the use of the mentioned treatment approach. High quality randomized controlled multicenter trials are urgently needed to validate the additional benefits of inhaled colistin in this setting.

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The use of mobile-aided learning in education of local anesthesia for the inferior alveolar nerve block

Primena učenja putem mobilnih uređaja u edukaciji izvođenja mandibularne anestezije

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Abstract

Background/Aim. Dental education has developed over the years, and various technologies have been included. Considering the fact that mobile devices are an imperative of modern time, the aim of our research was to evaluate effectiveness of Mobile-Aided Learning on practical administering the inferior alveolar nerve block (IANB). **Methods.** This prospective study involved 34 students who were randomly divided into two groups: G1 (control) group with 16 students and G2 (study) group with 18 students. Students of both groups previously successfully completed theoretical and practical training provided by the curriculum. For the purpose of additional education, students of the G2 group used a mobile application for 3D simulation of local anesthesia (Mobile-Aided Learning) outside the dental office for a period of one semester. After that, all students completed a post-clinical questionnaire. **Results.** The average time for performing anesthesia by participants in the G1 group was 70.54 ± 20.16 seconds, while in the G2 group it was 57.13 ± 17.45 seconds, which was significantly shorter ($p < 0.05$). A successful anesthesia application was higher in the G2 group (83.3%) compared to the G1 group (75%). The results of the post-clinical test questionnaire also indicated difference in the mean values of the responses to all questions, which was in favor of the G2 group participants. **Conclusion.** Application of Mobile-Aided Learning showed a significantly higher efficiency in student education for practical implementation of the IANB.

Key words:
anesthesia, dental; mandible; nerve block; students; cell phone; learning; computer simulation.

Apstrakt

Uvod/Cilj. Stomatološka edukacija razvijala se tokom godina uz uključenje različitih novih tehnologija. Imajući u vidu činjenicu da su mobilni uređaji imperativ modernog doba, cilj našeg istraživanja bio je da se proceni efikasnost primene mobilnog učenja na praktično izvođenje anestezije kod studenata koji prvi put sprovode mandibularnu anesteziju. **Metode.** U ovoj propektivnoj studiji učestvovala su 34 studenta koji su nasumce bili podeljeni u dve grupe: G1 (kontrolnu) grupu sa ukupno 16 studenata i G2 (studijsku) grupu sa 18 studenta. Studenti obe grupe uspešno su završili teorijski i praktični deo nastave predviđene nastavnim programom. Studenti G2 grupe su, u cilju dodatne edukacije, koristili mobilnu aplikaciju za 3D simulaciju lokalne anestezije (*Mobile Aided Learning*) van stomatološke ordinacije u trajanju od jednog semestra. Nakon toga, svi student su popunili postklinički upitnik. **Rezultati.** Prosečno vreme izvođenja anestezije kod ispitanika G1 (kontrolne) grupe bilo je 70,54 ± 20,16 sekundi, dok je kod ispitanika G2 (studijske) grupe vreme izvođenja anestezije bilo 57,13 ± 17,45 sekundi ($p < 0.05$). Iako bez statističke značajnosti, uspešnost davanja anestezije bila je veća u studijskoj grupi (83,3%), u odnosu na kontrolnu grupu (75%). Rezultati postkliničkog upitnika (testa), takođe su ukazali na razliku u srednjim vrednostima odgovora na sva pitanja, koja je bila u korist studijske grupe. **Zaključak.** Primena mobilnog učenja pokazala je veću efikasnost u edukaciji studenata za izvođenje mandibularne anestezije.

Ključne reči:
anestezija, stomatološka; mandibula; blokada živca; studenti; mobilni telefon; učenje; simulacije, kompjuterske.

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Introduction

The basic principle of modern dentistry today is painless dentistry. Application of local anesthesia allows patients maximum comfort and completely painless treatment. Therefore, mastering anesthesia techniques is an important aspect of the dental curriculum [1]. However, learning anesthesia techniques is still a complex process, and moving to work with patients is often very difficult for students [2].

Dental education has developed over the years, and various technologies have been included in the curriculum. In this sense, simulation models of dental education have been used for more than 100 years [3]. They have a significant impact on education in many areas of dentistry such as endodontics, oral hygiene and operative dentistry [3–5]. This education system contributes to improving psychophysical skills of students before their first clinical experience, their manipulative abilities, increasing patient safety during clinical trials conducted by inexperienced clinicians [3, 6].

Today, we became owners of personal computers, the Internet happened, and information and communication technologies (IT) experienced flourishing and irreversibly changed the whole world. Undoubtedly, they unwittingly permeate the sphere of dental education in form of simulation models, complementing conventional teaching in that way. Computer teaching in the health profession, also known as Computer-Aided Learning (CAL), has become a popular means of providing information to students, patients and practitioners [7].

Today, in the context of the widespread use and appearance of mobile devices, such as smartphones and tablets, people can communicate, work, entertain, access the Internet, and even explore and learn. Bearing in mind the fact that mobile devices are an imperative of modern times, the aim of our research was to evaluate the effectiveness of Mobile-Aided Learning on practical application of anesthesia by students who are dealing with implementation of the inferior alveolar nerve block (IANB) procedure.

Methods

Participants

The presented research was approved by the Institutional Review Commission. This prospective study involved 34 students of the fourth year at the Department of Dentistry, Faculty of Medicine, Kosovska Mitrovica, University of Priština, Serbia, who did not have any practical skills regarding application of the IANB on patients. The participants were randomly divided into two groups: G1 group (control group) with 16 students and G2 group (study group) with 18 students (Figure 1). The students of both groups then successfully completed the theoretical and practical part of education envisaged by the curriculum, and we applied a direct anesthetic technique for the IANB [8].

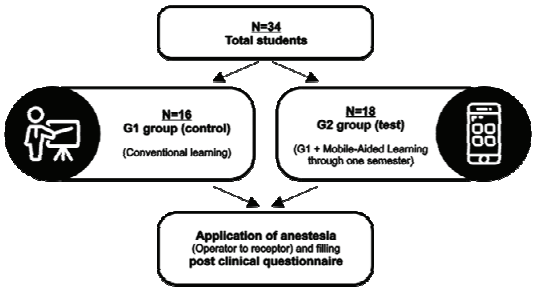


Fig. 1 – Learning protocol.

Mobile-aided learning

For the purpose of additional education, students of the G2 Group used the Dental Simulator mobile application (Campinas, SP 13083765, Brazil), which is available for iOS (App Store) and Android (Google Play Store), (Figure 2). After registering, students used an application outside the dental office *via* "University Mode" in Serbian through Study Mode (where dental students can read technical descriptions, watch clinical and simulation videos and practice) and Simulation Mode (students can simulate dental procedures and get feedbacks, so they can learn their mistakes in 3D) (Figure 3).

Fig. 2 – Home screen of Dental Simulator Application.

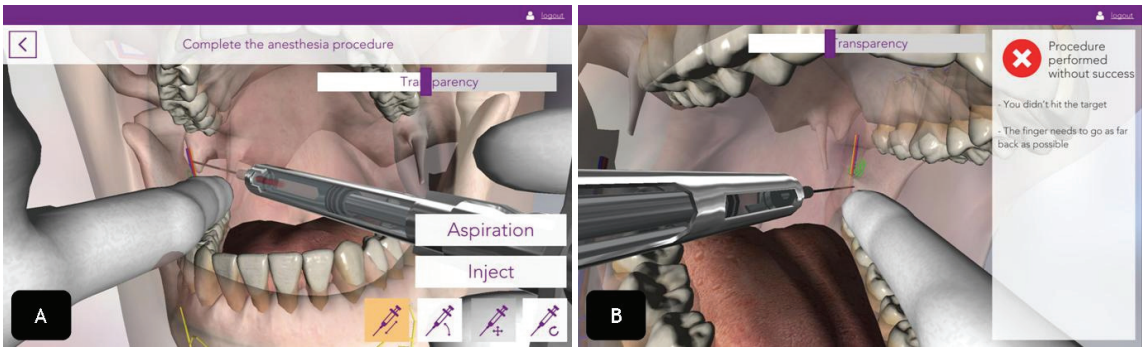
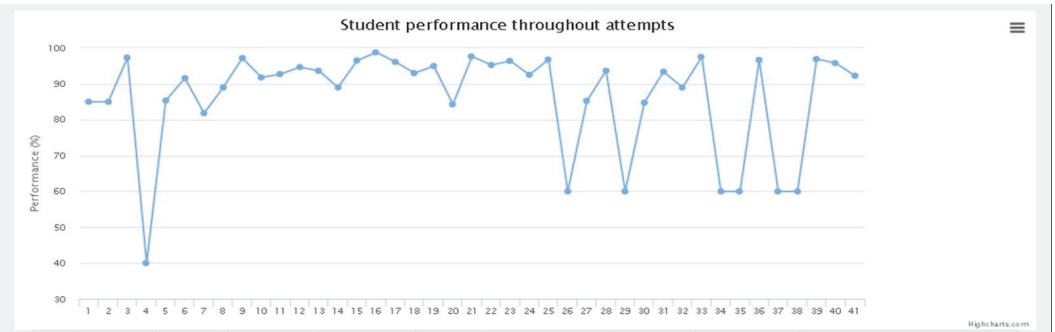


Fig. 3 – A) Simulation of dental procedure, and B) feedbacks.



n	Interval	Side	Finger	Aspiration	Sucess	Mode	Distance	Total Errors	Timestamp
1	55 s	Right	Thumb	No	No	Simulation	1.79	1	2016-12-14 12:42:14
2	41 s	Right	Thumb	No	No	Simulation	1.8	1	2016-12-14 15:21:05
3	50 s	Right	Thumb	No	Yes	Simulation	0.4	0	2016-12-14 15:22:18
4	65 s	Right	Index	No	No	Simulation	2.1	3	2016-12-14 15:23:36
5	86 s	Right	Index	No	No	Simulation	1.68	1	2016-12-14 15:25:55

Fig. 4 – Monitoring student education through the University Mode.

Through the University Mode, in order to monitor the success of the G2 student education, the educator had access to information about each student during exercise (Figure 4).

After completing their education and written consent, in the second phase of the research, students applied the IANB to each other (the operator to the receptor) by a direct method. We used 2% lidocaine with adrenaline (40 mg + 0.025 mg)/2mL (2% Lidokain®, Galenika AD, Serbia). The parameter for selecting the side for anesthesia was the presence of at least one tooth of a molar or premolar region with preserved pulp vitality. For this reason, as well as in order to monitor the success of an anesthesia, 15 minutes before and after anesthesia, the vitality of the teeth of these regions was checked by the standard procedure (Roeko Endo-Frost, Coltene Whaledent). The success of education was evaluated through the success of anesthesia, as well as the time of application that included the period from the moment of removal of needle protection until syringe aspirated for negative pressure and observed for the absence of blood. After that, the injection was administered at a rate of 0.4 mL over 30 seconds [9].

Post-clinical questionnaire

Additionally, the success of education was measured on the basis of post-clinical questionnaires. After application of anesthesia, participants completed a questionnaire that evaluated their knowledge and skills. Questions were quantified by a 5-point Likert scale, and possible answers and values were: I totally disagree = 1; I partially disagree = 2; abstained = 3; partially agree = 4; I totally agree = 5.

Statistical analysis

Statistical data analysis was performed using IBM SPSS Statistics 22 (IBM Corporation, Armonk, NY, USA). Results were presented as frequency (percentage), median (range) and mean ± standard deviation. The Fisher's exact test was used to test differences between nominal data (frequencies). For numeric data with normal distribution independent samples Student's *t*-test was used to test differences between groups. For numeric data with non-normal distribution and ordinal data Mann-Whitney U was used. All *p* values less than 0.05 were considered significant.

Results

The examined parameters showed a significant success of the participants in the G2 group compared to those in the G1 group. The average time for performing anesthesia by participants of the G1 group was significantly longer comparing to subjects who were using 3D simulation (Table 1). Also, after additional aided education, participants of the Group 2 performed the IANB more successfully, although it was not statistically significant (Table 1).

The results of the post-clinical test questionnaire also indicated differences in the mean values of responses to all questions in favor of the G2 group (Table 2), which was especialy notable (and statistically significant) for answers to the question "I easily identify the exact location of the sting".

Table 1

Average time for anesthetic procedure of the inferior alveolar nerve block (IANB)

Parameters	G1 (control) group (n = 16)	G2 (study) group (n = 18)	*p*
Time (seconds), mean ± SD	70.54 ± 20.16	57.13 ± 17.45	0.045*
Success of anesthesia, n (%)			
yes	12 (75)	15 (83.3)	0.609
no	4 (25)	3 (16.7)	

SD – standard deviation; *statistically significant difference.

Table 2

Post-clinical questionnaire and values classified according to a Likert scale

Question	G1 (control) group median (range)	G2 (study) group median (range)	*p*
I self-confident in the IANB anesthetic procedure	3 (1-5)	4.5 (3-5)	0.412
I easily identify the anterior border of the ramus	3 (1-5)	4 (2-5)	0.322
I easily identify the pterygomandibular raphe	3 (1-5)	3 (2-5)	0.197
I easily identify the exact location of the sting	3 (1-4)	4 (3-5)	0.033*
I can apply the IANB anesthesia next time without supervision	3.5 (1-5)	4 (1-5)	0.302

IANB – inferior alveolar nerve block; *statistically significant difference.

Discussion

Many dentistry students point to inadequate preparation for practical use of local anesthesia in clinical conditions [10], while studies show that even clinical dentists identify the administration of local anesthetics as one of the most stressful procedures in everyday clinical work [11]. It especially applies for the IANB, which is often complex for dentistry students to be understood and performed, primarily due to difficult and insufficiently clear identification of the sting location.

Researches show that an average person spend up to 5.5 hours with a mobile phone during the day, from that at least 2 hours with the so-called unnecessary content, such as social networking, games, etc. Also, several studies have found that mobile devices today play an important role in education and have the impact and benefits in relation to the point of pedagogical perspective [12, 13]. Therefore, our aim was to apply a 3D simulation of the IANB in education of students, beside conventional methods. Also, the learning process which includes simulation techniques allows students to critically evaluate how they felt during the exercise, to practice the same procedure repeatedly without the need for supervision and with synchronous computer feedback [14], and may have an impact on the level of reliability when applying the first anesthetic procedure [15]. Our study suggests that the model of student education which, in addition to conventional methods, includes mobile 3D simulation, gives better results than the conventional method alone considering skill of providing the IANB.

An important parameter that indicates knowledge of the IANB technique and the level of safety in its performance is time required for anesthesia. The procedure for giving anesthesia will be shorter in people with higher level of knowledge and education. In other research, higher education corresponds with shorter time of giving [16]. In our study, the time of anesthesia was statistically significantly shorter in the study group compared to the control which indicates that training with additional simulations can improve skill of students for the performance of the IANB. Similar results published López-Cabrera et al. [17], pointing the fact that students who were practicing on dental anaesthesia simulation model, besides the conventional methods, exhibited shorter time of the procedure for the anterior superior alveolar nerve.

Perception of students about the level of their knowledge and safety when performing the IANB was measured by a post-clinical questionnaire using the Likert scale. A similar instrument of research was used in other studies [15, 18]. Students of the study group had more positive answers to all questions of the post-clinical questionnaire, which was statistically significant for the question "I easily identify the exact location of the sting".

The effectiveness of anesthesia was also one of the tested parameters. In our study, the G1 group had a failure rate of 25%, while in the G2 group it was 16.7%. Although the difference was not statistically significant, it could indicate a better knowledge of the technique and self-confidence in performing the IANB.

Numerous studies have dealt with the effect of simulation models on education of students in the field of local anesthesia. Marei and Al-Jandan [15] compared theoretical and practical knowledge of students with conventional methods of learning in relation to knowledge when conventional methods were used together with a simulation model (electric phantom). Their results point to a better level of knowledge of students in which the simulation model was used, but the statistical significance existed only in terms of theoretical knowledge. López-Cabrera et al. [17], who used the phantom as a simulation model, also highlighted significantly higher level of self-confidence among students who used simulation models in addition to classical methods.

Our study confirms benefits of the use of simulation models as supplemental methods of education of students in providing the IANB. However, we would like to point out that, within the various types of simulation models, the aided learning model used in our study shows numerous benefits. First of all, the advantages of mobile learning are that mob ile phones are always at hand (having in mind the fact that daily use of a mobile phone is growing day by day) and, financially, they are more profitable because they do not need additional phantoms and tools for exercising. For the development of effective skills, awareness of reasons when and how the error occurred is more important than the final result [19], and the "University Mode" of the mobile application provides all the information and shows the most common student errors at any time during exercise.

Conclusion

The use of mobile-aided learning exhibited several benefits for student education concerning practical IANB application. Students who used a combination of conventional method and virtual simulation model exhibited shorter time of anesthesia, showed more self-confidence and had a higher percentage of successful anesthesia. This type of simulation model can be recommended for regular student education.

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Influence of applied CD34$^+$ cell dose on the survival of Hodgkin's lymphoma and multiple myeloma patients following autologous stem cell transplants

Uticaj primenjene doze CD34$^+$ ćelija na preživljavanje bolesnika sa Hodgkin-ovim limfomom i multiplim mijelomom nakon autologne transplantacije matičnih ćelija

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Abstract

Background/Aim. Autologous stem cell transplants (ASCTs) improve the rate of overall survival (OS) in patients with hematological malignancies such as multiple myeloma (MM) after induction chemotherapy, aggressive non-Hodgkin's lymphomas (NHL), and relapsed, chemotherapy-sensitive Hodgkin's lymphoma (HL). The study aim was to evaluate influence of applied CD34$^+$ cell quantity on clinical outcome, as well as early post-transplant and overall survival (OS) of HL and MM patients following ASCT. **Methods.** This study included a total of 210 patients (90 HL/120 MM) who underwent ASCT. Stem cell (SC) mobilization was accomplished by granulocyte-colony stimulating factor (G-CSF) 10–16 μg/kg body mass (bm) following chemotherapy. For proven poor mobilizers, mobilization with G-CSF (16 μg/kgbm) and Plerixafor (24 or 48 mg) was performed. To our best knowledge, it was the first usage of the Plerixafor in our country in the ASCT-setting. Harvesting was initiated merely at "cut-off-value" of CD34$^+$ cells ≥ 20 × 10^6/L in peripheral blood with "target-dose" of CD34$^+$ cells ≥ 5 × 10^6/kgbm in harvest. The CD34$^+$ cell count and viability was determined using flow cytometry. **Results**. The majority of HL patients (76.7%) were infused with > 5.0 × 10^6/kgbm CD34$^+$ cells, while 68.3% of MM patients were treated by approximately 4.0–5.4 × 10^6/kgbm CD34$^+$ dose, respectively. Beneficial response (complete/partial remission) was achieved in 83.3% (HL) and 94.2% (MM) patients. Among parameters that influenced survival of HL patients with positive response to the therapy, multivariate analysis (pre-ASCT performance status, CD34$^+$ cell quantity applied, rapid hematopoietic, i.e. lymphocyte and platelet recovery) indicated that higher CD34$^+$ cell dose used, along with pre-ASCT performance status correlated with superior event-free survival (EFS) and OS following ASCT. In MM patients, multivariate analysis (renal impairment, infused CD34$^+$ cell quantity, early platelet recovery) indicated that the number of CD34$^+$ cells infused was the most important parameter that influenced both EFS and OS after ASCT. **Conclusion**. Data obtained in this study undoubtedly confirmed that CD34$^+$ cell dose applied is an independent factor that may contribute to superior clinical outcome and OS of HL and MM patients following ASCT.

Key words:
hematologic neoplasms; stem cells; transplantation, autologous; survival; flow cytometry.

Apstrakt

Uvod/Cilj. Autologna transplantacija matičnih ćelija (ATMĆ) poboljšava učestalost ukupnog preživljavanja (UP) kod bolesnika sa hematološkim malignitetima kao što su multipli mijelom (MM) nakon indukcione hemoterapije, agresivni non-Hodgkin-ovi limfomi (NHL) i recidivantni hemiosenzitivni Hodgkin-ov limfom (HL). Cilj ove studije je bila procena uticaja primenjene doze CD34$^+$ ćelija na klinički ishod, kao i na rano post-transplantacijsko i UP bo-

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lesnika sa Hodgkin-ovim limfomom i multiplim mijelomom posle ATMĆ. **Metode**. Ova studija obuhvatila je ukupno 210 bolesnika (90 HL/120 MM) koji su bili lečeni primenom ATMĆ. Mobilizacija matičnih ćelija (MĆ) izvedena je pomoću stimulišućeg faktora granulocitnih kolonija (G-CSF) [10–16 μg/kg telesne mase (tm)] posle hemioterapije. Za dokazane "slabe-mobilizatore" izvedena je dodatna mobilizacija upotrebom G-CSF (16 μg/kgtm) uz dodatak *Plerixafora* (24 ili 48 mg). Po našem saznanju, ovo je bila prva primena *Plerixafora* u našoj zemlji u okvirima ATMĆ. Prikupljanje ćelija je započeto jedino pri graničnoj, odnosno "*cut-off*" vrednosti $CD34^+ \geq 20$ /μL u perifernoj krvi, sa "ciljnom dozom" $CD34^+ \geq 5 \times 10^6$/kg telesne mase (tm) ćelija u afereznom produktu (*harvest*). Broj $CD34^+$ ćelija i vijabilnost bili su određivani primenom protočne citometrije. **Rezultati**. Većini bolesnika sa HL (76,7%) infundovano je > $5{,}0 \times 10^6$/kgtm $CD34^+$ ćelija, dok je 68,3% MM bolesnika tretirano dozom od $4{,}0–5{,}4 \times 10^6$/kg tm $CD34^+$ ćelija. Povoljan terapijski odgovor (potpuna/parcijalna remisija) postignut je kod 83,3% (HL) i 94,2% bolesnika (MM). Od parametara koji su individualno uticali na preživljavanje bolesnika sa HL i povoljan odgovor na terapiju, multivarijantna analiza (status pre ATMĆ, primenjena doza $CD34^+$ ćelija, rani oporavak hematopoeze, tj. oporavak limfocita i trombocita) ukazali su na to da primena većih doza $CD34^+$ ćelija, zajedno sa karakteristikama pre ATMĆ, pozitivno korelira sa boljim preživljavanjem i izostanakom neželjenih događaja (IND), kao i UP posle ATMĆ. Kod bolesnika sa MM, multivarijantna analiza (oštećenje bubrega, doza primenjenih $CD34^+$ ćelija, rani oporavak trombocita) pokazala je da je broj infundovanih $CD34^+$ ćelija najznačajniji parametar koji ima uticaja na IND i UP bolesnika posle ATMĆ. **Zaključak**. Podaci dobijeni u ovoj studiji neosporno ukazuju na to da je infundovana doza $CD34^+$ ćelija nezavisan faktor koji može doprineti boljem kliničkom ishodu i UP bolesnika sa HL i MM posle ATMĆ.

Ključne reči:
hematološke neoplazme; matične ćelije; transplantacija, autologna; preživljavanje; citometrija, protočna.

Introduction

Autologous stem cell transplants (ASCTs) improve the rate of overall survival (OS) in patients with hematological malignancies such as multiple myeloma (MM) after induction chemotherapy, aggressive non-Hodgkin's lymphomas (NHL) [1], and relapsed, chemotherapy-sensitive Hodgkin's lymphoma (HL) [2]. As a result, ASCT has become the standard therapeutic option for these malignancies [3, 4]. In order to identify patients benefiting from ASCT, several clinical parameters were reported to be of prognostic importance in HL [5], and MM [6]. Moreover, some ASCT parameters may also influence OS of transplanted patients including early lymphocyte, neutrophil and platelet recovery, infused lymphocyte dose, and the number of infused $CD34^+$ cells [7]. Of particular importance is the number of $CD34^+$ cells received by patients, which is a common predictor of the potential engraftment [8]. Moreover, there may be a correlation between the number of given $CD34^+$ cells, and disease relapse, transplant-related mortality and OS. However, the role of an infused autograft $CD34^+$ cell dose and early lymphocyte, neutrophil, and platelet recovery following ASCT has not been firmly established as standard procedure [1, 2, 7].

The present study aimed to evaluate the influence of applied $CD34^+$ cell dose and various clinical parameters that might influence early post-ASCT and OS of HL or MM patients following transplants.

Methods

This retrospective study included a total of 210 patients who underwent ASCT between November of 2005 and January of 2017. Ninety patients were diagnosed with HL and 120 with MM.

Each patient with HL went through an initial standard staging according to the Ann Arbor classification evaluation before treatment [8], with calculation of the International Prognostic Score (IPS) for risk stratification [9].

MM patients were, after initial evaluation, staged according to the Durie and Salmon clinical staging system, and risk groups were determined according to the International Scoring System (ISS) [10]. Chromosomal abnormalities were revealed using interphase fluorescence *in situ* hybridization (iFISH) [11].

All HL patients were initially treated according to ABVD protocol (adriamycin, bleomycin, vinblastine and dacarbazine) and were evaluated according to current response criteria [12]. Platinum-based salvage chemotherapy was given at relapse.

Stem cell (SC) mobilization was completed by granulocyte-colony stimulating factor (G-CSF) at standard dose of 10–16 μg *per* kg of body mass (kgbm) in all patients with previously application of chemotherapy [salvage regimen in HL and cyclophosphamide, adriamycin and dexamethasone (CAD) or high dose (HD)-cyclophosphamide in MM)].

Collections of autologous SCs – using Cobe-Spectra and Spectra-Optia (Terumo-BCT, USA) – were initiated merely at "cut-off-value" of $CD34^+$ cells $\geq 20 \times 10^6$/L in peripheral blood. The "target-value" of harvested $CD34^+$ cells was $\geq 5 \times 10^6$/kgbm. Among of all patients, 6 (2.9%) were proven poor mobilizers. The second mobilization using G-CSF (16 μg/kgbm) and with Plerixafor [24 or 48 mg (one or two doses/bottles), approximately 6–11 hours prior to harvesting] was performed. For all of these patients, $\geq 4 \times 10^6$/kgbm $CD34^+$ cells were collected. To our best knowledge, it was the first usage of the Plerixafor in the ASCT setting in our country.

Finally, cells were cryopreserved using our original controlled-rate freezing procedure by optimized dimethyl sulfoxide (10% DMSO) and stored at -140 ± 5 °C (mechanical freezer) or at -196 °C (liquid nitrogen) and thawed immediately prior clinical use in a water bath at 37 ± 3 °C, as described previously [13, 14].

The $CD34^+$ cell quantity in harvest was determined with a flow cytometer (Beckman-Coulter, USA). Cell viability (i.e. the ratio of "non-apoptotic" $CD34^+$ cells) was also estimated on the basis of the 7-aminoactinomycin D (7-AAD) flow cytometry assay (Immunotech, France), as earlier described [15].

The BEAM [total dose (TD) – carmustine 300 mg/m^2, etoposide 800 mg/m^2, cytarabine 1600 mg/m^2 and melphalan 140 mg/m^2] conditioning-protocol was given in 76 HL patients (84.4%), while 14 patients (15.6%) received the CBV (TD-cyclophosphamide 6,000 mg/m^2, carmustine 300 mg/m^2, etoposide 750 mg/m^2). G-CSF was administered after autologous SCs infusion and was continued until the absolute neutrophil count (ANC) was at least 1.0×10^9/L on two consecutive days. Platelet (PLT) transfusions were administered empirically for patients with PLT counts of 20×10^9/L or lower, or in patients who experienced bleeding. Mediastinal radiation was applied after ASCT on initially bulky mediastinal mass, if post ASCT positron emission tomography / computed tomography (PET/CT) was positive. Within post-transplant relapse, five patients received brentuximab-vedotin (anti-CD30 antibody), and two more cases, as post-transplant consolidation due to high risk of relapse.

Regarding MM patients, a historical VAD regimen, as initial treatment was given in 36 patients (30.0%), Thalidomide-based combinations in 80 (66.7%) patients, and bortezomib-based regimes in 4 (3.3%) patients. Peripheral blood SCs were collected during 1–2 consecutive aphereses following mobilization protocol CAD. In poor mobilizers (6 patients), second mobilization was conducted with addition of Plerixafor with a sufficient number of $CD34^+$ cells for transplant ($\geq 4 \times 10^6$/kgbm). In 5 patients, who underwent "tandem" ASCT, a target $CD34^+$ cell dose of 8.0×10^6/kgbm was collected. The conditioning regiment consisted of high dose melphalan, as a single agent at a dose of 200 mg/m^2, and at reduced dose of 100 or 140 mg/m^2 for patients with reduced creatinine clearance (30–60 mL/min) or with a high comorbidity index. Patient therapeutic response was evaluated according to criteria of the International Myeloma Working Group [16]. Relapsed patients were treated with bortezomib-based combinations if they did not receive bortezomib initially.

The study was performed according to the guidelines of the Declaration of Helsinki and was approved by the local Ethics board.

Following ASCT, the OS was measured from the date of ASCT until the last follow-up or until death from any cause, while event free survival (EFS) was measured from the date of ASCT until the disease progression/relapse or the last follow-up. OS functions were calculated using the Kaplan-Mayer approach, while a log-rank test was used to compare statistical differences between curves. The cutoff points for recovery of absolute lymphocyte count (ALC) of 500×10^6/L or greater (ALC500), ANC $\geq 500 \times 10^6$/L (ANC500), and PLT count $\geq 20 \times 10^9$/L (PLT20), by Day +20, Day +11, and Day +13, respectively, were calculated according to previously published data [7]. The Spearman's correlation coefficient was used to analyze correlations among variables of interest. In order to predict OS after ASCT, cutoff values of $CD34^+$ cells for HL and MM, were determined as 25th and 75th percentile values of its distribution, respectively. Statistical analyses were done using IBM SPSS statistical package (Version 21). All statistical tests were two-sided. The level of significance (alpha level) in all analyses was set at $p < 0.05$.

Results

Patient characteristics and cellular research

The clinical and laboratory characteristics of HL and MM patients are summarized in Tables 1 and 2. A total of 90 patients with HL, and 120 patients with MM were analyzed.

The mean dose of transplanted $CD34^+$ cells in HL patients was 7.1×10^6/kgbm (range $2.5–8.0 \times 10^6$/kgbm) in 250 mL harvest volume in average (range 100–650 mL). Twenty one patients (23.3%) had $CD34^+$ cell doses of $\leq 5.0 \times 10^6$/kgbm (25th percentile value). After administration of a conditioning regimen, the aplasia duration was 11 days in average (range 4–28 days). The median time for ALC500 recovery was 16 days (range 9–31 days), ANC500 was 12 days (range 6–26 days), and PLT20 was 12 days (range 5–44 days). After ASCT, 12 patients (13.3%) had progressive disease (PD), 3 had developed signs of stable disease (SD) (3.3%), 31 had a partial response (PR) (34.4%), and 44 had a complete response (CR) (48.9%) to therapy. There was not a strong correlation between achievement of CR and $CD34^+$ cell doses, nor with recovery of ALC500, ANC500 and PLT20, or disease relapse. There was no difference regarding the clinical characteristics of patients who had received $\leq 5.0 \times 10^6$/kgbm $CD34^+$ cell dose compared to those who had received $> 5.0 \times 10^6$/kgbm $CD34^+$ cells.

Regarding MM patients, the mean $CD34^+$ cell dose administered was 5.0×10^6/kgbm (range $2.5–7.73 \times 10^6$/kgbm) in 300 mL harvest volume of (range 100–660 mL). Eighty two patients (68.3%) had $CD34^+$ cell doses of $4.0–5.4 \times 10^6$/kgbm (75^{th} percentile value). After applying a conditioning regimen, the average aplasia duration was 8 days (range 4–21 days). The median time until ALC500 recovery was 15 days (range 7–23 days), until ANC500 was 12 days (range 7–24 days) and until PLT20 was 11 days (range 5–26 days). Following ASCT, five (4.2%) patients had PD, two (1.7%) had SD, 32 (26.7%) had PR, 52 (43.3%) patients had very good partial remission (VGPR), and 29 (24.2%) patients had CR. The number of infused cells was not predictive for the time required for lymphocyte, neutrophil or PLT engraftment. Disease relapse was confirmed in 62/113 (54.9%) patients. Bortezomib-based combinations in relapsed disease received 23/59 (40.0%) patients who were not initially treated with proteasome inhibitors.

Finally, the use of original cryopreservation protocol resulted with an acceptable $CD34^+$ recovery (74.2 ± 12%) and cell viability. Namely, the mean fraction of non-viable harvested (fresh) and cryopreserved (post-thawed) 7-AAD positive cells was 2.58 ± 1.2% and 4.58 ± 2.9%, respectively.

Table 1

Clinical and laboratory characteristics of 90 patients with Hodgkin's lymphoma

Clinical characteristics	Patients, n (%)
Age at diagnosis (years), median [range]	28 [18–46]
Age at ASCT (years), median [range]	31 [20–52]
Male/female ratio, n	50/40 (56/44)
Ann Arbor stage, n (%)	
III–IV	57 (63.3)
B symptoms, n (%)	77 (85.6)
Bulky disease, n (%)	44 (48.9)
BM infiltration, n (%)	4 (4.4)
IPS, n (%)	
low	28 (31.1)
high	62 (68.9)
Pre-ASCT ECOG PS ≤ 1, n (%)	73 (81.1)
Initial therapy, n (%)	
ABVD	90 (100.0)
Conditioning regimen, n (%)	
BEAM	76 (84.4)
CBV	14 (15.5)
$CD34^+$ cell dose (mean ± SD = 7.1 ± 3.8 × 10^6/kgbm), n (%)	
< 5 × 10^6/kgbm	21 (23.3)
> 5 × 10^6/kgbm	69 (76.7)
ALC, n (%)	
≥ 20 × 10^9/L	24 (26.7)
ANC, n (%)	
≥ 11 × 10^9/L	63 (70.0)
PLT, n (%)	
≥ 13 × 10^9/L	32 (35.6)
Pre/after ASCT treatment response, n (%)	
CR/PR	71 (78.9)/ 75 (83.3)
SD/PD	19 (21.1)/ 15 (16.7)
Relapse after ASCT, n (%)	31/75 (41.3)
Vital status, n (%)	
alive	60 (66.7)
dead	30 (33.3)

ASCT – autologous stem cell transplant; BM – bone marrow; IPS – International Prognostic Score; ECOG PS – Eastern Cooperative Oncology Group Performance Status; ABVD – adriamycin, bleomycin, vinblastine and dacarbasin; BEAM – carmustine, etoposide, cytarabine and melphalan; CBV – cyclophosphamide, carmustine and etoposide; ALC – absolute lymphocyte count; ANC – absolute neutrophil count; PLT – platelets; CR – complete remission; PR – partial remission; PD – progressive disease; SD – stable disease

Table 2

Clinical and laboratory characteristics of 120 patients with multiple myeloma

Clinical characteristics	Patients, n (%)
Age at diagnosis (years), median [range]	54 [22–65]
Age at ASCT (years), median [range]	55.5 [23–65]
Male/female ratio, n	66/54 (55.6/45.4)
Type of multiple myeloma, n (%)	
IgG	75 (62.5)
IgA	23 (19.2)
Light chains	16 (13.3)
IgD	3 (2.5)
non-secretory	3 (2.5)
Clinical Stage (Salmon and Durie), n (%)	
I/II	26 (21.6)
III	94 (78.3)
Renal impairment, n (%)	
(serum creatinine ≥ 2 mg/dL; eGFR < 60 mL/min/1.73m^2)	
at diagnosis	12 (10.0)
pre-ASCT	9 (7.5)
ISS	
1+ 2	70 (61.4)
3	31 (27.2)
High risk cytogenetics [del17p or t(4;14) or t(14;16)], n (%)	7/34 (20.6)

Table 2 (*continued*)

Clinical characteristics	Patients, n (%)
Initial therapy, n (%)	
VAD	36 (30.0)
thalidomide-based	80 (66.7)
bortezomib-based in induction	4 (3.3)
Conditioning regimen, n (%)	
HD-melphalan	120 (100.0)
$CD34^+$ cell dose (mean ± SD = 5.0 ± 2.8 × 10^6/kgbm), n (%)	
2.5-4.0x10^6/kgbm	18 (31.7)
4.0–5.4 × 10^6/kgbm	82 (68.3)
ALC500, n (%) ≥ 20 × 10^9/L	21 (17.5)
ANC500, n (%) ≥ 11 × 10^9/L	68 (56.7)
PLT20, n (%) ≥ 13 × 10^9/L	24 (20.0)
Pre/after ASCT treatment response, n (%)	
CR/VGPR/PR	111 (92.5)/113(94.2)
SD/PD	9 (7.5)/7(5.9)
Relapse after ASCT, n (%)	62/113 (54.9)
Vital status, n (%)	
alive	76 (63.3)
dead	44 (36.7)

ASCT – autologous stem cell transplant; eGFR – estimated Glomerular Filtration Rate; ISS – International Scoring System; VAD – vincristine, adriamycin, dexamethasone; CAD – cyclophosphamide, adriamycin, dexamethasone; HD – high dose; ALC – absolute lymphocyte count; ANC – absolute neutrophil count; PLT – platelets; CR – complete remission; VGPR – very good partial remission; PR – partial remission; PD – progressive disease; SD – stable disease.

Transplant-related mortality of the patients was less than 1.0% (2/210), and was caused by parainfluenza viral infections. No high grade (III–IV) organ toxicity (cardiac, pulmonary, renal, or liver) was recorded.

Analysis of patients' survival

The median follow-up time for patients with HL was 67 months (range 12–192 months). Median EFS after ASCT was 20 months (range 1–119 months), and median OS after ASCT was 38 months (3–119 months). OS after ASCT wasn't influenced by gender, presence of B symptoms, bulky disease and Ann Arbor clinical stage at diagnosis ($p > 0.05$). Initial IPS influenced EFS ($p = 0.015$), but not OS ($p = 0.062$). Pre-ASCT Eastern Cooperative Oncology Group Performance Status (ECOG PS) influenced both EFS and OS ($p < 0.0001$). Favorable pre-ASCT treatment response, as well as after-ASCT, strongly influenced EFS and OS ($p < 0.0001$). OS of the patients with an unfavorable treatment response (PD, SD) was very poor with a median survival of less than 12 months.

The patients with a favorable pre-ASCT treatment response (CR or PR), who had received a lower dose of $CD34^+$ cells (≤ 5 × 10^6/kgbm) experienced inferior EFS (Log Rank = 5.84; $p = 0.016$; median 50 months vs. median not reached) and OS (Log Rank = 8.076; $p = 0.004$; median 50 months vs. median not reached) (Figure 1 A, B).

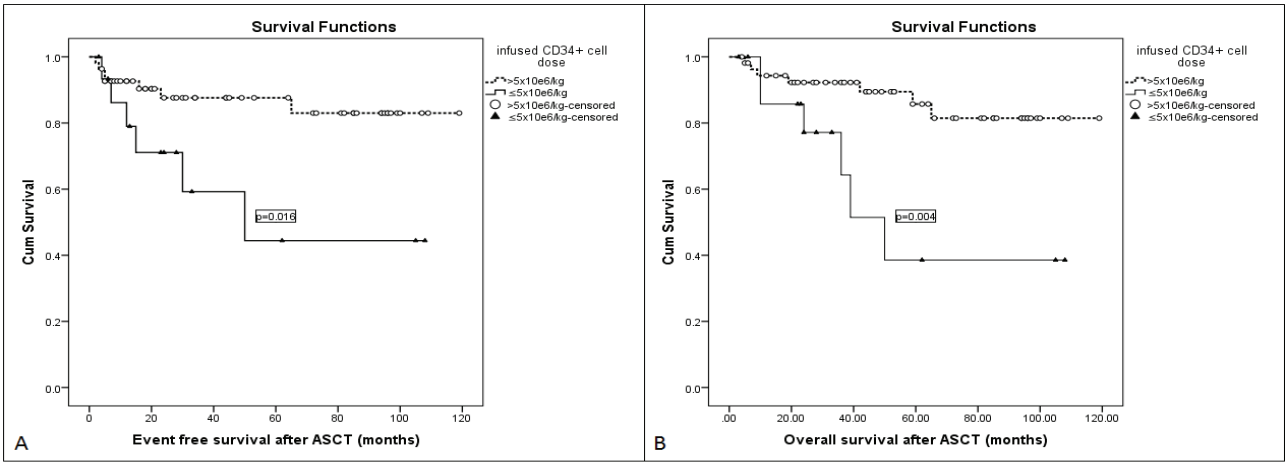

Fig. 1 – Event free survival (A) and overall survival (B) following autologous stem cell transplant (ASCT) according to applied $CD34^+$ cell dose in Hodgkin's lymphoma patients.

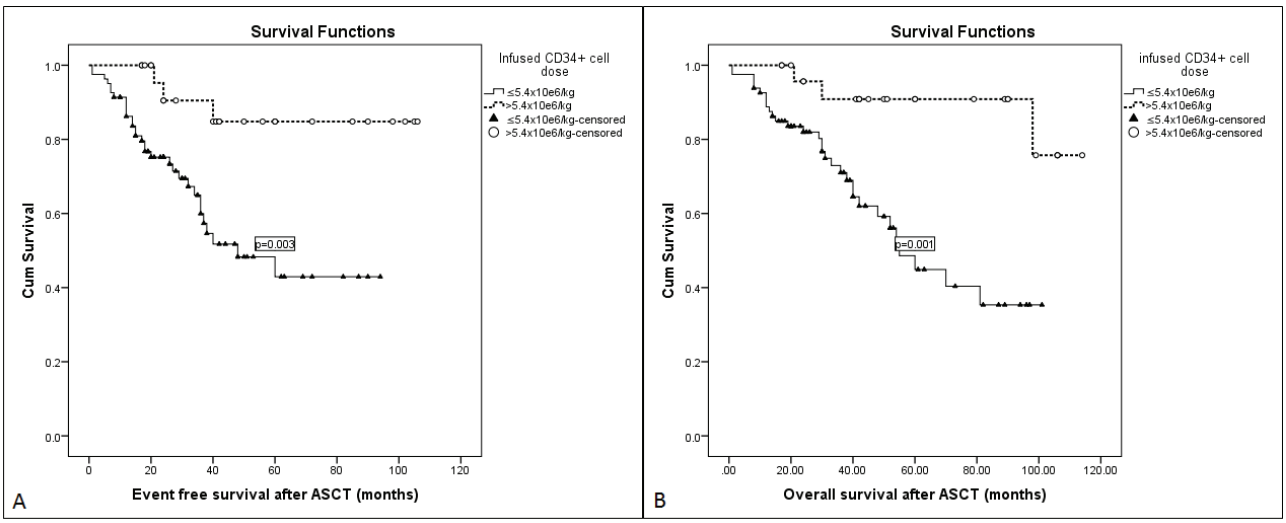

Fig. 2 – Event free survival (A) and overall survival (B) following autologous stem cell transplant (ASCT) according to applied CD34$^+$ cell dose in multiple myeloma patients.

In these patients, OS was influenced by a prolonged recovery of ALC500 ≥ 20 days (Log Rank = 6.44; $p = 0.011$) as well as EFS (Log Rank = 5.76; $p = 0.016$). Early cell recovery of ANC500 by Day +11 was not in correlation with OS or EFS ($p > 0.05$). However, early PLT recovery by Day +13 was associated with improved OS (Log Rank = 4.03; $p = 0.045$), but was of borderline significance regarding EFS (Log Rank = 3.59; $p = 0.058$). Different conditioning regimens (BEAM vs. CBV) influenced neither EFS nor OS ($p > 0.05$). Multivariate analysis was done for the following variables: pre-ASCT ECOG PS, CD34$^+$ cell dose (> 5 × 10^6/kgbm vs. ≤ 5 × 10^6/kgbm), ALC500 recovery by Day +20, and PLT20 by Day +13. The analysis concluded that CD34$^+$ cell dose was the most important parameter that influenced OS [hazard ratio (HR) = 6.67; 95% CI; 1.64–2.73; $p = 0.008$], along with ECOG PS [(HR = 9.64; 95% CI; 2.39–38.94; $p = 0.001$]. Regarding parameters that influenced EFS (pre-ASCT ECOG PS; CD34$^+$ cell dose > 5 × 10^6/kgbm vs. ≤ 5 × 10^6/kgbm; and ALC500 recovery by Day +20), again CD34+ cell dose [(HR) = 4.35; 95% CI; 1.165–16.13; $p = 0.029$], along with ECOG PS [(HR) = 10.0; 95% CI; 2.47–40.73; $p = 0.001$] significantly influenced OS.

The median follow-up time of MM patients was 52 months (range 10–190 months). The median EFS after ASCT was 25 months (range 1–106 months), and OS after ASCT was 34 months (1–114 months). Variables of gender, age, type of M protein, clinical stage, and ISS, didn't have influence on EFS or OS after ASCT ($p > 0.05$). However, the presence of renal impairment at diagnosis and pre-ASCT influenced EFS and OS ($p < 0.05$). Any favorable treatment response (CR, PR, VGPR) before and after ASCT strongly influenced both EFS and OS ($p < 0.0001$). OS of the patients with an unfavorable treatment response was very poor with a median of 14 months. Different induction regimens (VAD vs. Tthalidomide-based combinations vs. bortezomib-based combinations) influenced neither EFS nor OS ($p > 0.05$).

Regarding patients who achieved a pre-ASCT favorable treatment response, with respect to clinical parameters, only the presence of pre-transplant renal impairment affected EFS ($p = 0.009$) and OS ($p = 0.005$). Furthermore, patients who had an inferior CD34$^+$ cell dose applied (< 4 × 10^6/kgbm) had diminished EFS (Log Rank = 8.61; $p = 0.003$; median 48 months vs. median not reached) and OS (Log Rank = 10.67; $p = 0.001$; median 55 months vs. not reached) (Figure 2 A, B).

Early PLT recovery by Day +13 was associated with improvement in both OS (Log Rank = 6.98; $p = 0.008$), and EFS (Log Rank = 9.01; $p = 0.003$). Other parameters (ALC and ANC) weren't of OS significance.

Regarding OS, multivariate analysis was done concerning the following variables: infused CD34$^+$ cells (> 5.4 vs. ≤ 5.4 × 10^6/kgbm), PLT20 recovery by Day +13, and presence of the pre-ASCT renal impairment. The results of the analysis showed that a CD34$^+$ cell dose was the most important parameter that influenced OS (HR = 4.59; 95% CI; 1.314–16.057; $p = 0.017$) and EFS (HR = 3.55; 95% CI; 1.069–11.780; $p = 0.038$), while the presence of renal impairment correlated with inferior EFS (HR = 0.39; 95% CI; 0.167–0.953; $p = 0.039$), and was of borderline influence on OS (HR = 0.39; 95% CI: 0.159–0.999; $p = 0.05$).

Discussion

Previous reports showed that many clinical and laboratory variables after ASCT in hematological malignancies were associated with better OS [7, 17–19]. However, there is no firm evidence as to which parameter represents the best OS predictor.

Early lymphocyte, neutrophil and PLT recovery were reported to influence OS and EFS after ASCT in patients with HL [2], NHL [7], and MM [17]. Our results suggest that a delayed recovery of ALC500 after Day +20, and PLT after Day +13 were associated with inferior OS and EFS in HL, while prolonged PLT20 recovery in MM patients correlated with inferior OS.

Although CD34$^+$ cell dose was investigated as a potential factor that might affect early recovery of ALC500,

ANC500 and PLT20 after ASCT [7, 20], this was not the case as determined by our study. The absence of any strong correlation between blood cell recovery and $CD34^+$ cell dose in our study might be the result of additional variables such as different pre-transplant conditioning protocols that were administered to patients. Our data support the results of some previous studies that have suggested there might be an OS benefit from receiving higher $CD34^+$ cell dose [7, 21, 22]. The patients with HL who received lower $CD34^+$ cell dose had shorter EFS as well as OS. The administration of $CD34^+$ cell dose remained an independent predictive factor of OS in multivariate models, which is in accordance with the study of Gordan et al. [23], who have suggested the predictive role of $CD34^+$ cell dose on OS in a mixed population of patients with HL and NHL undergoing ASCT. Additionally, it was demonstrated that ALC by Day +15 was an independent prognostic marker for the progression free survival (but not prognostic for OS), indicating faster overall recovery caused by $CD34^+$ cell dose. Delayed ALC recovery and lower $CD34^+$ cell dose may allow minimal residual disease to outgrow and overcome immunologic activity [24]. Furthermore, current investigations in this field suggest the potential role of lymphocyte subsets that contribute to early immune reconstitution, and may have a protective role against residual disease progression, as well as possibility to better and safer mobilize lymphocyte subsets [25, 26].

Since in both HL and MM patients the higher $CD34^+$ cell doses correlated with an improved chance for OS, it is possible that receiving higher $CD34^+$ dose indicates a "healthy" marrow which could mobilize more $CD34^+$ cells, since better mobilization is not only represented by number of collected SCs [7]. Furthermore, in the present study, MM patients had lower median collected and infused $CD34^+$ cell dose compared to HL ones, which might be the consequence of age-related factors and poorer mobilization potential, since MM patients are more older compared to HL patients. Moreover, the bone marrow microenvironment likely has an additional, still unknown stimulating role in engraftment, especially in younger patients.

In HL patients, not only did $CD34^+$ cell dose independently influenced OS, but pre-transplant disease status showed prognostic significance on EFS and OS after ASCT. This may suggest that high dose chemotherapy followed by ASCT improves treatment response [5]. Of particular interest in MM patients is the presence of renal impairment, which was of borderline significance regarding OS, and correlated with unfavorable EFS, possibly due to disease aggressiveness and reduced-dose melphalan for conditioning. However, some previous studies reported that MM patients with impaired renal function may have outcomes comparable to those with normal renal function, despite the use of conditioning dose reduction [27]. This is mainly due to the usage of proteasome inhibitors in induction treatment, whose proportion in our study is rather small.

Although the current study has a few limitations including its retrospective nature, the fact that different conditioning regimens were used, the relatively limited number of patients and the inability to determine lymphocyte subsets, it points out the prognostic role of $CD34^+$ cell dose as an easy detectable parameter that correlate with OS after ASCT.

Conclusion

Although SC transplant represents standard procedure in relapsed/refractory HL and MM patients, there is no variable that might help in identifying high-risk patients who underwent ASCT. The results obtained in this study confirm that advanced response through pre-ASCT treatment, early recovery of ALC500 and PLT20 (HL patients), as well as PLT20 (MM patients) could influence the patients' OS. Also, superior $CD34^+$ cell dose could be a useful predictive factor for treatment efficacy. More precise evaluation of overall treatment effectiveness by ASCT required prospective $CD34^+$ cell and some lymphocyte subsets investigations using randomized, controlled and larger clinical studies.

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Evaluation of dental health among adolescents with mental disorders

Evaluacija zdravlja zuba kod adolescenata sa mentalnim poremećajima

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Abstract

Background/Aim. According to the World Health Organization (WHO), there is an increasing prevalence of mental disorders among children and adolescents worldwide. Previous studies have shown that people with mental disorders, regardless age, have an increased prevalence of dental caries due to several reasons. The aim of this study was to determine prevalence of dental caries in adolescents with mental disorders and to consider possible risk factors that might contribute to their current dental health status. **Methods.** The study was conducted as an observational cross-sectional study. The study group comprised 70 randomly selected hospitalized adolescents with mental disorders. The control group comprised 70 randomly chosen mentally healthy adolescents. They were matched to the study group by gender and age. All the participants were subjected to targeted dental examination according to criteria recommended by the WHO. Collection of data related to mental disorders of the study group was obtained from the patient's medical records. All collected data were organized and analyzed by descriptive statistical parameters and regression models. **Results.** Majority of the study group patients were diagnosed with schizophrenia, schizotypal and delusional disorders (F20-F29), as well as behavioral and emotional disorders usually occurring in childhood and adolescence (F90-F98). Almost 90% of them were treated with antipsychotics of the second generation, as monotherapy or in combination with first-generation antipsychotics. Adolescents with mental disorders had significantly more carious and extracted teeth and three times less filled teeth than mentally healthy adolescents in the control group. The mean value of the decay-missing-filled teeth (DMF) index in the study group patients was also significantly higher than the mean value of DMF index in the control group subjects. **Conclusion.** It seems that mental disorder among adolescents mainly affects oral health indirectly, decreasing motivation of patients in maintaining oral hygiene.

Key words:
mental health; adolescent; oral health.

Apstrakt

Uvod/Cilj. Prema podacima Svetske zdravstvene organizacije (SZO), sve više je mentalnih poremećaja među decom i adolescentima širom sveta. Ranije sprovedena istraživanja pokazala su da osobe sa mentalnim poremećajima, bez obzira na starost, imaju veću učestalost karijesa, što se objašnjava na više načina. Cilj ovog istraživanja je bio da se odredi prevalencija karijesa kod adolescenata sa mentalnim poremećajima i razmotre mogući faktori rizika koji bi mogli doprineti zdravlja njihovih zuba. **Metode.** Istraživanje je sprovedeno po tipu opservacione studije preseka. Studijsku grupu je činilo 70 slučajno odabranih hospitalizovanih adolescenata sa mentalnim poremećajima. Kontrolnu grupu je činilo 70 slučajno odabranih mentalno zdravih adolescenata, koji su po polu i starosti odgovarali bolesnicima studijske grupe. Svim bolesnicima je izvršen detaljan stomatološki pregled, prema kriterijumima preporučenim od strane SZO. Podaci o mentalnim poremećajima adolescenata prikupljeni su iz istorija bolesti. Svi dobijeni podaci analizirani su deskriptivnim statističkim parametrima i regresionim modelima. **Rezultati.** Većina ispitanika studijske grupe bolovala je od shizofrenije, shizotipskih poremećaja i sumanutih poremećaja (F20-F29), kao i od poremećaja ponašanja i poremećaja emocija sa početkom u detinjstvu i adolescenciji (F90-F98). Skoro 90% ispitanika studijske grupe je lečeno antipsihoticima druge generacije, u vidu monoterapije ili u kombinaciji sa antipsihoticima prve generacije. Adolescenti sa mentalnim poremećajima imali su znatno

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više karijesnih i ekstrahovanih zuba od zdravih adolescenata i tri puta manje zuba sa postavljenim ispunima. Srednja vrednost karijes, ekstrahovan, plombiran zub (KEP) indeksa, bila je, takođe, statistički značajno veća u studijskoj nego u kontrolnoj grupi ispitanika. **Zaključak.** Čini se da mentalni poremećaji kod adolescenata uglavnom indirektno utiču na oralno zdravlje, smanjujući motivaciju za održavanjem oralne higijene.

Ključne reči:
mentalni poremećaji; adolescenti; usta, zdravlje.

Introduction

According to the latest reports of the World Health Organization (WHO), mental disorders are the 3rd leading cause of disability of European citizens [1], and previous study suggests an increasing prevalence of mental disorders among children and adolescents worldwide [2].

Adolescence is a period between childhood and adulthood, from 14 to 18 years of age, and according to some studies, up to 25 years of age [3]. In this specific part of life, many problems and psychological disorders reach their peak [4]. The most common mental disorders in this period are depressive and anxiety disorders, obsessive-compulsive disorders and posttraumatic stress disorders [4]. Many of these patients are treated solely by psychotherapeutic methods, but some of them also need to receive pharmacological agents that are not approved for persons less than 18 years of age. Moreover, some severe mental disorders, like schizophrenia, depression, anxiety, attention deficit hyperactivity disorder and bipolar disorder, frequently require pharmacological treatment in adolescents [5].

Dental caries is a major public health problem globally and it is the most widespread non-communicable disease [6]. Previous studies have shown that people with several mental disorders have an increased prevalence of dental caries [7–10]. This can be explained by several reasons: mental disorders lead to lack of motivation, lack of oral hygiene, fear to visit a dentist, difficulty to access health services and adverse effects of antipsychotic medication, mainly xerostomia, could be also present [10].

In Serbia no research has been conducted to oral and dental health of this vulnerable group of psychiatric patients. Therefore, the aim of this study was to determine prevalence of dental caries, to register condition of teeth still present, and to consider possible risk factors that have possibly contributed to the current dental health status of adolescents with mental disorders.

Methods

The study was conducted as an observational cross-sectional study. It was adjusted to the Strengthening the Reporting of Observational Studies in Epidemiology (STROBE) statement for improving the quality of observational studies [11]. Also, it received approval of the Ethics Committee of the Clinic for Mental Disorders "Dr. Laza Lazarević" Belgrade and Community Health Center "Vračar" in Belgrade, Serbia. The research was conducted in accordance to the Declaration of Helsinki [12]. The participation of all participants was voluntary. Each participant and their legal representative (for persons under 18 years old) was informed, through a special brochure, of the type of the research, data collection procedure, and other aspects of the study; written consent was obtained from all participants or their legal representatives to use personal data for research purposes.

Two groups of participants were formed: the study group comprised 70 randomly selected adolescents with mental disorders, hospitalized at the Clinic for Mental Disorders "Dr. Laza Lazarević" in Belgrade ("bias-coin" randomization). The inclusion criteria for entering the study were that the patient was hospitalized, younger than 25 years and diagnosed with mental disorders (according to the 10th Revision of the International Classification of Diseases [13]) at least two years prior to the study. The exclusion criteria were hospitalized patients older than 25 years diagnosed with mental disorder in the period shorter than two years from the time of the survey, the simultaneous presence of severe somatic illnesses or severe disability, and inability to communicate or a refusal to cooperate. The control group comprised 70 randomly chosen mentally healthy adolescents ("bias-coin" randomization) who were being treated at the Community Health Center "Vračar" in Belgrade. They were matched to the study group by gender and age. The exclusion criteria were the diagnosis of any mental or somatic illness and the use of drugs that can cause oral changes or xerostomia (antibiotics, antifungals, blood pressure medication, corticosteroids, diabetes medication, etc.) [14].

All the participants were subjected to targeted dental examination according to criteria recommended by the WHO [15]. Dental check-ups were carried out by the dentist (VDj) at the Clinic for Mental Disorders "Dr. Laza Lazarević" in Belgrade, and the Community Health Center "Vračar" in Belgrade. Examinations were performed in the daylight, using flat dental mirrors and sharp probes. Dental check-ups were carried out with the aim of measuring parameters of oral and dental health and assessing the decayed, missing, filled (DMF) index, which is used for oral health assesment [16]. Clearly visible lesions with cavities on tooth surfaces were registered as caries; teeth having only changes in transparency, but with intact surface and without cavitation were registered as being healthy.

Collection of data related to mental disorder of the study group was obtained from the patient's medical records.

All collected data were organized and evaluated using dedicated software (SPSS 23.0 Inc., Chicago, IL, USA) and were analyzed by descriptive statistical parameters and regression models. The descriptive statistical parameters were represented by the measures of central tendency

(mean value and median), measures of variability (standard deviation and variation interval) and were expressed in percentages. The methods for testing the differences in numerical data (age, DMF index) were represented by the *t*-test of independent groups. If there were no grounds for application of parametric statistical methods, the Mann-Whitney test was applied. For testing data of different categories (gender, parents education level, etc.), the Pearson's χ^2-test was used. The relationship between the DMF index and independent variables used in this study was evaluated using a linear regression model - univariate (individually for each of the independent variables) and multivariate (if any of independent variables was statistically significant in univariate regression analysis). Level of significance was set at $p \leq 0.05$.

Results

Socio-demographic characteristics of all participants are shown in Table 1. The groups were comparable in terms of age and gender (Table 1). Statistically significant difference between these groups was observed only in terms of place of residence; all patients from the control group lived in urban area, while only 52% of the study group lived in this type of area. Only 29.3% of the study group patients had both of parents with high school education, which was quite opposite to the control group patients.

Majority of the study group patients were diagnosed with schizophrenia, schizotypal and delusional disorders (F20-F29), as well as behavioral and emotional disorders, with onset usually occurring in childhood and adolescence (F90-F98) (Figure 1).

Table 1

Socio-demographic characteristics of all participants

Socio-demographic characteristics	Study group	Control group	*p*
Gender, n (%)			
male	36 (48.0)	38 (50.7)	[a]0.435
female	39 (52.0)	37 (49.3)	
Age (years), mean ± SD; Med (min-max)	18.87 ± 3.05; 18 (15–25)	19.21 ± 3.15; 19 (15–26)	[b]0.494
Place of residence, n (%)			
urban area	39 (52.0)	75 (100,0)	[a]0.000
peri-urban area	17 (22.7)	0 (0)	
rural area	19 (25.3)	0 (0)	
Father education level, n (%)			
without any school	0 (0)	0 (0)	[a]0.168
elementary school	8 (10.7)	0 (0)	
high school	22 (29.3)	10 (13.3)	
college	13 (17.3)	16 (21.3)	
faculty	1 (1.3)	32 (42.7)	
there is no father figure in family	20 (26.7)	7 (9.3)	
don't know/didn't sure	11 (14.7)	10 (13.3)	
Mother education level, n (%)			
without any school	1 (1.3)	0 (0)	[a]0.238
elementary school	11 (14.7)	3 (4.0)	
high school	22 (29.3)	13 (17.3)	
college	10 (13.3)	29 (38.7)	
faculty	0 (0)	27 (36.0)	
there is no mother figure in family	20 (26.7)	3 (4.0)	
don't know/didn't sure	11 (14.7)	0 (0)	

n (%) – number (percentage) of patients; *p* – significance; SD – standard deviation; Med – median; min – minimum; max – maximum; [a]χ^2 – test; [b]*t* – test of independent groups.

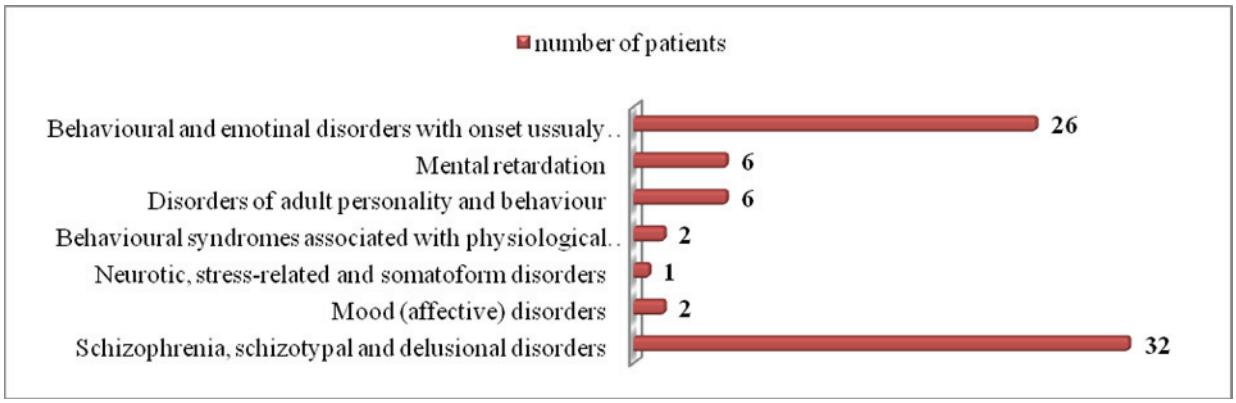

Fig. 1 – Distribution of mental disorder diagnosis among the study group patients.

Concerning schizophrenia, schizotypal and delusional disorders (Figure 2), majority of patients were diagnosed with acute and transient mental disorders. Among the study group patients with behavioral and emotional disorders, with onset usually occuring in childhood and adolescence (Figure 3), most of them were diagnosed with mixed behavioral disorders and emotions.

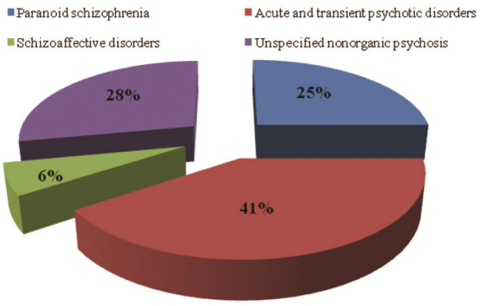

Fig. 2 – Distribution by diagnosis categories of schizophrenia, schizotypal and delusional disorders among the study group patients.

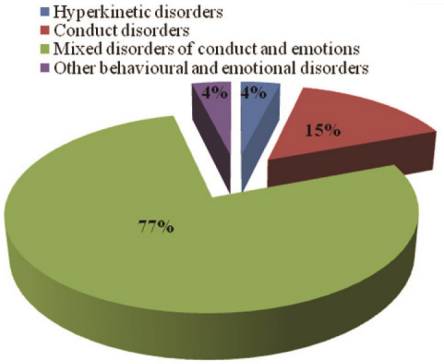

Fig. 3 – Distribution by diagnosis categories of behavioral and emotional disorders among the study group patients, usually occurring in childhood and adolescence.

All patients from the study group were treated with an average of 3.27 ± 0.83 (2 to 5) psychiatric drugs (Table 2). Almost 90% of them were treated with antipsychotics of the second generation, as monotherapy or in combination with first-generation antipsychotics (Table 2). Also, almost half of the study group patients were treated with antidepressant drugs, while 70.7% were treated with anxiolytics.

In addition to the above mentioned groups of psychiatric drugs, 40% of the patients received hypnotics, 65.3% mood stabilizers, while only 6.7% of study group patients received anticholinergics (Table 2).

Table 2

Medical data of the study group patients

Medical characteristics	Study group
Number of medications per patient, mean ± SD; Med (min-max)	3.27 ± 0.83; 3 (2–5)
Typical antipsychotics, n (%)	
yes	21 (28.0)
no	54 (72.0)
Atypical antipsychotics, n (%)	
yes	67 (89.3)
no	8 (10.7)
Antidepressants, n (%)	
yes	34 (45.3)
no	41 (54.7)
Anxiolytics, n (%)	
yes	53 (70.7)
no	22 (29.3)
Hypnotics, n (%)	
yes	30 (40.0)
no	45 (60.0)
Mood stabilizers, n (%)	
yes	49 (65.3)
no	26 (34.7)
Anticholinergics, (%)	
yes	5 (6.7)
no	70 (93.3)

n (%) – number (percentage) of patients; *p* – significance; SD – standard deviation; Med – median; min – minimum; max – maximum.

Adolescents with mental disorders had significantly more carious and extracted teeth, and three times less filled teeth than mentally healthy adolescents in the control group (Table 3). The mean value of the DMF index in the study group patients (6.45 ± 3.48) was also significantly higher than the DMF index in the control group subjects (2.75 ± 2.12). The difference in all four observed source variables between the groups was statistically significantly different and significantly less favorable for the study group (Table 3).

In terms of socio-demographic characteristic, there was no statistically significant difference in the value of the DMF index among participants in both groups (Table 4). Also, in relation to the characteristics of the mental disorder, no statistically significant significance was found in mean values of the DMF index within the study group patients (Table 5).

Table 3

Distribution of carious, extracted and filled teeth and the value of the decay-missing-filled teeth (DMF) index

Variables	Study group		Control group		p^a
	mean ± SD; Med (min-max)	%	mean ± SD; Med (min-max)	%	
Carious teeth	4.31 ± 2.69; 4 (0–8)	66.7	0.64 ± 0.86; 0 (0–3)	23.3	0.000
Extracted teeth	1.45 ± 1.51; 1 (0–4)	22.5	0.37 ± 0.65; 0 (0–2)	13.6	0.000
Filled teeth	0.69 ± 1.14; 0 (0–5)	10.8	1.68 ± 1.53; 1 (0–5)	61.1	0.000
DMF index	6.45 ± 3.48; 7 (0–12)	100	2.75 ± 2.12; 2 (0–9)	100	0.000

% – percentage of patients; *p* – significance; SD – standard deviation; Med – median; min – minimum; max – maximum; [a]Mann-Whitney test.

Table 4

The mean value of the decay-missing-filled teeth (DMF) index among patients in both groups by socio-demographic characteristics

Socio-demographic characteristics	Study group		Control group	
	mean ± SD	*p*	mean ± SD	*p*
Gender				
male	6.25 ± 3.17	[a]0.823	3.00 ± 2.27	[a]0.402
female	6.64 ± 1.82		2.49 ± 2.16	
Place of residence				
urban area	6.49 ± 3.23	[b]0.846	–	–
peri-urban area	6.00 ± 3.89			
rural area	6.79 ± 3.75			
Father education level				
without any school	7.75 ± 2.91	[b]0.602	–	[b]0.314
elementary school	6.27 ± 3.60		3.30 ± 1.64	
high school	5.15 ± 3.65		2.25 ± 2.57	
college	–		2.59 ± 2.37	
faculty	6.95 ± 3.55		2.43 ± 2.07	
there is no father figure in family	6.27 ± 3.44		3.70 ± 1.57	
don't know/didn't sure	–		–	
Mother education level				
without any school	7.73 ± 3.61	[b]0.518	2.00 ± 2.00	[b]0.863
elementary school	6.59 ± 3.39		2.38 ± 1.90	
high school	5.70 ± 3.83		3.03 ± 2.10	
college	–		2.74 ± 2.57	
faculty	6.70 ± 3.33		2.33 ± 2.31	
there is no mother figure in family	5.18 ± 3.47		–	
don't know/didn't sure	–		–	

SD – standard deviation; *p* – significance; [a]Mann-Whitney test; [b]Kruskal-Wallis test.

Table 5

The mean value of the decay-missing-filled teeth (DMF) index among the study group patients by medical data

Medical data	mean ± SD	*p*
Diagnostic category		
F20-F29	5.94 ± 3.78	[a]0.198
F30-F39	2.50 ± 2.12	
F50-F59	9.50 ± 2.12	
F60-F69	8.17 ± 1.48	
F70-F79	8.50 ± 1.52	
F90-F98	6.46 ± 3.48	
Number of medications per patient		
2	6.43 ± 3.65	[a]0.848
3	6.13 ± 3.30	
4	6.77 ± 3.59	
5	7.00 ± 4.69	
Typical antipsychotics		
yes	6.71 ± 3.91	[b]0.553
no	6.35 ± 3.33	
Atypical antipsychotics		
yes	6.39 ± 3.51	[b]0.653
no	7.20 ± 3.42	
Antidepressants		
yes	6.00 ± 3.45	[b]0.292
no	6.83 ± 3.51	
Anxiolytics		
yes	6.04 ± 3.39	[b]0.062
no	7.45 ± 3.70	
Hypnotics		
yes	6.60 ± 3.69	[b]0.636
no	6.36 ± 3.37	
Mood stabilizers		
yes	6.88 ± 3.36	[b]0.155
no	5.65 ± 3.62	
Anticholinergics		
yes	7.60 ± 3.21	[b]0.462
no	6.37 ± 3.51	

SD – standard deviation; [a]Mann-Whitney test; [b]Kruskal-Wallis test; *p* – significance.

By defining the mean value of the DMF index of adolescents with mental disorders as a outcome, none of independent variables were statistically significant in the univariate regression analysis (Table 6), so the multivariate regression model was not formed.

Table 6

The value of the decay-missing-filled teeth (DMF) index among adolescents with mental disorders analyzed by the univariate linear regression model

Independent variables	Univariate linear regression model	
	#B (95% CI)	*p*
Gender	0.391	0.630
Age	0.173	0.761
Place of residence	0.096	0.843
Father educational level	-0.008	0.969
Mother educational level	-0.242	0.221
Diagnostic category	0.143	0.267
Number of medications per patient	0.255	0.605
Typical antipsychotics	-0.362	0.688
Atypical antipsychotics	0.612	0.642
Antidepressants	0.829	0.308
Anxiolytics	1.417	0.109
Hypnotics	-0.244	0.768
Mood stabilizers	-1.224	0.149
Anticholinergics	-1.229	0.450
Psychoactive substances	-0.310	0.232

#Unstandardized coefficient B; CI – confidence interval; *p* – significance.

Discussion

In both groups, approximately the same number of subjects was gender-related, which indicates homogeneity of the sample and allows adequate interpretation of the results of the study group. Gender and age are individual characteristics who determined general and oral health [17]. A study on the global burden of diseases, injuries and risk factors from 2015, indicates that the incidence of dental caries of permanent teeth is the greatest in the age group of 15 to 19 years and gradually decreases in older age groups [18].

However, in addition to individual characteristics, environmental factors also influence health, as well as the interaction of individual characteristics with environmental factors and *vice versa* [17]. Thus, poor economic conditions are recognized as factors that have negative effects on health; education is also a factor that plays a significant role in developing skills and knowledge needed for positive lifestyle changes [17]. As the sample of this study consisted of adolescents that are in the educational period of life, it is important to analyze the parent or guardian education level as well, because they can contribute to development of positive lifestyles if they themselves understand their importance. This study showed that in most adolescents with mental disorders both parents had a high school education (29.3%). Also 26.7% of the study group patients did not have father or mother figure in their family. Cianetti et al. [19] have shown that dental caries presence was higher in children where the mothers' and fathers' educational level was lower. Also, Crocombe et al. [20] have shown that children, whose parents had higher education level have approximately half of the relative risk of caries, compared to children whose parents had low levels of education.

Also, nearly 50% of adolescents with mental disorders lived in periurban and rural areas, in opposite to participants of the control group, where 100% of patients were living in urban area. Many previous studies have shown a significantly higher prevalence of dental caries with rural residence location [21–23]. The latest national research on the health of citizens of the Republic of Serbia, precisely points to the importance of access to health services, which depends on many factors, and also on the distance of the health service [17]. Moreover, the results of this national survey have shown that the rural population often experience barriers to obtain the dental health care (19.3%) [17].

In the present study, the most common diagnostic category of mental disorders in the study group were schizophrenia, schizotypal and delusional disorders (42.7%), which is similar to previous studies about oral health of psychiatric patients [24–26]. Velasco-Ortega et al. [24] have shown in their research about actual dental status and treatment needs of older adults with and without chronic mental disorders in Spain, that 56% of the study group patients suffered from schizophrenia. Also, research about prevalence of bucco-dental pathologies in patients with psychiatric disorders in Venezuela showed that even 60% of patients had schizophrenia as most common mental disorder [25]. On the other hand, Bertaud-Gounot et al. [26] have shown that 36.6% of psychiatric inpatients in Rennes, France, had schizophrenia. Djordjevic et al. [27] in their research have come to conclusion that oral diseases, especially dental caries and periodontal disease, are much more prevalent in patients with schizophrenia than in healthy population, possibly due to the nature of this psychiatric disorder, length of hospital treatment and oral-side effects of psychotropic medications used for schizophrenia [27]. Schizophrenia is a chronic mental disorder characterized with disturbances in thoughts, behavioral changes, and impaired cognitive functions. All this affect a person's ability to carry out daily activities and maintain oral hygiene [28].

The patients of the study group in the present study were treated with the average number of 3.27 ± 0.73 psychotropic drugs (2 to 5), and the most used medications were antipsychotics of the second generation (89.3%), anxiolytics (70.7%) and mood stabilizers (65.3%). Okamoto et al. [29] have reported that patients with schizophrenia, who used antipsychotics, and especially anxiolytics, show higher level of hypo-salivation. Hyposalivation consequently leads to a buildup of dental plaque on marginal gingiva, which is a major etiologic factor for the occurrence of caries [30].

The mean value of DMF index of the study group patients in our study was 6.45 ± 3.48, which is two times higher than in participants of the control group (0.64 ± 0.86). Also, results of our study point that the study group patients had seven times higher mean value of carious teeth, four times higher mean value of extracted teeth, and even four times lower mean value of filled teeth than the control group participants. This indicates that adolescents with mental disorders and their parents have a lack of motivation for rehabilitation of carious teeth and weak habits in maintaining oral hygiene, which is confirmed by previous studies [31, 32].

Conclusion

Results of this study suggest that mental disorders mainly affect oral health indirectly, decreasing motivation of patients in maintaining oral hygiene.

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Impact of educational intervention for correct inhaler technique on the quality of life of children with asthma

Uticaj sprovođenja edukacije za pravilnu inhalatornu tehniku na kvalitet života dece sa astmom

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Abstract

Background/Aim. Asthma is the most common chronic disease in children and adolescents and has shown an apparent increase in incidence in recent years. The first purpose of the study was to evaluate the influence of education about proper use of inhalers on quality of life in children with asthma. Secondly, we aimed to understand which aspects of quality of life in children with asthma can be significantly improved after education and to identify factors that may affect the level of that improvement. **Methods.** In this prospective, before-and-after interventional study, a total of 147 children with asthma were enrolled. The Pediatric Asthma Quality of Life Questionnaire (PAQLQ) was used to measure the functional problems that are most troublesome to children with asthma. We used the Asthma Control Test (ACT), based on a series of question about symptoms and daily functioning, to identify patients with poorly controlled asthma. Forced expiratory volume in one second (FEV1) and peak expiratory flow (PEF) were also determined. Trained educators estimated patients' inhaler technique and collected questionnaire information. **Results.** Multivariate analysis of covariance indicated significant differences between PAQLQ and ACT scores which all were significantly higher after education about proper use of inhalers ($p < 0.001$). A number of children demonstrating a correct inhalation technique improved from 28 (19%) to 127 (86.4%) ($p < 0.001$). Asthma severity accounted for the largest proportion of variability PAQLQ and ACT scores (38.4%). **Conclusion.** Inhaler technique improvement contributes to better asthma control in children with asthma rather than to their quality of life. Asthma severity proved to be a major contributor to variations in PAQLQ and ACT scores and significant obstacle for quality of life improvement in children with asthma.

Key words:
asthma; child; nebulizers and vaporizers; quality of life; education, medical; respiratory function tests.

Apstrakt

Uvod/Cilj. Astma je najčešće hronično oboljenje kod dece i adolescenata čija se incidencija stalno povećava u poslednje vreme. Pimarni cilj ovog rada bio je da se utvrdi uticaj edukacije o pravilnoj upotrebi inhalatora na kvalitet života dece sa astmom. Drugi cilj je bio razumevanje koji aspekti kvaliteta života mogu biti značajno unapređeni posle edukacije i identifikacija faktora koji utiču na nivo tog unapređenja. **Metode.** Ukupno 147 dece sa astmom je bilo uključeno u ovu prospektivnu i intervencijsku (pre - posle), studiju. Za merenje funkcionalnih problema koji se najčešće javljaju kod dece sa astmom korišćen je *The Pediatric Asthma Quality of Life Questionnaire* (PAQLQ). Test za kontrolu astme (ACT), koji se bazira na nizu pitanja u vezi sa simptomima i dnevnim funkcionisanjem, korišćen je za utvrđivanje loše kontrolisane astme. Takođe, mereni su i forsirani ekspiratorni volumen u 1 sekundi (FEV1) i vršni ekspiratorni protok (PEF). **Rezultati.** Multivarijantna analiza kovarijanse pokazala je da postoje statistički značajne razlike u vrednosti PAQLQ i ACT skorova pre i nakon sprovedene edukacije o pravilnoj upotrebi inhalatora ($p < 0,001$). Broj dece koja su pravilno koristila inhalator povećao se sa 28 (19%) na 127 (86,4%) ($p < 0,001$). Stepen astme identifikovan je kao faktor koji je najviše doprinosio varijabilnosti u vrednostima skorova (38,4%). **Zaključak.** Bolja inhalaciona tehnika kod dece sa astmom više doprinosi boljoj kontroli astme u odnosu na unapređenje kvaliteta života. Najveći uticaj na vari-

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jacije u ACT i PAQLQ skorovima ima stepen astme koji se pokazao kao najveća prepreka za unapređenje kvaliteta života kod dece sa astmom.

Ključne reči:
astma; deca; nebulizatori i vaporizatori; kvalitet života; edukacija, medicinska; respiratorna funkcija, testovi.

Introduction

Asthma is the most common chronic disease in children and adolescents and has shown an apparent increase in incidence in recent years [1]. Health professionals are challenged to find effective responses to the influence of chronic disease such as asthma on the health and quality of life of children and their families. It is known that asthma manifests emotional and social effects on children. In addition to regular visits to the doctor, children need education to understand the disease, avoid triggers and to manage medication.

There are guidelines that address asthma management in children: the Practical Allergy (PRACTALL) consensus report, the Global strategy for asthma (GINA) and the International consensus on (ICON) pediatric asthma [2–4]. Despite all, asthma is a disease that is still poorly controlled. The reasons for poor control of asthma are numerous, but one of the main reasons is the poor inhalation technique [5]. Regardless of the type of inhaler, the importance of proper application, regular education and training of medical staff are the most effective strategy for the reduction of errors in the application of an inhalation technique. Also, the regular control technique of taking the drug in each subsequent visit to the doctor is of particular importance. Considering that errors in the inhalation process are very frequent and that may affect the availability of the drug to lungs, correct inhalation technique is essential for the adequate bronchodilatory effect.

Possible errors include those which do not depend on an inhaler type (an inadequate exhalation just before the inhalation or by inhalation through the nose) and errors originating from a device itself (inadequately prepared inhaler) [6]. It has been shown that the improper use of different inhalers is associated with poor control of the asthma [7]. Incorrect inhalation technique may lead to decrease of lung deposition of inhaled drug up to 50% [8]. When a bronchodilator is applied, the increase in FEV 1 (forced expiratory volume in one second) may be lower for a third if the drug has not been adequately taken. Also, incorrect inhaler technique correlates with a poorer control of asthma in patients treated with inhaled corticosteroids [9].

Parents frequently report being unsure and confuse on how to manage the child's asthma. Also, the family caregiver's perception of managing asthma has been shown to affect child health outcomes, including hospitalizations and emergency department visits [10]. The impact of asthma on children's daily activities, including sports and play as well as their emotional status is very significant. Studies have shown that incorporation of asthma education plans can be quite beneficial [11]. Appropriate education has proved to be very useful for both individual and group programming to improve asthma self-management skills in children and their parents [12]. One of the main tasks for asthma educators is to determine what is preventing the patient from achieving asthma control. When an educator understands where the patient make a mistake, he or she should teach him or her to use the inhaler in such a way that all steps are correct. The aim of this study was to evaluate the influence of education about proper use of inhalers in children on their quality of life. The specific objective was to understand which aspects of the quality of life in children with asthma can be significantly improved after education and to identify factors that may affect the level of that improvement.

Methods

Study design and participants

Between January 2016 and June 2017, interventional study was performed in 147 juvenile patients with mild, moderate and severe persistent asthma aged between 7 and 17 years. It was a prospective, before-and-after, interventional study in which each patient was his/her own control. Exclusion criteria were enrollment in education program in the past and chronic disease in addition to asthma.

The diagnosis of asthma was accepted when a patient with common clinical symptoms of the disease and airflow limitation had a positive bronchodilator test or a daily peak expiratory flow variability > 20% or a positive methacholine challenge test documented in the medical record. The level of severity of asthma was defined according to the Global Initiative for Asthma criteria which was based on asthma symptom frequency, medication use, FEV1 and PEF values. Uncontrolled asthma was defined as the Asthma Control Test (ACT) score < 20. The duration of the study was 18 months; during the first 6 months data were collected and all patients included were consecutively enrolled from the primary care center. Next, during one year, education which lasted for three months, was conducted. Education on inhalation technique was performed by certified nurses in three stages: in first session, children were taught about importance of proper inhaler use and inhaled medications. Also, demonstration of the proper use of different types of inhalers was performed. Second session was consisted of workshops and training for proper use of inhalers. In the third phase of the education checking of inhalation techniques was carried out. All participants received theoretical lessons with audiovisual aids, practical exercise and written instructions containing important guidelines for the treatment of asthma. Although education was referred to different types of inhalers, it was standardized because nurses were equally trained for each inhaler used in the study and training was conducted according to manufacturer's instructions. The study was conducted in accordance with the Declaration of Helsinki principles and was approved by the Ethics Committee of the

Health Center Rakovica. Written informed consent was obtained from child's parents. Personal identification data were anonymous.

Measurements and questionnaires

On inclusion in the study, a record was made of the patients' general and socio-demographic characteristics (age, gender, anthropometric data, type of habitat environment, exposure to tobacco smoke, financial status, type of asthma inhaler). At the visit to a pediatrician, results of functional respiratory tests (FRT), FEV1 and peak expiratory flow (PEF), were obtained. The children included in the study used some of the following inhalers: MDI (metered-dose inhaler with a spacer or without it), Autohaler, Accuhaler/Diskus and Turbohaler (dry powder inhaler). Trained educators in presence of pediatrician requested children to demonstrate their inhaler technique and if any of the steps was missing or done wrong according to the checklist, it was assigned as incorrect inhaler use. Also, as a relative inhaler technique improvement measurement, we calculated the percentage of correct steps for each patient and his/her inhaler. The limitations in daily life (physical, emotional and social) associated with asthma were assessed using the Serbian version of the Pediatric Asthma Quality of Life Questionnaire (PAQLQ) [13]. Translation into Serbian and linguistic validation of PAQLQ(S) was made by the MAPI Research Institute (1996) in Lyon, France. To determine if patients'asthma symptoms are well controlled we used the ACT. The children were accompanied with parents but the first author of this article conducted all the interviews. After the first data collection, the patients have attended education and were followed-up for a period of one-year at the primary care center.

Statistics

The Kolmogorov-Smirnov test was used to determine if the distribution of variables was normal. Equality of variances was controlled by the Levene's test. The estimated sample size of the study was 111 patients with a confidence interval of 95% and a random error of 5%. The PEF and emotional status score – were not normally distributed ($p < 0.05$) for the pooled samples. Therefore, logarithmic transformations were performed for both of these variables. After logarithmic transformations, both variables, PEF and emotional status score, were tested for normality of distribution. As they achieved normal distribution, these transformed values were used in all subsequent analyses. The χ^2-test was used to determine distributions of type of environment, exposure to tobacco smoke and financial status towards FEV1 and PEF (less or more than 80%) and absolute inhaler technique improvement. To determine whether there was a statistically significant difference in quality of life and asthma control after training and education we used the general linear model of analysis of variance. Multivariate analysis of covariance (MANCOVA, Wilks' lambda) was performed to test the hypotheses that education (fixed factor), asthma severity, FEV1 and relative inhaler technique improvement (covariates) have a significant effect on the normally distributed scores for symptoms, activity limitation, emotional function, overall PAQLQ and ACT (dependent variables). Univariate ANCOVA was then performed for each of the individual parameters. Partial eta-squared (η^2) values, which describe the proportion of variability attributable to a factor, were included to provide an intuitive measure of effect size. Pearson's correlation was employed to establish possible relationships between scores for symptoms, activity limitation, emotional function, overall PAQLQ and ACT and covariates. Differences were considered statistically significant at $p < 0.05$. All analyses were performed using Statgraphics 4.2 software (STSC, Inc. & Statistical Graphics Corporation 1985–1989) and CBstat 4.3.2 version software (K. Linnet, Risskov, Denmark).

Results

Anthropomorphological data of the patients are shown in Table 1.

Table 1
Anthropomorphological and demographic characteristics of patients before education

Characteristics	Values
Age (years), median (interquartile range)	9 (8.0–13.0)
Height (cm), median (interquartile range)	139 (129.0–160.0)
Weight (kg), median (interquartile range)	32.0 (36.0–52.0)
Gender, n (%)	
males	90 (61)
females	57 (39)
Type of environment, n (%)	
urban	99 (67)
rural	48 (33)
Exposure to tobacco smoke, n (%)	
no	97 (66)
yes	50 (34)
Financial status, n (%)	
very bad	15 (10)
bad	66 (45)
good	55 (37)
very good	11 (7)
Asthma severity, n (%)	
mild	92 (63)
moderate	45 (31)
severe	10 (7)

n (%) – number (%) of patients.

ANOVA indicated significant differences between the PAQLQ (symptoms, activity limitation, emotional function and overall PAQLQ) and ACT scores which all were significantly higher after patient education conducted ($p < 0.001$) (Table 2).

Also, FEV1 ($p = 0.048$) and relative inhaler technique improvement ($p < 0.001$) were significantly higher after patient education conducted. A number of children demonstrating a correct inhalation technique improved from 28 (19%)

to 127 (86.4%) ($p < 0.001$). When we tested the distribution of type of environment, exposure to tobacco smoke and financial status according to FEV1 and PEF no significant differences were found (Figure 1).

Because we assumed that some other parameters could potentially moderate the impact of the education about proper inhaler use on quality of life, we used multivariate analysis of covariance (MANCOVA). We used the MANCOVA test to establish whether the groups of independent variables (before and after education) were significantly different in relation to dependent variables (symptoms, activity limitation, emotional function, overall PAQLQ and ACT scores, collectively), after controlling for covariates: asthma severity and FEV1, as well as relative inhaler technique improvement. MANCOVA revealed that education ($p = 0.004$), FEV1 ($p = 0.019$), asthma severity ($p < 0.001$) and relative inhaler technique improvement (< 0.001) were significant covariates (Table 3). Based upon η^2 values, asthma severity accounted for the largest proportion of variability of the PAQLQ and ACT scores (38.4%). Less but significant proportion of variability of the PAQLQ and ACT scores was accounted by relative inhaler technique improvement (23.7%). Age, gender, type of habitat environment, exposure to tobacco smoke, financial status, type of asthma inhaler were not significant as covariates.

Table 2
Values of scores for quality of life, asthma control test, FEV1, PEF and correct inhaler technique during inhaler use

Parameters	Before education	After education
PAQLQ scores		
Activity limitation	22.1 ± 4.5	23.5 ± 4.0**
Symptoms	44.8 ± 8.8	48.0 ± 8.1**
Emotional function	35.0 (32.0–40.0)	39.0 (34.0–42.0)**
Overall PAQLQ	102.5 ± 18.2	109.7 ± 18.2**
ACT score	19.4 ± 2.1	21.8 ± 1.8**
FEV1 (%)	83.7 ± 7.6	85.2 ± 7.4*
PEF (L/min)	335.0 (290.0–385.0)	345.0 (294.0–386.0)
Correct steps (%)	76.0 ± 11.0	97.6 ± 1.6**
Incorrect / Correct inhaler technique†, n (%)	119 (81) / 28 (19)	20 (13.6) / 127 (86.4)**

Data following the normal distribution were presented as means ± standard deviation, and data not following the normal distribution were presented as median (interquartile range).
PAQLQ – Pediatric Asthma Quality of Life Questionnaire; ACT – Asthma Control Test; FEV1 – forced expiratory volume in one second; PEF – peak expiratory flow; n (%) – number (%) of patients.
***$p < 0.05$; **$p < 0.001$; †χ^2 test.**

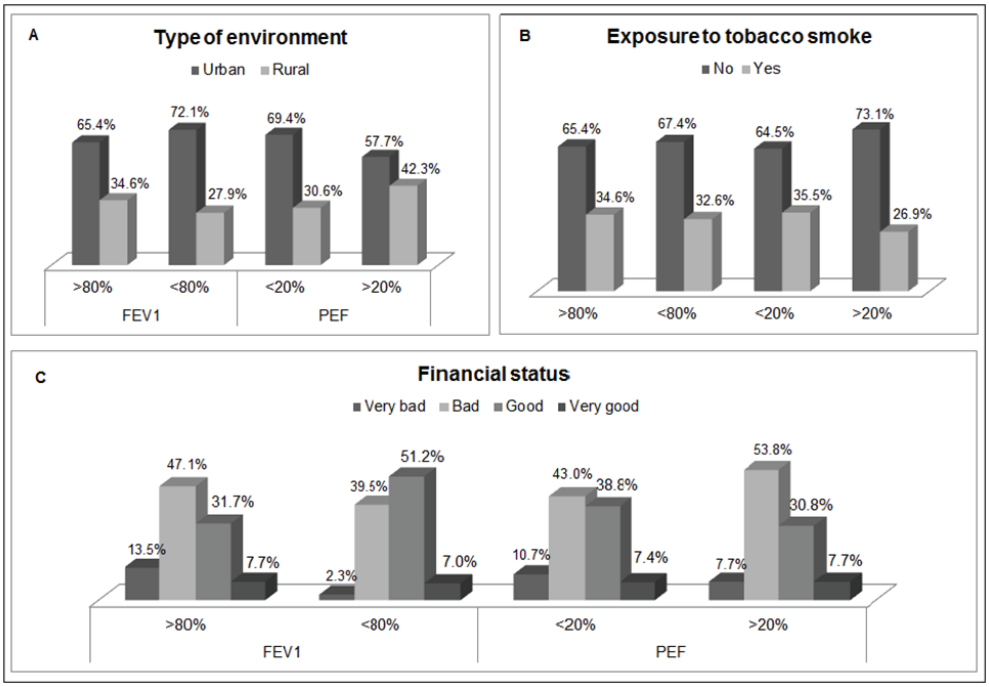

Fig. 1 – Distribution of type of environment (A), exposure to tobacco smoke (B), and financial status (C) according to forced expiratory volume in one second (FEV1) and peak expiratory flow (PEF).

Table 3

Multivariate and univariate analysis of covariance (ANCOVA) results depended on education

Multivariate ANCOVA					
Effect		Wilks' Lambda	F	Partial η^2	*p*
Asthma severity		0.616	44.599	0.384	< 0.001
FEV1 (%)		0.96	3.013	0.04	0.019
Relative inhaler technique improvement		0.763	22.227	0.237	< 0.001
Education		0.948	3.947	0.052	0.004
Univariate ANCOVA					
Dependent variable	Parameter	B	Observed power	Partial η^2	*p*
Activity	Asthma severity	-4.574	1	0.354	< 0.001
	FEV1 (%)	0.06	0.529	0.014	0.042
	Education	1.31	0.634	0.018	0.022
Symptoms	Asthma severity	-9.165	1	0.363	< 0.001
	FEV1 (%)	0.14	0.678	0.02	0.016
	Education	2.488	0.605	0.017	0.026
Emotions	Asthma severity	-6.938	1	0.329	< 0.001
	Education	2.593	0.811	0.027	0.005
Total score	Asthma severity	-20.677	1	0.373	< 0.001
	FEV1 (%)	0.275	0.575	0.016	0.032
	Education	6.39	0.735	0.023	0.010
Asthma control score	Asthma severity	-1.594	1	0.247	< 0.001
	FEV1 (%)	0.041	0.871	0.032	0.002
	Relative inhaler technique improvement	0.067	1	0.177	< 0.001
	Education	0.986	0.971	0.049	< 0.001

PAQLQ – Pediatric Asthma Quality of Life Questionnaire; ACT – Asthma Control Test; FEV1 – forced expiratory volume in one second.

Table 4

Pearson's correlations between parameters of quality of life and asthma control test and FEV1, asthma severity and relative inhaler technique improvement

Parameters	Correlations, rho (*p*)				
	Activity limitation	Symptoms	Emotional function[a]	Overall PAQLQ	ACT
Before education					
FEV1	0.224** (0.006)	0.208* (0.011)	0.227** (0.006)	0.226** (0.006)	0.163* (0.049)
Relative inhaler technique improvement	0.227** (0.006)	0.276** (0.001)	0.234** (0.004)	0.260** (0.001)	0.595** (< 0.001)
Asthma severity	-0.628** (< 0.001)	-0.631** (< 0.001)	-0.587** (< 0.001)	-0.637** (< 0.001)	-0.554** (< 0.001)
After education					
FEV1	0.224** (0.006)	0.224** (0.006)	0.246** (0.003)	0.238** (0.004)	0.126 (0.128)
Relative inhaler technique improvement	0.056 (0.502)	0.064 (0.438)	0.039 (0.638)	0.055 (0.507)	0.222** (0.007)
Asthma severity	-0.617** (< 0.001)	-0.624** (< 0.001)	-0.626** (< 0.001)	-0.639** (< 0.001)	-0.494** (< 0.001)

PAQLQ – Pediatric Asthma Quality life Questionaire; ACT – Asthma Control Test; FEV1 – forced expiratory volume in one second.
***p* < 0.05, ***p* < 0.01; [a]logarithmic transformed variable.**

ANCOVA, however, found that asthma severity and FEV1were significant for all the PAQLQ and ACT scores but relative inhaler technique improvement was significant only for asthma control score. Relative inhaler technique improvement and FEV1 provided positive model coefficients (B) and this equated to positive relationships between covariates and the PAQLQ and ACT scores.

The education positive model coefficient was equated to higher score values after patient education relative to values before education was conducted. The negative model coefficient for the asthma severity implied a negative relationship between this covariate and dependent variables (PAQLQ and ACT scores).

FEV1 was positively correlated with activity limitation, symptoms, emotional function and overall the PAQLQ and ACT scores before and after education was conducted (Table 4). The ACT score positively correlated with FEV1 only before education was conducted but not after it. Asthma sever-

ity was negatively correlated with all dependent variables before and after education was conducted. Relative inhaler technique improvement was positively correlated with all parameters of quality of life and the ACT score before education was conducted but after education the only significant correlation was with the ACT score.

Discussion

Maintaining control of asthma in children continues to be a problem, despite the advancements in its therapy. Individual factors such as genetics, smoking, type of inhaler, improper compliance, as well as family and environmental factors such as pets in the home, air pollution, and pollen exposure were identified as important factors that determine poorly controlled asthma [14, 15]. In many cases, asthma is poorly controlled due to incorrect use of inhaler, especially in children. Considering that children with asthma depend on their caregivers for help in managing their illness, participation in training programs for better asthma control was proved to be very useful for both children and parents [16]. Also, it is worth mentioning that asthma management improvement could result in decreasing asthma medication costs [17].

Numerous studies have focused on the importance of proper training for an inhaler use [18–20]. Few have examined the additional impact of age, obesity and limited parental health literacy [21–23]. Considering that relatively high rate of incorrect handling of inhalers has been reported in asthmatic children [24], we intend to examine the potential of education in improving inhaler technique and, consequently, better asthma control. Also, we aimed to investigate whether quality of life of children, in addition to asthma control, depend on other factors such as FEV1 values, asthma severity and relative inhaler technique improvement given as the percentage of correct steps. We initiated our study using an ethnically homogenous group of asthmatic children who were under different life circumstances (type of environment, exposure to tobacco smoke and financial status) and we found that profile of the study participants was equally distributed towards FEV1 and PEF values. At the beginning of the study children with asthma demonstrated a number of errors in device use. We revealed that 81.0% of the patients used the inhaler incorrectly, which means that only 19.0% of children were treated properly. Such a large number of errors occured most likely because the proper use of an inhaler was not well understood by patients. Also, physicians often have limited time to properly educate patients during regular medical check-up. All the patients in the study had received training program including practical demonstration and re-check of inhaler technique. After the training was completed correct inhaler technique was found to be present in 86.4% (n = 127) and incorrect inhaler technique was recorded in 13.6% (n = 20) of the patients included in the study. Our results showed that training reduced errors and improved outcomes. All scores of quality of life, determined in this study, asthma control and FEV1were significantly higher after educational interventions were performed. These results were in accordance with a review of controlled trials that demonstrated that a broad range of inhaler devices are very effective in delivering therapy when patients use them properly [6].

Education program contributed 5.2% to variability of quality of life with the largest single influence in the asthma control score (4.9%). When we look at differences observed among parameters of quality of life, as well as asthma control, before and after education, it is clear that the improvement of the disease management is accomplished. Although significant, the impact of education on 4.9% of the asthma control score variability suggests that training program had a relatively modest influence in asthma control improvement. However, it should be noted that the main effect of education was significant improvement of inhaler technique, which contributed to 23.7% variability of the quality of life and ACT scores. The positive model coefficient (B = 0.067) for the percentage of correct steps implied a positive relationship to the ACT score with contribution of 17.7%. On the other hand, when ANCOVA was performed, the relative inhaler technique improvement did not have a direct impact on quality of life scores. Based on these findings we could conclude that education improved asthma control, which is, in turn, positively affect quality of life.

Further analysis of physical, emotional and social issues and overall PAQLQ and ACT scores in asthmatic children as dependent variables, revealed a significant, but negative effect of asthma severity on quality of life (38.4%). This actually means that the level of asthma severity was major factor that affected quality of life of these children and to those with severe asthma we could expect only slight or no increase in scores after education regardless of inhaler technique improvement. Several studies have demonstrated that poorly controlled asthma was found to be associated with lower quality of life and ACT scores [25, 26]. It is not surprising that significant negative relationship was found between the level of asthma severity and activity limitation, symptoms and emotional function. When asthma is well controlled, symptoms are rare, activity is not limited, and sleep is not interrupted. At the same time the positive relationship between the PAQLQ and ACT scores and FEV1 and percentage of correct steps indicates an increase in quality of life, which is, at least partly, a consequence of the education and inhaler technique improvement.

Strategies to decrease the impact of asthma on quality of life in children should be focused on both, choosing an appropriate inhaler device and patient education for its proper use. It would certainly be useful not only for the patient's health but it would also have positive economic consequences.

Limitations of the study were relatively small number of children and having no information whether the level of proper use was followed after the study. Also, it is important to note that there was difference in age among children who took part in this study.

Conclusion

Education apparently plays a significant role in processes that lead to the PAQLQ and ACT scores increase in

asthmatic children. Correct use of an inhaler contributes to better asthma control rather than quality of life. Asthma severity proved to be a significant contributor to variations in the PAQLQ and ACT scores and major obstacle for quality of life improvement in children with asthma. Future studies addressing our observations are duly warranted.

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Anterior intra-pelvic approach and *corona mortis* vascular anastomoses: A clinical anatomical study shows high frequency

Prednji intra-karlični pristup i *corona mortis* vaskularne anastomoze: kliničko anatomska studija pokazuje visoku učestalost

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Abstract

Background/Aim. *Corona mortis* vascular anastomoses (CMVA) must be located during surgical gold standard treatment method for displaced acetabular fractures. This study aimed to answer the following questions: What is the clinical frequency observed of CMVA? What is the composition of CMVA: arterial, venous or a combination? **Methods.** A retrospective review was made of 31 patients (24 males, 7 females; mean age 43.5 years) who underwent surgery for acetabular fractures between 2011 and 2015. The anterior intra-pelvic (AIP) approach was applied to all patients. By examination of the intraoperative CMVA compositions, the frequency of CMVA was determined together with identification of venous or arterial formation and distance from the pubic symphysis. **Results.** CMVA was observed during dissection in 29 (94%) patients and was ligated. In 14 (45%) patients, CMVA was recorded as venous, in 7 (23%) patients as arterial and in 8 (26%) patients as both. The mean distance of CMVA from the pubic symphysis was 35.9 mm (range 21.6–48.7 mm). **Conclusion.** The results showed very high CMVA frequency in the AIP approach, higher than previously reported in the English literature. Orthopedic surgeons should be aware about CMVA while doing this approach in surgical treatment of acetabular fractures.

Key words:
arteriovenous anastomosis; anatomy; orthopedics; acetabulum; wounds and injuries; pubic symphysis.

Apstrakt

Uvod/Cilj. *Corona mortis* vaskularne anastomoze (CMVA) moraju biti identifikovane i locirane u toku hirurškog zahvata koji predstavlja zlatni standard u lečenju dislociranih preloma acetabuluma. Cilj rada bio je da se odgovori na sledeća pitanja: kolika je klinička učestalost CMVA, kao i kakva je struktura CMVA – arterijska, venska ili kombinovana. **Metode.** Izvršena je retrospektivna analiza 31 bolesnika (24 žene i sedam muškaraca, prosečne starosti 43,5 godina) koji su operisani zbog frakture acetabuluma u periodu od 2011. do 2015. godine. Prednji intrakarlični (PIK) pristup je bio primenjen kod svih bolesnika. Intraoperativno su bili praćeni i beleženi: struktura CMVA i učestalost, istovremeno sa identifikacijom venske ili arterijske formacije, kao i udaljenost od pubične simfize. **Rezultati.** CMVA su bile uočene tokom disekcije kod 29 (94%) bolesnika i podvezane. Kod 14 (45%) bolesnika CM, god sedam (23%) bolesnika arterijske i kod njih osam (26%) kombinovane. Prosečna udaljenost CMVA od pubične simfize iznosila je 35,9 mm (opseg 21,6–48,7 mm). **Zaključak.** Rezultati su pokazali veoma visoku učestalost CMVA kod PIK pristupa, višu od ranije objavljenih u literaturi na engleskom jeziku. Ortopedi bi trebali da ovo imaju u vidu kod PIK pristupa u hirurškom lečenju fraktura acetabuluma.

Ključne reči:
anastomoze, arteriovenske; anatomija; ortopedija; acetabulum; povrede; pubična simfiza.

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Introduction

Surgical treatment is the gold standard treatment method for displaced acetabular fractures and successful clinical results have been reported in the long-term following internal fixation where anatomic reduction has been achieved [1, 2]. The most frequently used surgical approaches are the Kocher-Langenbeck and ilioinguinal approaches [1–5]. The extended iliofemoral approach is recommended for complex fractures, but this approach also has high rates of complications and morbidity [2, 6]. In the last few decades, the anterior intrapelvic (AIP) approach has become known as a relatively less invasive approach for complex fractures, especially those involving the load-bearing roof and medial wall [7–11]. There has continued to be increasing popularity of the technique due to highly encouraging studies [5, 7, 9, 11].

In the AIP and ilioinguinal approaches, vascular anastomoses which provide the connection between the external and internal iliac vascular system on the posterior side of the superior pubic ramus, may be the cause of significant bleeding. Obturator vessels and nerves are the most important structures requiring attention because of their direct contact with the quadrilateral surface [10]. These vessels, which are known as *corona mortis* vascular anastomoses (CMVA), must be located during surgical exposure and appropriately tied or cauterized. First described by Albrecht von Haller (1708–1777), various studies have been conducted on the frequency of observation of these vessels, the anatomic variations and structural properties. The rate of frequency of observation has been reported as ranging from 1% to 100% [9, 12–17].

The aim of this study was to answer the following questions: What is the clinical frequency observed of CMVA? In clinical cases, what is the composition of CMVA: arterial, venous or a combination?

Methods

A retrospective evaluation was made from the records of patients who had been treated for acetabular fractures with the AIP approach, between 2011 and 2015, in two different centers. Children fractures and geriatric age patients were excluded and a total of 31 patients' records were included in the study. The AIP approach had been applied to all patients and the operations were performed by two surgeons experienced in the field of trauma and pelvis surgery. Approval for the study was granted by the local Ethics Committee and the study was performed in accordance with the ethical standards laid down in the Declaration of Helsinki from 1964 and all subsequent revisions.

Preoperative anteroposterior (AP), iliac and obturator oblique pelvis radiographs and computed tomography (CT) images were taken. The fractures were classified according to the Judet et al. [18] classification. All the fractures were evaluated clinically and radiologically as unstable and the decision was taken for surgery. In three patients, fractures were bilateral and extended to both hemipelvis. In four patients, there was acetabulum fracture together with a displaced pelvic fracture.

Surgical technique

The AIP approach technique was applied as defined in detail in the articles of Cole and Bolhofner [3] and Hirvensalo et al. [19]. Patients were placed supine on the operating table to allow adequate visualization on AP and Judet radiographs (pelvic). Prophylactic antibiotic (cefazolin, 1 g *iv*) was routinely administered. The presence of CMVA was investigated during exposure in all cases. In this process, the anatomic properties of CMVA were recorded (placement, distance from the pubic symphysis, arterial, venous or both). Then, the vessels were appropriately tied.

Results

Demographic data of the included patients are shown in Table 1.

Table 1

Demographic data of included patients

Parameters	Values
Mean age in years (range)	43.5 (21–65)
Male : female, n (%)	24 : 7 (77.4 : 22.6)
Letournel classification, n	
anterior column	15
both columns	2
anterior column plus posterior hemi-transverse	6
transverse	6
T shaped	2

Very different rates related to CMVA visualization have been reported in cadaver and endoscopic studies (Table 2). The patients were operated on at mean 3.9 days, range: 1 to 9 days (Figure 1). Before the reduction of the particular fracture, any CMVA was found and ligated to prevent extensive bleeding. CMVA were determined during dissection in 29 (94%) patients. In respect of vascular composition of CMVA, three types were identified (Figure 2): type I, purely arterial CMVA (n = 7/31, 23%); type II, purely venous CMVA (n = 14/31, 45%) and type III, a combination of both arterial and venous connections located on the behind of superior ramus of the pubic bone (n = 8/31, 26%). The average distance of CMVA from the pubic symphysis was 35.9 mm (range 21.6 – 48.7 mm).

Postoperative foot drop was observed in one patient, obturator nerve palsy in two ptients, partial iliac vein damage in one patient, and external femoral vein damage also in one patient. All vascular injuries were treated with primary sutures during the surgery. Drop foot was resolved after six months and all obturator nerve palsies resolved within 3 months after the index surgeries.

Table 2

Incidence of various connections (vascular, arterial and venous) from the references and the present study

Study	Corona mortis (%)	Arterial connections (%)	Venous connections (%)	Arterial and venous connections (%)	Specimens
Berberoğlu et al. [12]	-	8	96	-	7 cadaver dissection and 28 patients endoscopic
Karakurt et al. [24]	-	28.5	-	-	98 patients, angiography
Sarikcioglu et al. [17]	-	20	14	-	54 cadaver halves
Okcu et al. [22]	61	19	52	-	150 cadaver halves
Hong et al. [28]	72	-	-	-	50 cadaver halves
Pungpapong et al. [29]	77.27	-	-	-	66 pelvic halves
Darmanis et al. [13]	83	-	-	-	80 cadaver halves
Rusu et al. [16]	80	31	18	53	40 cadaver halves
Kacra et al. [10]	40	-	40	-	10 cadaver halves
Stavropoulou-Deli and Anagnostopoulou [23]	28.5	40	50	-	70 cadaver halves
Elmadağ et al. [9]	100	29.4	70.6	-	17 patients (AIP)
Current study	94	24	48	28	31 patients (AIP)

AIP – anterior intra-pelvic.

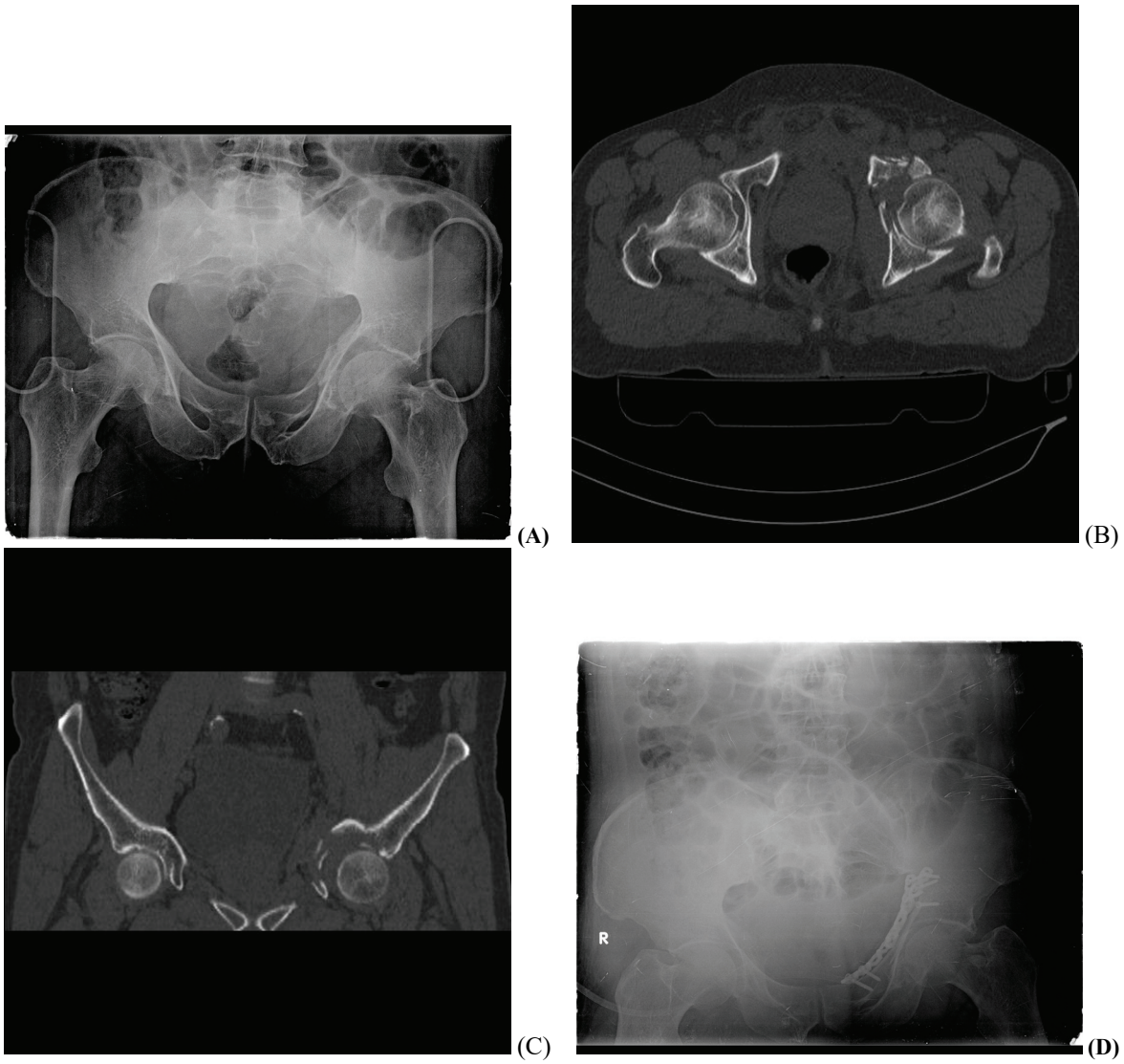

Fig. 1 – A 65-year-old male patient suffered with an acetabular fracture of the left hip after a simple fall (A);Tomography scans show dome impaction and displaced anterior column fracture on the left acetabular bone (B, C); Anatomic surgical reduction of the fracture and restoration of the dome impaction can be seen on the postoperative pelvic x-ray (D).

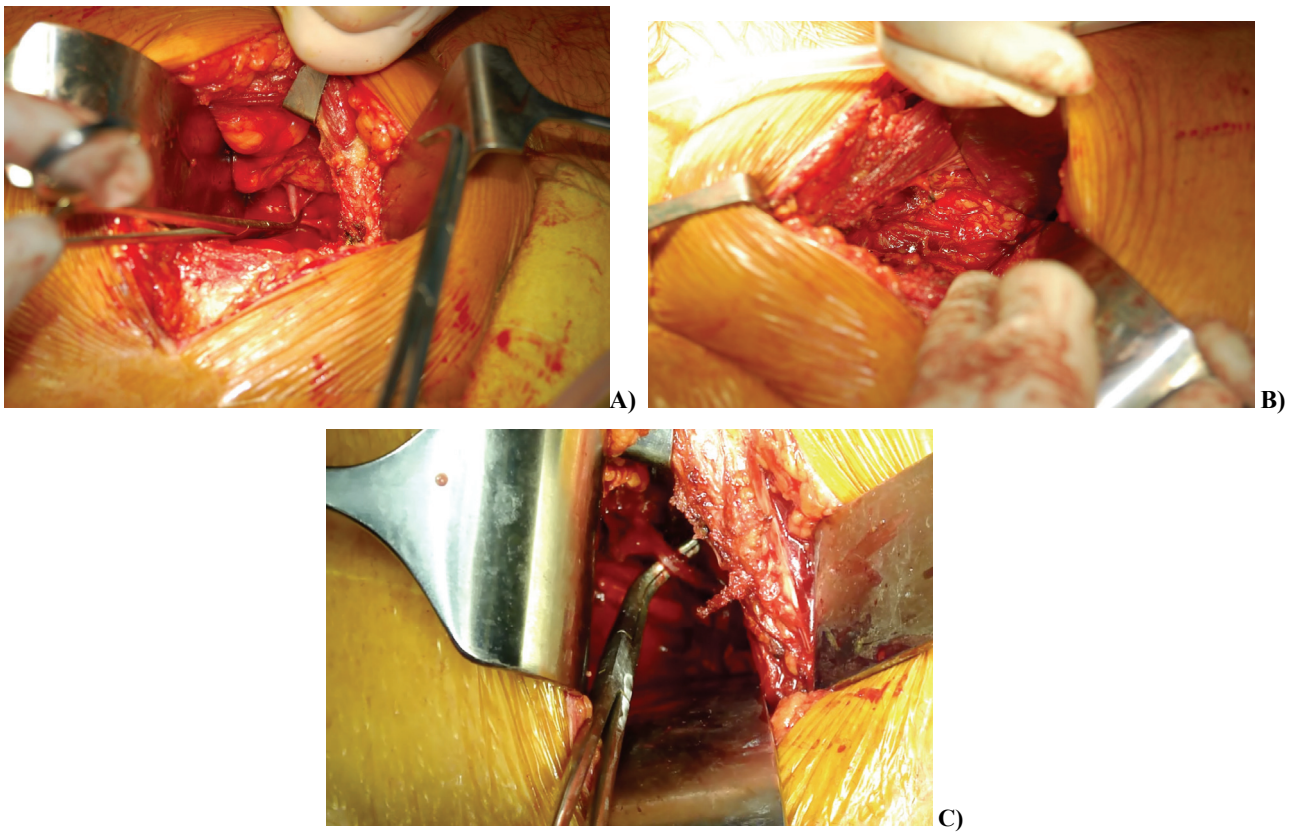

Fig. 2 – Surgical exploration of *corona mortis* vascular anastomoses (CMVA): A) arterial; B) venous; C) both.

Discussion

While *corona mortis* has a place in some studies as an anatomic variation, other researchers have stated that there are anatomic variations within CMVA. In this study, the AIP approach was applied to 31 patients and CMVA were identified in the majority of the patients (29/31, 94%). There was some form of anastomosis in almost all the hemi-pelvises. The thickness varied but nearly all were large enough to be a cause of bleeding.

In studies of 50 cadaver halves by Tornetta et al. [20], anastomosis was determined between the obturator and external iliac system vessels at the rate of 84%. In these cases, the arterial type was determined together with *corona mortis* at 34%, venous at 70% and a combination of both types at 20%. In dissections of 7 cadavers by Berberoğlu et al. [12], and in additional endoscopic evaluations of 28 cases, venous anastomosis was seen in 96% and in 8% accessory branches of the obturator artery. In the endoscopic examination of 141 hemi-pelvises of 121 patients by Lau and Lee [21], *corona mortis* was encountered as arterial in 22%, aberrant obturator vein in 27%, and as arterial or venous in 40%. Sarikcioglu et al. [17] determined venous anastomosis at a rate of 20% in 27 cadavers (54 cadaver halves) and the obturator artery was seen to originate from the inferior epigastric artery in 14%. In dissections of 150 cadaver halves of 75 cadavers, Okcu et al. [22] determined vascular anastomoses between the obturator and external iliac systems in 91 of 150 sides (61%), and anastomotic veins in 78 of 150 exposures (52%), arterial connections were seen in 29 (19%) of the exposures.

Rusu et al. [16] noticed the differences and systematically recorded the possibilities of CMVA, thereby determining in a study of 40 hemi-pelvis dissections from 20 cadavers, 32 (80%) CMVA, of which 10 (31%) were arterial, 16 (53%) arterial and venous and 6 (18%) venous. In the dissection of 10 hemi-pelvic cadavers, Kacra et al. [10] determined 4 (40%) venous CMVA. In the dissection of 20 hemi-pelvis of 10 cadavers by Stavropoulou-Deli and Anagnostopoulou [23], eight arterial and 10 venous CMVA were determined. In the current study, CMVA were present in 94% and determined as venous in 45% (n = 14/31), arterial in 23% (n = 7/31) and a combination of both in 26% (n = 8/31) of the patients. Darmanis et al. [13], in an examination of the hemipelvis of 80 cadavers, any vessel was determined crossing the superior pubic ramus in 83%, arterial anastomosis was determined in 36% and venous anastomosis in 60%, but in 492 operations applied with an anterior approach (ilioinguinal or AIP), *corona mortis* was encountered in only 5 cases. Findings in the operational group could be interpreted in complete contrast to those of the current study. However, there are few studies in literature presenting data supporting this.

When clinical studies have been examined, Elmadağ et al. [9] determined CMVA in all of 17 acetabular fractures operated on with the AIP approach, 70.6% of which were reported as venous and 29.4% as arterial CMVA. In a series of 55 cases, Cole and Bolhofner [3], who first defined the AIP

approach, first reported that anatomic vascular blockage related to the technique was anastomosis between the obturator vessels and the inferior epigastric artery and these anastomoses are often to be found but they are sometimes of different dimensions. From clinical studies, Cole and Bolhofner [3] and Elmadağ et al. [9] determined CMVA in every case at rates similar to those of the current study. There are angiographic studies of *corona mortis* in literature, but angiographic studies only evaluate arterial anastomoses and do not give information about venous connections [16, 24]. Advanced radiological techniques and fine slice thicknesses can provide the determination of higher incidence of *corona mortis*.

When examined anatomically, CMVA are immediately behind the superior pubic ramus and lateral of the pubic symphysis. In various studies in literature there are a series of findings about the thickness of CMVA and the distance to the pubic symphysis (Table 3). Rusu et al. [16] classified CMVA into four arterial subtypes, three venous subtypes and the combined type of arterial and venous anastomosis together. In studies by Sakthivelavan et al. [25] in which the origin of the obturator artery was examined in 116 hemi-pelvis, the obturator artery was determined to originate from the internal iliac system in 60.3% and from the external iliac system in 39.7% of cases. It was determined that, in 90% of the hemi-pelvises, the superior pubic ramus was crossed by various shapes and numbers of veins, to be drained from external iliac vein to obturator foramen. Similarly, Pai et al. [26] reported that in the majority of cases, the superior pubic ramus was traversed by multiple venous vessels but a percentage was not reported, whereas the rate of obturator artery crossing the superior pubic ramus was stated as 21% in total (19% originating from the external iliac system and 2% of dual origin, n = 98). There are studies in literature stating that the condition is less important when vascular diameter is < 1 mm [12]. The high incidence of CMVA obtained in the current study and that these vessels were of a thickness which could lead to bleeding, raises the question of whether very small diameter CMVA (< 1 mm) have been disregarded by many researchers or could not be determined. The importance of this question is further increased in studies not reflecting the findings of vessels below 2 mm [27–29].

The area of this study offering enlightenment can be considered to be not the presence of CMVA but that there may be variations in origins and thickness of the veins which comprise CMVA. In addition, CMVA not seen in some cases in clinical studies may be due to injury during trauma, and not visualized in some cases in cadaver studies may be due to vascular collapse occurring due to the lack of blood circulation in the veins which form CMVA or because of a fixation technique and time elapsed since the fixation. Examination of fresh cadavers in anatomic studies in this area would raise rates of CMVA encountered by researchers. One of the strengths giving importance to the current study is that CMVA could be seen in the majority of the cases in a living population. In this respect, need to make a careful surgery is essential for the AIP approach.

Limitations of this study are following: the number of cases was low, vascular diameters were not measured quantitatively, and detailed origins of the vessels were not determined. As the incision did not allow for it during the operation and because of the inherent risk, vessel origins were not determined. However, strong aspect of the study is that it drew attention to the high presence of CMVA. In addition, showing live CMVA which did not collapsed during the operation is strength of this study compared to previous cadaver and angiographic studies.

Table 3

The distance between the *corona mortis* and the pubic symphysis

Study	Arterial *corona mortis*		Venous *corona mortis*		Arterial or venous connecting vessel	
	Diameter	Distance from pubic symphysis	Diameter	Distance from pubic symphysis	Diameter	Distance from pubic symphysis
Berberoğlu et al. [12] mean (range), mm	0.98 (0.6–1.2)	-	3.3 (2.2–4.9)	-	-	40.4 (33.2–52.7)
Hong et al. [28] mean (range), mm	-	-	-	-	2.60 (2.0–4.2)	52 (38–68)
Karakurt et al. [24] mean (range), mm	-	33.4 (21.4–41)	-	-	-	-
Okcu et al. [22] mean (range), mm	-	64 (45–90)	-	56 (37–80)	-	-
Tornetta et al. [20] mean (range), mm	-	-	-	-	-	62 (30–90)
Darmanis et al. [13] mean (range), mm	-	71 (42–88)	-	65 (39–82)	-	-
Stavropoulou-Deli and Anagnostopoulou [23] (mean), mm	3	52.4	3.13	46.7	-	-
Current study mean (range), mm	-	-	-	-	-	35.9 (21.6–48.7)

Conclusion

As this study was the clinical one with the very high observed frequency of CMVA, higher than previously reported in the English literature, it can be considered necessary to take great care with these vessels during surgical exposure. Anastomoses have a different anatomic structure and include variations in size and origin.

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CASE REPORT
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Castleman's disease associated with mixed connective tissue disorder and cerebral ischaemia and vasculitis: A rare case and a diagnostic challenge for an infectologist

Kastlemanova bolest udružena sa mešanim poremećajem vezivnog tkiva i cerebralnom ishemijom i vaskulitisom: redak slučaj i dijagnostički izazov za infektologa

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Abstract

Introduction. Castleman's disease (CD) or angiofolicullar lymph node hyperplasia is a rare pathologic process characterized by non-neoplastic reactive proliferation of lymphoid tissue. Mimicking clinical and laboratory signs of infection, it could be a great diagnostic problem for an infectologist. **Case report.** We report a case of a 39-year old man who was initially clinically suspected to have an infectious central nervous system (CNS) affection, having most similar appearance to neurotuberculosis. Malignancy with bone metastases and lymphoma were also among many possible diagnoses. The patient was later histologically confirmed to have Castleman's disease, analyzing the enlarged inguinal lymph node, which was the key point in rejecting the suspicion of malignancy and tuberculosis. By further analyses, the patient was diagnosed to have mixed connective tissue disorder (MCTD). Vasculitis of mesencephalon and thalamus was detected by magnetic resonance imaging. **Conclusion.** CD with CNS involvement is very rare as well as CD with MCTD association, making this case even more unique. This case report underlines the importance of definitive histological diagnosis in patients with lymphadenopathia associated with systemic involvement and the need of additional immunological and radiological examinations, as well.

Key words:
castleman disease; diagnostic techniques and procedures; diagnosis, differential; neurologic manifestations; histology.

Apstrakt

Uvod. Kastlemanova bolest (KB) ili angiofolikularna hiperplazija limfnih čvorova je redak patohistološki proces koji se karakteriše ne-neoplastičnom reaktivnom proliferacijom limfnog tkiva. S obzirom da imitira kliničke i laboratorijske znake infekcije, može predstavljati značajan dijagnostički problem za infektologa. **Prikaz bolesnika.** Predstavljamo tok bolesti tridesetdevetogodišnjeg muškarca kod koga je u početku bila postavljena klinička sumnja na infekciju centralnog nervnog sistema (CNS), koja je najviše podsećala na neurotuberkulozu. Među ostalim mogućim dijagnozama našli su se i malignitet sa metastazama u kostima i limfom. U daljem toku, kod bolesnika je histološkom analizom limfnog čvora utvrđena KB, što je bilo presudno u odbacivanju sumnje na malignitet i tuberkulozu. Dodatnim analizama je kod bolesnika utvrđena mešovita bolest vezivnog tkiva (MBVT). Magnetnom rezonancom otkriven je vaskulitis mezencefalona i talamusa. **Zaključak.** Kastlemanova bolest sa zahvatanjem CNS-a veoma je retka, kao i KB udružena sa MBVT, što zajedno ovaj slučaj čini još jedinstvenijim. Ovim prikazom slučaja naglašava se važnost definitivne histološke dijagnoze kod bolesnika sa limfadenopatijom i pridruženim sistemskim manifestacijama i potreba za dodatnim imunološkim i radiološkim analizama.

Ključne reči:
kastlemanova bolest; dijagnostičke tehnike i procedure; dijagnoza diferencijalna; neurološke manifestacije; histologija.

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Introduction

Castleman's disease (CD) represents angiofolicullar lymph node hyperplasia. It is a rare pathologic process of undetermined etiology. It is characterized by non-neoplastic reactive proliferation of lymphoid tissue [1]. CD is one of many causes of the fever of unknown origin [2]. This disease belongs to the field of research of hematology, oncology, rheumatology and virology because it includes episodic systemic inflammatory symptoms, reactive proliferation of morphologically benign lymphocytes and multiple organ system impairment as a result of excessive interleukin-6 (IL-6) and other proinflammatory cytokines. Regarding viral ethiology, there is a human herpes virus 8 (HHV-8) positive and human immunodeficiency virus (HIV) positive, HHV-8 positive and HIV negative, and HHV-8 negative and HIV negative variant of the disease (idiopathic CD) [3]. Unicentric CD (UCD) implies enlargement of one group of lymph nodes. Multicentric CD (MCD) implies enlargement of two and more groups of lymph nodes and it is associated with systemic symptoms appearance, unlike UCD [4].

The latest diagnostic criteria (2017) [5] for diagnosing HHV-8-negative/idiopathic multicentric CD are established by an international working group of 34 pediatric and adult pathology and clinical experts. The group came up with the following major and minor diagnostic criteria for idiopathic multicentric CD. Major diagnostic criteria (need both present to diagnose) are: histopathologically confirmed CD, and enlarged lymph nodes (> 1 cm in short-axis diameter) in two or more lymph node stations. Minor diagnostic criteria are (need at least two out of eleven criteria and at least one laboratory criterion present): elevated C-reactive protein (CRP) (greater than 10 mg/L) or erythrocyte sedimentation rate (greater than 15 mm/hr); anemia (hemoglobin less than 12.5 g/dL for males, and less than 11.5 g/dL for females); thrombocytopenia (platelet count less than 150 k/μL) or thrombocytosis (platelet count greater than 400 k/μL); hypoalbuminemia (albumin less than 3.5 g/dL); renal dysfunction (estimated glomerular filtration rate < 60 mL/min/1.73 m^2) or proteinuria (total protein > 150 mg/100 mL); polyclonal hypergammaglobulinemia (total gamma globulin or immunoglobulin G > 1700 mg/dL); constitutional symptoms: night sweats, fever (> 38 °C), weight loss or fatigue; large spleen and/or liver; fluid accumulation: edema, anasarca, ascites, or pleural effusion; eruptive cherry hemangiomatosis or violaceous papules; lymphocytic interstitial pneumonitis [5].

Case report

A 39-year-old man presented with a 2-month history of predominantly low grade fever (37.2 °C–37.6 °C), weight loss (approximately 15 kg), incoherent speech, intensive headache with nausea and vomitting. Several days before admission to hospital, he had constant feeling of intense neck pain and his family noticed right eyelid drooping and weakness of his arms and legs with consequent movement difficulty. One day before admisson, he was sleepy and confused, according to his family. After examining in a local hospital, he was sent to the Clinic for Infectious Diseases of the Clinical Center Niš, Serbia, as suspected meningoencephalitis.

The patient's past medical history was significant for a couple of rheumatologist visits due to suspected Reynaud's syndrome owing to periodic feeling of numbness in hand fingers (which occurred one year before current illness and the examining plan was not completed). Concerning family medical history, the patient's father died of myasthenia gravis.

Clinical examination at admission revealed somnolence, disorientation, slurred speech, neck stiffness, positive Brudzinski's neck sign, right eyelid ptosis, bilaterally sligthly reduced breath sound, left pretibial edema, billateral inguinal lymphadenopathy, cachexia, maculopapular rash on trunk and proximal lower extremities. During 7-week hospitalization, the periods of normal body temperature, and slightly raised body temperature up to 38 °C and fever ≤ 39 °C were shifting. The level of consciousness varied between full consciousness and sopor, altogether with the right eyelid ptosis, the degree of which increased and decreased in paralel with neck stifness intensity fluctuation until complete regression. During full consciousness, he has permanently complained of pain in bones. Pretibial edema was present throughout the complete hospital stay. The rash dissapeared after the first hospitalization week.

Routine blood investigations revealed increased white blood cells count (14.9 × 10^9/L) and platelet count (736 × 10^9/L), anemia with red blood cells (RBC) count of 3.25 × 10^{12}/L, hemoglobin level of 91 g/L, hematocrit of 28%, decreased albumines (25 g/L), increased CRP level (118 mg/L), increased procalcitonin level (0.13 ng/mL), low sodium level (128 mEq/L), increased gamma glutamyl transferase (203 U/L), prolonged prothrombin time (20.6%). Autoantibodies levels were within normal range (anti-Sjogren's syndrome-related antigen A and B, anti-scleroderma 70 kD topoisomerase antigen, antisynthetase antibodies, anti-centromeric B, anti-double stranded DNA, antiphospholipid antibodies), except anti-ribonucleoprotein 70 (anti-RNP 70) which was > 200 U/mL and antinuclear antibodies (ANA) screen (6.7 U/mL, cut-off value for positive result is 1.2 U/mL). Level of β2 microglobulin was increased (4.55 mg/mL), however, myeloma was excluded when serum protein electrophoresis detected no monoclonal band, there was a polyclonal increase in gamma-globulins (20.8%). Interleukine-6 (IL-6) value was 12.23 pg/mL (reference range is < 5 pg/mL).

Two cerebrospinal fluid (CSF) analyses (on admission and eleven days after admission) revealed low sugar (0.2 mmol/L and 2.9 mmol/L, respectively), increased microprotein level (3.36 g/L and 1.43 g/L, respectively), decreased chlorine (116 mmol/L and 106 mmol/L, respectively), 265 RBC and 39 RBC, respectively, 159 polymorphonuclear neutrophils (PMN) cells and 0 PMN cells, respectively 15 lymphocytes and 2 lymphocytes. Blood sugar levels were 5.1 and 5.5 mmol/L, respectively. The CSF was xantochromic both times.

Microbiologic analyses findings of the CSF, on admission and eleven days after admission, (routine CSF bacterial and fungal culture, *Mycobacterium tuberculosis* CSF culture and microscopic exams), as well as of the blood (serology

for borreliosis, HIV, syphilis, brucellosis, leishmaniosis, Cytomegalovirus, Epstein-Barr virus, hepatitis B and C and PCR for HHV8) were negative. Findings of the three urine and three blood analyses for *Mycobacterium tuberculosis* (culture and microscopic exam) were negative, likewise.

Bone marrow biopsy sample and tumor markers analyses results were normal (human chorionic gonadotropin beta, alpha-feto protein, gastrointestinal 19-9 antigen, carcinoembryogenic antigen) except prostate specific antigen (11.43 ng/mL) which was explained as reactive benign prostatic hyperplasia by further analysis (prostatic exam, echosonographic and MSCT findings, prostate biopsy results).

Electromyoneurography (EMNG) of the lower limbs detected distal symmetric sensorimotor polyneuropathy.

Magnetic resonance imaging (MRI) of the brain, showed mesencephalic and thalamic lesions suggestive of ischaemia and vasulitis (Figures 1–3).

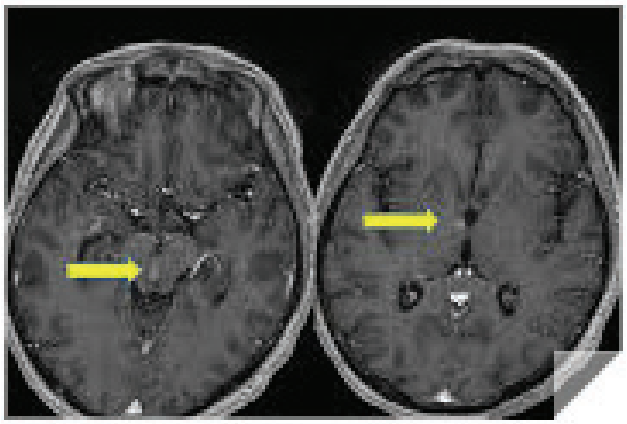

Fig. 1 – T1 weighted (T1W) axial section presenting areas of altered signals without expansive process, in the right mesencephalic (picture left) and the right thalamic region (picture right), which show post-contrast signal enhancement indicative of vasculitis and consequent cerebrovascular ischemia.

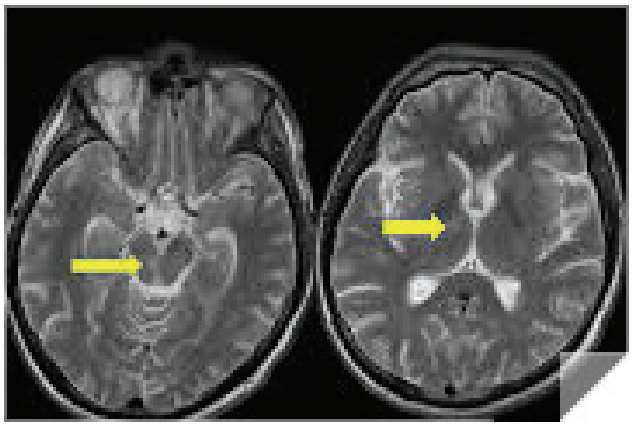

Fig. 2 – T2 weighted (T2W) axial section presenting areas of altered signals without expansive process, in the right mesencephalic (picture left) and the right thalamic region (picture right), which show post-contrast signal enhancement which is indicative of vasculitis and a consequent cerebrovascular ischemia.

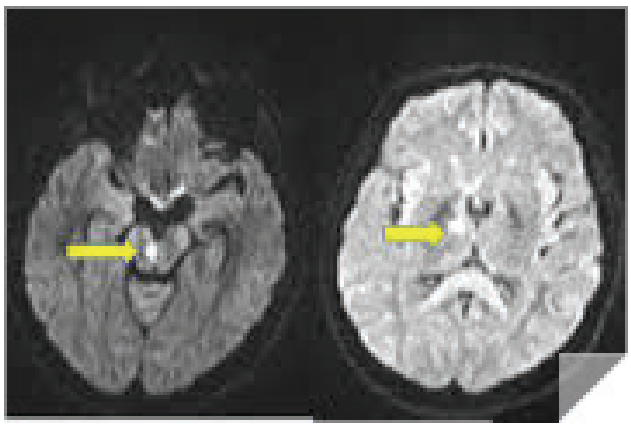

Fig. 3 – Diffusion-weighted imaging (DWI) sequence showing a restricted diffusion in the right mesencephalic (picture left) and the right thalamic region (picture right) which confirms ischaemic process.

Multislice computed tomography (MSCT) of chest and abdomen showed lung fibrosis (Figure 4), bilateral pleural effusion, pericardial effusion and mediastinal lymph nodes up to 15 mm; enlarged liver (181 mm in the midclavicular line), and enlarged inhomogenous spleen (maximal height of 140 mm, vertical height of 122 mm). MSCT of the pelvis showed bilaterally enlarged inguinal lymph nodes, both sized 26 mm.

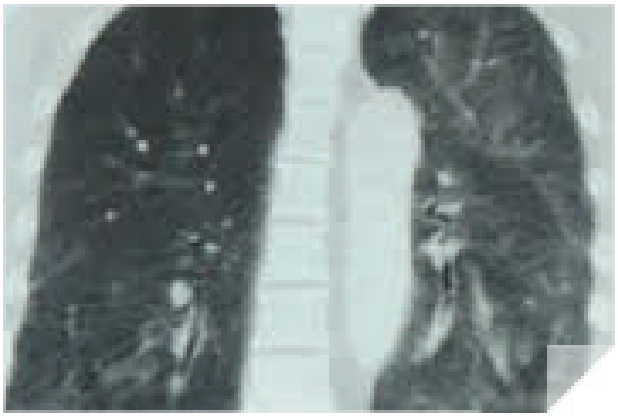

Fig. 4 – Multislice computed tomography (MSCT) showing an irregulary contoured, 25 × 22 mm and a tape-like, 15 × 8 mm reticular abnormalities of the lungs, in the right middle lobe medial segment, representing pulmonary fibrosis.

Bone scintigraphy with 99m Technetium marked diphosphono-propanodicarboxylic acid (99mTc – DPD) demonstrated enhanced accumulation of radiopharmaceutical in the mandibular ramus, right sternoclavicular joint, fifth lumbar vertebra, the both femoral necks, left iliac bone, left ischial bone and right knee joint (Figure 5).

Pathohistological analysis of the extirpated inguinal lymph node showed a lymphoproliferative process (angiofolicullar hyperplasia of the lymphoid tissue with hyalinization), suggesting multicentric CD. Bioptic material was analyzed on serial histological sections, colored by hematoxylin-eosin (HE) metod.

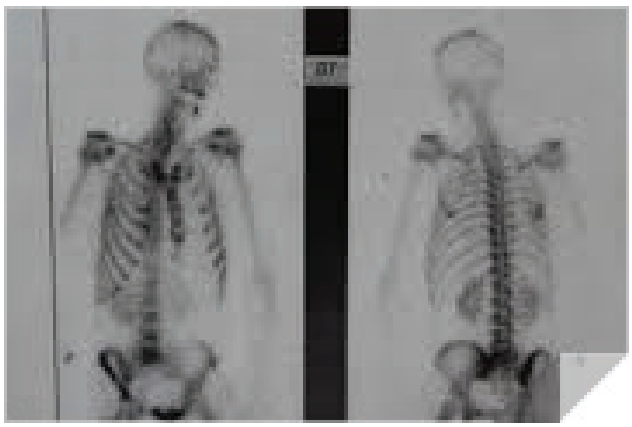

Fig. 5 – Bone scintigraphy with 99m Technetium marked diphosphono-propanodicarboxylic acid (99mTc – DPD), 3 h after application of 740 MBq osteotropic radiopharmaceutic.

It contained tissue of the lymph node with globally preserved morphology, with proliferation of the fibrous tissue, which was adequate to its localization (inguinal lymph nodes) and markedly multiplied small blood vessels with slightly expanded and hyalinized walls in places. The finding suggested non-neoplastic lymphoproliferative disorder with regression of germinative centers, abnormal vascular proliferation and hyalinization, and concentric arch-like lymphocytes areas only in places. Prominent interfollicular area contained multiplied non-neoplastic plasma cells, immunoblasts, plasmocytoid monocytes and hystiocytes. Expression of the used immunohistochemical markers did not show neoplastic proliferation. Morphological findings and immunohistochemical analyses corresponded to non-tumorous angiofolicullar hyperplasia of lymphoid tissue that is multicentric CD. Additional application of immunochemistry methods excluded possibility of neoplastic process (the tissue was analyzed for expression of CK AE1/AE3, CD20, CD3, bcl-2, Ki67, CD23 and CD 138 markers) (Figures 6 and 7).

The patient was empirically treated for central nervous system (CNS) infections (parenterally administred – ceftriaxone 2 g/12 h the first day, acyclovir 500 mg/8 h the first day, metronidazole 500 mg/8 h the first day; dexamethason, minimum 8 mg daily, maximum 32 mg daily during first 24 days; mannitol in reducing doses during first 14 days), including *Mycobacterium tuberculosis* brain infection (perorally administred – isoniazid 300 mg daily, rifampin 600 mg daily, pyrazinamide 2,500 mg daily daily, during first 6 weeks; streptomycin 1 g daily during first 10 days and ethambutol 800 mg daily during first 4 weeks) until the proper diagnosis was made.

After hospital discharge and obtaining the correct diagnosis, the patient was referred to a haematologist and started treatment with prednisone, starting with 50 mg daily to 10 mg daily nowdays (in lack of therapy of choice – anti-IL-6 monoclonal antibodies). In first six months after discharge from the hospital the corticosteroid therapy induced only moderate disease remission and symptomatic relief. Twelve months after the discharge, the patient's condition ameliorated significantly in terms of normal body temperature, normal weight, no palpable lymphadenopathy, normal level of consciousness, complete regression of eyelid ptosis, no rash, no edema, achieved walking and speech ability (with the help of the companion due to a slight limb instability) and laboratory markers of inflammation within reference range.

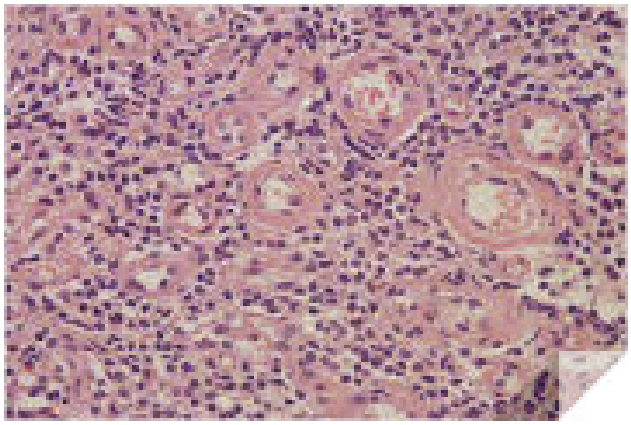

Fig. 6 – Vascular proliferation and hyalinization of the lymph node (hematoxylin & eosin stain, magnification ×40).

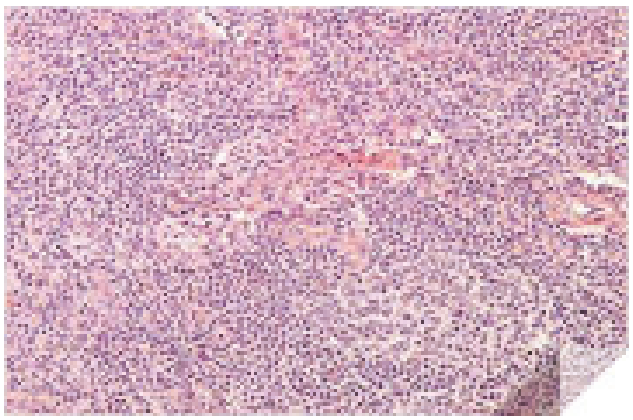

Fig. 7 – Germinal center regression with hyalinization of the lymph node (hematoxylin & eosin stain, magnification ×20).

Discussion

Castleman disease is a very rare disease. Unicentric CD (UCD) is the most common at 16 *per* million person years and occurs at every age. Idiopathic MCD is a less frequent disease with an estimated incidence of 5 *per* million person years [6]. The estimated US 10-year prevalence of MCD was 2·4 per million which is information obtained from data analyses of 59 MCD patients identified between 2000 and 2009 at two the United States MCD referral centres [7]. Until now, there was no presentation of MCD (which is even more rare than UCD) from the Serbian authors, there was only one presentation of an UCD case, published in 2011 [8].

Our patient's findings could be consequent to a number of diseases, including bacterial meningitis, tuberculosis with tuberculous meningitis, cerebritis, cancer with bone and brain metastases, lymphoma, myeloma, systemic lupus erythematosus (SLE), mixed connective tissue disease (MCTD)

and other systemic autoimmune disorders. Fever and meningeal syndrome were the reason for admission to the Clinic for Infectious Diseases.

The diagnosis of CD was obtained after the histopathological and immunohistochemical analysis. This was probably the consequence of IL-6 overproduction which induced anaemia, increase of immunoglobulins, increase in inflammatory activity parameters and the formation of autoantibodies, explaining the positive antinuclear antibodies (ANA) test along with physical findings and symptoms [9].

Having in mind our patients bone scintigraphy findings, it was very hard to differentiate them from disseminated bone metastases. However**,** there were some case reports of CD which mimicked MCTD [10]. Further more, SLE is often linked with anti ribonucleoprotein (RNP) positivity and osteopenia [11], and 32% of the patients with multicentric CD have criteria for POEMS syndrome (peripheral neuropathy, organomegaly, endocrinopathy, monoclonal protein, skin changes) [12]. Our patient fulfills the criteria for multicentric CD associated with the osteosclerotic variant of POEMS syndrome.

Most patients, as our patient, with the generalized form present with systemic symptoms such as fever, weight loss, anemia and hyperglobulinemia. The systemic involvement and histological presentation were the elements for the diagnosis of the multicentric CD. A review of the presence of autoimmune diseases concomitant to CD revealed an association with rheumatoid arthritis, myasthenia gravis, SLE/polymyositis overlap syndrome, MCTD and SLE. The fact that the patients father died due to suspected myasthenia gravis is another brick that straightens the wall of the CD diagnosis [9].

Castleman's disease may occur at any site with lymph nodes and extranodal areas. Castleman's disease is rarely diagnosed in the CNS, with only 13 cases in the literature until year 2005. The origin of intracerebral CD was explained by dendritic cells' participation in immune disregulation in MCD [13, 14].

Cerebral ischaemia, vasculitis and CSF alteration in our patient were most likely the result of POEMS syndrome, presenting as aseptic meningitis [15].

A range of systemic therapies have been utilized in MCD, including cytotoxic chemotherapy agents and antibodies directed against CD20 as well as IL-6 and its receptor (rituximab and siltuximab). Corticosteroids may offer effective symptom relief but, as the duration of response is typically limited, their main role is in combination with chemotherapy or other MCD treatments [16]. We suppose that initial dexamethasone treatment (administred as therapy against intracranial swelling) gave improvement of the patient's immunologically induced symptoms, incidentally targeting IL-6 pathways as a corticosteroid, but not as successful as targeted anti IL-6 therapy would have done it).

Siltuximab has a greater proportion of complete responses and longer progression-free survival for iMCD than rituximab [17]. Our patient fullfied the criteria for iMCD, so the right therapy of choice should have been siltuximab. Castleman's disease can transform into variety of malignancies, particularly non-Hodgkin's lymphoma, and Hodgkin's disease especially if targeted anti-IL-6 antibody therapy has not been implemented [18].

Conclusion

This case brings together two very rare presentations associated with CD – the MCTD presentation and the cerebral affection, making it even more unique. The whole clinical picture and the laboratory findings make this particular case, as well as any case of CD, a diagnostic challenge for various medical fields specialists.

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Primeri referenci:

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Tabele

Sve tabele pripremaju se sa proredom 1,5 na posebnom listu. Obeležavaju se arapskim brojevima, redosledom pojavljivanja, u desnom 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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