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Movember – "moustache month"
***Movember* – „brkati mesec"**

For eighteen years, the world has been celebrating Movember – a month with a moustache, dedicated to preserving men's health. The term Movember is a combination of the English words November and moustache. It is an initiative, launched in Australia, whose goal is to raise awareness about the prevention of malignant diseases in men – prostate and testicular cancer. The importance of preventive examinations for men is best illustrated by the data that every year in Europe, about 450 thousand men get prostate cancer, and about 100 thousand people die. In Serbia, about three thousand men get prostate cancer every year, and more than a thousand die.

The Editorial Board of the Vojnosanitetski Pregled invites its associates to engage in the promotion of the basic goals of Movember this month.

U svetu se već osamnaest godina obeležava *Movember* – „brkati mesec", posvećen očuvanju zdravlja muškaraca. Izraz *Movember* je nastao kombinacijom engleskih reči November i moustache (brkovi). Reč je o inicijativi, pokrenutoj u Australiji, čiji je cilj podizanje svesti o prevenciji malignih bolesti kod muškaraca – raka prostate i testisa. O značaju preventivnih pregleda kod muškaraca najbolje govore podaci da svake godine u Evropi oko 450 000 muškaraca dobije rak prostate, a premine oko 100 000 ljudi. U Srbiji svake godine rak prostate dobije oko 3 000 muškaraca, a premine više od 1 000.

Uređivački odbor Vojnosanitetskog pregleda poziva svoje saradnike da se tokom ovog meseca angažuju u promociji osnovnih ciljeva *Movember*-a.

ORIGINAL ARTICLES

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Contact sensitization in patients with chronic venous insufficiency and the impact of the disease duration on the risk of occurence of contact sensitization

Kontaktna senzibilizacija kod bolesnika sa hroničnom venskom insuficijencijom i uticaj dužine trajanja bolesti na rizik od nastanka kontaktne senzibilizacije

Ljuba Vujanović*†, Marina Jovanović *†, Milan Matić*†, Sanja Jakovljević†, Zoran Golušin*†

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Abstract

Background/Aim. Development of allergic contact dermatitis as a complication of treatment of chronic venous insufficiency (CVI) is well known. The aim of this study was to determine the incidence and risk of eczematous contact sensitization in patients with CVI, as well as the correlation between disease duration and contact sensitization. **Methods.** The study involved 266 subjects examined during three-year-period who were divided into two groups: the study group included patients with CVI referred for allergy testing due to suspected contact dermatitis, and the control group included the ones without CVI patch tested for suspected contact dermatitis. The severity of CVI was assessed by Clinical Etiology-Anatomy-Pathophysiology (CEAP) classification. Thereafter, each patient underwent patch testing. **Results.** The incidence of contact sensitization among patients with CVI was 49.3%. In these patients, the incidence of contact sensitization to the European standard battery of allergens was 31.55%; to the battery specific for CVI it was 28.45%. Patients with CVI had a 2.45-fold higher risk for developing contact sensitization to two or more allergens, and a 3.69-fold higher risk for developing contact sensitization to five or more allergens compared to those without CVI. The prevalence of contact sensitization in patients with CVI was not significantly different from those without CVI. There was a positive correlation between the incidence of contact sensitization and the duration of the disease. **Conclusion.** Patients with CVI had no statistically significantly distinct contact sensitization prevalence and had 2.45 and 3.69 times higher risk to manifest contact sensitization to two and more allergens and five and more allergens, respectively, than patients with no CVI. The positive correlation between frequency of contact sensitization and disease duration was found.

Key words:
dermatitis, contact; incidence; risk assessment; venous insufficiency.

Apstrakt

Uvod/Cilj. Poznato je da se tokom lečenja hronične venske insuficijencije (HVI) može razviti, kao komplikacija, alergijski kontaktni dermatitis. Cilj rada je bio da se utvrde rizik i učestalost kontaktne senzibilizacije ekcemskog tipa kod obolelih od HVI, kao i postojanje korelacije između dužine trajanja bolesti i kontaktne senzibilizacije. **Metode.** Istraživanjem su obuhvaćena 266 ispitanika. Formirane su dve grupe: eksperimentalna grupa (bolesnici sa HVI, upućeni na alergološko testiranje pod sumnjom na postojanje kontaktnog dermatitis) i kontrolna grupa (ispitanici bez HVI, epikutano testirani pod sumnjom na postojanje kontaktnog dermatitisa). Težina HVI procenjivana je na osnovu *Clinical Etiology-Anatomy-Pathophysiology* (CEAP) klacifikacije. Svaki ispitanik je bio podvrgnut alergološkom epikutanom testiranju *patch* testom. **Rezultati.** Učestalost kontaktne senzibilizacije među obolelima od HVI iznosila je 49,3%. Učestalost kontaktne senzibilizacije kod osoba sa HVI na alergene iz sastava Evropske standardne baterije iznosila je 31,55%, a na alergene iz baterije specifične za HVI 28,45%. Prevalencija kontaktne senzibilizacije kod osoba sa HVI nije se statistički značajno razlikovala od prevalencije kod osoba bez HVI. Bolesnici sa HVI su imali 2,45 puta viši rizik od nastanka kontaktne senzibilizacije na dva i više alergena, a 3,69 puta viši rizik od nastanka kontaktne senzibilizacije na pet i više alergena u odnosu na bolesnike bez HVI. Učestalost

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kontaktne senzibilizacije je bila u pozitivnoj korelaciji sa dužinom trajanja bolesti. **Zaključak.** Bolesnici sa HVI nisu imali statistički značajno veću prevalenciju senzitizacije, a imali su 2,45 puta, odnosno 3,69 puta viši rizik od nastanka kontaktne senzibilizacije na dva i više alergena i na pet i više alergena, redom, u odnosu na bolesnike bez HVI. Nađena je pozitivna korelacija između učestalosti kontaktne senzibilizacije i dužine trajanja bolesti.

Ključne reči:
dermatitis, kontaktni; incidenca; rizik, procena; venska insuficijencija.

Introduction

Chronic venous insufficiency (CVI) is a consequent condition to incompetence of the lower extremity veins [1].

According to statistics of the World Health Organization (WHO), around 15% of the general population suffer from chronic venous insufficiency, with increased prevalence in older age [2, 3]. In 70–80% of cases, CVI is the crucial etiological factor in the occurrence of venous ulcerations [4].

CVI is a disease characterized by chronic recurrent course demanding long-term therapy and monitoring. Adverse reactions and complications may develop during the treatment of CVI. The most common complication due to the local therapy is contact allergic dermatitis manifested either on the site of the drug application or on the other parts of body in the form of disseminated lesions. Allergic contact dermatitis emerges during the treatment in 60% to 80% in patients with CVI, including patients with venous ulcerations [5–7].

The aim of this research was to determine the frequency of contact sensitization, prevalence of contact sensitization to studied allergens, polyvalent sensitization and possible risk for development of contact sensitization with respect to the length of duration of CVI. The study included patients with symptoms of contact dermatitis.

Methods

Patients

This study was cross-sectional and included 266 patients suspected to have contact derimatitis (CD), treated at the Clinical Centre of Vojvodina in Novi Sad (Serbia) in a three-year-period, from 2010 to 2013. The patients were divided into two groups: the experimental group which encompassed patients suffering from CVI suspected to have CD (CVI group) and the control group involving subjects suspected to have CD without presence of CVI (CD group). The CVI group counted 150 cohorts (96 women and 54 men, of average age 64.24 ± 12.01 years), while the control group involved 116 subjects (89 women and 27 men, of average age 45.55 ± 17.00 years). Patients with CVI were older than those from the control group ($p < 0.001$), with small but statistically significant difference. There was statistically significant difference in gender structure between examined groups ($p < 0.001$) due to higher percent of females in the control group than in the experimental group.

All the patients were thoroughly examined; the venous duplex ultrasound of lower extremities was performed, Ankle Brachial Pressure Index (ABPI) was done as well as the Clinical-Etiology-Anatomy-Pathophysiology (CEAP) classification in patients with CVI.

Informed consent was obtained from all patients in accordance with the Institutional Review Board Policy, and the research protocol followed the ethical guidelines of the 1975 Declaration of Helsinki.

Each of the subjects fulfilled appropriate questionnaire as well as written consent for the further investigation. Questionnaires were adapted to the research and included personal data, family and professional details, anamnesis, disease course, duration of disease, potential deteriorating factors, signs and symptoms indicative to allergic contact dermatitis. Excluding criteria were: data about atopic diathesis such as presence of allergic conjunctivitis, rhinitis, asthma and atopic dermatitis; patients suffering from any systemic disease; patients on the immunosuppressive therapy in the previous six months; patients exposed to intensive sunlight during last four weeks before testing; systemic and local application of corticosteroids during last four weeks before the testing; active dermatitis at the time of testing; pregnancy and breastfeeding.

Patch test

Allergy test was conducted in all subjects with allergens from the European Standard Series of Allergens (28 allergens) (Table 1) and locally modified standard series for leg ulcer (23 allergens) (Table 2) which are of production from Chemotechnique Diagnostics® (Vellinge, Sweden). The test site was intact upper back skin. Allergens were applied on skin, while their occlusion was aided by specific chambers and hypoallergenic adhesive test tape: Curatest® from Lohmann & Rauscher, Neuwied, Germany. They were removed and read at D2, D3, D4 and D7. According to International Contact Dermatitis Research Group (ICDRG) reactions of intensity + and above were regarded as positive.

Statistical analysis

In the statistical data processing the calculation of the percentage structure, arithmetic mean, and standard deviation were used. During the further analysis, the χ^2 test was done to compare means proportion; *t*-test for independent samples to differ contact sensitization between studied groups; Population Adjusted Frequency of Sensitization (PAFS) standardization, in order to overcome differences in frequency of contact sensitization relative to gender and age of subjects; the Pearson`s r and Spearman's ρ correlation coefficient for assessment of the association between disease duration and contact sensitization (IBM SPSS Statistics 20.0).

Table 1

Standard European battery of contact allergens (Chemotechnique Diagnostics® Vellinge, Sweden, 2013)

1.	Potassium dichromate petrolatum 0.5%
2.	Neomycin Sulphate petrolatum 20.0%
3.	Thiuram Mix petrolatum 1.0%
4.	Fragrance Mix II petrolatum 14.0%
5.	Cobalt chloride petrolatum 1.0%
6.	Paraphenylenediamine free base petrolatum 1.0%
7.	Benzocaine petrolatum 5.0%
8.	Formaldehyde aqua 1.0%
9.	Colophony petrolatum 20.0%
10.	Clioquinol petrolatum 5.0%
11.	Balsam of Peru petrolatum 25.0%
12.	N-Isopropil-N-phenyl paraphenylenediamine petrolatum 0.1%
13.	Wool alcohols petrolatum 30.0%
14.	Epoxy resin petrolatum 0.1%
15.	Mercapto Mix petrolatum 1.0%
16.	Budesonid petrolatum 0.1%
17.	Paraben Mix petrolatum 16.0%
18.	Paratertiarybutyl phenol formaldehyde resin petrolatum 1.0%
19.	Fragrance Mix petrolatum 8.0%
20.	Quaternium-15 petrolatum 1.0%
21.	Nickel Sulphate, $6H_2O$ petrolatum 5.0%
22.	5-Chloro-2-methyl-4-isothiazolin-3-one + 2-Methyl-4-isothiazolin-3-one (3 : 1 in Water) aqua 0.01%
23.	Mercaptobenzothiazole petrolatum 2.0%
24.	Sesquiterpene lactone Mix petrolatum 0.1%
25.	Tixocortol pivalate petrolatum 1.0%
26.	Dibromodicyanobutane petrolatum 0.3%
27.	Hydroxy-methyl-pentylcyclohexene- carboxaldehyde (HMPCC or HICC) (Lyral®) petrolatum 5.0%
28.	Primin petrolatum 0.01%

Table 2

Specific battery of contact allergens for chronic venous insufficiency

1.	Amerchol petrolatum 50.0%
2.	Fusidic acid sodium salt petrolatum 2.0%
3.	Chlorhexidine digluconate aqua 0.5%
4.	Benzalkonium chloride petrolatum 0.1%
5.	Bacitracin petrolatum 20.0%
6.	Cetyl/stearil alcohol petrolatum 20.0%
7.	Butyl hydroxytoluene (BHT) petrolatum 2.0%
8.	Chloramphenicol petrolatum 5.0%
9.	Benzoyl peroxide petrolatum 1.0%
10.	Propyleneglycol petrolatum 5.0%
11.	Propolis petrolatum 10.0%
12.	Thiomersal petrolatum 0.1%
13.	Sorbic acid petrolatum 2.0%
14.	Chlorocresol (PCMC) petrolatum 1.0%
15.	Trolamine petrolatum 2.5%
16.	Sorbitan sesquioleate petrolatum 20.0%
17.	Tixocortol pivalate petrolatum 1.0%
18.	Phenylmercuric acetate petrolatum 0.01%
19.	Chloracetamide petrolatum 0.2%
20.	Diazolidinyl urea petrolatum 2.0%
21.	Imidazolidinyl urea petrolatum 2.0%
22.	Gentamycin sulphate petrolatum 20.0%
23.	Sulphanilamide petrolatum 5.0%

Results

Positive reactions during the patch test were revealed in 60.7% of patients with CVI and in 50% of patients belonging to the control group. There was no statistically significant difference between rates ($\chi^2 = 0.063$; df = 1; $p = 0.731$). PAFS standardization was done in order to overcome significant difference between groups with regard to gender and age of the subjects. Sensitivity rate to at least one allergen in the examined series in the experimental group was 49.3%, while it was 40.5% in the control group. The difference between the groups did not reach significant level ($z = 0.6870 < 1.96$; $p > 0.05$). The prevalence of sensitization to allergens from the European Standard Series of Allergens

was estimated and amounted 31.55% in the experimental group and 32.07% in the control group with no statistically significant difference ($z = 0.9280 < 1.96$; $p > 0.05$). The frequency of sensitization to allergens of locally modified standard series for leg ulcers was 28.45% in the experimental group, while it counted 21.62% in the control group with no statistically significant difference ($z = -0.82 < 1.96$; $p > 0.05$) as well as with regard to gender.

The most common contact sensitizers of the European Standard Series of Allergens in the CVI group and control group are presented in Table 3, while those from locally modifed standard series for leg ulcers are shown in Table 4.

The monosensitization rate to one of the examined allergens was 49.3% in the CVI group and 40.5% in the control group. Sensitivity to more than two allergens was determined in 25.3% subjects in the CVI group and in 10.3% of patients with contact dermatitis (the control group). Sensitivity to more than five allergens accounted 9.6% in the CVI group and 2.6% in the control group (Table 5).

The difference in sensitivity rates to at least one allergen was not statistically significant ($\chi^2 = 1.71$; $p > 0.05$); for two and more allergens (≥ 2) it reached statistical significance ($\chi^2 = 8.671$; $p < 0.05$), as well the difference between positive sensitivity rates to five and more allergens (≥ 5) p ($\chi^2 = 3.914$; $p < 0.05$).

Besides the *t*-test for independent samples related to distribution of reactivity in examined groups as well as single values of χ^2 test used to present statistically significant difference, the relative risk (RR) [odds ratio (OR), 95% confidence interval (CI)] for developing contact dermatitis in patients in the CVI group versus patients in the control group was determined. It was not estimated higher risk for contact sensitization to at least one allergen (RR 1.217; 95% CI 0.921-1.609; $p > 0.05$). However, subjects in the CVI group had 2.5 times higher risk for manifesting contact sensitization to at least two allergens (RR 2.456, 95% CI 1.664-3.627; $p < 0.05$); and 3.5 times higher risk for polysensitization (RR 3.692, 95% CI 1.961- 6.951; $p < 0.05$).

Average disease duration was 18.72 years, ranging from three months to 60 years. The correlation between disease duration and contact sensitization is shown in Table 6. The weak, positive correlation between CVI duration and contact sensitization was estimated ($\rho = 0.165$), but accomplished statistical significance ($p = 0.044$).

Table 3

Standardized sensitivity rates to allergens from standard battery in the experimental (CVI) group

	Allergen	USR	SRA	SRF	SRM	SR
1	Potassium dichromate	0.67	0.35	0.51	0.00	0.30
2	Neomycin sulphat	5.33	3.91	3.36	4.37	3.76
3	Thiuram mix	3.33	1.44	1.31	1.59	1.43
4	Fragrance mix II	4.00	2.38	2.00	1.87	1.95
5	Cobalt chloride	1.33	1.25	1.87	0.00	1.12
6	Paraphenylenediamine free base	1.33	0.59	0.00	1.59	0.64
7	Benzocaine	0.00	0.00	0.00	0.00	0.00
8	Formaldehyde	3.33	1.44	1.80	1.11	1.52
9	Colophony	7.33	3.64	4.70	2.56	3.84
10	Clioquinol	2.00	0.83	0.00	2.08	0.83
11	Balsam of Peru	9.33	4.29	2.45	6.90	4.23
12	N-isopropyl-N-phenyl paraphenylenediamine	1.33	5.81	5.88	0.00	3.53
13	Wool alcohols (lanolin)	11.33	11.08	13.25	3.92	9.51
14	Epoxy resin	0.67	0.35	0.00	1.11	0.44
15	Mercapto mix	0.67	0.26	0.33	0.00	0.20
16	Budesonid	6,67	3.89	3.54	5.18	4.20
17	Paraben mix	3.33	1.94	0.83	2.36	1.44
18	Paratertiarybutyl phenol formaldehyde resin	0.67	0.35	0.00	1.11	0.44
19	Fragrance mix	12.67	12.05	11.47	7.86	10.02
20	Quaternium-15	0.00	0.00	0.00	0.00	0.00
21	Nickel sulphate, $6H_2O$	6.00	4.72	6.31	0.48	3.98
22	5-chloro-2-methyl-4-isothiazolin-3-one+2-methyl-4-isothiazolin-3-one (3:1 in water)	4.67	7.93	6.71	5.42	6.20
23	Mercaptobenzothiazole	0.67	0.24	0.00	0.48	0.19
24	Sesquiterpene lactone mix	2.67	1.77	0.65	4.19	2.07
25	Tixocortol pivalate	2.00	0.95	0.83	1.11	0.94
26	Dibromodicyanobutane	4.67	7.07	6.21	3.44	5.10
27	Hydroxymethylpentylcyclohexenecarboxaldehyde (Lyral)	2.00	1.51	0.33	4.19	1.87
28	Primin	2.67	6.91	5.88	1.87	4.28

CVI – chronic venous insufficiency; USR– unstandardized rates; SR – standardized rate; SRA – SR to age; SRF – SR for females; SRM – SR for males.

Table 4

Standardized sensitivity rates to allergens from specific battery for chronic venous insufficiency (CVI) in the experimental (CVI) group

	Allergen	USR	SRA	SRF	SRM	SR
1	Amerchol®	13.33	9.34	11.37	7.11	9.66
2	Fusidic acis sodium salt	9.33	4.50	4.64	3.94	4.36
3	Chlorhexidine digluconate	1.33	5.80	5.56	048	3.53
4	Benzalkonium chloride	0.00	0.00	0.00	0.00	0.00
5	Bacitracin	4.00	7.50	8.59	1.59	5.79
6	Cetyl/stearil alcohol	5.33	2.39	1.66	3.19	2.27
7	Butyl hydroxytoluene (BHT)	3.33	2.10	0.83	4.67	2.37
8	Chloramphenicol	2.00	0.76	0.00	2.95	1.18
9	Benzoyl peroxide	2.00	6.14	6.04	1.11	4.07
10	Propyleneglycol	0.67	0.24	0.00	0.48	0.19
11	Propolis	14.00	11.89	9.17	11.80	10.22
12	Thiomersal	1.33	0.59	0.00	1.59	0.64
13	Sorbic acid	3.33	2.12	1.14	4.81	2.61
14	Chlorocresol (PCMC)	0.00	0.00	0.00	0.00	0.00
15	Trolamine	4.00	2.27	1.98	1.87	1.93
16	Sorbitan sesquioleate	7.33	6.49	7.20	4.91	6.28
17	Tixocortol pivalate	0.00	0.00	0.00	0.00	0.00
18	Phenylmercuric acetate	0.00	0.00	0.00	0.00	0.00
19	Chloracetamide	0.00	0.00	0.00	0.00	0.00
20	Diazolidinyl urea	1.33	0.50	0.33	0.48	0.39
21	Imidazolidinyl urea	2.00	0.95	0.83	1.11	0.94
22	Gentamycin sulphate	3.33	2.15	3.05	2.22	2.72
23	Sulphanilamide	1.33	0.69	0.00	2.22	9.66

USR – unstandardized rates; SR – standardized rate; SRA – SR to age; SRF – SR for females; SRM - SR for males.

Table 5

Distribution of standardized sensitization rates to one, two and more allergens among studied groups

Studied groups	One positive reaction (%)		Two and more positive reactions (%)		More than five positive reactions (%)	
	yes	no	yes	no	yes	no
CVI (n = 150)	49.30	50.70	25.30	74.70	9.60	90.40
CD (n =116)	40.50	59.50	10.30	89.70	2.60	97.40

CVI group – patients with chronic venous insufficiency (CVI) suspected to have contact dermatitis (CD); CD group – subjects without CVI suspected to have CD.

Table 6

Correlation between disease duration and contact sensitization (Spearman's coefficient of correlation - ρ)

Parameters	ρ (p-value)
Contact sensitization (n = 150)	0.165 (0.044)
Contact sensitization (normalized) (n = 150)	0.165 (0.044)

Discussion

Considering age of all subjects (from 17 years to 86 year), the average age was more than 50 years, precisely 54.89 years [standard deviation (SD) 14.53], which is in accordance with data from previous population and clinical research [8, 9]. The average age of patients suffering from chronic venous disease was 64.25 years (SD 12.06) as it has been reported in other studies (63.1 to 74.2 years) [5, 6, 10–16]. In literature data review, average age of patients with contact dermatitis is between 40.3 and 51 years as it was in our control group with age of 45.55 years in mean [16].

The prevalence of contact sensitization was assessed at 60.67% before PAFS standardization. According to the literature, frequency of contact sensitization among patients with CVI is from 46% to 80%, although most authors studied only patients suffering from venous ulcers as severe form of the disease. The lowest prevalence of contact sensitization of 46% has been reported in Canadian study among patients with venous ulcers [17] due to misreading of allergy test, while Jindal et al. [18] estimated it at 50%. Having done PAFS standardization, the prevalence of contact sensitivity in our research was 49.3% in the CVI group and 40.5% in CD group. We could not find data in available literature about standardized contact sensitization prevalence within patients suffering from CVI. German authors reported prevalence rate of 53.8% [19]. In the CVI group, most of subjects who manifested positive reaction were 70–79 years old, with

contact sensitivity rate of 50% which is in compliance with results reported in England, America, Poland and Croatia − 51%, 52%, 56% and 48%, respectively [5, 7, 11, 15].

Having analyzed unstandardized and standardized sensitization rates (regarding to gender and age) in the CVI group, differences were established. Significantly higher standardized sensitization rates were determined for N'N'' isopropyl-phenyl paraphenylenediamine (IPPD), dibromodicyanobutane, while standardized rates for fragrance mix, wool alcohols, colophony, balsam of Peru, nickel sulphate were significantly lower. Sensitization rates for cobalt chloride and hydroxy-methyl-pentylcyclohexene-carboxaldehyde (HMPCC or HICC) (Lyral®) were evidently not affected by either gender or age as they remained stable after standardization.

Following standardization, among the most common sensitizers were still Fragrance mix and wool alcohols with incidence of 10.2% and 9.51%, respectively, then, dibromodicyanobutane, methyl-chloro-isothiazolin, dibromodicyanobutane and primin.

The results could not be compared with otherstudies as there was no standardized relative incidence to gender and age in available literature data.

Apart from differences in sensitization rates (unstandardized and standardized), distinctions among some standardized sensitization rates to gender were observed. Females compared to males mostly reacted to wool alcohols (13.25% *vs* 3.92%, respectively), Fragrance mix (11.47% *vs* 7.86%, respectively), nickel sulphate (6.31% *vs* 0.48%, respectively), methyl-chloro-isothiazolin (6.71% *vs* 5.42%, respectively) and dibromodicyanobutane (6.21% *vs* 3.44%, respectively). These allergens are usual constituents in skin care products as well as in cosmetics, while nickel sulphate is found in alloys for bijouterie making, decorative hairpins, buckles, and other metal products widespread in everyday use. Because of that, it was expected that women would show a higher frequency of sensitization to previously mentioned allergens. Men in our study presented most frequent reactions to Fragrance mix (7.76%), balsam of Peru (6.90%), methyl-chloro-isothiazolin (5.42%), budesonide (5.18%), neomycin sulphate (4.37%), which are compounds in skin care products as well as in some local terapeutics. An intriguing and surprising paradox is that female subjects, although at low incidence, responded to potassium dichromate and cobalt chloride. Standardized relative incidences of those allergens were 0%. It is well known that sensitivity to metals such as potassium dichromate, nickel sulphate and cobalt chloride is increasingly common day by day [18, 20]. Hypersensitivity to potassium dichromate and cobalt chloride is usually related to professional exposure of men in engineering and construction, but there is also non-professional exposure in everyday life. The sensitization rate for those allergens in our research patently indicates sparing exposure due to disease [19]. The results were not compared to data reported in other research because of no information about standardized relative incidence to gender among patients suffering from CVI.

Analyzing unstandardized and standardized sensitization rates to allergens specific for CVI in the experimental group (CVI), standardized rates for sulfanilamide (9.66%), bacitracin (5.79%), benzoyl peroxide (4.07%), chlorhexidine digluconate (3.53%) were significantly higher, while standardized sensitization rates for propolis (10.22%), Amerchol® (9.66%), sorbitan sesquioleate (6.28%), fusidic acid (4.36%) were lower than in the control group..

Despite the increase and decrease in contact sensitization frequency caused by standardization of relative incidence, order of the most common sensitizers remained unchanged including propolis (10.22%), Amerchol® (9.66%), sorbitan sesquioleate (6.28%), bacitracin (5.79%) and fusidic acid (4.36%).

Processing standardized sensitivity rates, it was noticed that women were more likely to have an eczematous reaction than men to Amerchol® l (11.37% vs 7.11%, respectively), bacitracin (8.59% vs. 1.59%, respectively), sorbitan sesquioleate (7.20% vs. 4.91%, respectively), benzoyl peroxide (6.04% vs. 1.11%, respectively). Contrasting, men reacted to propolis in more cases (11.8% vs. 9.17%). All the mentioned allergens are contained in various pharmaceuticals for skin care or in topical medicaments used in CVI treatment as emulators and emolliens, or as an active agent (antibiotics). Contact sensitization to these allergens indicates to therapeutic habits of treating CVI in our population. The results have not been collated with as there were no comparable reports in available literature data.

According to standardization, the most common sensitizers were: wool alcohols (9.92%), paraphenylendiamine (7.34%), colophony (6.90%), nickel sulphate (5.93%) and potassium dichromate (4.23%).

Having processed data, the dissimilarity in standardized sensitization frequency among individual allergens were detected. Female subjects far more reacted to paraphenylendiamine, nickel sulphate, potassium dichromate, balsam of Peru, while male subjects showed more prevalent reaction to colophony and wool alcohols [21, 22].

Following standardization in regard to gender and age, various results about frequency of contact sensitization can be found in literature. Top ten allergens in research of Israeli, Turkey, Czech, Chinese, European and USA authors [23–26] are equal with ours. Freireich-Astman et al. [27] established that women more often reacted to nickel sulphate, paraphenylendiamine, potassium dichromate, balsam of Peru than men, which match our report except that our rates were quite lower. The separation of men in terms of frequency of contact hypersensitivity to colophony, wool alcohols and Fragrance mix taking into account occupational exposure to other allergens. In their research, Israeli authors [27] registered higher frequency of contact hypersensitivity to these allergens in men, while a statistically significant difference was evident only for wool alcohols. In contrast, Brasch et al. [26] in their study in Germany, had completely opposite results. The men in that research reacted to these three allergens more rarely than women but with no statistically significant difference.

Distinctions between unstandardized and standardized rates of sensitization were determined. The lower standardized rates were for Amerchol®, benzoyl peroxide, thiomersal, trolamine, sorbitan sesquioleate, imidazolidinyl urea, while the higher rates related to propolis, sorbic acid, chloracetamide, diazolidinyl urea and sulphanilamid. The sensitization rates of fusidic acid, chlorhexidine digluconate, cetyl/stearil alcohol, butyl hydroxytoluene, chloramphenicol stayed consistent after standardization process due to not been affected by gender or age.

The most common sensitizers were propolis (7.96%), sulphanilamid (5.00%), benzoil peroxyde (4.18%), diazolidinyl uea (3.25%) and Amerchol® (2.70%).

Only female subjects had positive reaction to fusidic acid, cetyl/stearil alcoho, butyl hydroxytoluene, sorbic acid, sorbitan sesquioleate, chloracetamide and imidazolidinyl urea. Reactivity to chlorhexidine digluconate, trolamine and sulphanilamid were registered only in men. The most specific difference according to gender was assessed for propolis and sulphanilamid for the benefit of men.

The battery of allergens used in other research are quite distinguish [28]. The series of allergens used by Austrian authors [28] and the one used in our study have two mutual allergens, propolis and propyleneglycol. There was no difference, in regard to gender, within male cohorts in Austrian report, while our male subjects were much more sensitized to propolis than women, accompanied by no presence of sensitization to propyleneglycol among men [23].

Conclusion

Contact sensitization prevalence in patients with CVI was not statistically significantly distinct from rates in subjects that have not presented CVI. Patients suffering from CVI had 2.45 times higher risk to manifest contact sensitization to two and more allergens, and 3.69 times higher risk for contact sensitization to five and more allergens than patients with no CVI. Furthermore, we established the positive correlation between frequency of contact sensitization and disease duration.

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Predictive value of the specific radiographic signs at panoramic radiography indicating possible close relationship of posterior teeth and surrounding anatomical structures: A CBCT study

Prediktivna vrednost radiografskih znakova ortopantomografije koji ukazuju na mogući bliski odnos bočnih zuba sa okolnim anatomskim strukturama: CBCT studija

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Abstract

Background/Aim. In planning the extraction of posterior teeth, it is necessary to determine their position and correlation with the surrounding anatomical structures. The aim of this study was to perform Cone Beam Computed Tomography (CBCT) analysis in order to evaluate the predictive value of specific orthopantomography (OPT) radiographic signs in determining the proximity of posterior upper teeth to the maxillary sinus and lower teeth to the mandibular canal. **Methods.** In a prospective study, 460 cases out of 423 patients were analyzed. Seven OPT radiographic signs were assessed to determine the correlation of the third molars to the mandibular canal, while five radiographic signs were followed to determine the correlation of the maxillary sinus floor to the upper posterior teeth. For each OPT radiographic sign, a precise analysis of the axial slices of the CBCT was performed. **Results.** Interruption of the white line is a radiographic sign showing statistically significant interruption of the integrity of the mandibular canal on the CBCT axial slices (p = 0.002). Also, diversion of the inferior alveolar canal at the axial sections showed statistically significant interruption of the continuity of the mandibular canal (p = 0.003). A radiographic sign, Darkening of the root, showed superimposition of the anatomical structures, a close relationship with the tooth but often with preserved mandibular canal integrity (p < 0.001). Absence of *lamina dura*, interruption of the maxillary sinus floor's cortex, and projection of the root apices in the sinus cavity were radiographic signs that were statistically the most commonly associated with interruption of maxillary sinus integrity on CBCT axial slices (p < 0.001). **Conclusion.** The results of our study indicate that the presence of certain radiographic signs on the OPT may have predictive significance. The exact relationship between anatomical structures and posterior teeth cannot be accurately estimated on OPT. The precise position of the molars and correlation to the mandibular canal and the maxillary sinus can be accurately determined by CBCT imaging.

Key words:
radiography, panoramic; cone beam computed tomography; mandible; maxillary sinus.

Apstrakt

Uvod/Cilj. U planiranju ekstrakcije bočnih zuba neophodno je odrediti njihov tačan položaj i odnos sa okolnim anatomskim strukturama. Ciljevi istraživanja bili su izvršiti procenu prediktivne vrednosti radiografskih znakova ortopantomografije i utvrditi značaj *Cone Beam Computed Tomography* (CBCT) u određivanju odnosa bočnih zuba sa madibularnim kanalom u donjoj vilici i odnosa bočnih zuba sa maksilarnim sinusom u gornjoj vilici. **Metode.** U prospektivnoj studiji analizirano je 460 slučajeva od 423 pacijenta. Na ortopantomografskim snimcima praćeno je sedam radiografskih znakova za određivanje odnosa bočnih zuba sa mandibularnim kanalom dok je pet radiografskih znakova praćeno za određivanje odnosa poda maksilarnog sinusa sa korenovima bočnih zuba. Za svaki radiografski znak vršena je precizna CBCT analiza na aksijalnim presecima. **Rezultati.** Prekid bele linije je radiografski znak koji je na aksijalnim presecima statistički značajno pokazivao prekide kontinuiteta madnibularnog kanala (p = 0.002). Skretanje kanala na aksijal

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nim presecima pokazao statistički značajno čest prekid kontinuiteta mandibulanog kanala ($p = 0.003$). Radiografski znak zatamnjenja korenova pokazao je superponiranje anatomskih struktura, blizak odnos sa bočnim zubom ali često bez prekida kontinuiteta ($p < 0.001$). Odsustvo lamine dure, prekid kontinuiteta poda maksilarnog sinusa i projekcija vrha korena u lumen sinusa su radiografski znaci koji su statistički najčešće bili povezani sa prekidom kontinuiteta poda maksilarnog sinusa na aksijalnim presecima ($p < 0.001$). **Zaključak.** Rezultati naše studije pokazali su da prisustvo određenih radiografskih znakova na ortopantomografskim snimcima može imati prediktivni značaj. Takođe, na ortopantomografskim snimcima se ne može sa sigurnošću proceniti tačan odnos anatomskih struktura i bočnih zuba. Precizan položaj bočnih zuba u odnosu na mandibularni kanal i maksilarni sinus moguće je izvršiti na trodimenzionalnim snimcima CBCT.

Ključne reči:
ortopantomografija; tomografija, kompjuterizovana, konusna; mandibula; maksilarni sinus.

Introduction

In planning posterior teeth extraction, it is necessary to determine their exact position and relationship with the surrounding anatomical structures to prevent intraoperative and postoperative complications [1, 2].

During preoperative preparation and planning of the extraction of the lower molars, it is essential to determine the exact position of the tooth and its relationship with the mandibular canal to avoid damaging the neurovascular contents of the mandibular canal [3, 4]. The particular importance for the upper posterior teeth extraction is the position of the tooth root apices and their relation to the floor of the maxillary sinus [5, 6].

The most commonly used radiographic method for analyzing the posterior teeth position and the relationship with mentioned anatomical structures is orthopantomography (OPT) [7, 8]. On OPT images, referring the relationship of the posterior teeth to the surrounding anatomical structures for diagnosis, treatment plan, and preoperative preparation were analyzed [9, 10].

Rood and Shehab [11] recommended altogether seven radiographic signs with the aim of determining the correlation of the posterior tooth and the mandibular canal on the OPT. Four of them occur on the tooth (darkening of the root, deflected roots, narrowing of the root, dark and bifid root) and the other three on the canal (interruption of the white line(s), diversion of the inferior alveolar canal, narrowing of the inferior alveolar canal).

According to Lopes et al. [12], for evaluating the relationship between the upper molars and the maxillary sinus floor, the presence or absence of the following radiographic signs should be followed: projection of the root apices in the sinus cavity, interruption of the maxillary sinus floor's cortex, *lamina dura*, darkening of the root apical region, upward curving of the sinus floor enveloping the tooth root partially or completely.

Cone Beam Computed Tomography (CBCT) has been widely used in dentistry in recent years, more than the conventional computed tomography (CT), due to lower radiation dose [13, 14]. However, higher radiation dose compared to OPT, additional costs, and lesser availability were responsible that CBCT has not been introduced as a routine additional diagnostic method [15, 16].

The aim of this study was to perform CBCT analysis in order to evaluate the predictive value of the specific OPT radiographic signs used in determining the proximity of posterior upper teeth to the maxillary sinus and lower teeth to the mandibular canal.

Methods

This prospective study was conducted at the Department of Dentistry, Faculty of Medical Sciences, University of Priština/Kosovska Mitrovica, on a sample of 423 patients (460 cases analyzed). A radiographic sign on OPT was identified as an inclusion criterion for the study. The patients identified with the radiographic sign on OPT were referred for CBCT imaging. The exclusion criteria for the study were relative or absolute contraindication for radiographic imaging, unclear images, and the absence of mandibular canal on OPT or CBCT (plexiform canal). Written informed consent was obtained from all participants for their data to be used in the study.

All of the panoramic radiographs were taken with Sirona Orthophos XG3D (Dentsply Sirona, USA), while the Sidexis XG 2.61 software, recommended by Sirona Dental System GmbH, was used to analyze the images. The Cone Beam CT scanner was Cranex 3Dx (Sorodex, KaVo). The field of view was 50 × 50 mm, 90 kVp, 6.1-sec exposure length with a radiation dose of 320.8 mGy/cm^2. The software used to analyze the three-dimensional radiographic images was the OnDemand3D CD viewer.

Having identified the radiographic sign on OPT, the marking and the mapping of the mandibular canal were performed using the CBCT software (Figures 1 and 2). Analysis was performed on the CBCT axial sections (Figure 2). Depending on the findings, the case was classified into one out of seven groups (twelve subtypes) based on the classes formed according to Maglione et al. [17] CBCT classification (Table 1).

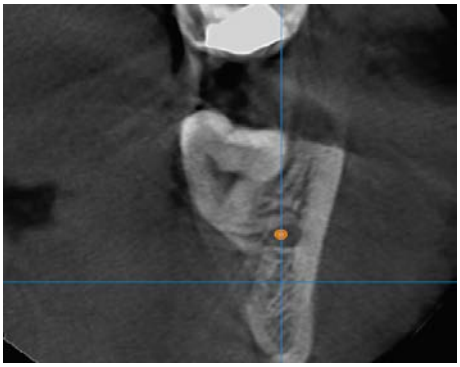

Fig. 1 – The marking and the mapping of mandibular canal.

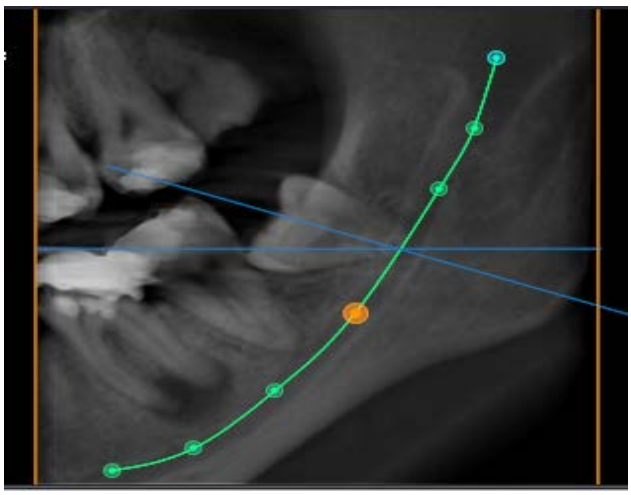

Fig. 2 – The axial cross-section analysis of the Cone Beam Computed Tomography (CBCT) image shows preserved diameter of the mandibular canal (class 2, subtype 2b).

To evaluate the relationship between the maxillary molars and maxillary sinus floor, radiographic signs, according to the criteria established by Lopes et al. [12], were used.

Having identified the radiographic sign using OPT, a detailed analysis was performed on CBCT slices. Depending on the relationship of upper posterior teeth and maxillary sinus floor on CBCT, cases were classified into one out of four groups based on the classes according to the recommendations of Shahbazian et al. [6]. CBCT classifications were used for the precise definition of the three-dimensional topographic relationship between anatomic structures (Table 2).

Statistical data analysis

The proportions of radiographic signs between the classes were analyzed using the χ^2-test. All p values less than 0.05 were considered significant. The Statistical Package for the Social Science Program (version 22, SPSS Inc., Chicago, IL, USA) was used in the statistical analysis.

Table 1
Cone Beam Computed Tomography (CBCT) radiological classification for determining the relationship of mandibular canal and the lower posterior teeth by Maglione et al. [17]

Classes	Subtypes
Class 0: the mandibular canal is not visible on the images (plexiform canal).	/
Class 1: the mandibular canal runs apically or buccally with respect to the tooth but without touching it (the cortical limitations of the canal are not interrupted).	1A: the distance IAN/tooth is greater than 2 mm. 1B: the distance IAN/tooth is less than 2 mm
Class 2: the mandibular canal runs lingually with respect to the tooth but without touching it (the cortical limitations of the canal are not interrupted).	2A: the distance IAN/tooth is greater than 2 mm. 2B: the distance IAN/tooth is less than 2 mm.
Class 3: the mandibular canal runs apical or buccal touching the tooth.	3A: in the point of contact, the mandibular canal shows a preserved diameter. 3B: in the point of contact, the mandibular canal shows a smaller calibre and/or an interruption of the corticalization.
Class 4: the mandibular canal runs lingually touching the tooth.	4A: in the point of contact, the mandibular canal shows a preserved diameter. 4B: in the point of contact, the mandibular canal shows a small calibre and/or an interruption of the corticalization.
Class 5: the mandibular canal runs between the roots but without touching them.	5A: the distance IAN/tooth is greater than 2 mm. 5B: the distance IAN/tooth is less than 2 mm.
Class 6: the mandibular canal runs between the roots touching them.	6A: in the point of contact, the mandibular canal shows a preserved diameter. 6B: in the point of contact, the mandibular canal shows a small calibre and/or an interruption of the corticalization.
Class 7: the mandibular canal runs between fused roots	/

IAN – inferior alveolar nerve.

Table 2
Cone Beam Computed Tomography (CBCT) radiological classification for determining the relationship of the maxillary sinus floor and upper posterior teeth

Class 1:	when there was a distinct space between the root tip and the sinus floor
Class 2:	when the roots were in close contact with the floor of the maxillary sinus (< 0.5 mm away)
Class 3:	when the roots were projected onto the sinus but were actually lateral or medial to it
Class 4:	when the roots were protruded into the maxillary sinus cavity.

Results

The total of 460 cases out of 423 patients with an identified OPT radiographic sign were analyzed. The proximity of posterior teeth to the maxillary sinus was analyzed on 234 (50.87%) images and 226 (49.13%) radiographic images implied an analysis of the inferior alveolar nerve canal position in relation to mandibular molars.

Results of OPT and CBCT analysis of the relationship between the mandibular canal and the molar teeth

Taking into account the total number of analyzed images, 13.3% or 30 cases belong to the first and second class with subclasses (1a, 1b, 2a, 2b). The statistical analysis revealed that radiographic sign 'Darkening of the root' was statistically significantly more frequent ($p < 0.001$) in cases without direct contact of anatomic structures (Classes 1a, 1b, 2a, 2b) comparing to the other classes (Figure 3).

The finding of radiographic sign 'Narrowing of the inferior alveolar canal' was confirmed in 18.5% of cases in the Class 3a according to the CBCT. Narrowing of the inferior alveolar canal was statistically significantly more frequent in the Class 3a comparing the other classes ($p = 0.050$).

The radiographic sign 'Interruption of the white line' was confirmed in 38% of patients having a CBCT finding corresponding to the Class 3b. The radiographic sign 'Interruption' of the white line was statistically significantly more frequent in the Class 3b than in the other classes ($p = 0.002$).

Diversion of the inferior alveolar canal was confirmed in 23.5% of subjects belonging to Class 3b. The frequency of this finding was statistically significantly different in the Class 3b compared to the other classes ($p = 0.003$).

Radiographic sign 'Darkening of the root' was confirmed in 52.4% of cases in the Class 4a. There was statistically significantly more frequency in the Class 4a compared to the presence in other classes ($p = 0.002$) (Figure 4).

In determining the bucco-mesial localization of the mandibular canal, the frequency of lingual localization of the mandibular canal was observed in 59 cases, while the occurrence of buccal-localization appeared in 50 cases. In 117 cases, the apical localization of the mandibular canal was observed.

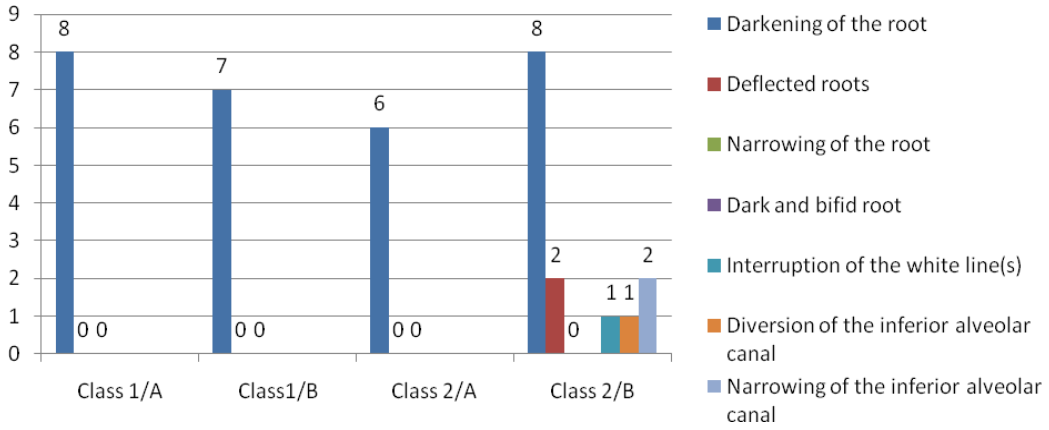

Fig. 3 – Frequency of radiographic signs in the first and second class (without the direct contact of anatomical structures).

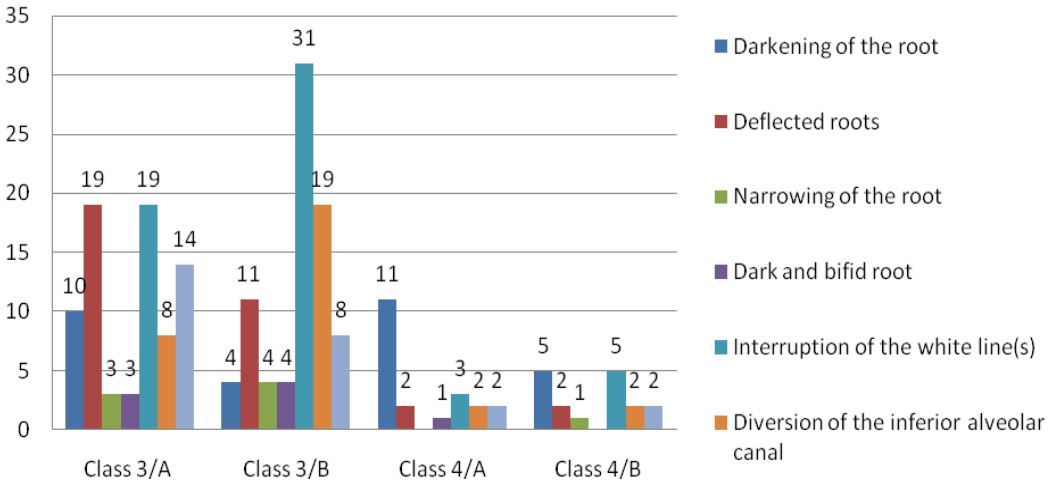

Fig. 4 – Frequency of radiographic signs in the third and fourth class (with the direct contact of anatomical structures).

The OPT and CBCT results analysis of the relationship between the roots of maxillary teeth and the maxillary sinus

The ʻDarkening of the rootʼ apical region was confirmed in 71% of subjects in the group with Type 1 finding on the CBCT. It was statistically significantly more frequent in the group with Type 1 findings on the CBCT compared to other findings on the CBCT ($p = 0.007$).

Upward curvature of the sinus floor enveloping the tooth root partially or completely was confirmed in 39% of subjects in the group with Type 2 finding on the CBCT and was statistically significantly more frequent in this group compared to other types ($p < 0.001$).

Interruption of the maxillary sinus floor's cortex was present in 15% of patients in the group with Type 4 findings on the CBCT. Interruption of the maxillary sinus floor's cortex was statistically significantly more frequent in the group with Type 4 findings on the CBCT compared to other types ($p < 0.001$).

The presence of radiographic findings ʻAbsence of *lamina dura*ʼ was also diagnosed in 15% of patients in the group with Type 4 findings on the CBCT and was significantly more common than other types ($p < 0.001$).

Projection of root apices in the sinus cavity was confirmed in 66 (55%) cases in the group with Class 4 findings on the CBCT. Projection of the root apices in the sinus cavity was statistically significantly more frequent in the group with Class 4 findings on the CBCT compared to other types (Table 3).

and ʻInterruption of the white lineʼ are associated with an increased risk of postoperative neurosensory complications. Our study found the frequent interruption of the mandibular canal diameter by monitoring CBCT axial sections for the OPT radiographic sign diversion of the inferior alveolar canal. Likewise, the OPT radiographic sign ʻInterruption of the white lineʼ showed statistically significant interruptions of the mandibular canal diameter, confirmed by the CBCT analysis. This may be one of the reasons for more frequent neurosensory postoperative complications when these radiographic signs occur.

In our study, the presence of the radiographic sign ʻDarkening of the rootʼ indicated the superimposition of the evaluated anatomical structures in most cases. The axial section images confirmed high-frequency of the close relationship of the two anatomical structures, but with preserved mandibular canal continuity.

According to the analogous clinical trials, assessing the importance of this radiographic sign, a frequent close relationship and direct contact between anatomical structures can be seen on radiograph images, as well as the appearance of clinical postoperative complications, such as paraesthesia [10, 20].

The American Dental Association (ADA) study [19] recognizes superimposition as a sign leading to neurosensory complications in some cases. Their research has been supported by the findings of other authors indicating the presence of two or more signs on panoramic radiography and the deep horizontally impacted mandibular molar represent factors associated strongly with the proximity of the impacted

Table 3

The frequency of orthopantomography (OPT) radiographic signs in classes based on the classification of Shahbazian et al. [25] after analysis on Cone Beam Computed Tomography (CBCT)

Radiographic signs	The position of the floor of the maxillary sinus at the axial cross-section				Total n (%)
	Class 1 n (%)	Class 2 n (%)	Class 3 n (%)	Class 4 n (%)	
Total	17 (5.3)	83 (26.1)	98 (30.8)	120 (37.7)	318 (100)
Projection of the root apices in the sinus cavity	2 (11.8)	6 (7.2)	14 (14.3)	66 (55.0)*	88 (27.7)
Interruption of the maxillary sinus floor's cortex	0 (0)	2 (2.4)	4 (4.1)	18 (15.0)*	24 (7.5)
Absence of *lamina dura*	0 (0)	3 (3.6)	0 (0)	18 (15.0)*	21 (6.6)
Darkening of the root apical region	12 (70.6)	40 (48.2)	60 (61.2)*	14 (11.7)	126 (39.6)
Upward curving of the sinus floor enveloping the tooth root partially or completely	3 (17.6)	32 (38.6)*	20 (20.4)	4 (3.3)	59 (18.6)

***there is a statistically significant difference in the frequency of radiographic sign compared to other classes ($p < 0.001$).**

Discussion

Several studies, which have dealt with a relationship among different anatomical structures on OPT images, have shown that the presence of certain radiographic indicators indicates a possibility of postoperative complications [18, 19].

The use of radiographic signs during the analysis of two-dimensional OPT images may point out a close relationship between the mandibular canal and impacted third molars, based on the recommendations of Rood and Shehab [11]. The authors claim that radiographic signs referring to diversion of the inferior alveolar canal, ʻDarkening of the rootʼ, tooth to the mandibular canal and an increased risk of complications [20–24]. Our study showed that it is impossible to determine the bucco-oral localization of the mandibular canal on the OPT, while at the axial sections of CBCT it is possible to determine the exact position and precise interrelation of the anatomical structures.

Lopes et al. [12] used similar OPT radiographic signs for evaluating the relationship of the upper posterior teeth with the maxillary sinus. The findings of their research indicate that the presence or absence of some OPT radiographic signs may have a predictive value in expectation of the occurrence of oro-antral communication during the procedure. The re-

sults of our study indicate that the absence of the *lamina dura*, interruption of the maxillary sinus floor's cortex, and projection of the root apices in the sinus cavity represent radiographic signs that are most frequently associated with the interruption of the maxillary sinus floor at axial sections on CBCT. Also, the findings indicate the possibility of the appearance of two or more radiographic signs in the same case. The study showed that OPT radiographic sign 'Interruption of the maxillary sinus floor's cortex', combined with the 'Absence of the *lamina dura*' positively correlated with the cortical interruption of the sinus flor at the axial sections of the CBCT.

According to the CBCT analysis in our study, the 'Interruption of the white line' and the diversion of the inferior alveolar canal at the OPT are radiographic signs pointing out a frequent interruption of the mandibular canal. The radiographic sign 'Darkening of the root' shows the superimposition of the anatomical structures, proximity of the mandibular canal and posterior teeth, but often with preserved diameter. The absence of the *lamina dura*, the interruption of continuity of the maxillary sinus floor, and projection of the apex of the root into the sinus lumen are the radiographic signs most commonly associated with the interruption of cortical bone of the maxillary sinus floor. The OPT radiographic signs may be predictive, but the precise position of the posterior teeth with the mandibular canal or maxillary sinus can only be determined on CBCT radiographs.

Conclusion

The results of our study indicate that the presence of certain radiographic signs on the OPT may have predictive significance. The exact relationship between anatomical structures and posterior teeth cannot be accurately estimated on the OPT since the precise position of the molars and correlation to the mandibular canal and the maxillary sinus can be accurately performed only on the CBCT.

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Similar fatty acid status of plasma lipids in postmenopausal women newly diagnosed with breast cancer and those receiving aromatase inhibitor therapy

Sličan masnokiselinski status kod žena sa novodijagnostikovanim karcinomom dojke i onih koje su na terapiji inhibitorima aromataze

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Abstract

Background/Aim. Dysregulation of fatty acid (FA) metabolism is recognized as a component of malignant transformation in many cancers, including breast cancer (BC), and is often related to disease progression and prognosis. Adjuvant endocrine BC therapy using aromatase inhibitors may also influence FA metabolism. Thus, the aim of our study was to compare plasma total lipids FA status in newly diagnosed postmenopausal patients with BC and in postmenopausal women with BC receiving aromatase inhibitors at least 2 years after completing chemotherapy with healthy women. **Methods.** The study included 17 newly diagnosed postmenopausal BC patients (ND group) and 21 postmenopausal women with BC receiving aromatase inhibitor therapy 2 years after ending chemotherapy (AI group), while a total of 15 apparently healthy women without a family history of BC, comparable in age and body mass index, served as a control group. **Results.** In both patient groups, we found significantly lower levels of vaccenic acid (18:1n-7), alpha-linolenic acid (18:3n-3), gamma-linolenic acid (GLA, 18:3n-6), and docosapentaenoic acid (22:5n-3), and a significantly higher level of dihomo-gamma-linolenic (20:3n-6), when compared with the control group. On the other hand, a significantly lower level of stearic acid (18:0) was observed only in AI patients, while the level of linoleic acid (18:2n-6) was significantly higher in ND women than in the control group. Reduced estimated activities of D6 and D5 desaturases were found in both patient groups than in the control group. **Conclusion.** Our results indicate that FA profiles of plasma lipids of the newly diagnosed, untreated BC patients are very similar to those of cured BC patients who underwent all sessions of chemotherapy and received aromatase inhibitors for at least two years. Additionally, according to our results, their FA profiles markedly differ from those of healthy women. Therefore, supplementation with omega-3 FA and GLA could have beneficial effects in these patients, and further studies should address the potential clinical benefits of the supplementation.

Key words:
aromatase inhibitors; antineoplastic agents; breast neoplasms; fatty acids; postmenopause; women.

Apstrakt

Uvod/Cilj. Promene u metabolizmu masnih kiselina prepoznate su kao komponenta maligne transformacije u mnogim različitim vrstama kancera, uključujući i karcinom dojke (KD), a često su povezane sa progresijom i prognozom bolesti. Adjuvantna endokrina terapija KD, korišćenjem inhibitora aromataze, takođe može uticati na metabolizam masnih kiselina (MK). Zbog toga je cilj ovog rada bio da se uporede MK profili iz ukupnih lipida plazme kod žena u menopauzi, a kojima je dijagnostikovan KD, i onih sa KD koje su na terapiji inhibitorima aromataze najmanje dve godine, sa MK profilima kontrolne grupe. **Metode.** U studiju je bilo uključeno 17 žena sa novodijagnostikovanim KD (ND grupi) i 21 žena sa KD koje su na terapiji inhibitorima aromataze (AI grupi). Kontrolnu grupu je činilo 15 uslovno zdravih žena u menopauzi koje nisu imale porodičnu anamnezu za KD i koje su po godinama i indeksu telesne mase poređene sa bolesnicama. **Rezultati.** Procenat vakcenske (18:1n-7), alfa-linolenske (18:3n-3), gama-linolenske (18:3n-6) i dokozapentaenske (22:5n-3) kiseline bio je značajno niži, dok je procenat dihomo-gama-

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linolenske kiseline (20:3n-6) bio značajno viši, kod obe grupe bolesnica u odnosu na kontrolnu grupu. Sa druge strane, niži nivo stearinske kiseline (18:0) pronašli smo u samo AI grupi, dok je visok nivo linolne kiseline (18:2n-6) utvrđen samo u ND grupi u odnosu na kontrolu. Smanjena procenjena aktivnost D6 i D5 desaturaze nađena je kod obe grupe bolesnica u odnosu na kontrolnu grupu. **Zaključak.** Naši rezultati su pokazali veoma sličan MK profil kod žena sa tek dijagnostikovanim KD i onih koje su na terapiji inhibitorima aromataze najkraće dve godine, ali i da se njihovi MK profili u velikoj meri razlikuju od MK profila kontrolne grupe. Zbog toga, suplementacija n-3 masnim kiselinama i gama linolenskom kiselinom može biti korisna za ove bolesnice, i buduće studije bi mogle ispitivati potencijalnu korist ovih suplemenata.

Ključne reči:
aromataza, inhibitori; antineoplastici; dojka, neoplazme; masne kiseline; postmenopauza; žene.

Introduction

Breast cancer (BC) is one of the most common causes of mortality worldwide and the second leading cause of cancer-related death in women [1]. Numerous factors, such as genetic constitution, immune system function, metabolism, hormone levels, the lack or short duration of breastfeeding, as well as environmental factors, are associated with BC development [2]. Nutritional status, amount and type of dietary fats and obesity, are also linked to the etiology of BC [3, 4].

Almost 70% of all BC are hormone-dependent, and hormones play a key role in the formation and progression of cancer. Some BC produce hormones and/or growth factors, which can act locally to stimulate or inhibit tumor growth. In addition to hormonal status, hormone receptor status is closely related to carcinogenesis. Specifically, the estrogen receptor (ER) plays a critical role in cancerogenesis since estrogen binding to the ER leads to altered expression of genes responsible for cell growth, differentiation, apoptosis, and angiogenesis [5]. In addition, overexpression in human epidermal growth factor receptor-2 (HER-2) leads to enhanced aromatase activity and estrogen production [6]. Accordingly, based on their receptor status, BCs are classified into four subgroups as follows: luminal A ER-positive (ER+) and progesterone receptor (PR) positive (PR+) tumors with luminal HER2 negative (HER2-), luminal B (ER+, PR-, HER2-), HER2 positive (ER-, PR- and HER2+), and basaloid (triple-negative) (ER-, PR-, HER2-) types [7–9].

BC treatment involves surgery, chemotherapy, radiotherapy, and adjuvant endocrine therapies in some types of BC. Aromatase inhibitor, which profoundly decreases plasma and intratumoral estrogen levels, is used as a standard endocrine treatment for early-stage, hormone receptor-positive BC in postmenopausal women [10, 11]. It blocks the final step in estrogen biosynthesis, ie., the bioconversion of androstenedione to estrone, catalyzing by enzyme aromatase [6]. Substantially, a decrease in both circulating plasma estrogens and intratumoral estrogen levels using aromatase inhibitors can lead to a reduction in BC mortality by almost one-third throughout the first 15 years [12].

The rapid growth and expansion of tumor tissue often leads to a poor blood supply of nutrients but also alters *de novo* synthesis of many macromolecules, including lipids and fatty acids (FA) [13]. Several studies have suggested that the malignant phenotype is characterized by alterations in FA metabolism pathways and the expression of enzymes included in FA metabolism. Thus, overexpression of FA synthase (FAS), the rate-limiting enzyme in the FA synthesis pathway, has been reported for a variety of cancers, including prostate, liver, ovary, colon, endometrium, and breast [14]. Furthermore, overexpression of stearoyl CoA-desaturase (SCD), responsible for conversion of saturated FA (SFA) to monounsaturated FA (MUFA), is confirmed in HER2+ BC cells [15] and in mucin-1 overexpressing BC cells [16]. Modified activities of these enzymes lead to altered distributions and concentrations of FA in cell membranes. In addition to tumor cells, abnormal FA profile has been shown in plasma in patients with a new diagnosis of pancreatic, non-small-cell lung and stomach or esophageal cancer [17], bladder cancer [18], non-Hodgkin's lymphoma [19], uterine cervical cancer [20], suggesting that changes in FA metabolism in patients with cancer are systemic [21].

Besides the tumor itself, cytotoxic chemotherapy in BC patients results in increased oxidative stress and selective depletion of long-chain polyunsaturated fatty acids (PUFA) in plasma and membrane of phospholipids [22, 23], and may limit the endogenous production of long-chain PUFA [24]. Long-term endocrine therapy with aromatase inhibitors, as a systemic treatment for many patients with BC, may also influence lipid metabolism and blood level of some lipoproteins [25].

Although some studies reported impaired serum FA profiles in BC women [2], literature data are not consistent. Moreover, it is not clear whether and how plasma FA status changes during BC development, after the surgery and chemotherapy, and especially during aromatase inhibitors treatment, when patients are considered cured. Therefore, the aim of our study was to compare plasma total lipids FA status in newly diagnosed postmenopausal patients with BC and in postmenopausal women receiving aromatase inhibitors at least 2 years after completing chemotherapy with the control group.

Methods

Subjects

Seventeen newly diagnosed postmenopausal BC patients (ND group), together with 21 postmenopausal women with BC receiving aromatase inhibitor therapy 2 years after the end of chemotherapy (AI group), were included in the study. Patients were recruited from the Department of Hematology at the Military Medical Academy

(MMA) in Belgrade, Serbia, during 2018 and 2019. The clinical diagnosis was histologically proven BC. Immunohistochemical measurement of ER expression was performed at the MMA. All newly diagnosed BC patients were included in this study immediately after the cancer diagnosis and before surgery. Inclusion criteria were postmenopausal women with ER+ and HER-2 negative BC. Additional criteria were the absence of chemo- and/or radiotherapy for the ND group and receiving adjuvant hormone therapy with aromatase inhibitors at least 2 years after the end of chemotherapy for the AI group. Excluding criteria for both groups were a metastatic or locally advanced disease of HER-2 positive, previous stroke or heart attack, presence of significant neurological deficit and consciousness disorder, dementia, presence of other malignancies, thyroid disease, and use of statins. The study protocols were approved by the Ethics Committee of the MMA in accordance with the Declaration of Helsinki and principles of Good Clinical Practice. All participants provided written informed consent at the time of their inclusion in the study. None of the study participants received supplements that may influence lipid and FA metabolism in the 3 months prior to entering the study. Fifteen apparently healthy women without a family history of BC, comparable in age and body mass index (BMI) with patients from the ND and AI groups, served as a control group.

Analytical methods

The blood was collected in the morning after 12 h of fasting. Plasma samples were immediately stored at -80 °C until the determination of FA profiles. Total plasma FA were isolated by Glaser's method with some modifications as previously reported by Nikolić Turnić et al. [26]. FA methyl esters were analyzed by gas chromatograph SHIMADZU 2014, which was equipped with capillary column RESTEK Rtx 2330. The temperature program was 140–210°C for 3°/min. Individual FA was identified compared with retention time FA methyl esters commercial standards PUFA-2 (Supelco, Inc., Bellefonte, Pennsylvania, USA). The results are presented as a percentage of total FA [27]. The activities of enzymes involved in long-chain FA syntheses were estimated as we previously described [28].

Statistical analysis

Statistical analysis was performed using the statistical package SPSS 20.0 for Windows. The results are presented as means ± standard deviation (SD). The Shapiro-Wilk's test was used to determine the normality of data distribution. Comparison between groups was assessed with ANOVA test with Tukey's *post hoc* test, for normally distributed variables. Kruskal-Wallis test and Mann-Whitney U test were used for comparisons between non-normally distributed variables. The alpha level for significance was set to $p < 0.05$.

Results

FA composition of total plasma lipids in the ND group, AI group, and healthy control women are presented in Table 1. Our results indicated significant differences between both groups of BC patients and healthy persons. Thus, a

Table 1
Fatty acid composition (%) in total plasma lipids in patients with breast cancer and controls

Fatty acid	ND group	AI group	Control group
16:0	27.87 ± 2.09	28.10 ± 1.86	27.24 ± 1.01
18:0	12.56 ± 0.61	12.42 ± 1.63*	13.58 ± 1.40
SFA	40.43 ± 2.21	40.52 ± 2.01	40.82 ± 1.56
16:1n-7	1.10 ± 0.44	1.40 ± 0.46	1.51 ± 0.52
18:1n-9	12.40 ± 1.31	12.76 ± 2.33	13.19 ± 1.55
18:1n-7	1.31 ± 0.20**	1.39 ± 0.23*	1.60 ± 0.24
MUFA	14.18 ± 1.69	15.55 ± 2.74	16.30 ± 1.58
18:2n-6	27.60 ± 5.51*	25.27 ± 2.94	24.29 ± 2.51
18:3n-6	0.50 ± 0.19**	0.58 ± 0.25**	0.85 ± 0.22
20:3n-6	3.35 ± 1.10**	3.89 ± 0.25***	2.38 ± 0.66
20:4n-6	9.52 ± 3.71	10.34 ± 2.57	11.15 ± 1.87
22:4n-6	0.43 ± 0.42	0.52 ± 0.38	0.46 ± 0.20
n-6 PUFA	41.40 ± 2.83	40.60 ± 3.16	39.13 ± 2.23
18:3n-3	0.21 ± 0.15***	0.18 ± 0.05**	0.44 ± 0.20
20:5n-3	0.41 ± 0.28	0.34 ± 0.26	0.48 ± 0.28
22:5n-3	0.38 ± 0.09**	0.42 ± 0.10**	0.58 ± 0.19
22:6n-3	2.36 ± 0.67	2.38 ± 0.70	2.24 ± 0.82
n-3 PUFA	3.36 ± 0.84	3.33 ± 0.96	3.75 ± 1.12
PUFA	44.76 ± 2.81	43.93 ± 3.52	42.88 ± 1.97
n-6/n-3 ratio	13.31 ± 4.62	13.11 ± 3.71	11.36 ± 3.55

Data are presented as a mean ± standard deviation.
ND – new diagnosed group; AI – aromatase inhibitors group; SFA – saturated fatty acids; MUFA – monounsaturated fatty acids; PUFA – polyunsaturated fatty acids.
***p* < 0.05; ****p* < 0.01; *****p* < 0.001 compared to the control group.**

significantly lower level of stearic acid (18:0) was observed in AI patients than in the control group. Further, among MUFA, only the levels of vaccenic acid (18:1n-7) were significantly lower in both groups of patients than in the control group.

Among n-6 PUFA, we found a significantly higher level of linoleic acid 18:2n-6 (LA, 18:2n-6) in ND women than in the control group. Furthermore, the level of gamma-linolenic acid (GLA, 18:3n-6) was significantly lower, while the level of di-homo-gamma linolenic acid (DGLA, 20:3n-6) was significantly higher in both patient groups than in the control group (Table 1).

When we compared levels of individual and total n-3 PUFA, we found lower levels of alpha-linolenic acid (ALA, 18:3n-3) and docosapentaenoic acid (DPA, 22:5n-3) in both patient groups than in the controls (Table 1).

As shown in Table 2, there were significant differences between the groups regarding estimated activities of enzymes desaturases. Reduced activities of D6 and D5 desaturases were found in both patient groups compared to the control group.

No differences between ND and AI groups were found in any FA or desaturase/elongase system.

Table 2

The estimated plasma desaturase and elongase activities in patients with breast cancer and controls

Desaturase and elongase	ND group	AI group	Control group
18:0/16:0 (elongase)	0.45 ± 0.04	0.44 ± 0.07	0.50 ± 0.06
16:1n-7/16:0 (SCD-16)	0.04 ± 0.02	0.05 ± 0.01	0.06 ± 0.02
18:1n-9/18:0 (SCD-18)	0.98 ± 0.16	1.06 ± 0.30	0.98 ± 0.16
18:3n-6/18:2n-6 (D6 desaturase)	0.02 ± 0.01**	0.02 ± 0.01**	0.04 ± 0.01
20:4n-6/20:3n-6 (D5 desaturase)	3.27 ± 1.87*	2.88 ± 1.08***	5.08 ± 1.69

Data are presented as a mean ± standard deviation.
ND – new diagnosed group; AI – aromatase inhibitor group; SCD – stearoyl-CoA desaturase.
***$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control group.**

Several authors have found a positive association between oleic acid in plasma and erythrocyte lipids and BC, mostly as a result of overexpression of the genes encoding SCD 18 [31, 32], but there are some studies that showed that MUFA were unrelated to BC [33]. Here we found a lower level of vaccenic acid in both patient groups than in controls, while other MUFA had similar levels in all groups. These results may be due to decreased activity of elongase 5, which is involved in the synthesis of vaccenic from palmitoyl acid [34].

Among PUFA, we found a significantly higher level of linoleic acid in the ND group than in the control group. Linoleic acid is an essential FA, a precursor of the n-6 PUFA family, and its level in plasma lipids in the healthy population depends on dietary intake. Several prospective epidemiological studies demonstrated no evidence of an association between LA and BC risk [35, 36], while a significant inverse association has been reported in other studies [15, 33, 37]. On the other hand, a meta-analysis of 12 case-control studies [38] and a cohort study [39] suggests a positive relationship between PUFA intake and BC. Our finding on LA is consistent with Pouchieu et al. [40], who also found higher plasma levels of LA in BC patients than in healthy women. Furthermore, some investigators suggested that LA is necessary for the growth of some tumors, such as prostate cancer [15].

In addition, GLA is synthesized from LA. In our study, the level of GLA was significantly lower in both groups of patients than in controls, which is consistent with the results of other authors [40]. The enzyme directly involved in this process is D6 desaturase, and the estimated activity of this enzyme was lower in patients than in the control group. Our results indicate that the synthesis of long-chain PUFA is disturbed in both patient groups. Since GLA has been shown to inhibit the overexpression and hyperactivity of FAS

Discussion

This study investigated possible differences between FA profiles in plasma total lipids in newly diagnosed BC women and women taking aromatase inhibitor during the 2 years after completing BC chemotherapy, and we compared these two groups with apparently healthy women without a family history of BC. Our results showed no differences between the two BC groups, however, there were significant differences when we compared them with the control group. Namely, we found a significantly lower level of stearic acid in the AI group (women who take aromatase inhibitors) than in the control group, while the level of linoleic acid was significantly higher in ND patients than in the controls. In the past two decades, many researchers have been investigating the association between serum or plasma phospholipid FA and BC risk. However, the results are inconsistent. While some authors reported no association between individual SFA in plasma phospholipids and risk of BC [2], several studies suggested an inverse association between the saturation index and the risk [14, 15]. Moreover, there is evidence that serum total SFA shows a significant positive association with BC risk in postmenopausal women [16]. Nevertheless, data on the FA composition in total plasma lipids in patients with untreated BC or during/after the therapy is rather sparse. Similar to our results, a reduced level of stearic acid has been found in cancers at different sites [19, 29, 30]. *In vitro* studies demonstrated inhibition of proliferation and induction of apoptosis in BC cells lines by stearic acid [3]. Since stearic acid is considered cardioprotective, a low level of this FA may lead to additional complications in BC patients.

oncogene, which is closely linked to malignant transformation of mammary cells [41], these results may suggest poor prognosis for our patients and the need for increased intake of this FA.

Additionally, the level of DGLA (20:3n-6) was significantly higher in both patient groups than in the control group. Similar results have been found in gastric adenocarcinoma [42] and prostate cancer [43]; and some authors have observed inverse associations between DGLA and breast cancer risk. The possible reason for higher levels of DGLA could be decreased activity of D5 desaturase, which converts DGLA into arachidonic acid. Moreover, DGLA is a substrate for cyclooxygenase and lipoxygenase and can be converted by inflammatory cells to prostaglandin E1 (PGE1), which possess both antiinflammatory and antiproliferative properties. PGE1 also inhibits the growth and differentiation of cancer cells [44], suggesting possibly beneficial effects in our patients.

Omega-3 PUFA are usually low in cancer patients. BC patients in this study had a lower level of essential alpha-linolenic acid and its indirect product docosapentaenoic acid in both patient groups than in controls. A meta-analysis of three prospective cohort studies on circulating FA showed that total n-3 PUFA were associated with decreased BC risk [37]. Also, while animal studies have shown that large amounts of fish oil (n-3 PUFA) in the diet may decrease the incidence and inhibit the growth rate of mammary carcinomas in rodents, some authors found no association between n-3 PUFA and BC risk, suggesting that a beneficial effect of n-3 PUFA on BC development may require a relatively high intake of seafood [40]. Since the control group in this study had low levels of n-3 PUFA as well, the lower levels in BC patients can not be attributed to lower intake, but rather impaired metabolism. Nevertheless, the obtained results indicate that supplementation with n-3 FA may be beneficial for BC patients.

The weakness of this study is the relatively small number of patients. Nevertheless, these preliminary results suggest that there is a pattern in alterations in FA profiles that should be confirmed in a larger study. Based on these results, dietary interventions studies in BC patients are recommended.

Conclusion

FA profiles of plasms lipids of newly diagnosed, untreated BC patients are the same as those of cured BC patients who underwent all sessions of chemotherapy and received aromatase inhibitors for at least two years. In addition, their FA profiles markedly differ from those in healthy women. Therefore, supplementation with omega-3 FA and GLA could have beneficial effects in these patients, and further studies should address the potential clinical benefits of the supplementation.

Conflict of interests

The authors declare that they have no competing interests.

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Training/detraining-induced gender specific functional adaptations of isolated rat heart

Polno specifične funkcionalne adaptacije izolovanog srca pacova uzrokovane treningom/prekidom treninga

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Abstract

Background/Aim. Mechanisms responsible for the beneficial effects of aerobic exercise training on cardiovascular function are well known, but detraining effects on myocardial parameters have not been adequately elucidated. Therefore, the study aimed to determine the occurrence and speed of cardiac adaptation reversibility after the cessation of aerobic exercise and to reveal gender differences in achieved effects of training/detraining. **Methods.** Female and male Wistar albino rats were divided into the following groups: control, trained, and two detrained groups. Hearts were perfused according to the Langendorff technique and the following cardiodynamic parameters were determined: the maximum and minimum rate of pressure development in the left ventricle (dp/dt max and dp/dt min, respectively), systolic and diastolic left ventricular pressure (SLVP and DLVP, respectively), heart rate (HR), and coronary flow. **Results.** Training significantly reduced values of dp/dt max, dp/dt min, and SLVP in males and females, and coronary flow in males. Detraining caused a reversion of those changes, which was gender-specific. In females, levels of SLVP were higher after 4 weeks of detraining compred to training, and after 2 weeks of detraining. Values of SLVP were lower in both detraining periods compared to training in males. Males had higher coronary flow after 2 weeks of detraining. Simultaneously, coronary flow was reduced in the 4th week of detraining in females. **Conclusion.** By using a model of the isolated rat heart, the present study confirmed the existence of training-induced changes in cardiac function. Cessation of training was followed by the loss of those adaptations, faster in males than females.

Key words:
adaptation, physiological; exercise; rats; heart; gender.

Apstrakt

Uvod/Cilj. Mehanizmi odgovorni za blagotvorno dejstvo aerobnog treninga na funkciju kardiovaskularnog sistema su dobro poznati, ali efekti prekida treninga na parametre srčane funkcije nisu dovoljno razjašnjeni. Studija je imala za cilj da utvrdi pojavu i brzinu reverzibilnosti srčane adaptacije nakon prestanka aerobnog treninga, kao i da otkrije postojanje razlike među polovima postignute delovanjem treninga/prekida treninga. **Metode.** Pacovi soja Wistar (ženke i mužjaci) su bili podeljeni u sledeće grupe: kontrolnu grupu, grupu podvrgnutu treningu i dve grupe kod kojih je trening prekinut. Izolovana srca su perfundovana prema Langendorff-ovoj metodi, a praćeni su sledeći kardiodinamski parametri: maksimalna i minimalna stopa razvoja pritiska u levoj komori (dp/dt max i dp/dt min), sistolni i dijastolni pritisak u levoj komori (SLVP i DLVP), frekvenca otkucaja srca (HR) i koronarni protok. **Rezultati.** Trening je značajno smanjio vrednosti dp/dt max, dp/dt min i SLVP i kod mužjaka i kod ženki, kao i koronarni protok kod mužjaka. Prekid treninga je doveo do vraćanja vrednosti postignutih tokom treninga na vrednos

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ti pre treninga kod ženki pacova, nivo SLVP je bio viši nakon 4 nedelje od prekida treninga u poredjenu sa vrednostima tokom treninga i 2 nedelje nakon prekida treninga. Vrednosti SLVP su kod mužjaka bile niže u periodima prekida treninga u poređenju sa periodom treninga. Mužjaci su imali veće vrednosti koronarnog protoka nakon 2 nedelje od prekida treninga. Istovremeno, koronarni protok se smanjio u 4. nedelji od prekida treninga kod ženki. **Zaključak.** Na modelu izolovanog srca pacova, je potvrđeno postojanje promena srčane funkcije pod uticajem treninga. Prestanak treninga je bio praćen gubitkom detektovanih adaptacija, koji je bio brži kod mužjaka nego kod ženki pacova.

Ključne reči:
adaptacija, fiziološka; vežbanje; pacovi; srce; pol.

Introduction

Regular physical activity brings numerous benefits which are associated with reduced risk of cardiovascular diseases. These benefits of regular physical activity (exercise) were also noticed in patients with established cardiovascular disease [1–4]. Regular exercise induces changes in hemodynamic and loading conditions of the heart, which can lead to a series of positive changes in the heart's structure and function [5]. Improved oxygen supply and myocardial contractility, both in health and disease, represent exercise-related cardiac adaptations [6]. In addition, the amelioration of cardiovascular capacity due to aerobic exercise training is associated with increased left ventricular (LV) mass and volume, myocyte hypertrophy, increased LV stroke volume, and lower resting and submaximal heart rate (HR) [1, 6–10].

These training-induced anatomical and physiological cardiovascular adaptations are partially or completely lost as a result of reduction or cessation of training. Significant changes in cardiovascular function occur after detraining and it is related to decreased peak oxygen uptake (peak VO_2) and cardiomyocyte length [11, 12]. Previous studies pointed out that exercise training induced myocardial remodeling and improved myocardial contractile state, but these changes disappeared after a short period of detraining [12-14]. While the mechanisms responsible for the beneficial effects of aerobic exercise training on cardiovascular function and dimensions of cardiomyocytes are well known [6, 10], detraining effects on myocardial parameters has not been adequately elucidated.

Another important variable in training/detraining responses is gender. Recently, it has been reported that cardiovascular response to exercise is sex-dependent [15–17]. Investigations in rodents have shown that females have a more pronounced hypertrophic response to exercise than males. Furthermore, there are differences in the pathways leading to cardiac hypertrophy between the sexes. It is certain that the genetic and hormonal differences modify cardiac adaptations and improve cardiovascular capacity [18–20].

Despite the growing number of studies investigating the gender differences in the exercise-induced response of the cardiovascular system, data are still inconsistent and not sufficiently reliable. Moreover, detraining effects on heart function between males and females are almost unknown and remain to be elucidated as well. Therefore, the present study aimed to assess the presence and speed of reversibility of cardiac adaptation after the cessation of aerobic exercise as well as to determine gender differences in achieved effects of training/detraining on isolated rat heart.

Methods

Ethical approval

The study was performed in the Laboratory for Cardiovascular Physiology of the Faculty of Medical Sciences, University of Kragujevac, Serbia. The experimental protocol was approved by the Faculty of Medical Sciences Ethics Committee for the welfare of experimental animals, University of Kragujevac, number 01-275916, and by the Ministry of Agriculture, Forestry, and Water Management, Authority for Veterinary of Serbia number 323-07-02882/2014-05. All experiments were also performed according to the European Union Directive for the welfare of laboratory animals (86/609/EEC) and principles of Good Laboratory Practice (GLP).

Animals

Sixty-four Wistar albino rats (32 males and 32 females, eight weeks old at the beginning of the experiment, body weight 180–200 g, obtained from the Military Medical Academy, Belgrade, Serbia) were subjected to the study's protocol. Rats were housed with a temperature adjusted to 22 ± 1 °C with a 12:12 light/dark cycle and free access to food and water (*ad libitum)*.

Exercise training protocol

Rats were subjected to swimming according to the training protocol described below. Rats were divided into 4 groups, while each group consisted of 2 subgroups, males (M) and females (F). The first group was the control group (C), containing subgroups CM and CF (n = 8 for each subgroup). The second group was the trained group (T), containing subgroups TM and TF (n = 8 for each subgroup). The third group included 2 weeks detrained animals (D2), i.e., animals subjected to training, followed by 2 weeks of detraining period, subgroups DM2 and DF2 (n = 8 for each subgroup). The fourth group consisted of 4 weeks detrained animals (D4), ie., animals subjected to training followed by 4 weeks of detraining, subgroups DM4 and DF4 (n = 8 for each subgroup). Rats from the control group were placed in the pool 5 times a week for 3 minutes to achieve water induced-stress [21]. Rats from the groups T, D2 and D4 were subjected to moderate-intensity exercises, such as swimming training (8 weeks, 5 days/week, 60 min/day). A week before the experiment, rats were gradually exposed to

swimming training from 5 to 15 minutes in order to familiarize them with the swimming exercise. Subsequently, they started with 8 weeks training process. Rats from the group T (TM and TF) were sacrificed a day after accomplishing the training process. On the same day, rats (the same age as in the T group) from the C group were sacrificed as well. Animals from the DM2, DF2, DM4 and DF4 groups were sacrificed after 2 and 4 weeks of training cessation, respectively.

Rats swam in a specially constructed swimming pool made of glass (80 × 60 × 100 cm). Water temperature (34 °C) was maintained by an electric heater, and a pump continuously made waves in order to prevent rats from floating. The swimming was continuously supervised.

Preparation of isolated rat hearts

The hearts of male and female Wistar albino rats (n = 64, 8 in each experimental subgroup) were excised and retrogradely perfused according to the Langendorff technique (Experimetria Ltd, 1062 Budapest, Hungary). After short-term narcosis induced by intraperitoneal application of ketamine (10 mg/kg) and xylazine (5 mg/kg), the animals were sacrificed by cervical dislocation (Schedule 1 of the Animals/ Scientific Procedures, Act 1986, UK), and premedicated with heparin as an anticoagulant. After emergency thoracotomy and rapid cardiac arrest by superfusion with ice-cold isotonic saline, hearts were rapidly excised, the aortas were cannulated and retrogradely perfused at gradually increased coronary perfusion pressure (CPP) from 40 to 120 cm H_2O in order to establish coronary autoregulation.

The composition of the non-recirculating Krebs-Henseleit perfusate was as follows (mM): NaCl 118, KCl 4.7, $CaCl_2x2H_2O$ 2.5, $MgSO_4x7H_2O$ 1.7, $NaHCO_3$ 25, KH_2PO_4 1.2, glucose 11, pyruvate 2, equilibrated with 95% O_2 plus 5% CO_2, and warmed to 37 °C (pH 7.4).

Immediately after the restoration of normal heart rhythm, through the created entrance to the left atrium of the heart and damaged mitral valve, the sensor (transducer BS473-0184, Experimetria Ltd, Budapest, Hungary) was inserted into the left ventricle for continuous monitoring of cardiac function.

After placing the sensor in the left ventricle, the following parameters of myocardial function have been continuously registered: maximum rate of pressure development in the left ventricle (dp/dt max); minimum rate of pressure development in the left ventricle (dp/dt min); systolic left ventricular pressure (SLVP); diastolic left ventricular pressure (DLVP); heart rate (HR).

The above-mentioned cardiodynamic parameters were recorded during every CPP. Furthermore, during every CPP, the coronary flow was measured by flowmetry.

Statistical analysis

IBM SPSS Statistics 20.0 for Windows was used for statistical analysis. Descriptive statistics were used to calculate the arithmetic mean with dispersion measures (standard deviation – SD and standard error – SE). The distribution of data was checked by the Shapiro-Wilk test. Where distribution between groups was normal, statistical comparisons were performed using the one-way ANOVA tests with a Tukey's post hoc test for multiple comparisons. Kruskal-Wallis test was used for comparison between groups where the distribution of data was different than normal. Values of $p < 0.05$ were considered to be statistically significant.

Results

Maximum rate of pressure development in the left ventricle (dp/dt max)

Trained groups (TM, TF) had significantly decreased levels of this parameter compared to their controls (CM, CF). This difference was observed only when CPP was high (80, 100, and 120 cm H_2O). Significantly higher values of dp/dt max were noticed in the DF4 group compared to the DM4 group during CPP of 80 and 100 cm H_2O (Figure 1, A–D).

Minimum rate of pressure development in the left ventricle (dp/dt min)

Values of dp/dt min were also lower in trained groups (TM, TF) compared to their controls (CM, CF). Statistical significance was observed during high CPP (80, 100, and 120 cm H_2O). Significantly higher values of dp/dt min were noticed in the DF4 group compared to the DM4 group during CPP of 80, 100, and 120 cm H_2O (Figure 2, A–D).

Systolic left ventricular pressure (SLVP)

Lower levels of SLVP were noticed in trained groups (TM, TF) compared to their controls (CM, CF) at CPP of 80, 100, and 120 cm H_2O. The TM group had higher levels of SLVP compared to the DM2 at all CPP values. A significant increase of SLVP in the DF4 group was found compared to the TF group, while the DM4 group had lower levels of SLVP than the TM group (CPP 80, 100, and 120 cm H_2O). Significantly higher values of SLVP in the DF4 group were noticed compared to the DM4 group during all CPP values. When comparing the DF2 group with DF4, the DF4 group had significantly higher levels of SLVP at all CPP values (Figure 3, A–D).

Diastolic left ventricular pressure (DLVP)

There were no significant changes in values of this parameter in any group (Figure 4, A–D).

Heart rate (HR)

TM group had lower HR than CM group during CPP 40 and 120 cm H_2O. HR in the TM group was significantly lower compared to DM2 and DM4 groups at CPP between 40 and 120 cm H_2O (Figure 5, A–D).

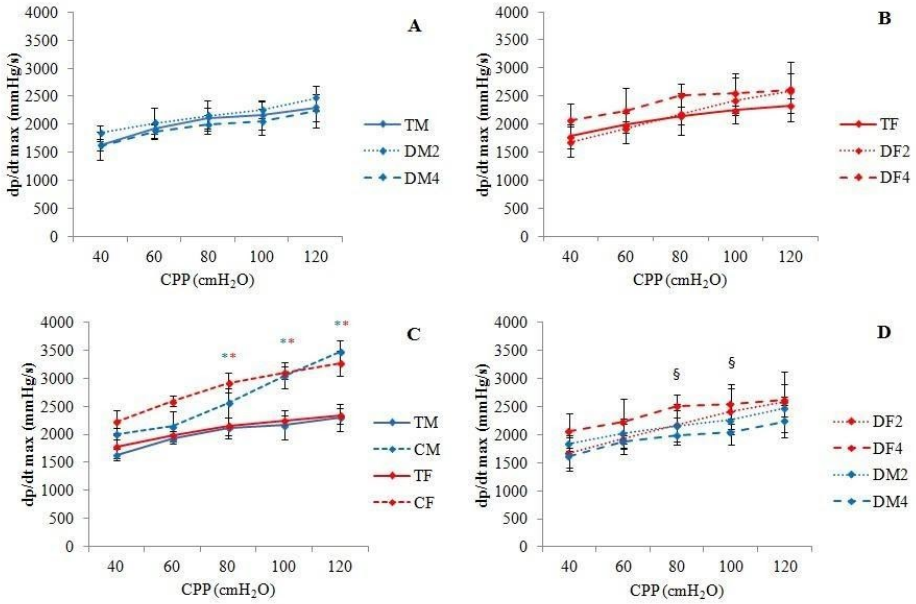

Fig. 1 – Effects of training/detraining on maximum rate of left ventricular pressure development (dp/dt max): A) TM vs. DM2 vs. DM4; B) TF vs. DF2 vs. DF4; C) CF vs. TF vs. CM vs. TM; D) DF2 vs. DF4 vs. DM2 vs. DM4.

Statistical significance at the level of $p < 0.05$: **TM (TF) vs. CM (CF); §DM4 vs. DF4. Data are presented as means ± SD.
CPP – coronary perfusion pressure; TM – trained males; DM2 – 2 weeks detrained males; DM4 – 4 weeks detrained males; CM – control males; TF – trained females; DF2 – 2 weeks detrained females; DF4 – 4 weeks detrained females; CF – control females.

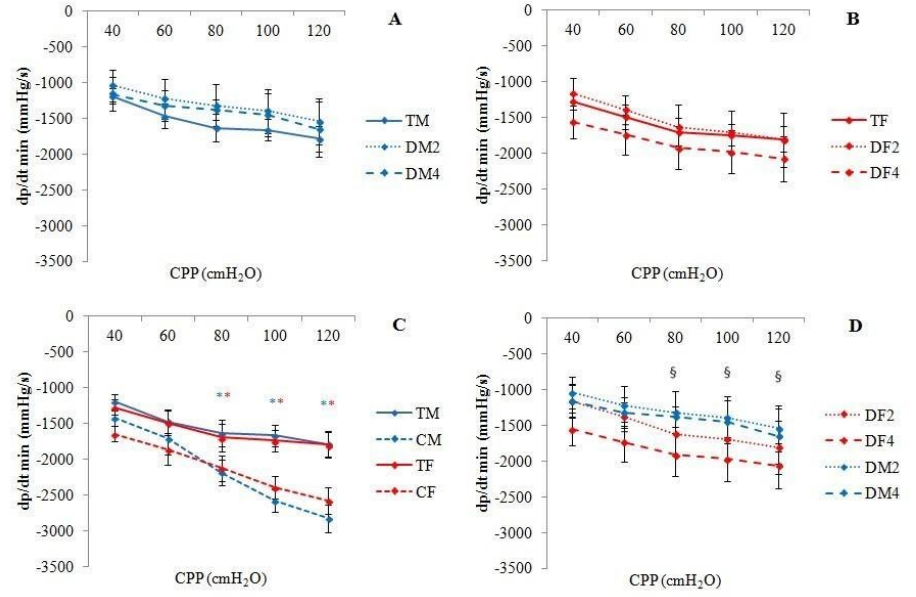

Fig. 2 – Effects of training/detraining on minimum rate of left ventricular pressure development (dp/dt min): A) TM vs. DM2 vs. DM4; B) TF vs. DF2 vs. DF4; C) CF vs. TF vs. CM vs. TM; D) DF2 vs. DF4 vs. DM2 vs. DM4.

Statistical significance at the level of $p < 0.05$: **TM(TF) vs. CM(CF); §DM4 vs. DF4. Data are presented as means ± SD.
CPP – coronary perfusion pressure; TM – trained males; DM2 – 2 weeks detrained males; DM4 – 4 weeks detrained males; CM – control males; TF – trained females; DF2 – 2 weeks detrained females; DF4 – 4 weeks detrained females; CF – control females.

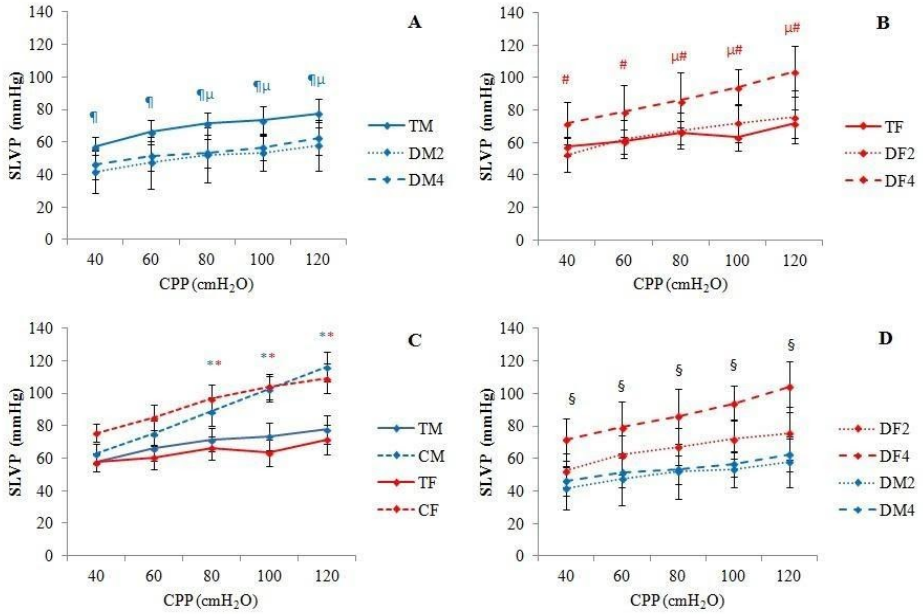

Fig. 3 – Effects of training/detraining on systolic left ventricular pressure (SLVP): A) TM vs. DM2 vs. DM4; B) TF vs. DF2 vs. DF4; C) CF vs. TF vs. CM vs. TM; D) DF2 vs. DF4 vs. DM2 vs. DM4.

Statistical significance at the level of $p < 0.05$: *TM(TF) vs. CM(CF); #DM2 (DF2) vs. DM4(DF4); ¶ TM(TF) vs. DM2(DF2); µTM(TF) vs. DM4 (DF4); £DM2 vs. DF2; §DM4 vs. DF4.

Data are presented as means ± SD.

CPP – coronary perfusion pressure; TM – trained males; DM2 – 2 weeks detrained males; DM4 – 4 weeks detrained males; CM – control males; TF – trained females; DF2 – 2 weeks detrained females; DF4 – 4 weeks detrained females; CF – control females.

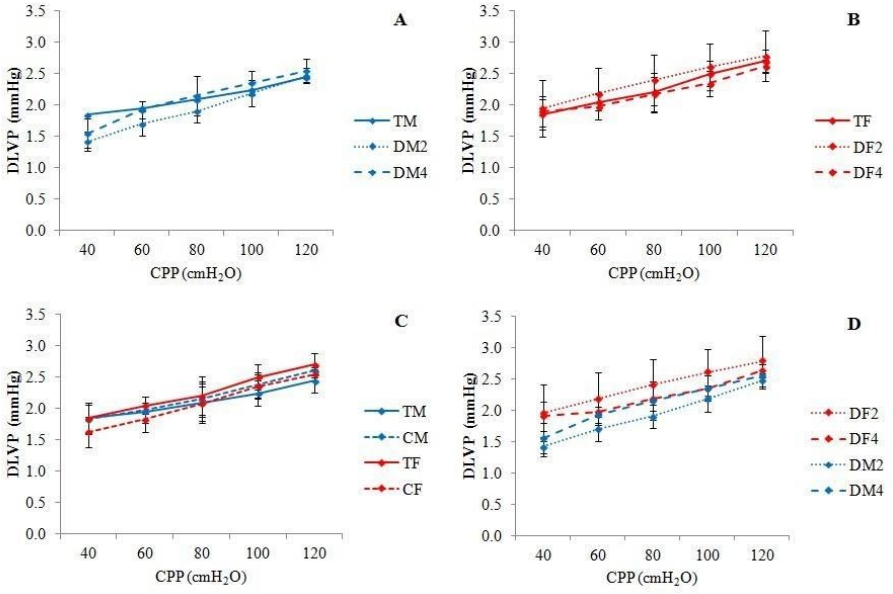

Fig. 4 – Effects of training/detraining on diastolic left ventricular pressure (DLVP). A) TM vs. DM2 vs. DM4; B) TF vs. DF2 vs. DF4; C) CF vs. TF vs. CM vs. TM; D) DF2 vs. DF4 vs. DM2 vs. DM4.

Statistical significance at the level of $p < 0.05$: **TM(TF) vs. CM(CF); #DM2 (DF2) vs. DM4(DF4); ¶ TM(TF) vs. DM2(DF2); µTM(TF) vs. DM4 (DF4); £DM2 vs. DF2; §DM4 vs. DF4.

Data are presented as means ± SD.

CPP – coronary perfusion pressure; TM – trained males; DM2 – 2 weeks detrained males; DM4 – 4 weeks detrained males; CM – control males; TF – trained females; DF2 – 2 weeks detrained females; DF4 – 4 weeks detrained females; CF – control females.

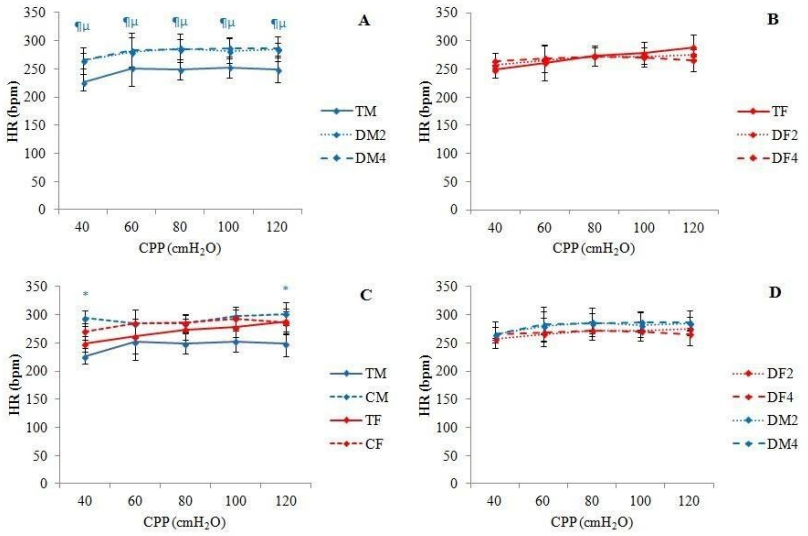

Fig. 5 – Effects of training/detraining on heart rate (HR): A) TM vs. DM2 vs. DM4; B) TF vs. DF2 vs. DF4; C) CF vs. TF vs. CM vs. TM; D) DF2 vs. DF4 vs. DM2 vs. DM4. Statistical significance at the level of $p < 0.05$: *TM(TF) vs. CM(CF); ¶ TM(TF) vs. DM2(DF2); ᵻTM(TF) vs. DM4 (DF4).
Data are presented as means ± SD.
CPP- coronary perfusion pressure; TM – trained males; DM2 – 2 weeks detrained males; DM4 – 4 weeks detrained males; CM – control males; TF – trained females; DF2 – 2 weeks detrained females; DF4 – 4 weeks detrained females; CF – control females.

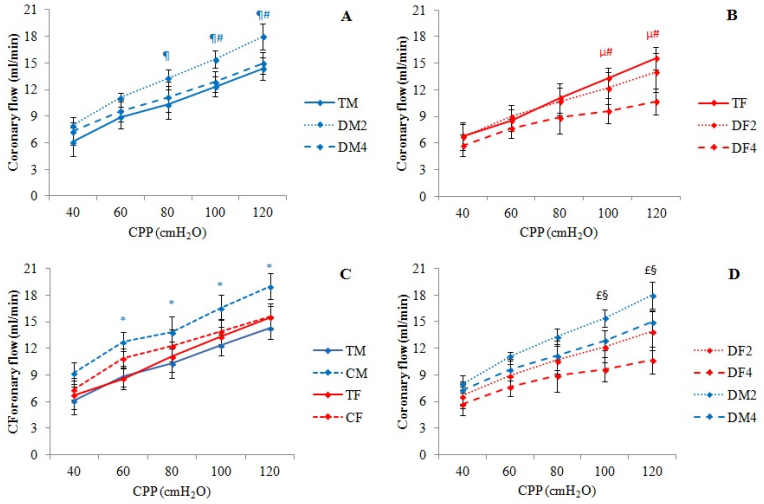

Fig. 6 – Effects of training/detraining on coronary flow (CF): A) TM vs. DM2 vs. DM4; B) TF vs. DF2 vs. DF4; C) CF vs. TF vs. CM vs. TM; D) DF2 vs. DF4 vs. DM2 vs. DM4. Statistical significance at the level of $p < 0.05$: **TM(TF) vs. CM(CF); #DM2 (DF2) vs. DM4(DF4); ¶ TM(TF) vs. DM2(DF2); ᵻ TM(TF) vs. DM4 (DF4); £DM2 vs. DF2; §DM4 vs. DF4.
Data are presented as means ± SD.
CPP- coronary perfusion pressure; TM – trained males; DM2 – 2 weeks detrained males; DM4 – 4 weeks detrained males; CM – control males; TF – trained females; DF2 – 2 weeks detrained females; DF4 – 4 weeks detrained females; CF – control females.

Coronary flow

The TM group had significantly lower levels of coronary flow than the CM group at CPP 60–120 cm H_2O. Coronary flow was significantly higher in the DM2 group compared to the TM at CPP 80–120 cm H_2O. The DF4 group had significantly lower levels of coronary flow than the TF group at CPP 100 and 120 cm H_2O. Comparing the DF2 with DM2 group, females had lower coronary flow than males at CPP 100 and 120 cm H_2O. The same results were recorded after 4 weeks of detraining between males and females. Coronary flow was higher in the DM2 group than in the DM4 and in the DF2 group than in the DF4 at 100 and 120 cm H_2O CPP (Figure 6, A–D).

Discussion

The magnitude of cardiovascular training-induced adaptations depends on mode, intensity, duration, and frequency of exercise. The best way to ensure a good training program is to ensure a gradual increase of load and enough time between exercise sessions for muscle regeneration, but not for regression of supercompensation [13, 21, 22]. Those adaptations are associated with the promotion of physiological cardiac hypertrophy (PCH), which is linked to less cardiac fibrosis and better systolic and diastolic function when compared to pathological hypertrophy. Ventricular dilatation represents a short-term adaptive response, while hypertrophy of the cardiac muscle fibers appears after a long time of regular physical activity [8, 9, 12, 23, 24].

In our experimental model, the heart was retrogradely perfused through the aorta, thus normal cardiac output and ejection fraction were not present. SLVP and dp/dt max describe systolic function in our research, while the diastolic function is described by dp/dt min and DLVP parameters. Our results show that 8 weeks of exercise induce slight depression of coronary function (lower SLVP and dp/dt max) in both males and females, keeping heart function within physiological limits. The reason for this might be the adaptation of the myocardium in terms of the rationality of its work at rest, proving its better response when exposed to physical effort. wIn our previous study, 12 weeks of training improved heart function, which could be related to the duration and intensity of the training program in this experimental protocol [21]. Values of HR in our study were significantly lower in the TM group compared to the control. This sinus bradycardia is in correlation with other cardiodynamic parameters and the abovementioned hypothesis and represents another physiological response of cardiac muscle to exercise, recently proved by D'Souza et al. [25] on a mice model.

Ishida et al. [26] investigated the influence of electrical stimulation on contractile parameters of the triceps during training and detraining and showed that 8 weeks of strength training did not induce significant changes in contractile properties, while during detraining, the muscles contracted faster. It was suggested that the release of Ca^{2+} and sarcoplasmic reticulum (SR) response during the strength training could be deferred and these effects might improve after strength training. This could be in agreement with our results which showed that levels of SLVP were higher after 4 weeks of detraining compared to training, and 2 weeks of detraining in females. On the contrary, values of this parameter in males were lower in both detraining periods than after the training period. HR in males was higher after both 2 and 4 detraining weeks compared to HR after regular training. Evangelista et al. [27] demonstrated that lower intrinsic HR is associated with low resting HR in trained rats. Detraining increased resting HR, approaching the basal values, as well as intrinsic HR. This bradycardia at rest tends to be associated with increased vagal activity and decreased sympathetic activity [28–30].

Furthermore, our results indicate that training-induced adaptations were lost after detraining in males and that value of tested parameters returned to the value similar to the control. Achieved adaptations persisted longer in females. The mechanism underlying the increased contractility in training has been reported as higher activity of Ca^{2+} ATPase and of Na^{+}/Ca^{2+} exchange [31, 32], therefore, the activity of this enzyme is probably lower in detraining. Opposite to our results, Bocalini et al. [13] found that the training-induced improvement in females was abolished after 2 weeks of detraining and returned to the values observed in the untrained group. A possible explanation for disproportion with the present investigation may be a different experimental protocol (investigation on isolated papillary muscle). In that sense, other authors determined that reduction of myocardial remodeling after detraining in rats and humans may be due to the duration of detraining [33]. For instance, a group of authors showed that after about 3 weeks of physical inactivity, the cardiac mass in rats regressed to baseline [34]. This is in correlation with the results of Kemi et al. [35], who determined that fractional shortening regressed with only 2 weeks of detraining. It was also proved that training-induced ventricular adaptation decline after detraining in humans [36].

Results regarding coronary flow showed that training protocol induced a decrease in heart perfusion which is in accordance with depression of myocardial function. In addition, this drop in coronary flow did not compromise the working capacity of the heart, allowing it to work in a lower physiological manner, as described above. On the other hand, detraining effects were gender-specific. While males had higher coronary flow after 2 weeks of detraining, in females, the reduction of coronary flow occurred in the 4th week of detraining. Based on this, we assume that training had a longer effect on females than males.

We did not find any significant differences in cardiodynamics between males and females who trained. Nevertheless, after 4 weeks of detraining, the heart function improved more in females than in males. An explanation for this might be a more pronounced hypertrophic response in females than in males, which was proved by many investigations [19, 37]. Ogawa et al. [38] noticed that gender difference is a result of a greater percentage of body fat in women. Some authors demonstrated that lipolytic activity in white adipose tissue, as well as plasma free fatty acid (FFA) levels, were higher in women after training than in men [39]. Furthermore, increased catecholamine-induced cardiac FFA uptake which leads to PCH in women is exercise-dependent. Investigations on rodents also identified pathways that may contribute to sexual dimorphism in exercise and cardiac adaptation to exercise. That mechanism underlying the development of PCH in females involves Ca^{2+}/calmodulin-dependent kinase (CaMK) and Akt/glycogen synthase kinase-3 (GSK-3) pathways [37]. Recent investigations proved that sex hormones could affect cardiac function and training-induced cardiac adaptations in rats [17, 40, 41]. Furthermore, since both estrogen receptors are expressed in the heart, female cardiac tissue activation of these receptors could lead to increased adaptive lipolytic activity. Moreover, it has been shown that reduction of cardiomyocytes contractility may be due to reduction of circulating testosterone which is in correlation with our results [14, 16, 19].

As expected, our study possesses some limitations. They are reflected in absence of the determination of histological and biochemical parameters within the heart muscle which could confirm obtained functional changes.

Conclusion

Findings of the present study pointed out that applied type of physical load induced functioning of the heart at a lower level of its cardiodynamic parameters, thus improving the rationality of heart work at rest. While training-induced cardiac responses were similar in males and females, cessation of training caused a reversion of those changes, which was gender-specific. Achieved adaptations were lost faster in males than in females. Results of the present study may be of practical interest in terms of obtaining an excellent basis for future reliable investigations on humans.

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Influence of biological markers on overall survival in surgically treated patients with non-small cell lung cancer

Uticaj bioloških markera na ukupno preživljavanje operisanih bolesnika sa nesitnoćelijskim karcinomom pluća

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Abstract

Background/Aim. Non-small cell lung cancer (NSCLC) is one of the most common malignant tumors and a leading cause of cancer-related deaths. The aim of this study was to assess the impact of biological markers on the overall survival rate in surgically treated NSCLC patients who received adjuvant chemotherapy and/or radiation therapy. **Methods.** This retrospective case series study included patients with NSCLC treated in the period between 2008 and 2017 at the Pulmonology Clinic and the Clinic for Chest Surgery, Military Medical Academy, Belgrade, Serbia. The survival analysis performed was based on immunohistological findings, histology type, and tumor, node, metastasis (TNM) stages. **Results.** The mortality rate was higher in the adenocarcinoma patient group compared to the squamous cell carcinoma group, albeit without statistical significance (58.3% vs. 31.2%, respectively; $p = 0.175$). Overall survival was shorter in the adenocarcinoma patient group compared to the squamous cell carcinoma group by approximately 750 days. Likewise, overall survival was shorter in the adenocarcinoma patient group compared to the squamous cell carcinoma group for CD31 positive ($p = 0.029$), p-63 positive ($p = 0.049$), MMP-9 positive ($p = 0.032$), and matrix metalloproteinase (MMP)-2 positive patients ($p = 0.016$). **Conclusion.** Adenocarcinoma is a more aggressive cancer type compared to squamous cell carcinoma with shorter overall survival. Our research showed a poorer overall survival in the adenocarcinoma group of patients compared to the squamous cell carcinoma group in CD31, p-63, MMP-9, and MMP-2 positive patients.

Key words:
biomarkers; carcinoma, non-small-cell lung; immunohistochemistry; survival; neoplasm staging; surgical procedures, operative.

Apstrakt

Uvod/Cilj. Nesitnoćelijski karcinom pluća (NSĆKP) je jedan od najčešćih malignih tumora i vodeći je uzrok smrti povezane sa karcinomima. Cilj ove studije bio je da se analizira uticaj bioloških markera na stopu ukupnog preživljavanja kod bolesnika sa NSĆKP koji su posle operacije dobijali adjuvantnu hemioterapiju i/ili radioterapiju. **Metode.** Sprovedena je retrospektivna studija tipa serije slučajeva na Klinici za pulmologiju i Klinici za grudnu hirurgiju Vojnomedicinske akademije u Beogradu, Srbija. Bolesnici sa NSĆKP lečeni su tokom perioda od deset godina (2008–2017). Analiza preživljavanja je bila zasnovana na imunohistohemijskim nalazima, patohistološkom tipu i tumor, nodus, metastaze (TNM) stadijumu. **Rezultati.** Stopa mortaliteta bila je viša u grupi bolesnika sa adenokarcinomom u poređenju sa grupom bolesnika sa skvamocelularnim karcinomom pluća, ali razlika nije bila statistički značajna (58,3%, odnosno 31,2%; $p = 0,175$). Ukupno preživljavanje bilo je kraće kod bolesnika sa adenokarcinomom u odnosu na bolesnike sa skvamocelularnim karcinomom za oko 750 dana. Ukupno preživljavanje je bilo kraće kod bolesnika sa adenokarcinomom u poređenju sa preživljavanjem bolesnika sa skvamocelularnim karcinomom kod CD31 pozitivnih ($p = 0,029$), p-63 pozitivnih ($p = 0,049$), metaloproteinaze matriksa (MMP)-9 pozitivnih ($p = 0,032$) i MMP-2 pozitivnih bolesnika ($p = 0,016$). **Zaključak.** Adenokarcinom pluća je značajno agresivniji karcinom u poređenju sa skvamocelularnim karcinomom pluća i oboleli imaju kraće vreme ukupnog preživljavanja.

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Ukupno preživljavanje je bilo kraće kod bolesnika sa adenokarcinomom u poređenju sa bolesnicima sa skvamocelularnim karcinomom kod CD31, p-63, MMP-9 i MMP-2 pozitivnih bolesnika.

Ključne reči:
biomarkeri; pluća, nesitnoćelijski karcinom; imunohistohemija; preživljavanje; neoplazme, određivanje stadijuma; hirurgija, operativne procedure.

Introduction

Lung cancer is one of the most common malignant tumors and a leading cause of cancer-related deaths [1–3]. About 80% of all lung cancers are non-small cell lung cancer (NSCLC), ie. squamous cell carcinoma and adenocarcinoma [4, 5].

The NSCLC significantly decrease overall survival, life quality, and working ability of patients, but increase direct and indirect medical cost [6]. Treatment options for patients with NSCLC consist of combined surgical treatment, radiation therapy, and/or one of the chemotherapy treatment protocols based on the stage of illness, histology type and tumor marker findings, and other parameters [6].

Surgery represents a treatment of choice for patients with NSCLC stage I-IIIA according to the tumor, node, metastasis (TNM) 8th edition classification [7, 8]. In addition to surgery, patients with resected NSCLC stage II-IIIA, who have a high risk of relapse, are treated with adjuvant chemotherapy and/or radiation therapy [6, 9]. Patients with stage IIIB and IV NSCLC are generally treated with chemotherapy and radiation therapy. For NSCLC stages I and II, radiation therapy alone is considered effective only when surgical resection is not possible either due to limited pulmonary reserve or the presence of comorbidities [10].

Histology and immunohistochemistry (IHC) analyses, as well as specific gene expression assessment may become a predictive factor for response to chemotherapy in future clinical research and patient treatment [11]. Expression of biomarkers [for example, human epidermal growth factor receptor (HER)-2, B-cell lymphoma (BCL)-2, cluster of differentiation 31(CD31), p-63, v-raf murine sarcoma viral oncogene homolog B1 (BRAF), Kirsten rat sarcoma 2 viral oncogene homolog (KRAS), etc.] can be tested at a protein level using IHC, while messenger ribonucleic acids (mRNA) levels can be determined through reverse transcriptase-polymerase chain reaction (RT-PCR)-based assays. Therefore, these biomarkers are currently not in use in daily practice. Many biomarkers in lung cancer were point mutations and rearrangements in specific genes including epidermal growth factor receptor (EGFR), anaplastic lymphoma kinase, HER-2, BCL-2, CD-31, p-63, matrix metalloproteinases (MMPs), BRAF, NUT, MET, ROS1, DDR2, fibroblast growth factor receptor 1 (FGFR1), KRAS, and phosphatase and tensin homolog (PTEN) [11]. These biomarkers might potentially provide additional information for clinical decision-making.

The overall five-year survival rate for all lung cancer in stage with localized disease is about 52.2%, in stage with the regional metastatic disease 25%, and in stage with distant metastatic disease 4% [12].

The aim of this study was to assess the impact of biological markers on the overall survival rate in surgically treated NSCLC patients, both after the adjuvant chemotherapy and/or radiation therapy.

Methods

Patients' data

This retrospective case series study of patients with NSCLC was designed as survival analysis based on immunohistological findings, histology type, and TNM stages. Forty NSCLC patients (17 females and 23 males; average age 59.22 ± 8.31 years) were treated at the Pulmonology Clinic and Chest Surgery Clinic of the Military Medical Academy in Belgrade, Serbia and were followed up over the period between 2008 and 2017.

Clinical files from all patients with clinically confirmed lung cancer admitted between 2010 and 2015 to the institutional healthcare network of the Military Medical Academy were accessed in both hard and electronic copies from the hospital registries. The following data were analyzed: demographic characteristics (age, gender), overall survival rate, immunohistological findings, histology type, and TNM stages of NSCLC.

TNM stage and patients treatment

The first step done in our hospital was to classify patients with NSCLC according to the TNM stages. T1N0M0 was classified as stage IA; T2N0M0 as stage IB; T1N1M0 as stage IIA; T2N1M0 and T3N0M0 as stage IIB; and T3N1M0, T1N2M0, T2N2M0, T3N2M0 as stage IIIA. Stage IIIB was classified as T4 any N M0 and any T N3M0, whereas stage IV was classified as any T any N M1 [13].

The second step consisted of treating patients with NSCLC according to their TNM stages. TNM stage I patients were only treated surgically. TNM stage IIA to IIIA patients were treated surgically and with adjuvant chemotherapy (etoposide and cisplatin – EP/PE protocol) and/or radiation therapy.

The above chemotherapy protocol was applied as follows: cisplatin at 60 mg/m^2 intravenous (*iv*) administered on day 1, plus etoposide at 120 mg/m^2 *iv* administered on days 1, 2, and 3, every 21 days for 4 cycles. Alternatively, cisplatin at 80 mg/m^2 *iv* was administered on day 1, plus etoposide at 100 mg/m^2 *iv* on days 1, 2, and 3, every 28 days for 4 cycles.

Radiotherapy was applied in patients with positive resection surface for malignancy and with N2 TNM stage [6].

Histology and IHC

Following tumor excision, collected tissue was formalin-fixed and paraffin-embedded (FFPE) as described below. Tissue slides were morphologically diagnosed at the Institute for Pathology and Forensic Medicine of the Military Medical Academy in Belgrade, Serbia and subsequently tested for a series of biomarkers using IHC standard protocol developed at the Laboratory for Immunohistochemistry and Electron Microscopy of the Institute for Medical Research of the Military Medical Academy in Belgrade, Serbia.

Excised tissue was fixed in 5% neutral-buffered formalin and processed in VIP Sakura apparatus for automatic fixation, dehydration, and paraffin embedding. Tissue blocks were cut at 5–7 μm and sections mounted on separate adherent chips (Super-Frost) and then dried at 56 °C for one h prior to staining.

Antibodies for immunostaining were applied according to manufacturers' recommendations. The following primary antibodies were used for IHC: HER-2, BCL-2, CD31, p-63, MMP-2, MMP-9, and MMP-14.

Statistical analyses

Continuous variables were presented as median with interquartile range (IQR). Categorical variables were reported as frequencies. Differences between categorical variables were tested by the χ^2 test, while the significance of the difference between continuous variables was tested by the non-parametric Mann-Whitney *U* test. Overall survival estimates were calculated using the Kaplan-Meier method [mean with 95% confidence interval (CI)] and log-rank (Mantel-Cox) test to assess differences between NSCLC groups. A *p*-value of less than 0.05 was considered statistically significant.

Ethics Committee approval

The study was conducted in accordance with the Declaration of Helsinki, and the protocol was reviewed and approved on September 6, 2015, by the Ethics Committee of the Military Medical Academy.

Results

We analyzed 40 patients with NSCLC (16 patients with squamous cell carcinoma and 24 with adenocarcinoma). Females were frequently in the adenocarcinoma group (13 or 54.2% out of 24 patients), while males were frequently in the squamous cell carcinoma group (12 or 75.0% out of 16 patients) (χ^2 test; $p = 0.133$). Differences according to age were not shown between groups [median age 61.04 (IQR 52.90-65.64) in patients with squamous cell carcinoma vs. median age 58.57 (IQR 54.16-66.09) in patients with adenocarcinoma; Mann-Whitney test: $p = 0.924$].

Overall survival for all patients followed according to biomarker type was not statistically different (log-rank (Mantel-Cox) test: $p > 0.05$) (Table 1).

The mortality rate was higher in the group of patients with adenocarcinoma compared to the squamous cell carcinoma patient group (58.3% or 14 out of 24 patients, and 31.2% or 5 out of 16 patients, respectively). However, this difference was not significant (χ^2 test: $p = 0.175$).

Overall survival of patients according to histology type of NSCLC (adenocarcinoma vs. squamous cell carcinoma) was not statistically different (log-rank (Mantel-Cox) test: $p = 0.057$) (Figure 1). However, cumulative survival was lower by approximately 750 days in the patient group with adenocarcinoma in comparison to the group with squamous cell carcinoma (estimated mean of 817.0 (561.2–1,072.7) vs. 1,566.4 (1,149.4–1,983.4) days respectively with 95% CI).

Overall survival of patients with adenocarcinoma and squamous cell carcinoma according to the interval from surgery to recurrence was presented in Table 2. A statistically significant difference was not observed between the groups. Cumulative survival was lower in the patient recurrence group with adenocarcinoma in comparison to the group of patients with squamous cell carcinoma by approximately 810 days (Figure 2).

Table 1

Overall survival in all patients with lung cancer according to biological markers tested (censored – alive at the end of the follow-up period)

Marker tested	Total number	Number of death events	Censored n (%)	Survival (days) – estimated mean (95% CI)	*p*-value*
HER-2 negative	32	14	18 (55.2)	1262.8 (929.7–1596.0)	0.316
HER-2 positive	8	5	3 (37.5)	655.0 (354.2–955.8)	
BCL-2 negative	1	1	-	540.0 (540.0–540.0)	0.447
BCL-2 positive	39	18	21 (53.8)	1230.9 (928.8–1533.1)	
CD31 negative	5	2	3 (60.0)	766.8 (506.1–1027.5)	0.728
CD31 positive	35	17	18 (51.4)	1194.4 (881.4–1507.4)	
p-63 negative	2	1	1 (50.0)	756.5 (456.4–1056.5)	0.875
p-63 positive	38	18	20 (52.6)	1206.6 (901.6–1511.5)	
MMP-9 negative	7	4	3 (42.9)	720.6 (533.6–907.5)	0.829
MMP-9 positive	33	15	8 (54.5)	1240.9 (911.8–1570.0)	
MMP-2 negative	19	7	12 (63.2)	1113.6 (817.0–1410.2)	0.303
MMP-2 positive	21	12	9 (42.9)	1017.7 (625.4–1410.0)	
MMP-14 negative	33	15	18 (54.5)	1243.7 (919.4–1568.0)	0.594
MMP-14 positive	7	4	3 (42.9)	663.3 (329.6–923.3)	

HER – human epidermal growth factor receptor; BCL – B-cell lymphoma; CD – cluster of differentiation; MMP – matrix metalloproteinase; CI – confidence interval.
***log-rank (Mantel-Cox) test.**

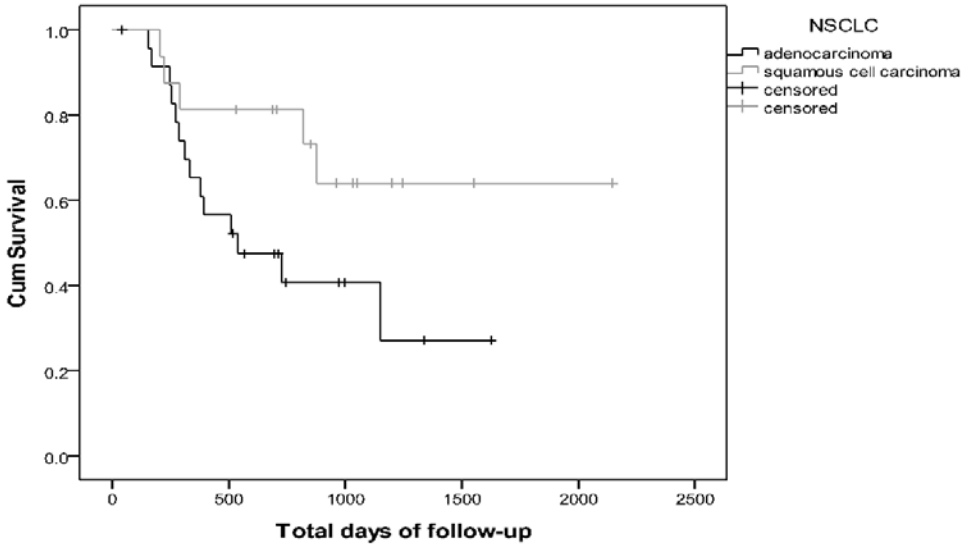

Fig. 1 – Kaplan-Meier analysis – survival curves of patients according to histology type of non-small cell lung cancer (NSCLC) (censored – alive at the end of the follow-up period).

Table 2

Distribution of overall survival of patients with non-small cell lung cancer (NSCLC) according to the interval from surgery/surgical resection to recurrence

Type of NSCLC	Recurrence	Total number	Number (%) of death events	Censored n (%)	Survival (days) – estimated mean (95% CI)	*p*-value*
Adenocarcinoma	Yes	17	11 (64.7)	6 (35.3)	803.4 (521.7–1,085.1)	0.940
	No	7	3 (42.9)	4 (57.1)	616.7 (328.4–904.9)	
Squamous cell carcinoma	Yes	6	2 (32.3)	4 (66.7)	1,615.8 (995.1–2,236.3)	0.814
	No	10	3 (30.0)	7 (70.0)	949.0 (707.0–1,191.0)	

CI – confidence interval.

***Log-rank (Mantel-Cox) test; yes adenocarcinoma/ yes squamous cell carcinoma log-rank (Mantel-Cox) test: $p = 0.147$; no adenocarcinoma/ no squamous cell carcinoma log-rank (Mantel-Cox) test: $p = 0.316$.**

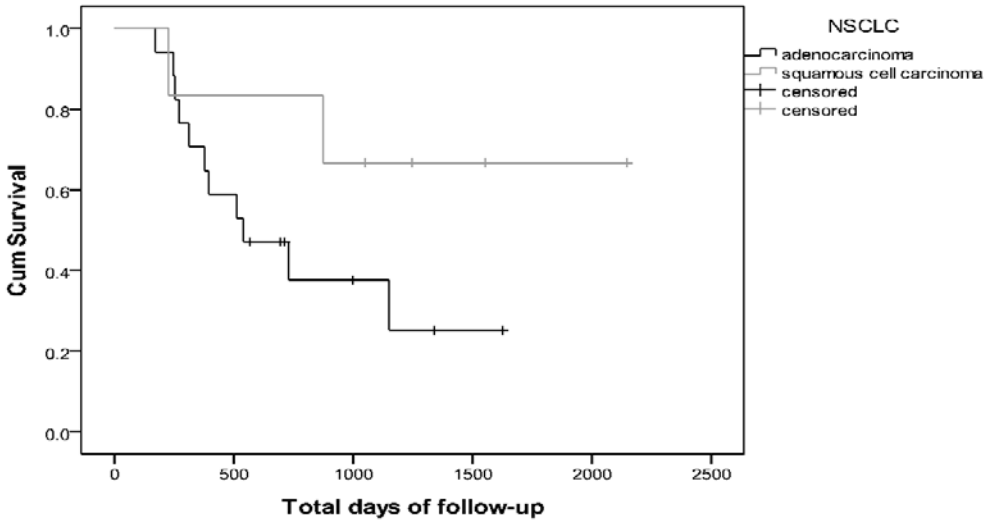

Fig. 2 – Kaplan-Meier analysis – survival curves in patients with recurrence according to histology type of non-small cell lung cancer (NSCLC) (censored – alive at the end of the follow-up period).

Table 3

Distribution of overall survival in patients with non-small cell lung cancer (NSCLC) according to clinical initial tumor, node, metastasis (TNM) stage

Type of NSCLC	TNM stage	Total number	Number (%) of death events	Censored n (%)	Survival (days) – estimated mean (95% CI)	*p*-value*
Adenocarcinoma	IA,IB	6	3 (50.0)	3 (50.0)	1101.5 (708.8–1494.2)	
	IIA,IIB	12	6 (50.0)	6 (50.0)	810.4 (508.9–1112.0)	0.060
	IIIA	6	5 (83.3)	1 (16.7)	374.4 (186.6–562.2)	
Squamous cell carcinoma	IA,IB	-	-	-	-	
	IIA,IIB	9	3 (33.3)	6 (66.7)	971.0 (702.1–1239.9)	0.970
	IIIA	7	2 (28.6)	5 (71.4)	1586.4 (325.6–947.9)	

CI – confidence interval.
***Log-Rank (Mantel-Cox) test; IIA, IIB adenocarcinoma/ IIA, IIB squamous cell carcinoma log-rank (Mantel-Cox) test: $p = 0.380$; IIIA adenocarcinoma/ IIIA squamous cell carcinoma log-rank (Mantel-Cox) test: $p = 0.007$;**

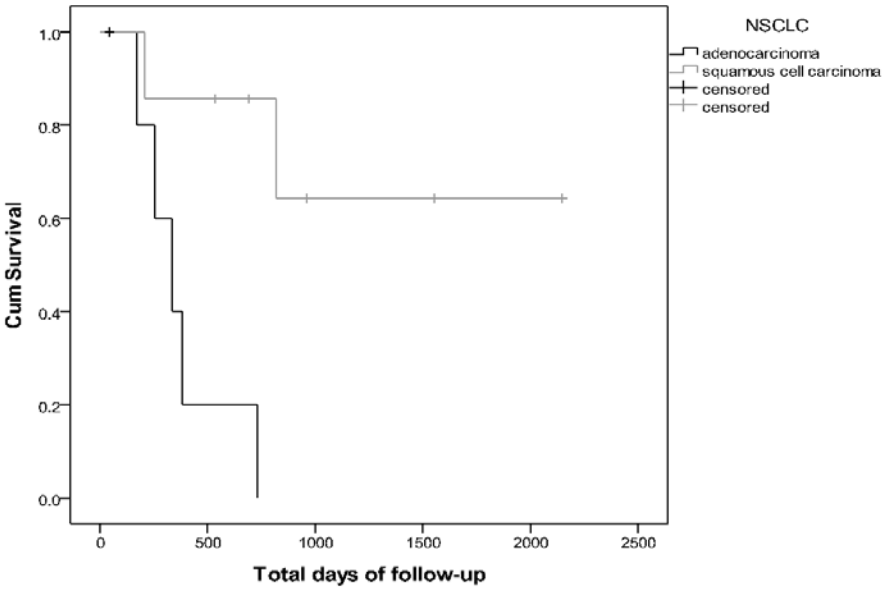

Fig. 3 – Kaplan-Meier analysis – survival curves in patients with non-small cell lung cancer (NSCLC) in IIIA tumor, node, metastasis (TNM) stage according to histology type (censored – alive at the end of the follow-up period).

Overall survival was estimated and compared among patients according to preoperative TNM stage in both patient groups (Table 3). There was no statistically significant difference in survival between patients in stages I, II, and IIIA within the adenocarcinoma ($p = 0.060$) and the squamous cell carcinoma group ($p = 0.970$). However, overall survival between patients with adenocarcinoma and those with squamous cell carcinoma according to the initial TNM stage showed that patients with adenocarcinoma had a statistically significantly lower survival rate compared to the patients with squamous cell carcinoma in TNM stage IIIA [log-rank (Mantel-Cox) test: $p = 0.007$; mean 374.4 days vs. 1,586.4 days, respectively] (Figure 3).

No significant differences were observed between adenocarcinoma and squamous cell carcinoma by the distribution of patients according to biological markers (Table 4). The patients were most frequently positive for BCL-2, CD31, p-63, MMP-9, and MMP-2, but rarely for HER-2 and MMP-14.

Overall survival was estimated and compared among NSCLC patients according to the status of biological markers in two patient groups studied (Table 4). There was a statistically significant difference in survival between patients with positive and negative biological markers regardless of NSCLC type. Cumulative survival was lower in the adenocarcinoma patient group compared to the squamous cell carcinoma group for CD31 positive (log-rank (Mantel-Cox) test: $p = 0.029$) (Figure 4), p-63 positive ($p = 0.049$) (Figure 5), MMP-9 positive ($p = 0.032$) (Figure 6) and MMP-2 positive patients ($p = 0.016$) (Figure 7).

Table 4

Overall survival in the patients with non-small cell lung cancer (NSCLC) according to biological markers (censored – alive at the end of the follow-up period)

Type of NSCLC	Marker[1] tested	Total, n (%)	*p*-value#	Number (%) of death events	Censored n (%)	Survival (days) – estimated mean (CI 95%)	*p*-value*
Adenocarcinoma	HER-2 negative	18 (75.0)	0.572	9 (50.0)	9 (50.0)	922.8 (621.3–1224.2)	0.103
	HER-2 positive	6 (25.0)		5 (83.3)	1 (16.7)	398.0 (241.9–554.1)	
Squamous cell carcinoma	HER-2 negative	14 (87.5)		5 (35.7)	9 (64.3)	All censored patients in HER-2 positive group	0.307
	HER-2 positive	2 (12.5)		-	2 (100.0)		
Adenocarcinoma	BCL-2 negative	1 (4.2)		1 (100.0)	-	540.0 (540.0–540.0)	0.735
	BCL-2 positive	23 (95.8)	1.000	13 (56.5)	10 (43.5)	833.0 (566.8–1099.2)	
Squamous cell carcinoma	BCL-2 negative	-		-	-	-	-
	BCL-2 positive	16 (100.0)		5 (31.2)	11 (68.8)	1566.4 (1149.4–1983.4)	
Adenocarcinoma	CD31 negative	3 (12.5)		1 (33.3)	2 (66.7)	847.3 (601.4–1093.2)	0.339
	CD31 positive	21 (87.5)	1.000	13 (61.9)	8 (38.1)	772.1 (504.6–1039.5)	
Squamous cell carcinoma	CD31 negative	2 (12.5)		1 (50.0)	1 (50.0)	572.5 (182.4–962.6)	0.451
	CD31 positive	14 (87.5)		4 (28.6)	10 (71.4)	1624.5 (1197.6–2051.5)	
Adenocarcinoma	p-63 negative	2 (8.3)		1 (50.0)	1 (50.0)	756.5 (456.4–1056.5)	0.618
	p-63 positive	22 (91.7)	0.657	13 (59.1)	9 (40.9)	799.4 (531.8–1067.0)	
Squamous cell carcinoma	p-63 negative	-		-	-	-	-
	p-63 positive	16 (100.0)		5 (31.2)	11 (68.8)	1566.4 (1149.4–1983.4)	
Adenocarcinoma	MMP-9 negative	4 (16.7)		2 (50.0)	2 (50.0)	749.7 (530.8–968.7)	0.531
	MMP-9 positive	20 (83.3)	1.000#	12 (60.0)	8 (40.0)	785.7 (505.5–1065.9)	
Squamous cell carcinoma	MMP-9 negative	3 (18.8)		2 (66.7)	1 (33.3)	681.7 (239.6–1123.7)	0.215
	MMP-9 positive	13 (81.2)		3 (23.1)	10 (76.9)	1710.4 (1278.8–2141.9)	
Adenocarcinoma	MMP-2 negative	9 (37.5)		3 (33.3)	6 (66.7)	1152.2 (725.0–1580.0)	0.141
	MMP-2 positive	15 (62.5)	0.219#	11 (73.3)	4 (26.7)	609.7 (384.7–834.7)	
Squamous cell carcinoma	MMP-2 negative	10 (62.5)		4 (40.0)	6 (60.0)	1041.7 (659.5–1423.9)	0.276
	MMP-2 positive	6 (37.5)		1 (16.7)	5 (83.3)	1882.4 (1416.8–2348.0)	
Adenocarcinoma	MMP-14 negative	18 (75.0)		10 (55.6)	8 (44.4)	852.6 (554.6–1150.6)	0.721
	MMP-14 positive	6 (25.0)	0.269#	4 (66.7)	2 (33.3)	572.2 (329.6–814.7)	
Squamous cell carcinoma	MMP-14 negative	15 (93.8)		5 (33.3)	10 (66.7)	All censored patients in MMP-14 positive group	0.494
	MMP-14 positive	1 (6.2)		-	1 (100.0)		

[1]For explanation see under Table 1.

 # – χ^2 test; * – log-rank (Mantel-Cox) test; HER-2 positive adenocarcinoma/ HER-2 positive squamous cell carcinoma log-rank (Mantel-Cox) test: $p = 0.092$; HER-2 negative adenocarcinoma/ HER-2 negative squamous cell carcinoma log-rank (Mantel-Cox) test: $p = 0.330$; BCL-2 positive adenocarcinoma/ BCL-2 positive squamous cell carcinoma log-rank (Mantel-Cox) test: $p = 0.072$; CD31 positive adenocarcinoma/ CD31 positive squamous cell carcinoma log-rank (Mantel-Cox) test: $p = 0.029$; p-63 positive adenocarcinoma/ p-63 positive squamous cell carcinoma log-rank (Mantel-Cox) test $p = 0.049$; MMP-9 positive adenocarcinoma/ MMP-9 positive squamous cell carcinoma log-rank (Mantel-Cox) test $p = 0.032$; MMP-9 negative adenocarcinoma/ MMP-9 negative squamous cell carcinoma log-rank (Mantel-Cox) test: $p = 0.730$; MMP-2 positive adenocarcinoma/ MMP-2 positive squamous cell carcinoma log-rank (Mantel-Cox) test: $p = 0.016$; MMP-2 negative adenocarcinoma/ MMP-2 negative squamous cell carcinoma log-rank (Mantel-Cox) test: $p = 0.837$; MMP-14 positive adenocarcinoma/ MMP-14 positive squamous cell carcinoma log-rank (Mantel-Cox) test: $p = 0.330$; MMP-14 negative adenocarcinoma/ MMP-14 negative squamous cell carcinoma log-rank (Mantel-Cox) test: $p = 0.130$.

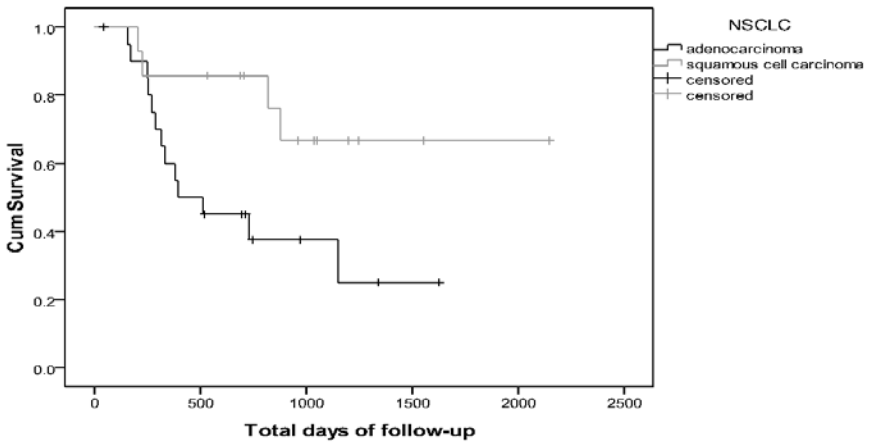

Fig. 4 – Kaplan-Meier analysis – survival curves in positive CD31 patients with non-small cell lung cancer (NSCLC) according to histology type (censored – alive at the end of the follow-up period).
CD – cluster of differentiation.

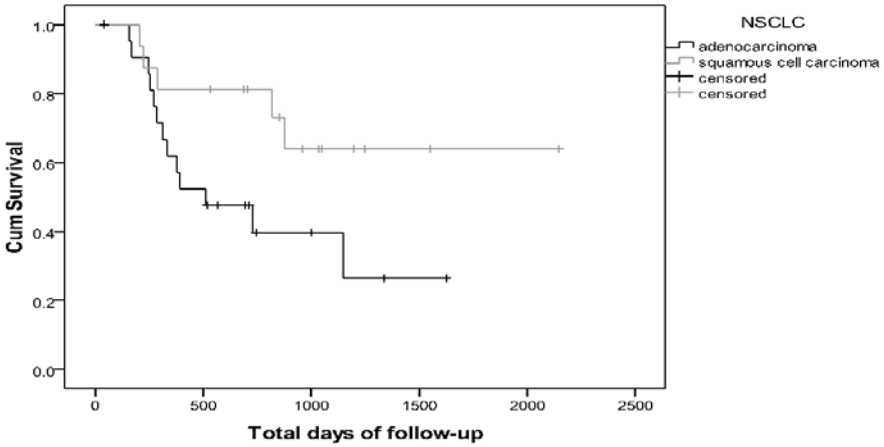

Fig. 5 – Kaplan-Meier analysis – survival curves in positive p-63 patients with non-small cell lung cancer (NSCLC) according to histology type (censored – alive at the end of the follow-up period).

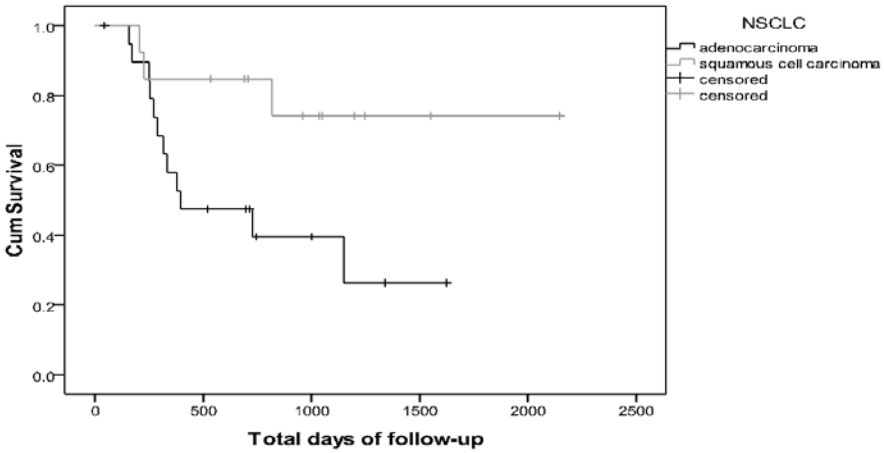

Fig. 6 – Kaplan-Meier analysis – survival curves in positive MMP-9 patients with non-small cell lung cancer NSCLC) according to histology type (censored – alive at the end of the follow-up period).
MMP – matrix metalloproteinase.

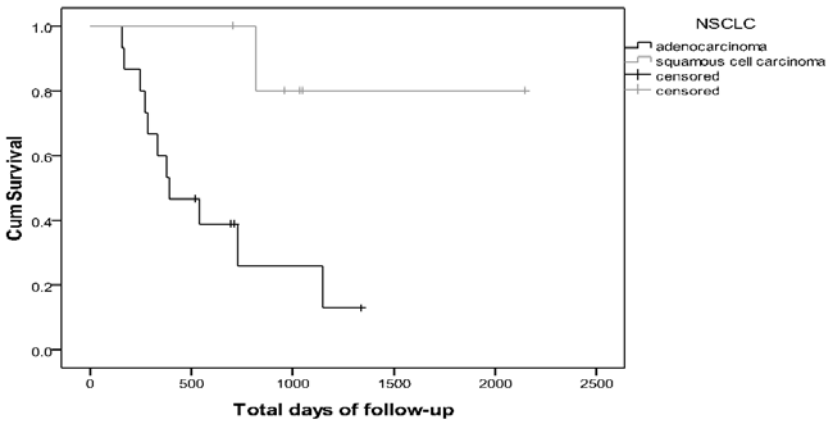

Fig. 7 – Kaplan-Meier analysis – survival curves in positive MMP-2 patients with non-small cell lung cancer (NSCLC) according to histology type (censored – alive at the end of the follow-up period). MMP – matrix metalloproteinase.

Discussion

NSCLC cancer is one of the major causes of cancer-related deaths [2]. To analyze survival in our NSCLC patients, we conducted a retrospective case-series study using the follow-up data from a period between 2008 and 2017. Our study design aimed at assessing the overall survival in NSCLC patients according to specific biomarkers expression, TNM stage, and histology type [14].

In the operable NSCLC patients, adjuvant chemotherapy has been considered a standard modality of treatment following surgical resection of the tumor [14–19]. Besides, molecularly targeted therapy has significantly improved outcomes of patients with a metastatic form of NSCLC [2, 18]. Nevertheless, for the majority of patients, platinum-based chemotherapy remains the gold standard treatment and has led to significantly improved survival outcomes with approximately 10–11 months median survival [20].

In our study, there were more male patients in the squamous cell carcinoma group, while female patients dominated the adenocarcinoma group. Men develop tracheal, bronchus, and lung cancer more often compared to women (1/18 for men; 1/45 for women) [3]. The estimated number of lung cancer cases worldwide has increased by 44% in men and 76% in women since 1985 [21]. The higher rate increase in women has been attributed to the fact that cigarette smoking in the female population peaked two decades later than in the male [21]. Our squamous cell carcinoma patients were not significantly older compared to the adenocarcinoma group (median age 61.04 vs. 58.57, respectively). Comparably, in another study, squamous cell carcinoma patients were slightly older when compared to adenocarcinoma patients (median age 69 vs. 65, respectively) [22, 23].

Patients with adenocarcinoma had a poorer prognosis compared to squamous cell carcinoma patients [22]. Similarly, in our study, the mortality rate was significantly higher in the adenocarcinoma group (58.3%) as opposed to the squamous cell carcinoma group (31.2%). According to the literature, generally, the five-year survival rate for all NSCLC patients in stage IA, IB, IIA, and IIB is about 49%, 45%, 30%, and 31%, respectively [24]. This rate for NSCLC patients in stage IIIA and IIIB is about 14% and 5%, respectively [24].

Overall survival of patients according to recurrence is of major significance [23]. Recurrence rates reported following surgical cancer resection range from 30% to 75% [25]. The majority of recurrent tumors are distant, and more than 80% of recurrences occur within the first two years after resection. Cumulative survival was lower in our patient recurrence group with adenocarcinoma compared to the squamous cell carcinoma group by about 810 days. This is in line with our findings showing adenocarcinoma as a more aggressive cancer type than squamous cell carcinoma.

The complete resection of early-stage NSCLC offers to patients high hopes for a successful therapeutic outcome. However, the recurrence rates postresection remain high [26]. For that reason, right from the beginning of therapy in NSCLC patients, a complete surgical removal needs to be ensured both macroscopically and microscopically. Often, occult micro-metastatic cancer cells, already present systemically at the time of surgery, remain undetected by standard staging methods suggesting an underestimation of the true tumor stage. Moreover, dissemination of cancer cells might occur during the handling of the tumor during surgery [26].

Overall survival according to TNM stages was observed among patients with adenocarcinoma, as well as those with squamous cell carcinoma. Statistically significant difference was not observed between our patients with adenocarcinoma and patients with squamous cell carcinoma in TNM stage groups IA, IB, IIA, and IIB, but this difference was shown between the groups in the IIIA stage. Overall survival was lower between stage IIIA adenocarcinoma patients compared to squamous cell carcinoma patients of the same stage (mean 374.4 days vs. 1,586.4 days, respectively). This evidence is also in line with adenocarcinoma being a more aggressive cancer type when compared to squamous cell carcinoma.

After curative resections, patients with lung cancer at the same TNM stage show wide variations in their recurrence onset and overall survival [26]. The current TNM staging system, based on clinical and pathological findings, may have achieved the limit of its relevance. Being able to predict the exact likelihood and timing of relapse can help guide the administration of adjuvant therapies. There are two methods for identifying factors related to recurrence and low overall survival following surgery: expression of tumor markers and molecular biological techniques. Excellent prognostic markers for predicting the postoperative recurrence of cancer are KRAS, Ki-67, p16, EGFR, and others. Since histological differentiation, vessel invasion, lymphatic permeation, and pleural invasion have been reported as poor prognostic factors for the disease-free survival [27–29], an extensive pathological investigation is also of high significance.

Personalized medicine by targeting appropriate molecular targets in tumors has helped improve survival in NSCLC patients [30]. MMPs and zinc-dependent endopeptidases play roles within various areas of cancer pathology. Tumor growth, metastasis, angiogenesis, and MMP activation are increased in nearly all human cancers when compared to normal tissue [31,32]. MMPs are involved in the degradation of the extracellular matrix. In addition, MMPs are known to influence lung cancer metastatic properties and are involved in several signaling pathways [ECM, collagen regulate polarization of Th1/Th2 inflammatory response; Springolipid and Ephrin receptor-signaling pathway (ET-1); N-cadherin; N-cadherin, vascular smooth muscle cell-extracellular matrix (VSMC-ECM) attachment; IGF-2, VEGF-B and VEGF signaling pathways; p38, JNK, and NF-κB pathways] [33]. Overall, increased levels of specific MMPs have been associated with NSCLC progression [33].

MMP-14 is a critical protein in cancer invasion and metastasis [33]. Invasion through collagen networks and subsequent collagenolysis relies principally on MMP-14 and not on secreted MMPs. MMP-14 expression has been correlated with primary tumor growth and metastasis as well as angiogenesis. Detailed analysis of MMP-14-promoted tumor growth has suggested that a cytoplasmic domain is required for the MMP-14 enhanced tumor growth.

MMP-2 has a role in extracellular matrix disassembly, increased cell proliferation, invasion/migration, and angiogenesis [34]. Strong immunohistochemical staining for MMP-2 in tumor tissue predicts poor survival in lung cancer patients [32, 35]. MMP-2 has been implicated in lymphatic and vascular invasion of NSCLC, thus the prognostic value of MMP-2 expression in NSCLC is of great significance [32, 33]. MMP-2 overexpression predicts a poor prognosis in early-stage NSCLC. This study shows that MMP-2 overexpression correlates with early cancer-related death. Other MMP subclasses are also associated with a degree of lung cancer aggressiveness. It is of note that one systematic review suggests that MMP-2 expression has a poor prognostic significance of NSCLC patient's survival [36].

MMP-9 has a role in extracellular matrix remodeling, increased cell proliferation, invasion/migration, and angiogenesis [37]. Highly expressed MMP-9 correlates with shortened survival of NSCLC patients [32, 38]. MMP-9 expression is an independent prognostic marker for resected stage NSCLC. Thus, MMP-9 is a novel biomarker significantly and independently predicting a worse prognosis of resected stage NSCLC. In a different study, tumor MMP-9 expression was associated with poor outcomes in adenocarcinoma but not in squamous cell carcinoma patients [39]. MMP-9 expression was identified as an independent marker of relapse in completely resected lung adenocarcinoma.

In NSCLC patients, genetic aberrations of the human epidermal growth factor-2 (HER-2) signaling pathway are associated with different sensitivity to EGFR tyrosine kinase inhibitors (TKIs) [40]. This is a plausible mechanism and prognostic role of acquired resistance to the EGFR TKIs in EGFR-mutated tumors. Although in our study a vast majority of patients in both cancer groups were HER-2 negative, our positive adenocarcinoma patients showed a lower survival rate compared to HER-2 negative adenocarcinoma patients. Gene amplification is a well-known mechanism of proto-oncogene activation and has been described in many human malignancies, including lung tumors. However, HER-2 amplification seems far less common in NSCLC compared to other cancers. Recently, the predictive role of HER-2 overexpression has been more extensively studied with the purpose to identify anti-HER-2 agents applicable in NSCLC patients.

BCL-2 overexpression is associated with better outcome and survival of the patients with NSCLC [41, 42]. Patients with positive BCL-2 expression have a better survival rate compared to patients with negative BCL-2 expression [43]. Our study showed comparable findings.

The intensity of neoangiogenesis in a tumor can be reliably evaluated by measuring the intratumoral microvessel density of CD31 cell membrane protein. CD31 is an integral endothelial membrane protein that mediates cell-to-cell adhesion. Statistics have shown a more significant survival rate in NSCLC patients with high CD-31 expression compared to patients with lower CD-31 expression [44].

Expression of p-63, an established marker of squamous differentiation, is also present in NSCLC patients [45]. P-63 is a transcription factor that transactivates p-53 target genes and induces apoptosis when expressed in cells. The p-63 gene amplification and overexpression may have important implications in tumorigenesis [46]. In our previous study, patients with weak p-63 expression had a significantly shorter overall survival than patients with no p-63 expression and a tendency of shorter overall survival than patients with p-63 expression [47]. Patients with negative p-63 expression tend to have a worse prognosis compared to patients with p-63 expression. On the other hand, in a study by Ko et al. [48], negative expression of p-63 was associated with a short recurrence interval of the disease and shorter survival in NSCLC.

Based on the results of our study, tumor (biological) markers represent significant negative prognostic indicators in all patients with NSCLC regardless of the histological tumor subtype. All patients with positive marker expression had a short recurrence interval of the disease, as well as a short overall survival. These data should be considered when deciding on patient treatment following surgical resections. We propose that patients with positive expression of BCL-2, CD31, p-63, MMP-9, MMP-2, HER-2, and MMP-14 should receive adjuvant chemotherapy irrespective of their TNM clinical stage and tumor histological type.

Limitation of the study

Our study is limited by a low size effect and a retrospective character; the optimal management of lung cancer patients according to biomarkers needs to be determined by prospective clinical trials in large patient cohorts.

Conclusion

Adenocarcinoma is more aggressive compared to squamous cell carcinoma showing a lower overall survival in patients. Cumulative survival was shorter by approximately 750 days in the adenocarcinoma patients in comparison with squamous cell carcinoma patients. In addition, cumulative survival was shorter in adenocarcinoma patient group in comparison with the squamous cell carcinoma group in CD31, p-63, MMP-9, and MMP-2 positive patients. Therefore, these biological markers have a significant prognostic value for NSCLC patient survival. Biological marker expression may be a useful clinical prognostic tool of therapeutic outcome.

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Subarachnoid and intracerebral hemorrhage in cocaine abusers

Subarahnoidalno i intracerebralno krvarenje kod korisnika kokaina

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Abstract

Background/Aim. Cocaine is an alkaloid extracted from the leaves of the plants *Erythroxylum coca* and *Erythroxylum novogranatense*. It can be taken orally, intranasally, intravenously, by inhalation, or intragenitally. Cocaine abuse can cause subarachnoid and intracerebral hemorrhage. The aim of this study was to determine cocaine and benzoylecgonine (BZ) concentrations in various body fluids and organs, the frequency of subarachnoid and intracerebral hemorrhage, and the relationship of concentration of cocaine and BZ in different body fluids with subarachnoid and intracerebral hemorrhage. **Methods.** The study analyzed a total of 26 autopsies reports from 2005 to 2018 with detected cocaine and/or BZ in the bodies during a forensic autopsy at the Institute of Pathology and Forensic Medicine, Military Medical Academy in Belgrade, Serbia. Brain tissue was taken for histopathological analysis and blood from the femoral vein, while urine, gastric content, brain, kidney, and liver with gallbladder samples were taken for toxicological analyses. **Results.** There were 26 autopsied patients aged 23 to 56 years (mean age 33.77±8.52); 20 (75.92%) were men and 6 (23.08%) were women. Cocaine was found in the blood of 12 (46.15%), in the urine of 15 (57.69%), and in the brain of 8 (30.77%) autopsied patients. BZ was found in the blood of 20 (76.92%), in the urine of 21 (80.77%), and in the brain of 10 (38.46%) autopsied patients. Subarachnoid hemorrhage was found in 10 (38.46%), intracerebral hemorrhage in 18 (69.23%), and both subarachnoid and intracerebral hemorrhage in 6 (23.07%) autopsied patients. Intracerebral (focal and perivascular) hemorrhage was more frequent. There were statistically significantly higher concentrations of both cocaine and BZ in most of the body fluids and organs of examinees with intracranial hemorrhage compared to examinees without hemorrhage. **Conclusion.** Subarachnoid and intracerebral hemorrhage were frequent findings in autopsied cocaine abusers. The correlation between subarachnoid and intracerebral hemorrhage and cocaine concentrations in blood was moderate. There were strong correlation between subarachnoid and intracerebral hemorrhage and BZ concentrations in almost all the samples.

Key words:
brain; cocaine; autopsy; blood; cerebral hemorrhage; cocaine-related disorders; subarachnoid hemorrhage.

Apstrakt

Uvod/Cilj. Kokain je alkaloid iz lišća biljki *Erythroxylum coca* i *Erythroxylum novogranatense*. Kokain se u organizam može uneti oralno, intranazalno, intravenski, inhalatorno i intragenitalno. Njegovom zloupotrebom mogu nastati subarahnoidalno i intracerebralno krvarenje. Cilj rada bio je da se prikažu koncentracije kokaina i benzoilekgonina (BZ) u telesnim tečnostima i organima, učestalost subarahnoidalnog i intracerebralnog krvarenja i povezanost tih krvarenja sa koncentracijama kokaina/BZ kod obdukovanih korisnika kokaina. **Metode.** Ispitivanje je obuhvatilo analizu 26 obdukcionih nalaza sa ustanovljenim prisustvom kokaina i/ili BZ iz baze podataka Instituta za patologiju i forenzičku medicinu Vojnomedicinske akademije u Beogradu, Srbija, za period 2005−2018. godine. Analizirane su vrednosti koncentracija kokaina i BZ u krvi, urinu, želudačnom sadržaju, mozgu, bubrezima i jetri sa žučnom kesom, kao i patohistološki nalaz mozga. **Rezultati.** Istraživanjem je obuhvaćeno 26 obdukovanih pacijenata starih od 23 do 56 godina (srednja vrednost 33,77 ± 8,52), 20 (75,92%) muškaraca i 6 (23,08%) žena. Toksikološko-hemijskom analizom nađen je kokain u krvi kod 12 (46,15%), u urinu kod 15 (57,69%) i u mozgu kod 8 (30,77%) obdukovanih pacijenata. U krvi je nađen BZ kod 20 (76,92%), u urinu kod 21 (80,77%) i u mozgu kod 10 (38,46%) obdukovanih pacijenata. Subarahnoidalno krvarenje ustanovljeno je kod 10 (38,46%), intracerebralno

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krvarenje kod 18 (69,23%), a subarahnoidalno i intracerebralno krvarenje zajedno kod 6 (23,07%) obdukovanih pacijenata. Intracerebralno (fokalno i perivaskularno) krvarenje bilo je češće. Koncentracije kokaina i BZ u telesnim tečnostima i organima kod obdukovanih pacijenata kod kojih je ustanovljeno intracerebralno i subarahnoidalno krvarenje bile su statistički značajno više nego kod onih koji nisu imali krvarenja. **Zaključak.** Subarahnoidalno i intracrebralno krvarenje su bili često zastupljeni kod obdukovanih pacijenata koji su koristili kokain. Korelacija između subarahnoidnog i intracerebralnog krvarenja i koncentracije kokaina u krvi bila je umerene jačine. Utvrđena je jaka korelacija između subarahnoidalnog i intracerebralnog krvarenja i koncentracija BZ u skoro svim analiziranim uzorcima.

Ključne reči:
mozak; kokain; autopsija krv; krvarenje, moždano; poremećaji izazvani kokainom; krvarenje, subarahnoidno.

Introduction

Cocaine is an alkaloid extracted from the leaves of the plants *Erythroxylum coca* and *Erythroxylum novogranatense*. There are two chemical forms of cocaine: hydrochloride salt and alkaloid, the so-called "freebase". It is usually taken orally, intranasally, intravenously, by inhalation, or intragenitally [1]. Cocaine is well absorbed, rapidly metabolized, and excreted. It is detected within 30 min in the blood or plasma. Maximal concentration of cocaine in plasma is reached between 50 and 90 min [2]. The elimination half-life of cocaine is between 30 min and 4 h, and it depends on the chronicity of abuse [3]. Most of cocaine is metabolized in the liver, except the amount of 1–9% that is excreted unchanged through the urine [4]. The two most important metabolites of cocaine are benzoylecgonine (BZ) and ecgonine methyl ester (EME) [5]. The effects of cocaine are dose-dependent and intake-dependent, and they depend on individual organism sensitivity of an user and other simultaneously used drugs. Low doses of cocaine lead to euphoria, increased motor activity, increased satisfaction, logorrhea, and rarely hallucinations. A high dose of cocaine causes hyperthermia, tachycardia, ventricular arrhythmia, elevated blood pressure, hallucinations, nausea, vomiting, anorexia, and suicidal ideas [6]. It is the second most commonly abused drug in Europe in the last twenty years with an increasing tendency. It is estimated that over 12,000,000 people in Europe aged 15 to 64 have tried cocaine at least once [7]. Cocaine abuse can frequently cause subarachnoid hemorrhage [8] and intracerebral hemorrhage [9].

The aim of this study was to analyze cocaine and BZ concentrations in the blood, urine, gastric content, liver with gallbladder, brain, and kidneys, as well as the frequency of subarachnoid and intracerebral hemorrhage, and the relationship of cocaine and BZ concentration in different body fluids with subarachnoid and intracerebral hemorrhage in cocaine abusers.

Methods

This retrospective study was conducted on archival material at the Institute of Pathology and Forensic Medicine, Military Medical Academy in Belgrade, Serbia. A total of 26 autopsy reports from 2005 to 2018 with detected cocaine and/or BZ in the organisms of autopsied patients during the forensic autopsy were analyzed. During the autopsies, brain tissue was taken to histopathological analysis and blood samples from the femoral artery, while urine, gastric content, brain, kidney, liver, and gallbladder tissue were taken for toxicological analyses.

Brain tissue samples were standardly processed. They were fixed in buffered 4% neutral formalin solution, dehydrated in increasing ethanol concentration, cleared by chloroform, infiltrated by wax in automatic tissue processing machine Leica ASP300S, (Germany) and paraffin-embedded by machine Thermo Scientific™ HistoStar™ (USA). Paraffin-embedded tissue blocks were clamped into a microtome Leica® RM2135 (Germany) for section cutting down to 4 µm thick, floated out on a water bath Leica HI1210 (Germany), picked up and placed on microscopic slides. The slides were then dried on a hot plate Leica HI1220 (Germany) for one h, dewaxed by xylene, hydrated by decreasing ethanol concentrations and water, and stained by hematoxylin and eosin (HE) staining.

Multistainer Leica ST5020 (Germany) did HE staining. When a stain was completed, the section was covered with a coverglass by DPX by automated glass coverslipper machine Leica® CV5030 (Germany). Microscopic slides were analyzed by microscope Olympus BX43 (Germany) with camera Olympus SC 50 (Germany) and software for digital photo analysis CellSense.

Brain, kidney, and liver with gallbladder tissues were prepared for toxicological analysis according to Stas-Otto-Ogier-Kohn-Abrest. Minced tissue was treated with 95% ethanol, previously acidified with tartaric acid. The proportion of the tissue sample to ethanol was 1 : 2. Alcoholic extract was filtered and evaporated into a syrup. It was distilled off under a vacuum, and the result was aqueous residue that was treated with petroleum-ether at the temperature of 60 °C to remove the fatty components. Each step of the procedure was repeated twice. The residue was treated with sodium hydrogen carbonate and taken to dryness by ether. The dry extract was mixed with 5% methanol and internal standards. The mixture was analyzed using High-Performance Liquid Chromatography (HPLC) with UV detection (Bio-Rad Diagnostics Group, Hercules, USA) and compared to the toxicological UV spectra library.

Blood, urine, and gastric content samples were prepared for toxicological analysis through the following steps: addition of 100 mL of ammonia solution (Merck, Germany) and 5 mL of chloroform (Merck, Germany), 20 min of mixing, centrifugation for 10 min at 3,000 rotations per min and evaporated to dry extract. The dry extract was analyzed using (HPLC) with UV

detection (Bio-Rad Diagnostics Group, Hercules, USA) and compared to the toxicological UV spectra library.

Data were statistically analyzed using the software package IBM SPSS Statistics Version 24. Descriptive statistical methods (minimal and maximal values, mean value ± standard deviation, frequencies) were used. Relation between variables was measured using nonparametric Mann-Whitney test and nonparametric correlation using Spearman's correlation test. The level of statistical significance was $p < 0.05$.

Results

There were 26 autopsied patients included in this study, aged 23 to 56 (33.77 ± 8,520) years, 20 (75.92%) men and 6 (23.08 %) women. BZ was detected in more blood, urine, and gastric content samples than cocaine; as well, BZ was detected in more tissue samples of the brain, kidney, and liver with the gallbladder. Cocaine was found in the blood in 12 (46.15%) autopsied patients, in the urine in 15 (57.69%), in the gastric content in 12 (46.15%), in the liver with gallbladder in 8 (30.77%), in the brain of 8 (30.77%), and in the kidneys in 8 (30.77%) of post-mortem examined patients. BZ was detected in the blood of 20 (76.92%) autopsied patients, in the urine of 21 (80.77%), in the gastric content of 16 (61.54%), in the liver with gallbladder of 14 (53.85%), in the brain of 10 (38.46%), and in the kidneys of 13 (50%) autopsied patients.

Cocaine and BZ concentrations in various samples were presented in Table 1.

The leading cause of death in all the autopsied patients was acute cocaine intoxication.

Intracranial (subarachnoid and intracerebral) hemorrhage was revealed during gross examination during autopsy and histopathologically confirmed by microscopic analysis of brain tissue in all samples. Subarachnoid hemorrhage was found in 10 (38.46%) autopsied patients. Intracerebral hemorrhage was found in 18 (69.23%) autopsied patients, including focal intracerebral hemorrhage in 4 (15.38%) (Figure 1), perivascular intracerebral hemorrhage in 6 (23.08%) (Figure 2), and both focal end perivascular intracerebral hemorrhage in 8 (30.77%) autopsied patients. Subarachnoid and intracerebral hemorrhage both were found in 6 (23.07%) autopsied patients.

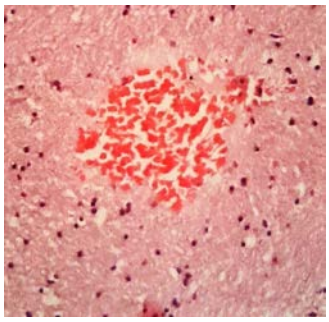

Fig. 1 – Acute focal intracerebral hemorrhage (hematoxylin eosin staining, 100×).

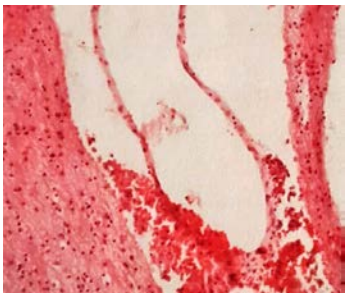

Fig. 2 – Acute perivascular intracerebral hemorrhage (hematoxylin eosin staining, 40×).

Frequencies of currently detected cocaine and BZ in various samples with subarachnoid and intracerebral hemorrhage are presented in Table 2.

Table 1

Cocaine and benzoylecgonine (BZ) concentrations in body fluids and organs

Sample	Cocaine			BZ		
	min	max	MV ± SD	min	max	MV ± SD
Blood (mg/L)	0.040	20.380	2.481 ± 5.766	0.009	10.420	1.278 ± 2.398
Urine (mg/L)	0.040	71.880	15.459 ± 25.279	0.350	684.720	63.984 ± 151.060
Gastric content (mg/L)	0.172	825.560	76.900 ± 236.008	0.003	26.190	3.441 ± 6.577
Brain (mg/g)	0.008	36.370	7.025 ± 13.562	0.0003	1154.520	118.652 ± 365.021
Kidneys (mg/g)	0.066	37.290	8.045 ± 14.620	0.005	539.550	43.022 ± 149.252
Liver with gallbladder (mg/g)	0.007	250.310	34.040 ± 87.697	0.0004	1309.750	98.933 ± 349.469

Min – minimum; Max – maximum; MV – mean values; SD – standard deviation.

Table 2

Frequencies of currently detected both cocaine and benzoylecgonine (BZ) in various samples of examinees with subarachnoid and intracerebral hemorrhage

Sample	Subarachnoid hemorrhage n (%)	Intracerebral hemorrhage n (%)	Subarachnoid and intracerebral hemorrhage, n (%)
Blood	9/10 (90)	7/18 (38.88)	5/6 (83.33)
Urine	10/10 (100)	11/18 (61.11)	6/6 (100)
Gastric content	8/10 (80)	10/18 (55.55)	6/6 (100)
Brain	5/10 (50)	8/18 (44.44)	5/6 (83.33)
Kidneys	6/10 (60)	8/18 (44.44)	6/6 (100)
Liver with gallbladder	5/10 (50)	8/18 (44.44)	5/6 (83.33)

The ratio between cocaine and BZ concentrations in samples of post-mortem examined patients with subarachnoid and intracerebral hemorrhage is shown in Table 3.

Vasculitis (infiltration of neutrophils in vessel's wall) was found histopathologically in one autopsied patient. In addition, blood vessel spasm was found in one autopsied patient, too. There was histologically revealed atherosclerosis in two cases, of which additionally thrombus was found in one, and microaneurism in the other.

Statistical analyses have shown statistically significant difference in the concentration of cocaine in the blood (p = 0.0088), gastric content (p = 0.244), liver with gallbladder (p = 0.0366), and brain (p = 0.03) between autopsied patients with and without subarachnoid hemorrhage.

There was no statistically significant difference in cocaine concentrations in the urine (p = 0.1141) and kidneys (p = 0.9296) between cases with and without subarachnoid hemorrhage.

Statistically significant difference was found in concentrations of cocaine in the blood (p = 0.034), urine (p = 0.0434), gastric content (p = 0.006), liver with gallbladder (p = 0.36), and brain (p = 0.0367) between autopsied patients with and without intracerebral hemorrhage.

There was no statistically significant difference in cocaine concentration in the kidneys (p = 0.1739) between cases with and without intracerebral hemorrhage.

Statistically significant difference was found in BZ concentrations in the blood (p = 0.027), urine (p = 0.0011), kidneys (p = 0.0041), liver with gallbladder (p = 0.0002), and brain (p = 0.0096) between cases with and without subarachnoid hemorrhage.

There was no statistically significant difference between BZ concentrations in the gastric content between cases with and without subarachnoid hemorrhage (p = 0.2187).

Statistically significant difference was found in BZ concentrations in the blood (p = 0.008), urine (p = 0.0006), kidneys (p = 0.0164), liver with gallbladder (p = 0.0002), and brain (p = 0.0025) between cases with and without intracerebral hemorrhage.

There was no statistically significant difference in BZ concentrations in the gastric fluid between cases with and without intracerebral hemorrhage (p = 0.5419).

Subarachnoid hemorrhage was statistically significantly often found in men (p = 0.0178). There was no statistically significant difference between genders in intracerebral hemorrhage presence (p = 0.63).

Correlation between cocaine and BZ concentrations in different body samples and subarachnoid and intracerebral hemorrhage presence was presented in Table 4.

Discussion

Most of the post-mortem examined patients in our study were men, which is similar to the data of the European Poison Control Centre [7]. The estimated cocaine concentrations in the blood of the autopsied patients in this study were between 0.040 mg/L and 20.380 mg/L, and those of BZ between 0.009 mg/L and 10.420 mg/L. A wide range difference of concentrations of cocaine and BZ could be attributable to cocaine pharmacokinetics, as it was confirmed in the study of Pilgrim et al. [10] in which concentrations of cocaine and BZ in the blood were found in the range between 0.01 and 3.0 mg/L in 49 autopsies of the cases with sudden death.

Having in mind the fact that blood fluctuation and metabolism stop at the moment of death, the ratio between cocaine and BZ concentration could lead to a conclusion

Table 3

Ratio between cocaine and benzoylecgonine (BZ) concentrations in samples of examinees with subarachnoid and intracerebral hemorrhage

Subarachnoid hemorrhage		Intracerebral hemorrhage	
cocaine/BZ in blood	cocaine/BZ in brain	cocaine/BZ in blood	cocaine/BZ in brain
0.320/0.170 (1.88)	18.950/1.590 (9.72)	4.060/10.420 (0.39)	18.950/1.590 (9.72)
4.060/10.420 (0.39)	36.370/0.020 (1.82)	0.070/0.610 (0.11)	36.370/0.020 (1.82)
0.410/1.490 (0.28)	0.064/0.238 (0.27)	0.410/1.490 (0.28)	0.064/0.238 (0.27)
20.380/4.120 (4.95)	0.071/0.125 (0.57)	20.380/4.120 (4.95)	0.071/0.125 (0.57)
0.040/0.040 (1.00)	0.070/0.005 (14.00)	1.235/0.204 (6.05)	0.008/0.003 (25.33)
0.120/0.260 (0.46)		1.870/2.070 (0.90)	0.123/0.016 (7.60)
0.1235/0.2043 (0.60)		0.130/1.830 (0.07)	0.070/0.005 (14.00)
0.280/0.400 (0.07)			

Note: concentrations in blood are expressed in mg/L and those in brain in mg/g.

Table 4

Correlation coefficient (r) as measure of the relationship between subarachnoid and intracerebral hemorrhage and concentrations of cocaine and benzoylecgonine (BZ)

Sample	Subarachnoid hemorrhage		Intracerebral hemorrhage	
	cocaine	BZ	cocaine	BZ
Blood	0.688	0.397	0.581	0.526
Urine		0.006	0.026	0.945
Gastric content	0.422		0.108	
Brain	0.356	0.635	0.071	0.711
Liver with gallbladder	0.101	0.775	0.052	0.971
Kidneys		0.770		0.365

r > 0.7 – strong correlation; 0.5 < r < 0.7 – moderate correlation; r < 0.5 – weak correlation.

about the duration of cocaine abuse. High cocaine and low BZ concentration or BZ absence indicate acute cocaine intoxication. BZ presence and cocaine absence in brain tissue indicates chronic cocaine abuse [11].

The ratio between cocaine and BZ concentrations can approximately indicate the time of cocaine intake of autopsied patients. Cocaine has a very short elimination half-life, thus it can be absent in samples while BZ is present. In a situation when the concentration of BZ in the brain is lower than in the blood, cocaine intake is estimated to be two hours or less before death. In our study, this was the case in seven autopsied patients, among which both subarachnoid and intracerebral hemorrhage were found in three cases and intracerebral hemorrhage in two cases. In the case of cocaine concentrations being a few times higher than BZ concentrations, the intake of cocaine was just before the death, as it was in two cases with subarachnoid hemorrhage and five cases with intracerebral hemorrhage in our study. BZ concentration in the brain higher than in the blood indicates chronic cocaine abuse, as it was in two autopsied patients in our study, one with subarachnoid and another with intracerebral hemorrhage [3]. According to the correlation coefficient, a moderate relationship was found between subarachnoid hemorrhage and cocaine concentrations in the blood, as well as between intracerebral hemorrhage and cocaine concentrations in the blood. A weak relationship was found between subarachnoid hemorrhage and cocaine concentration in gastric content and brain.

According to the correlation coefficient, a strong correlation between subarachnoid hemorrhage and BZ concentrations in the kidneys and liver with gallbladder was found. The correlation was also strong between intracerebral hemorrhage and BZ concentration in the brain, urine, and liver with gallbladder. A moderate correlation was found between subarachnoid hemorrhage and BZ concentrations in the brain and between intracerebral hemorrhage and BZ concentrations in the blood. A weak correlation was found between subarachnoid hemorrhage and BZ concentrations in the blood and intracerebral hemorrhage and BZ concentrations in the kidneys.

Both concentrations of cocaine and BZ in the blood had a moderate correlation with subarachnoid and intracerebral hemorrhage. The study on seven examinees has shown that the concentration of cocaine in the blood leads to an increase in systolic and diastolic blood pressure, which can cause blood vessel rupture and intracranial hemorrhage [12].

In a study on newborn pigs, the impact of cocaine and its metabolites on cerebral arterioles was studied. It was found that a greater amount of cocaine and BZ and higher concentrations in body fluids cause vasoconstriction of cerebral arterioles. Vasospasm was in a strong correlation with the dosage of cocaine intake and concentrations of cocaine and BZ in body fluids [13].

Stroke is more common in people that have used cocaine in alkaloid form than in the form of hydrochloride salt [14]. The relationship between cocaine and stroke leads to the risk of early onset of stroke. Some authors recommend toxicological analysis of urine and blood in stroke in young adults because of suspicious connection of cocaine and stroke [15]. The young age for stroke onset is between the 20s and 50s, as it was in our study with examinees aged between 23 and 56 years [16]. According to the literature data, arterial hypertension is the main reason for hemorrhagic stroke [17]. The indirect sympathomimetic effect of cocaine temporarily increases systolic arterial blood pressure, which can cause spontaneous bleeding from existing arteriovenous malformations, aneurysms, parts of the brain previously affected by stroke, or can cause new aneurysm [18]. Cocaine also causes an increase in blood pressure in people with hypertensive cerebral vasculopathy in subcortical brain regions, which leads to intracerebral hemorrhage, especially in those regions of the brain [19]. The results of this cohort study have shown that patients with toxicologically detected cocaine had higher arterial blood pressure values, increased risk of intraventricular cerebral hemorrhage, and greater mortality than the patients who had negative drug screens for cocaine. The most frequent localization of intracerebral hemorrhage in that study was in subcortical regions [20]. Cocaine usually leads to intracerebral hemorrhage in the basal ganglia and thalamus [3]. The way cocaine affects the intracranial hemorrhage development in autopsy cases of cocaine-related cerebrovascular disease, without histopathological changes on brain blood vessels, is still unknown [21].

Subarachnoid hemorrhage due to cocaine abuse is usually caused by arterial aneurysm rupture [22]. Aneurism rupture in cocaine abusers is more often localized in anterior and medial cerebral artery blood supply, unlike in other people where it is localized in posterior cerebral artery supply [23].

During a gross examination of autopsied patients with subarachnoid hemorrhage in our study, no arterial aneurysm was found. In spite of that, during the histopathological examination, wall thinning and dilatation of blood vessels were present in one case, which points to microaneurysm. Cocaine causes vasospasm in blood vessels, thrombosis, hypertensive vasculitis, necrotizing arteritis, thicking of the *tunica intima* of blood vessels, and atherosclerosis [24]. Vasospasm, which was detected in one case in our study, is considered a complication of subarachnoid hemorrhage, even though in cocaine abusers chronic vasospasm is caused by changes in *tunica media* and elastic lamina of blood vessels in the brain. Vasospasm leads to intimal hyperplasia by thrombocytes aggregation and activation. The wall of blood vessels becomes thicker and the obstruction occurs. Long-term cocaine abuse can cause atherosclerosis, intimal thinning, and periadventitial fibrosis of blood vessels [25]. In our study, atherosclerosis was found in two autopsied patients with BZ concentrations higher in the brain than in the blood. It can be concluded that the abuse of cocaine was chronic in those patients. Cocaine leads to an increase in plasma lipid concentrations, direct and indirect cholesterol concentrations, endothelial permeability, and more frequent inflammatory cells infiltration in atherosclerotic plaques [4]. Radiological studies using angiography examination during autopsy show vascular lesions in 78% of cocaine abusers with subarachnoid hemorrhage and 48% of cocaine abusers with intracerebral

hemorrhage. The most common vascular lesions in people with subarachnoid hemorrhages are saccular aneurysms of the anterior communicating artery, in spite of people with intracerebral hemorrhages who had arteriovenous malformations [26]. According to the study of Kibayushi et al. [27] on the evaluation of chronic changes of blood vessels in the brain, 36.84% of autopsied patients did not have vasculopathy. In our study based on histopathological examination of brain tissue, there were no morphological changes in blood vessels in the brain in 66.66% of cases. Therefore, vasculopathy is not the only cause of intracerebral hemorrhage in cocaine abusers.

In our study, mean values of cocaine and BZ concentrations in the blood and organs of post-mortem examined patients with intracranial hemorrhage were higher than in cases without hemorrhage. It can be concluded that a greater amount of cocaine intake can cause intracranial hemorrhage, regardless of the blood vessels' damage.

Histopathological analysis showed inflammatory infiltrate in the wall of blood vessels in one case. Cocaine-induced vasculitis is a rare complication of abuse and it consists of perivascular inflammatory infiltrate, partial damage of blood vessels' wall, and vascular thrombosis [28, 29]. Besides cocaine, adulterant levamisole can cause vasculitis [30]. In our study, levamisole was toxicologically detected in one case, in the so-called "body packer" [31].

Conclusion

Subarachnoid and intracerebral hemorrhage in autopsied patients, cocaine abusers, was frequent. Intracerebral (focal and perivascular) hemorrhage was more frequent. There were statistically significant higher concentrations of both cocaine and BZ in most of the body fluids and organs of autopsied patients with intracranial hemorrhage compared to the cases without hemorrhage. The correlation between subarachnoid and intracerebral hemorrhage and cocaine concentrations in the blood was moderate. There were strong correlation between subarachnoid and intracerebral hemorrhage and BZ concentrations in almost all samples.

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Composite bioscore is superior to routine biomarkers and established scoring systems in predicting mortality in adult critically ill patients with secondary sepsis

Kombinovani bioskor je superiorniji u odnosu na rutinske biomarkere i skorove u predviđanju mortaliteta kod odraslih kritično obolelih bolesnika sa sekundarnom sepsom

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Abstract

Background/Aim. Sepsis represents a significant global burden, with an estimated 48.9 million cases and 11.0 million sepsis-related deaths recently recorded worldwide. The aim of this observational study was to assess a prognostic value of some readily available routine biomarkers: presepsin, procalcitonin, C-reactive protein (CRP), white blood cell (WBC) count, platelet count, mean platelet volume (MPV), and lactate, as well as their combination regarding the outcome in a cohort of critically ill adult patients with secondary sepsis. **Methods.** A total of 86 critically ill patients with secondary sepsis due to peritonitis, pancreatitis, and severe trauma, admitted to the surgical intensive care unit, were enrolled in this prospective study. Blood samples for biomarker analysis were collected in three time points: on admission (the 1st day) and on the 3rd, and 5th day after admission. The Sequential Organ Failure Assessment (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 hours after admission (1st day). SOFA and SAPS II scores were recorded daily. The primary endpoint was hospital mortality. **Results.** Values of each applied score were expectedly significantly higher in non-survivors in all time points. Regarding investigated parameters, only presepsin levels were significantly higher in non-survivors in all time points; MPV levels on the 3rd and 5th day; serum lactate levels on the 3rd day; CRP levels and WBC count on the 5th day. Clinical accuracy of parameters in predicting lethal outcomes was investigated in all time points. On the 1st day, apart from all three scores, only presepsin demonstrated statistically significant discriminative power regarding outcome (AUC of 0.670). Apart from SAPS II and SOFA score, on the 3rd day presepsin, MPV, and lactate (AUCs of 0.716, 0.667, and 0.642, respectively) and on the 5th day presepsin, MPV, CRP, and WBC count (AUCs of 0.790, 0.681, 0.643 and 0.654, respectively) were good predictors of the lethal outcome. Composite bioscore (presepsin, MVP, and lactate) on the 3rd day had the highest AUC of 0.820 in comparison with individual scores and parameters. The independent predictor of the lethal outcome on the 1st day was presepsin ($p < 0.05$) and on the 3rd day MPV ($p < 0.01$). **Conclusion.** Composite bioscore is superior to routine biomarkers and established scoring systems in predicting mortality in adult critically ill patients with secondary sepsis.

Key words:
biomarkers; critical illness; intensive care units; mortality; sepsis; severity of illness index; prognosis.

Apstrakt

Uvod/Cilj. Sepsa predstavlja značajno globalno opterećenje, sa procenjenih 48,9 miliona slučajeva i 11 miliona smrtnih slučajeva povezanih sa sepsom godišnje širom sveta. Cilj prospektivne, opservacione studije bio je da se proceni prognostička vrednost nekih lako dostupnih, rutinskih biomarkera kao što su: presepsin, prokalcitonin, C-reaktivni

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protein (CRP), broj leukocita, srednji volumen trombocita (MPV) i laktati kao i njihove kombinacije, u smislu predviđanja ishoda sekundarne sepse kod odraslih kritično obolelih bolesnika. **Metode.** Prospektivnim istraživanjem obuhvaćeno je ukupno 86 kritično obolelih bolesnika sa sekundarnom sepsom kao komplikacijom peritonitisa, pankreatitisa i teške traume, koji su bili primljeni u hiruršku jedinicu intenzivne terapije. Uzorci krvi za određivanje biomarkera uzimani su u tri vremena: na dan prijema – prvi dan, zatim trećeg i petog dana. Prvog dana izračunati su i zabeleženi sledeći skorovi: *Sequential Organ Failure Assessment* (SOFA) skor, *Simplified Acute Physiology Score* (SAPS) II i *Acute Physiology and Chronic Health Evaluation* (APACHE) II skor. SOFA i SAPS II skorovi su izračunavani i beleženi svakodnevno. Primarni ishod bio je bolnički mortalitet. **Rezultati.** Vrednosti svih primenjenih skorova u sva tri vremena su, očekivano, bile značajno veće kod obolelih sa smrtnim ishodom. Od svih ispitivanih parametara, samo su vrednosti presepsina u sva tri vremena bile značajno veće kod umrlih; vrednosti MPV trećeg i petog dana; vrednosti laktata trećeg dana; vrednosti CRP-a i broj leukocita petog dana. U svim vremenima ispitivana je preciznost parametra u smislu predviđanja smrtnog ishoda. Prvog dana, osim sva tri skora, samo je presepsin bio statistički značajan prediktor ishoda (AUC 0.670). Osim SAPS II i SOFA skora, trećeg dana statistički značajni prediktori ishoda bili su presepsin (AUC 0.716), MPV (AUC 0.667) i laktati (AUC 0.642), a petog dana presepsin (AUC 0.790), MPV (AUC 0.681), CRP (AUC 0.643) i broj leukocita (AUC 0.654). Kombinovani bioskor (presepsin, MPV i laktati) je trećeg dana bio najbolji prediktor ishoda (AUC 0.820) u poređenju sa individualnim skorovima i parametrima. Nezavisni prediktor smrtnog ishoda prvog dana bio je presepsin ($p < 0.05$), a trećeg dana MPV ($p < 0.01$). **Zaključak.** Kombinovani bioskor je superiorniji od rutinskih biomarkera i skorova u predviđanju mortaliteta kod odraslih kritično obolelih bolesnika sa sekundarnom sepsom.

Ključne reči:
biomarkeri; kritična stanja; intenzivna nega, odeljenja; mortalitet; sepsa; bolest, indeks težine; prognoza.

Introduction

Sepsis represents a significant global burden, with an estimated 48.9 million cases and 11.0 million sepsis-related deaths recorded worldwide in 2017. The latest analysis for the global burden of disease study revealed that recorded death toll represented 19.7% of all global deaths [1]. It is evident that this life-threatening organ dysfunction, resulting from uncontrolled host response to infection, is responsible for one-fifth of all deaths despite all the latest technology and newer antibiotics. Bearing in mind the importance of mortality prediction in critically ill septic patients, over the years, investigators focused their attention on various potential biomarkers in this regard [2, 3]. So far, no specific biomarkers for mortality prediction in this patient population have been identified. Without a specific biomarker, it is difficult for clinicians to determine which patients are likely to improve and which will have a poor outcome. Interesting biomarkers in this regard encompass procalcitonin and a rather novel biomarker presepsin. Presepsin, which is a 13-kDa peptide, is another name for the soluble cluster of differentiation (CD)14 subtype (sCD14-ST). Membrane CD14 is a coreceptor for endotoxin, and during the systemic immunoinflammatory response, its soluble form is cleaved from immunocompetent cells like monocytes/macrophages [4]. Procalcitonin is a 116-amino acid polypeptide precursor of calcitonin released by the C cells of the thyroid gland [5].

The aim of this prospective, observational study was to assess the prognostic value of some readily available routine biomarkers: presepsin, procalcitonin (PCT), C-reactive protein (CRP), white blood cell count (WBC), platelet count, mean platelet (MPV) volume, and lactate regarding the outcome in a cohort of critically ill adult patients with secondary sepsis. In addition, the aim was to evaluate the combination of these biomarkers in the same regard and compare their ability to predict mortality with the use of clinical tools like established scoring systems.

Methods

Patients

A total of 86 critically ill patients with secondary sepsis due to peritonitis, pancreatitis, and severe trauma, admitted to surgical intensive care unit (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 the local Ethics Committee and informed consent from the patients or first-degree relatives. 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] [6]. The study lasted 3 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/hr for more than two hours despite adequate fluid resuscitation, acute lung injury with PaO_2/FiO_2 less than 250, blood creatinine level higher 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 normalized 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. 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. Out of 260 patients initially considered for enrolment, 174 were excluded.

Blood samples for biomarker analysis were collected in three time points: on admission (1st day) and on the 3rd, and 5th day after admission. Additionally, 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 hours after admission to the SICU (1st day) [7–9]. SOFA and SAPS II scores were recorded daily during SICU stay to assess the severity of organ dysfunction in secondary sepsis.

The use of antibiotics, circulatory volume replacement, vasoactive support, and source controlled were performed according to guidelines [10]. Various modes of mechanical ventilation and surgical procedures were performed if and when necessary in all patients. The outcome measure was hospital mortality; patients were followed until hospital discharge (survivors) or hospital death (non-survivors).

Sampling and analysis

The patient's venous blood was drawn by trained, qualified phlebotomists. The blood samples were taken into BD Vacutainer K_2 EDTA tubes and analyzed within 2 hours from venepuncture. A complete blood count was determined by Siemens Advia 120 hematology system, Siemens Healthineers Germany, which is a flow cytometry-based system. Differentiation of white blood cells is done by the peroxidase and basophil channel. On the Advia 120, the peroxidase method is a primary differential method. Advia 120 analyzer method of counting platelets is based on two-dimensional laser light scatter. The laser optics low angle and high angle scatter was used to determine the platelet count simultaneously with the red blood cells. MPV was a calculated parameter from the platelet volume histogram. For CRP determination ADVIA 1800, Siemens Healthineers Germany, was used and for procalcitonin measurement, CENTAUR Advia XP, Siemens Healthineers Germany, was used. Arterial lactate values were measured by blood gas analyzer GEM3000 Premier, Instrumentation Laboratory Werden Company Spain. For presepsin determination, Patfast compact immunoassay analyzer, Mitsubishi Chemical Europe Germany, was used. Normal ranges for these cells and biomarkers are as follows: leukocytes, 4–11.0 × 10^9/L; platelets, 130.0–400.0 × 10^9/L; CRP, 0.00–4.00 mg/L; procalcitonin, < 0.10 ng/mL; presepsin, < 360 pg/mL (reference values from our laboratory).

Statistical analysis

Complete statistical analysis of data was done with the statistical software package, SPSS Statistics 18. In the case of continuous data, variables were presented as mean value ± standard deviation (SD), median, minimal, and maximal values. Kolmogorov-Smirnov test was used for evaluating the distribution of continual data. Statistical significance between groups was tested by the Mann-Whitney or Friedman test. The Spearman's Rank Correlation analysis was used to establish the relationship between parameters. Receiving Operating Characteristics (ROC) curves were constructed and analyzed to determine the sensitivity and specificity of variables for predicting lethal outcomes (Youden index was used in all cases). Calculations of odds ratios (OR) and their 95% confidence intervals (CI) were done to determine the strength of the association between variables and outcomes. For that purpose, the most promising independent variables as single or combined risk factors were incorporated into binary logistic regression analyses. Mantel-Cox log-rank to analyze survival time between each tertile of presepsin concentration as well as Kaplan-Meier survival curve to analyze the probability of death between each tertile, were performed.

All the analyses were estimated at a $p < 0.05$ level of statistical significance.

Sample size calculation

The sample size of the study was calculated based on a 30% difference in presepsin levels between survivors and non-survivors and a 37% of mortality rate. The effect size was 0.535 and the allocation rate was 0.6. With a test power of 0.8 (80%) and a type I (alpha) error of 0.05, the analysis revealed that the sample size of 86 patients (54 survivors and 32 non-survivors) was sufficient to detect a statistically significant difference between groups. The calculation was performed by GPower 3.1 statistical program, using the Wilcoxon-Mann-Whitney test because of the high data variability.

Results

Baseline characteristics of the study population

During a 3-year period, out of 260 patients initially considered for enrolment, 174 patients were excluded. The remaining 86 patients (average age was 59 years; range 18–

Table 1

Demographic and clinical data of patients with sepsis

Parameter	Values
Patients	86
age (years), mean (range)	59 (18–89)
sex, n (%)	
male	56 (65.1)
female	30 (34.9)
SAPS II score (on the day 1) mean ± SD	37.28 ± 14.56
APACHE II score (on the day 1) mean ± SD	13.15 ± 5.96
SOFA score (on the day 1) mean ± SD	5.81 ± 3.80
Reason for ICU admission, n (%)	
severe sepsis due to:	
peritonitis	40 (46.5)
pancreatitis	18 (20.9)
trauma	28 (32.6)
Blood cultures, n (%)	
Gram-positive	8 (9.3)
Gram-negative	19 (22.1)
polymicrobial	14 (16.3)
negative blood cultures	45 (52.3)
Overall hospital mortality, n (%)	32 (37.2)

SD – standard deviation; SAPS – Simplified Acute Physiology Score; APACHE – Acute Physiology and Chronic Health Evaluation; SOFA – Sequential Organ Failure Assessment; ICU – Intensive Care Unit.

89 years; 30 females) with secondary sepsis and/or septic shock due to peritonitis (40 patients – 46.5%), pancreatitis (18 patients – 20.9%), and trauma (28 patients – 32.6%) as the underlying cause were enrolled. Out of the 86 patients, 8 patients (9.3 %) developed Gram-positive bacteraemia, 19 patients (22.1%) developed Gram-negative bacteraemia, and 14 patients (16.3%) had polymicrobial bacteraemia. In 45 patients (52.3%), no pathogen was isolated from blood culture. Injury Severity Score – ISS (determined using Abbreviated Injury Scale – AIS) was calculated and recorded in all polytrauma patients (mean ± SD): 33.82 ± 3.59. The demographic and clinical data are shown in Table 1.

Baseline laboratory characteristics of patients on the 1st, 3rd, and 5th day according to the outcome are shown in Table 2.

Values of each applied score were, expectedly, significantly higher in non-survivors in all time points. Regarding investigated parameters, only presepsin levels were higher in non-survivors, the difference reached high statistical significance in all time points. The second best were MPV levels. They were significantly higher in non-survivors in two out of three time points: on the 3rd and 5th day. Serum lactate levels were significantly higher in non-survivors on the 3rd day. Finally, CRP levels and WBC count were significantly higher in non-survivors on the 5th day.

Procalcitonin levels and platelet count did not differ significantly between survivors and non-survivors in any of the three time points.

A time course of presepsin according to hospital outcome is presented in Figure 1.

Table 2
Baseline laboratory parameters of patient with sepsis on the 1st, 3rd day, and 5th day according to outcome

Parameter	Survivors (n = 54) mean ± SD; median (min–max)	Non-survivors (n = 32) mean ± SD; median (min–max)	*p*-value
APACHE II score 1st day	11.94 ± 5.22; 11.50 (2–26)	15.19 ± 6.63; 14.50 (5–32)	0.023
SAPS II score			
1st day	34.74 ± 12.68; 35.00 (8–92)	41.56 ± 16.61; 39.50 (6–92)	0.034
3rd day	28.28 ± 12.08; 27.50 (0–58)	38.41 ± 14.34; 37.00 (12–67)	0.003
5th day	25.00 ± 12.03; 23.00 (0–58)	40.18 ± 14.07; 41.00 (0–62)	0.000
SOFA score			
1st day	5.13 ± 3.20; 4.00 (0–15)	6.97 ± 4.46; 6.00 (0–19)	0.040
3rd day	4.56 ± 3.24; 4.00 (0–12)	6.41± 3.68; 5.00 (0–15)	0.017
5th day	3.44 ± 2.90; 3.00 (0–10)	6.59 ± 3.14; 6.00 (0–11)	0.000
Presepsin (pg/mL)			
1st day	1,068.59 ± 1,105.38; 722.50 (101–5315)	1,710.78 ± 1,595.09; 1,160.50 (214.–8,144)	0.008
3rd day	920.98 ± 1,172.52; 530.00 (67.30–5880.00)	1,493.59 ± 1,816.20; 891.00 (425–9,419)	0.002
5th day	683.23 ± 991.49; 473.50 (52.60–7123.00)	1,323.86 ± 1,171.99; 836.00 (345.00–5,142)	0.000
Procalcitonin (ng/mL)			
1st day	5.51 ± 12.00; 0.92 (0.007–61.62)	7.22 ± 15.16; 1.38 (0.19–69.15)	0.281
3rd day	7.73 ± 28.26; 0.91 (0.03–185.57)	14.56 ± 57.68; 1.05 (0.08–259.48)	0.706
5th day	3.12 ± 9.42; 0.51 (0.06–60.36)	1.90 ± 3.27; 0.64 (0.11–11.10)	0.988
C-reactive protein (mg/L)			
1st day	179.65 ± 76.04; 170.37 (10.95–362.87)	174.24 ± 94.75; 175.44 (6.37–396.97)	0.818
3rd day	165.29 ± 80.36; 152.77 (14.97–412.79)	156.69 ± 83.78; 161.71 (10.15–312.08)	0.794
5th day	126.98 ± 70.39; 115.28 (11.16–305.34)	156.72 ± 53.85; 153.63 (46.08–250.38)	0.048
WBC count (10^9/L)			
1st day	14.62 ± 8.33; 13.50 (2.09–34.71)	14.26 ± 6.84; 13.55 (4.55–34.36)	0.993
3rd day	13.08 ± 7.39; 10.76 (3.24–38.45)	13.04 ± 3.87; 13.76 (4.81–23.76)	0.179
5th day	11.26 ± 4.21; 10.85 (3.25–21.90)	14.39 ± 5.63; 13.15 (8.28–30.61)	0.036
Platelet (10^9/L)			
1st day	241.42 ± 142.75; 199.00 (53.20–623.00)	194.87 ± 115.52; 165.50 (25.00–503.00)	0.211
3rd day	255.76 ± 169.12; 210.00 (61.10–735.00)	196.31 ± 87.92; 191.00 (48.00–409.00)	0.302
5th day	281.36 ± 167.18; 238.50 (52.60–724.00)	219.02 ± 107.78; 232.00 (49.10–399.00)	0.200
MPV (fL)			
1st day	9.00 ± 1.37; 8.55 (7.00–12.90)	9.22 ± 1.54; 9.05 (7.10–16.10)	0.437
3rd day	8.85 ± 1.31; 8.55 (6.60–13.00)	9.57 ± 1.49; 9.20 (7.10–14.60)	0.015
5th day	8.86 ± 1.65; 8.50 (6.50–14.30)	9.71 ± 1.77; 9.25 (7.00–15.00)	0.014
Lactate (mmol/L)			
1st day	2.00 ± 1.90; 1.20 (0.50–9.70)	2.03 ± 2.58; 1.20 (0.60–15.00)	0.707
3rd day	1.06 ± 0.66; 0.80 (0.20–4.30)	1.59 ± 1.42; 1.20 (0.30–7.40)	0.038
5th day	0.90 ± 0.26; 0.80 (0.50–1.60)	1.34 ± 0.97; 0.95 (0.20–4.30)	0.164

SD – standard deviation; WBC – white blood count; MPV –mean platelet volume.
For other abbreviations see under Table 1.

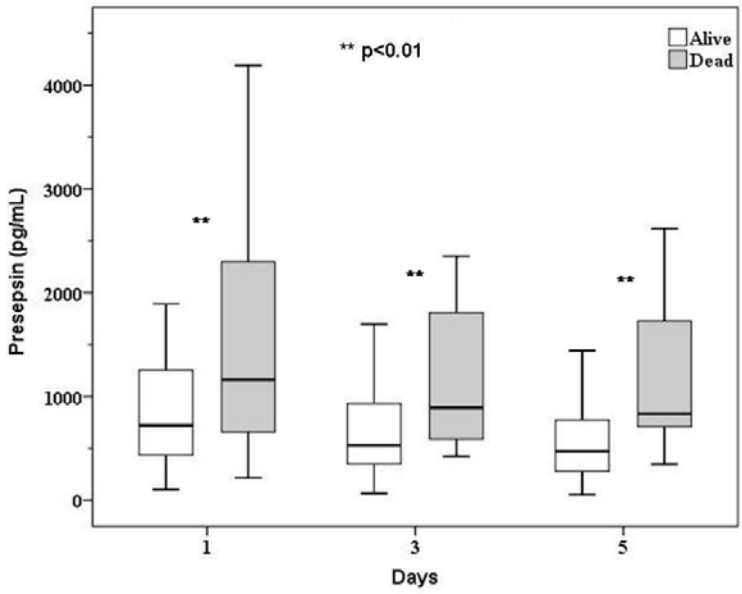

Fig. 1 – Levels of presepsisn in all three-time points according to hospital outcome.

Comparison and correlation of parameters in three time point intervals (1st, 3rd, and 5th day)

Of all measured parameters, only WBC count and MPV did not differ significantly between time point intervals within groups (survivors, non-survivors). Presepsin, PCT, CRP, and serum lactate values differ significantly within survivors; platelet count differs significantly within both survivors and non-survivors. *Post hoc* pairwise comparisons were determined exactly between which time points statistically significant difference in parameter levels occurred (Table 3). Interestingly, except for platelet count, which differed significantly within both survivors and non-survivors in all three time points, all other parameters differ significantly within survivors, but not within non-survivors.

The Spearman's test of correlation between investigated parameters in all three time points was performed. On the 1st day, there was a statistically significant positive correlation between serum lactate and PCT (rho = 0.414; $p = 0.005$) in survivors. Additionally, there was a statistically significant positive correlation between presepsin and PCT in non-survivors (rho = 0.466; $p = 0.014$).

On the 3rd day, there was a statistically significant positive correlation between presepsin and PCT (rho = 0.503; $p = 0.000$) and MPC and lactate (rho = 0.279; $p = 0.041$) in survivors. There were no statistically significant correlations between parameters in non-survivors at this time point.

wOn the 5th day, there was a statistically significant positive correlation between presepsin and PCT (rho = 0.504; $p = 0.000$) as well as between presepsin and WBC count (rho = 0.452; $p = 0.001$) in survivors. On the other hand, in non-survivors, there was a statistically significant positive correlation between presepsin and PCT (rho = 0.465; $p = 0.045$) and lactate and WBC count (rho = 0.662; $p = 0.001$).

In general, regarding all patients, there were several sta-

Table 3

Comparison of presepsin, procalcitonin, C-reactive protein, platelet count, and serum lactate levels between time points within groups

Time point comparison*	Presepsin		Procalcitonin		C-reactive protein		Platelet count		Serum lactates	
	survivors	non-survivors	survivors	non-survivors	survivors	non-survivors	survivors	non-survivors	survivors	non-survivors
All time points z (*p*-value)	23.284 (< 0.01)	0.273 (> 0.05)	9.682 (< 0.01)	3.763 (> 0.05)	19.241 (< 0.01)	1.263 (> 0.05)	19.411 (< 0.01)	6.909 (< 0.05)	28.583 (< 0.01)	1.848 (> 0.05)
3rd vs. 1st day z (*p*-value)	-3.225 (< 0.01)	n.s.	-0.755 (> 0.05)	n.s.	-0.984 (> 0.05)	n.s.	-1.567 (> 0.05)	-0.432 (> 0.05)	-4.591 (< 0.01)	n.s.
5th vs. 1st day z (*p*-value)	-3.687 (< 0.01)	n.s.	-2.210 (< 0.05)	n.s.	-3.684 (< 0.01)	n.s.	-3.200 (< 0.01)	-1.737 (< 0.05)	-4.817 (< 0.01)	n.s.
5th vs. 3rd day z (*p*-value)	-2.734 (< 0.01)	n.s.	-3.496 (< 0.01)	n.s.	-3.799 (< 0.01)	n.s.	-3.723 (< 0.01)	-2.078 (< 0.05)	-1.825 (< 0.05)	n.s.

***Fridman test; n.s. – non significant.**

tistically significant and highly significant positive correlations between some of the investigated parameters. Regardless of statistical significance, most of the correlations were weak (rho below or around 0.5) except for the correlation between lactate and WBC count on the 5th day in non-survivors, which was good (rho = 0.66).

Clinical accuracy of baseline parameters in predicting lethal outcome

Clinical accuracy of baseline parameters in predicting lethal outcome was investigated in all time points. On the 1st day, apart from all three scores, only presepsin demonstrated statistically significant discriminative power regarding the outcome. Levels of all three scores, as well as presepsin higher than cut-off values, were moderate predictors of lethal outcome (Table 4).

On the 3rd day, apart from SAPS II and SOFA score, presepsin, MPV, and lactate demonstrated statistically significant discriminative power regarding the outcome. Levels of two scores, as well as presepsin, MPV, and lactate higher than cut-off values, were good predictors of lethal outcome (Table 5).

On the 5th day, apart from SAPS II and SOFA score, presepsin, MPV, CRP, and WBC count demonstrated statistically significant discriminative power regarding the outcome. Levels of two scores, as well as presepsin, MPV, CRP, and WBC count higher than cut-off values, were very good predictors of lethal outcome (Table 6).

A combination of presepsin, MVP, and lactate into one composite bioscore on the 3rd day was performed in order to determine whether it would increase their discriminative power, which is prognostic ability regarding lethal outcome. Individual values were scored as 1 because they were all above previously determined ROC curve cut-off levels; this composite bioscore ranges from 0 to 3 points.

On the 3rd day, the composite bioscore demonstrated statistically highly significant discriminative power regarding the outcome. Levels higher than cut-off values were very good predictors of lethal outcome (Table 7).

A percentage of non-survivors according to each bioscore point value on the 3rd day is shown in Figure 2.

Table 4

Clinical accuracy of baseline parameters in predicting lethal outcome on the 1st day

Parameter	AUC ROC	*p*-value	95% confidence interval		Cut-off value	Sensitivity (%)	Specificity (%)	Youden index
			lower bound	upper bound				
APACHE II	0.647	0.023	0.524	0.770	15.50	43.8	81.5	0.25
SAPS II	0.637	0.034	0.511	0.764	47.50	34.4	92.6	0.27
SOFA	0.623	0.045	0.498	0.748	5.50	56.3	68.5	0.25
Presepsin	0.670	0.009	0.554	0.786	812.50	71.9	57.4	0.29

AUC ROC – area under the receiver operator characteristic curve.
For other abbreviations see under Table 1.

Table 5

Clinical accuracy of baseline parameters in predicting lethal outcome on the 3rd day

Parameter	AUC ROC	*p*-value	95% confidence interval		Cut-off value	Sensitivity (%)	Specificity (%)	Youden index
			lower bound	upper bound				
SAPS II	0.701	0.003	0.580	0.821	3 0.50	74.1	63.0	0.37
SOFA	0.662	0.018	0.539	0.784	3.50	88.9	64.8	0.35
Presepsin	0.716	0.002	0.606	0.825	539.50	88.9	51.9	0.41
MPV	0.667	0.015	0.546	0.788	8.65	85.2	53.7	0.39
Lactate	0.642	0.039	0.503	0.781	1.15	55.6	70.4	0.26

AUC ROC – area under the receiver operator characteristic curve; MPV – mean platelet volume.
For other abbreviations see under Table 1.

Table 6

Clinical accuracy of baseline parameters in predicting lethal outcome on the 5th day

Parameter	AUC ROC	*p*-value	95% confidence interval		Cut-off value	Sensitivity (%)	Specificity (%)	Youden index
			lower bound	upper bound				
SAPS II	0.813	0.000	0.696	0.929	39.00	68.2	9 0.7	0.59
SOFA	0.761	0.000	0.645	0.876	3.50	9 0.9	55.6	0.46
Presepsin	0.790	0.000	0.688	0.893	639.50	81.8	72.2	0.54
MPV	0.681	0.014	0.557	0.806	8.85	81.8	61.1	0.43
CRP	0.643	0.040	0.518	0.779	118.56	81.0	51.9	0.33
WBC count	0.654	0.036	0.518	0.791	14.90	45.5	85.2	0.31

AUC ROC – area under the receiver operator characteristic curve; MPV – mean platelet volume; CRP – C-reactive protein; WBC – white blood cell.
For other abbreviations see under Table 1.

Table 7

Clinical accuracy of composite bioscore in predicting lethal outcome on the 3rd day

Composite bioscore	AUC ROC	*p*-value	95% confidence interval		Cut-off value	Sensitivity (%)	Specificity (%)	Youden index
			lower bound	upper bound				
	0.820	0.000	0.701	0.895	2.00	75.8	78.0	0.51

AUC ROC – area under the receiver operator characteristic curve.

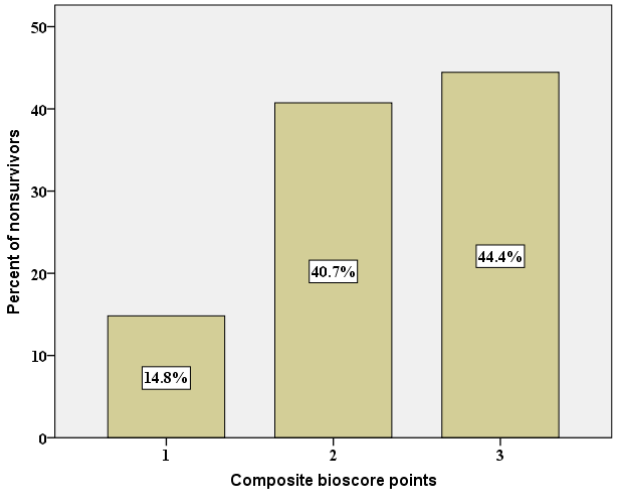

Fig. 2 – Percentage of non-survivors according to each bioscore point value on the 3rd day.

The association of investigated parameters with lethal outcomes was assessed by univariate logistic regression analyses. OR with 95% CI was calculated for each parameter. A 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 each time point. In Table 8 univariate and multivariate logistic regression analyses of parameters for predicting lethal outcome on the 1st, 3rd, and 5th day are shown.

The only independent predictor of lethal outcome by multivariate logistic regression analysis on the 1st day was presepsin.

In the second time point, on the 3rd day, univariate logistic regression analyses of all parameters showed statistical significance only for MPV. This biomarker remained an independent predictor of lethal outcome by multivariate logistic regression analysis on the 3rd day.

In the third time point, on the 5th day, univariate logistic regression analyses of all parameters showed statistical significance only for WBC count and lactate. Both WBC count and lactate lost statistical significance by multivariate logistic regression analysis on the 5th day, therefore, they were not independent predictors of lethal outcome in this time point.

Table 8

Univariate and multivariate logistic regression analyses of parameters for predicting lethal outcome on the 1st, 3rd, and 5th day

Parameter	OR	Univariate logistic regression analysis			OR	Multivariate logistic regression analysis		
		95% CI		*p*-value		95% CI		*p*-value
1st day								
presepsin	1.000	1.000	1.001	0.040	1.000	1.000	1.001	0.035
procalcitonin	1.010	0.974	1.046	0.596				
C-reactive protein	0.999	0.994	1.005	0.771				
WBC count	0.994	0.939	1.052	0.833				
MPV	1.113	0.822	1.507	0.489				
lactate	1.006	0.822	1.231	0.953				
3rd day								
MPV	1.440	1.017	2.039	0.030	1.634	1.110	2.405	0.008
5th day								
WBC count	1.146	1.025	1.280	0.016				
lactate	4.063	1.303	12.666	0.016				

OR – odds ratio; CI – confidence interval; MPV – mean platelet volume; WBC – white blood cell.

Mantel-Cox log-rank to analyze survival time between each tertile of presepsin concentration, as well as Kaplan-Meier survival curve to analyze the probability of death between each tertile, were performed. Analysis of medians of survival in days, in three presepsin concentration tertiles, revealed that for our patient population, day 50 was the critical one. On the 50th day, the estimated mortality was 50%.

Log-rank pairwise comparisons demonstrated that there was a statistically significant difference between tertiles in survival time (shown in Table 9 and Figure 3).

presepsin and PCT in non-survivors on the 1st and 3rd day, yet PCT levels, as well as platelet count, did not differ significantly between survivors and non-survivors in any of three time points. Clinical accuracy of baseline parameters in predicting lethal outcome was investigated in all time points. On the 1st day, apart from all three scores, only presepsin demonstrated statistically significant discriminative power regarding the outcome. Levels of all three scores, as well as presepsin, higher than cut-off values, are moderate predictors of lethal outcome. On the 3rd day, apart from SAPS II and

Table 9

Log rank pairwise comparisons of survival time between presepsin tertiles on the 1st day

	Presepsin tertiles on the 1st day	χ^2	*p*-value
Mantel Cox Log Rank	tertile 1/tertile 2	3.857	0.040
	tertile 1/tertile 3	4.671	0.020

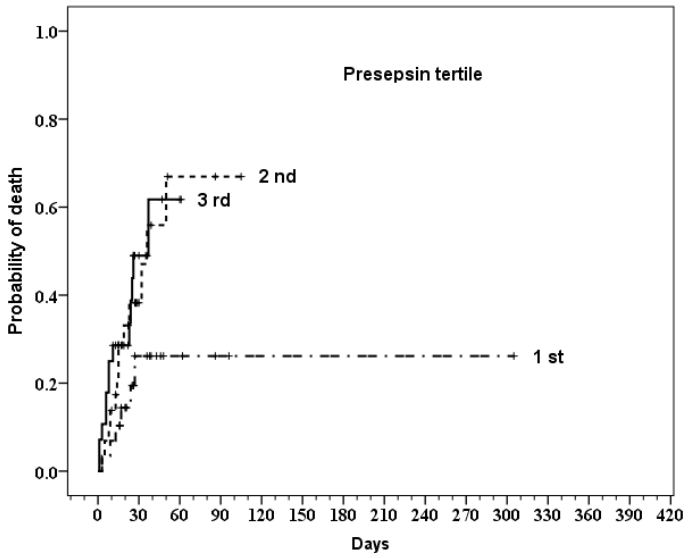

Fig. 3 – Kaplan-Meier survival curves for hospital mortality by tertiles of presepsin concentration on the 1st day.

Discussion

Treating everyone empirically with antibiotics and supportive measures is a difficult endeavor and often non-effective in some cases. Therefore, it is important to identify high-risk patients. Individual biomarkers and/or their various combinations can provide beneficial prognostic information regarding mortality in adult patients with sepsis. Mortality prediction is also an important factor in patient stratification. In our study, only presepsin levels were higher in non-survivors. The difference reached high statistical significance in all time points (on the 1st day – on admission, and then on the 3rd and 5th day after admission). The second best were MPV levels. They were significantly higher in non-survivors in two out of three time points: on the 3rd and 5th day. Serum lactate levels were significantly higher in non-survivors on the 3rd day. Finally, CRP levels and WBC count were significantly higher in non-survivors on the 5th day. There was a statistically significant positive correlation between SOFA score, presepsin, MPV, and lactate demonstrated statistically significant discriminative power regarding the outcome. On the 5th day, apart from SAPS II and SOFA score, presepsin, MPV, CRP, and WBC count demonstrated statistically significant discriminative power regarding the outcome. Levels of two scores, as well as presepsin, MPV, CRP, and WBC count higher than cut-off values, were very good predictors of lethal outcome. The combination of presepsin, MVP, and lactate into one composite bioscore on the 3rd day was performed in order to determine whether it would increase their discriminative power. On the 3rd day, composite bioscore had the highest AUC/ROC of 0.82 and the best combination of sensitivity and specificity, which is obvious from the high Youden index (above 0.5) compared to individual scores and parameters. Only on the 5th day, SAPS II score and presepsin reached the prognostic value of composite bioscore. It should be noted that it happened two days later, which is rather a long period of time when critically ill septic patients are concerned. Finally, the independ-

ent prognostic significance of parameters in predicting lethal outcome was assessed by univariate logistic regression analyses. A 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 each time point. Only presepsin was an independent predictor of lethal outcome on the 1st day and MVP on the 3rd day by multivariate logistic regression analysis.

Our group has been investigating the predictive value of various parameters in critically ill patients for over a decade [11]. It is obvious that, in our study, the best choice of a predictive biomarker in this clinical setting was presepsin. This immuno-biomarker has a complex biological role. Apart from pro-inflammatory properties, it should be noted that sCD14 receptors can prevent cytokine release and facilitate endotoxin transfer to lipoproteins, which are both anti-inflammatory actions, by the virtue of competing with the membrane-bound forms for the free ligand [12]. Presepsin has gained significant attention over the last five years primarily because of the availability of the point-of-care Mitsubishi Pathfast™ compact immunoassay analyzer, with the clinically acceptable turnaround time for obtaining results being less than 20 min. This is important for both diagnostic and therapeutic clinical decisions. Landmark study regarding presepsin as a biomarker in critically ill septic patients is the analysis of data from the multicenter Albumin Italian Outcome Sepsis (ALBIOS) trial [13]. In a multicenter ALBIOS trial, 997 critically ill septic patients were recruited. Presepsin was measured in three time points: on the 1st, 2nd, and 7th day after admission. This timeline was slightly different than ours. Higher presepsin levels on the 1st day were associated with lethal outcome, which is in accordance with our results. Unlike the 90-day mortality rate in the ALBIOS trial, our outcome measure was hospital mortality which is time-consuming but more comprehensive. Levels of presepsin in our study (divided into tertiles) were comparable to those in the ALBIOS trial. ALBIOS investigators reported that presepsin concentration on the 1st day is an independent predictor of lethal outcome by multivariate logistic regression analysis, same as in our study. In the ALBIOS trial, the authors added a clinical model (which included all significant risk factors for mortality) to presepsin concentration on the 1st day in order to improve prognostic accuracy (AUC/ROC). This is a similar approach to our composite bioscore (presepsin, MPV, lactate), which improved AUC/ROC to 0.80 but on the 3rd day, in comparison with presepsin alone on the 1st day with AUC/ROC of 0.67. Interestingly, ALBIOS authors never reported AUC/ROC for presepsin alone, but only for the combination of presepsin and clinical model or clinical model alone. AUC/ROC for presepsin and clinical model was 0.80 in the ALBIOS trial, which is the same as AUC/ROC for composite bioscore in our study. ALBIOS investigators achieved this earlier, on the 1st day; in our study, it was achieved on the 3rd day. However, this is not comparable because, in the ALBIOS trial, investigators used 9 clinical components in the clinical model to add to presepsin. Most of these nine components include the length of stay, duration of infection, time to change in one or another aspect of therapy, etc. Therefore, data can be obtained only after a considerable amount of time and retrospectively. In contrast, we added only two readily available laboratory parameters, MPV and lactate, in our composite bioscore.

Brodska et al. [14] conducted a comparable study regarding diagnostic and prognostic values of presepsin versus established biomarkers in critically ill patients with sepsis (n = 30) and SIRS after cardiac surgery (n = 30). Among other things, they tested the hypothesis that presepsin, as a novel biomarker, can outperform traditional biomarkers as a predictor of 28-day mortality. Similar to our study, they also analyzed procalcitonin, CRP, and lactate in this regard. Opposite to our results, authors reported that all investigated biomarkers were significantly associated with mortality on the 1st day with comparable values of AUC/ROCs. In our study, only presepsin demonstrated statistically significant discriminative power regarding outcome on the 1st day. Moreover, in contrast to our results, they reported that multiple regression analyses showed independent associations of CRP and lactate with mortality. In our study, the independent predictor of lethal outcome was presepsin on the 1st day and MPV on the 3rd day. Lactate showed statistical significance in mortality prediction on the 5th day by univariate logistic regression analysis, yet statistical significance was lost by multivariate logistic regression analysis. Presepsin is a novel biomarker to diagnose sepsis, but its prognostic value has not been comprehensively reviewed until recently. Yang et al. [15] performed a systematic review and meta-analysis of the prognostic value of presepsin in adult septic patients and concluded that the 1st-day presepsin levels had prognostic value to predict mortality in adult patients with sepsis regardless of sepsis severity. However, they noted that further research is warranted for unified clinical information. In accordance with our results are the findings from Behnes et al. [16], who assessed the prognostic utility of presepsin in 116 critically ill septic patients. They reported that presepsin levels on the 1st, 3rd, and 8th day revealed significant prognostic value for 30 days and 6 months all-cause mortality (presepsin: range of AUCs 0.64 to 0.71, $p < 0.02$). Furthermore, just like in our study, in all three-time points, levels of procalcitonin and CRP were not statistically significant predictors of outcome. We had only one exemption, the CRP had an AUC of 0.64 on the 5th day with borderline statistical significance ($p = 0.04$). A similar smaller study was performed by El-Shafie et al. [17]. They enrolled 31 patients and measured presepsin and CRP on admission, on the 2nd and 4th day. The authors reported that all presepsin values were significantly higher in non-survivors while none of the CRP levels were significantly different between survivors and non-survivors. Additionally, ROC analysis was performed and the authors reported slightly higher AUCs in all-time points, compared to ours: 0.75, 0.80, and 0.83, respectively. Recently, another study comparing presepsin with procalcitonin and CRP as predictors of sepsis outcome has been published [18]. Fifty-five patients were enrolled and presepsin, procalcitonin, and CRP were measured on ad-

mission, 24 and 72 hours later. As in our study, the primary outcome was hospital mortality. Our study does not agree with the findings of Mahnod et al. [18], who reported that none of the investigated biomarkers (including presepsin, which was a good predictor of the outcome on admission in our trial) showed predictive ability regarding outcome on admission. Both presepsin and CRP showed good discriminative power at 24 and 72 hours while procalcitonin reached that ability at 72 hours. This is partially in accordance with our data regarding presepsin being a good predictor of mortality on the 3rd day; yet, in contrast to our data were the results regarding CRP and PCT. We found no predictive ability for either one at this time point. The idea of adding biomarkers to improve predictive ability is gaining momentum. An interesting approach to this problem was presented by Kim et al. [19]. In a retrospective study, authors opted to measure several biomarkers (including presepsin and procalcitonin) in leftover blood samples of 157 septic patients; the outcome measure was 30-day mortality. In accordance with our results, their data showed that PCT could not predict 30-day mortality and that AUC for presepsin was 0.68. Their multi-marker panel had an AUC of 0.77, AUC for SOFA score was 0.61. In our study, AUC for composite bioscore was higher, 0.82. A narrative review regarding novel biomarkers for sepsis reiterated the fact that sepsis, as a heterogeneous complex syndrome, is still incompletely understood and that literature regarding many of the established and emerging sepsis biomarkers produced conflicting results so far [20]. Utility, performance and validity of these biomarkers should be extensively tested. Presepsin and PCT are often investigated in their capacity to differentiate between bacterial systemic inflammatory response syndrome and nonbacterial one, which is their usefulness in early infection detecting [21–24]. In a most recent study, the authors enrolled 31 patients who underwent emergency abdominal surgery with abdominal infections. This patient population is comparable to our peritonitis and pancreatitis subgroups. They investigated preoperative levels of presepsin, CRP, and PCT and their correlation with clinical course and 90-day mortality [25]. Moreover, as in our study, they performed a multi-marker approach which is, especially, our composite bioscore. They reported that presepsin had the highest predictive value (AUC of 0.86) for mortality as opposed to previously established blood biomarkers like PCT, which is in accordance with our results. However, opposite to our results, their multi-marker approach, which included presepsin, PCT and interleukin-6, showed no additional predictive value over presepsin alone. The authors noted a very interesting fact that, although presepsin outperformed PCT, the latter is approved in the United States by the Food and Drug Administration (FDA) as a predictive sepsis marker.

Roughly one-third of our patient population was a group of trauma critically ill patients who developed secondary sepsis. In a recent systematic review [26], the prognostic value of serum PCT in critically ill trauma patients was investigated with conflicting results: out of six studies regarding PCT outcome prediction ability, four found significantly higher levels of PCT in non-survivors, which is in contrast to our results, while two demonstrated no association between PCT levels and lethal outcome, which is in accordance with our data. In our present study, CRP levels showed a statistically significant difference between survivors and non-survivors only on the 5th day, which is, from the clinical point of view, a rather late predictor of lethal outcome with an AUC of 0.64. On the 1st and 3rd day, there were no statistically significant differences in CRP levels between survivors and non-survivors, which is in accordance with our previous study demonstrating AUC for this biomarker being < 0.55, thus CRP failed to predict lethal outcome in a similar patient population [27].

Our composite bioscore showed statistically highly significant discriminative power regarding the outcome. Apart from presepsin, MPV and lactate levels were included. We demonstrated, in our previous research, that MPV was an independent predictor of lethal outcome in critically ill and injured patients who developed secondary sepsis [3]. Lactate levels are routinely used to assess circulatory function and tissue perfusion. Higher levels are thought to be associated with circulatory dysfunction and impaired tissue perfusion. Nonetheless, there is no clear-cut relationship and interpretation of results should be performed cautiously. Persistent hyperlactatemia may be the result of decreased clearance, not increased production. Additionally, when adrenalin is administered to the patients, the production of lactate can be increased in the presence of adequate tissue oxygenation. Lactate may be a substrate for metabolism, may be increased in liver dysfunction, and finally, may persist with or without tissue hypoperfusion [28]. In our study, lactate levels were statistically significantly higher in non-survivors on the 3rd day and at that time point demonstrated statistically significant discriminative power regarding outcome; lactate levels higher than cut-off values were good predictors of lethal outcome with an AUC of 0.64. These results are in accordance with other similar studies [29, 30].

Although we calculated study power and complied with the computed sample size, roughly two-thirds of critically ill patients with secondary sepsis had to be excluded primarily because of malignant disease. Therefore, for confirmation of our findings, a larger trial is warranted.

Sepsis continues to be a leading cause of death in hospitalized patients, with nearly 30 million patients worldwide and nearly 6 million deaths due to sepsis each year. Despite exhaustive investigations, there are no specific markers of sepsis yet. Investigators and clinicians alike are working on developing algorithms for early sepsis detection in order to improve survival. A major problem for early sepsis diagnosis, as well as early prognosis of sepsis outcome is highly expressed heterogeneity and significant variability in this patient population. Recently, an interesting study regarding the early prediction of sepsis from clinical data was published [31]. Authors concluded that diverse computational approaches predict the onset of sepsis several hours before clinical recognition, but generalizability to different hospital systems remains a challenge. Currently, more than 175 biomarkers have been studied in sepsis; the majority are inflammatory proteins [32].

Conclusion

Our research demonstrated that composite bioscore (presepsin, MPV, and lactate) is superior to routine biomarkers like PCT and CRP, as well as established scoring systems (APACHE II, SAPS II, SOFA) in predicting mortality in adult critically ill patients with secondary sepsis. Independent predictors of lethal outcome were also components of composite bioscore: presepsin on the 1st day and MPV on the 3rd day. That is clinically relevant because it is early enough to identify high-risk patients in order to improve their survival.

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High number of CD14$^+$B7H4$^+$ monocytes is significantly associated with increased concentrations of IL-4, IL-13, IL-10, and TGF-β_1 in tumor microcirculation of lung carcinoma

Visoke vrednosti CD14$^+$B7H4$^+$ populacije monocita su značajno povezane sa povišenim koncentracijama IL-4, IL-13, IL-10 i TGF-β_1 u tumorskoj mikrocirkulaciji karcinoma pluća

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Abstract

Background/Aim. Lung cancer (LC) is one of the leading causes of mortality. Disease progression and advanced disease are characterized by the unprotective immune response due to M2 macrophage polarization, myeloid-derived suppressor cells (MDSC) activity, cytokine imbalance, and regulatory T lymphocyte activity. The aim of this study was to investigate the association between Th_1/Th_2 cytokines and CD14$^+$B7H4$^+$ monocyte (Mo) number in LC patients. **Methods.** We investigated principal Th_1/Th_2 cytokines and CD14$^+$B7H4$^+$ Mo number in blood and tumor microcirculation samples of 41 LC patients (III and IV clinical stage) and 30 healthy participants (control group). **Results.** The serum concentrations of investigated cytokines in all patients vs. heathy controls did not differ significantly. Stratification in groups according to tumor histology, disease extent, and tumor size revealed significant differences. LC patients with different histology types demonstrated significant differences, both in serum and tumor microcirculation samples. The presence of metastasis was associated with increased IFN-γ/IL-4 in blood and increased IL-13 in tumor microcirculation samples. Tumor microcirculation samples of the largest tumors were characterized by the Th_2 cytokine profile. Investigation of CD14$^+$B7H4$^+$ Mo in blood samples demonstrated a significant association of extreme value of this cell population with elevated IL-2/IL-13. Patients with the highest CD14$^+$B7H4$^+$ Mo number in tumor microcirculation samples demonstrated significant increments of IL-4, IL-13, IL-10, and TGF-β_1. **Conclusion.** LC patients demonstrated polarization of cytokine response associated with microenvironment origin, tumor histology type, tumor size, and disease extent. The highest number of CD14$^+$ B7H4$^+$ monocytes is significantly associated with the Th_2 cytokine profile.

Key words:
antigens, cd; blood; cytokines; histological techniques; lung neoplasms; monocytes.

Apstrakt

Uvod/Cilj. Karcinom pluća (KP) je jedan od vodećih uzroka smrtnosti. Napredovanje bolesti i uznapredovala bolest karakterišu se neprotektivnim imunološkim odgovorom zbog polarizacije M2 makrofaga, aktivnosti supresorskih ćelija mijeloidnog porekla (MDSC), neravnoteže citokina i regulatorne aktivnosti T limfocita. Cilj rada bio je da se utvrdi povezanost između Th_1/Th_2 citokina i CD14$^+$B7H4$^+$ broja monocita (Mo) kod bolesnika sa KP. **Metode.** Ispitali smo glavne Th_1/Th_2 citokine i broj CD14$^+$B7H4$^+$ Mo u uzorcima iz krvi i mikrocirkulacije tumora 41 bolesnika sa KP (III i IV klinički stadijum) i 30 zdravih ispitanika (kontrolna grupa). **Rezultati.** Koncentracija ispitivanih citokina u serumu bolesnika u odnosu na zdrave ispitanike nije se značajno razlikovala. Stratifikacija u histološkim grupama tumora, obimu bolesti i veličini tumora otkrila je značajne razlike. Kod bolesnika sa KP različitih histoloških tipova utvrđene su značajne razlike, kako u uzorcima seruma tako i u mikrocirkulaciji tumora. Prisustvo metastaza bilo je povezano sa povećanjem IFN-γ/IL-4 u krvi i povećanjem IL-13 u uzorcima mikrocirkulacije tumora. Uzorci

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mikrocirkulacije najvećih tumora okarakterisani su Th_2 citokinskim profilom. Ispitivanje $CD14^+B7H4^+$ Mo u uzorcima krvi pokazalo je značajnu povezanost ekstremne vrednosti te ćelijske populacije sa povišenim udelom IL-2/IL-13. Bolesnici sa najvećim brojem $CD14^+B7H4^+$ Mo u uzorcima mikrocirkulacije tumora pokazali su značajan porast IL-4, IL-13, IL-10 i TGF-β_1. **Zaključak.** Bolesnici sa KP pokazuju polarizaciju citokinskog odgovora povezanu sa vrstom mikrookoline, histološkim tipom tumora, veličinom tumora i proširenošću bolesti. Najveće vrednosti broja $CD14^+B7H4^+$ monocita značajno su povezane sa Th_2 citokinskim profilom.

Ključne reči:
antigeni, cd; krv; citokini; histološke tehnike; pluća, neoplazme; monociti.

Introduction

Lung cancer (LC) is one of the leading causes of mortality. Advanced disease is associated with a minimal survival rate and lack of therapeutic possibilities [1]. Two histologically distinct entities, small cell LC (SC-LC) and group of non-small cell LC (NSC-LC), differ significantly in biological behavior, clinical manifestations, therapeutic response, and outcome [2].

Although immune cells establish a dynamic relationship with LC cells from the initial lesion, various mechanisms induce an inadequate anti-tumor response, resulting in the disease progression. M2 macrophage polarization [3], myeloid-derived suppressor cells (MDSC) activity [4], cytokine imbalance [5, 6], and regulatory T lymphocyte activity [7] are recognized as important factors that contribute to immunosuppression essential for LC progression. More than 20 years ago, Asselin-Paturel et al. [8] demonstrated that immune response in NSC-LC patients (both intratumor and systemic) corresponds to the so-called Th2 cytokine profile. They found that both tumor cells and tumor-infiltrating leukocytes produced cytokines and that advanced disease is associated with high interleukin (IL)-6, IL-10, transforming growth factor (TGF)-β_1, and absent/low IL-2, IL-4, interferon (IFN)-γ, granulocyte macrophage-colony stimulating factor (GM-CSF) (mRNA presence). From that point, further studies demonstrated that there is a polarization of cytokine response towards Th_2 type, at least in the population of patients with NSC-LC tumor type. Investigation of serum samples of LC patients demonstrated that IL-4/IL-10 are significantly increased, while IL-2/IFN-γ are significantly decreased in NSC-LC patients [9–12], surgical tumor reduction resulted in Th_2 decrease/Th increase [11] and that peripheral blood mononuclear cells of LC progressors contained a high volume of IL-4 mRNA [12]. Several studies confirmed these findings in the tumor tissue of LC patients, demonstrating a very high concentration of IL-4/IL-10 in pleural effusion samples [13], a significant predominance of IL-4/IL-10 over IL-2/IL-12/IFN-γ mRNA in tumor samples of LC patients [14, 15], and absent/low mRNA content for IFN-γ mRNA in tumor-infiltrating lymphocytes (TIL) extracted for LC tumor tissue [16]. Similar to previous studies, patients with SC-LC type also demonstrate Th_2 type predomination, represented with the finding of significantly increased IL-6 is in their sera [17]. This finding is particularly emphasized in the group of SC-LC patients with fast disease progression and in those that with unsuccessful response to therapy. Both experimental and clinical data implicate that various populations of myeloid cells found in LC are significantly associated with the disease progression and outcome [18] and connect their immunosuppressive capacity with the production of their specific mediators [19–21]. We have demonstrated that the number of $CD14^+B7H4^+$ monocytes (Mo) is significantly increased in blood and tumor microcirculation of LC patients and was correlated to tumor size and number of involved lymph nodes [22].

The aim of this study was to investigate the association between the Th_1/Th_2 cytokine profile and the number of $CD14^+B7H4^+$ Mo in blood and tumor microcirculation samples of LC patients.

Methods

Patients

We investigated 41 patients diagnosed for LC (31 males, 10 females, aged 62 ± 8 years) and 30 healthy controls (22 males, 8 females, aged 57 ± 14 years) (Table 1). Patients were diagnosed and treated at the Clinic for Lung Diseases, Military Medical Academy, Belgrade, Serbia for 18 months. All necessary diagnostic procedures (clinical, bronchoscopy, laboratory, histological, and radiological) were performed at the Military Medical Academy, Belgrade,

Table 1
Demographic and clinical characteristics of the study participants

Characteristics	Patients	Healthy persons
Sex, n		
male	31	22
female	10	8
Age (years), mean ± SD	62 ± 8	57 ± 14
Histological type of tumor, n		
NSC-LC (adenocarcinoma)	13	
NSC-LC (squamous cell carcinoma)	9	
NSC-LC (squamous cell carcinoma)	10	
SC-LC	9	
Clinical TNM stage, n		
III	24	
IV	17	
Metastases, n		
M0	27	
M1	14	
Tumor size, n		
T1	7	
T2	15	
T3	12	
T4	7	

SD – standard deviation; NSC-LC – non-small cell lung cancer; SC-LC – small lung cancer; TNM – tumor, node, metastasis.

Serbia. All patients and healthy controls gave their consent, and this study was approved by the local Ethics Committee, Military Medical Academy (11-03/2014, 12-02/2015).

All investigated cytokines were analyzed in groups of patients according to tumor histology type (SC-LC, adenocarcinoma (Ad) NSC-LC, squamous (Sq) NSC-LC, NSC-LC), tumor size, presence of metastasis and $CD14^+B7H4^+$ Mo number in blood, and tumor microcirculation samples. According to the $CD14^+B7H4^+$ Mo value, blood samples were qualified as basal level (less than 1% $CD14^+B7H4^+$), low (1–5%), intermediate (5–10%), and extreme value of $CD14^+B7H4^+$ (more than 10%). Tumor microcirculation samples were qualified as basal (less than 2% $CD14^+B7H4^+$), low (2–20%), intermediate (20–40%), and extreme value of $CD14^+B7H4^+$ (more than 40%).

Samples

Blood samples were taken from the cubital vein, while tumor microcirculation samples were taken from accessible pathological blood vessels with needle aspiration during diagnostic bronchoscopy. Both types of blood samples were taken with BD Vacutainer® Plus Plastic Serum Tubes. Serum was separated from the samples after centrifugation (3,000 g, 10 min, RT) and frozen at -70 ºC until testing. IL-2, IL-12, IFN-γ, IL-4, IL-5, IL-6, IL-10, and IL-13 were quantified with commercial flow cytometric tests (eBioscience kits) on Beckman Coulter flow cytometer FC500.

Both blood and tumor microcirculation samples were taken in the same way as described, but with the BD Vacutainer® spray-coated K2EDTA Tubes. The procedure for cell staining and immunophenotype analysis strategy was basically the same as previously referenced [22]. After removing erythrocytes with lysing buffer (NH_4Cl, EDTA, $KHCO_3$, 10 min with constant mixing) and further washing of nucleated cells (two times centrifuged with RPMI 1,640 culture medium complemented with 5% of normal human serum), cells were resuspended, enumerated (Beckman Coulter ACT differ blood counter) and concentration corrected to final suspension of 1×10^6 cells/100 μL per test tube. Final cell suspensions were stained with a cocktail of monoclonal antibodies. Multicolor analysis was performed with different combinations of CD15-FITC or PECy7, CD33-PE or PECy7, CD45-ECD or PECy5, HLA-DR PE/Cy5, CD14-FITC, CD16-PE, CD11b-PE, CD10-PECy7, CD3-FITC, CD19-FITC, CD56-FITC, B7H4-PECy7 (Biolegend, USA). The flow cytometry was performed using a Beckman Coulter FC 500 flow cytometer with CXP analysis software. Wanted subpopulations were identified from the initial $CD45^+$ /Side Scatter cell population, which was negative for T, B, and NK antigens. This triple-negative population of every sample was further gated on a CD11b versus HLA-DR dot plot histogram, and Mo was analyzed as lineage triple-negative ($CD3^-$, $CD19^-$, $CD56^-$), $CD45^+$, HLA-$DR^{-/low}$, $CD11b^+$ population. After further classification of this population according to the expression of CD15 or CD14, the $CD14^+$ Mo population was further investigated for B7H4 expression. Value of $CD14^+B7H4^+$ Mo cell population was expressed as % of all $CD45^+$ analyzed cells.

Statistical analysis

Data analysis was performed using the GraphPad Prism 5 software. Patients were stratified into groups according to lung tumor histology type, disease extent, and tumor node metastasis (TNM) classification. The difference in cytokine values was analyzed in two directions, either between groups (Mann-Whitney test) or as a ratio of tumor/serum sample of a particular patient (Wilcoxon matched pairs test). Comparison among multiple groups was done with nonparametric Kruskal-Wallis test, while identification of differences was performed with Dunn's multiple comparison test.

Results

The concentration of investigated cytokines did not differ significantly between LC patients and healthy controls (Table 2). Due to ethical reasons, we did not have adequate samples from healthy controls to compare them to LC tumor microcirculation samples. All investigated cytokines values demonstrated a higher tumor/ serum ratio.

Table 2

Comparison of serum cytokine concentrations of lung cancer (LC) patients and healthy persons (controls)

Cytokine	LC patients	Controls	*p*
IL-2			
serum	29 ± 29	19 ± 28	ns
tumor	32 ± 20	nd	nd
IL-12			
serum	9 ± 7	11 ± 29	ns
tumor	35 ± 28	nd	nd
IFN-γ			
serum	42 ± 41	57 ± 45	ns
tumor	58 ± 36	nd	nd
IL-4			
serum	24 ± 21	30 ± 21	ns
tumor	25 ± 14	nd	nd
IL-5			
serum	12 ± 15	9 ± 19	ns
tumor	39 ± 20	nd	nd
IL-6			
serum	45 ± 50	39 ± 38	ns
tumor	101 ± 91	nd	nd
IL-10			
serum	25 ± 21	19 ± 29	ns
tumor	37 ± 22	nd	nd
IL-13			
serum	53 ± 45	45 ± 38	ns
tumor	118 ± 53	nd	nd
IL-17	16 ± 13	9 ± 15	ns
serum	19 ± 11	nd	nd
tumor			
TGF-β1			
serum	729 ± 561	1010 ± 865	ns
tumor	320 ± 257	nd	nd

All values are expressed as mean ± standard deviation in pg/mL.
IL – interleukin; IFN – interferon; TGF– transforming growth factor.
ns – not significant; nd – not determined (Mann-Whitney test).

Serum cytokine concentrations between groups with particular lung tumor histology varied significantly. Patients with SC-LC type demonstrated highly increased levels of IL-2, IL-12, IL-6, and IL-13 (Tables 3 and 4). Patients from the Ad NSC-LC group had a profile of elevated IL-2, IFN-γ, IL-4, IL-10, IL-13, IL-17, and the lowest level of IL-6, compared to others. Patients with Sq NSC-LC demonstrated the highest IL-6 level of all but together with the significantly decreased value of all investigated cytokines. Contrary to others, patients with large cell (Lc) tumor type demonstrated the highest values of IL-2, IFN-γ, IL-10, and TGF-β_1 in their serum samples.

Analysis of tumor microcirculation samples demonstrated a completely different cytokine profile compared to that of serum samples. Patients with SC-LC tumor type had significantly increased IL-2, IL-12, IFN-γ, IL-6, IL-10, and IL-13. NSC-LC patients from the Ad group demonstrated increased IL-4, IL-6, and IL-17, Sq NSC-LC group had significantly increased IL-5 and TGF-β_1 value, while patients with Lc NSC-LC type demonstrated only increment of IL-5.

Patients with detectable metastasis (M1) had significantly increased IFN-γ and IL-4 compared to the M0 group. Patients with metastasis (M1) in tumor samples demonstrated a significant increase of IL-13.

Patients with the largest tumor size had significantly increased serum values of IL-2, IL-12, IFN-γ, IL-4, IL-5, IL-10, IL-13, and TGF-β_1. Interestingly, the T4 group demonstrated the lowest IL-6 and modest IL-17 level.

From all analyzed parameters, tumor size was clearly associated with a particular cytokine profile in tumor microcirculation. Smaller lung tumors were significantly associated with increased IL-2, IFN-γ, IL12 (T1), and IL-6 (T2), while larger sized tumors were associated with Th2 profile, namely, with increased IL-4, IL-10 (T3), IL-5, IL-13 (T4), and TGF-β_1 (T3, T4).

Stratification of patients with LC in groups according to the number of $CD14^+B7H4^+$ Mo in blood samples demonstrated specific cytokine profiles (Tables 5 and 6). Patients with the basal/low number of the $CD14^+B7H4^+$ Mo had significantly higher levels of IFN-γ and IL-12, together with IL-4, IL-5, IL-10, and TGF-β_1 compared to intermediate/extreme groups. On the other side, patients with intermediate/extreme high numbers of the $CD14^+B7H4^+$ Mo demonstrated a significant increase of IL-2 and IL-13, together with insignificantly elevated IL-6/IL-13 compared to basal/low groups.

Table 3

Average value of investigated cytokines according to tumor histology type, presence of metastases and tumor, node, metastasis (TNM) classification

Cytokine	SC-LC	NSC-LC			Metastases		TNM stage			
		Ad	Sq	Lc	M0	M1	1	2	3	4
IL-2										
serum	26 ± 16	36 ± 43	6 ± 9	47 ± 46	37 ± 36	45 ± 55	31 ± 21	18 ± 22	58 ± 58	61 ± 16
tumor	46 ± 20*	32 ± 20	19 ± 19	31 ± 19	34 ± 22	34 ± 20	31 ± 26	33 ± 21	42 ± 5	43 ± 10*
IL-12										
serum	13 ± 10	9 ± 8	4 ± 2	11 ± 7	11 ± 10	6 ± 9	1 ± 1	11 ± 10	6 ± 5	28 ± 5
tumor	92 ± 77*	24 ± 23*	15 ± 7**	8 ± 6	30 ± 28*	31 ± 28*	56 ± 15*	19 ±22**	63 ± 43*	15 ± 5
IFN-γ										
serum	31 ± 20	55 ± 55	23 ± 45	59 ± 47	26 ± 23	192 ± 212	22 ± 20	23 ± 19	48 ± 33	47 ± 7
tumor	87 ± 64*	72 ± 58	43 ± 14	30 ± 9	123 ± 110*	55 ±28	77 ± 37*	74 ± 51***	65 ± 41	41 ± 44
IL-4										
serum	24 ± 19	46 ± 45	11 ± 8	15 ± 14	19 ± 14	90 ± 75	15 ± 12	40 ± 51	11 ± 11	55 ± 16
tumor	9 ± 11	40 ± 13	27 ± 20	23 ± 14	8 ± 17	38 ± 20	2 ± 15	33 ± 21	49 ± 12**	34 ±8*
IL-5										
serum	18 ± 20	14 ± 19	1 ± 1	14 ± 19	20 ± 11	35 ± 35	2 ± 1	15 ± 24	5 ± 12	49 ±5
tumor	20 ± 20	31 ± 23	51 ± 13*	55 ± 26**	29 ± 24*	34 ± 25	43 ± 8*	23 ± 23	48 ± 13**	40 ± 31
IL-6										
serum	78 ± 98	4 ± 4	88 ± 92	5 ± 4	26 ± 38	60 ± 90	2 ± 3	74 ± 112	41 ± 86	8 ± 3
tumor	146 ± 125	158 ± 155**	93 ± 80	7 ± 4	124 ±134**	87 ± 94	21 ± 13*	123 ± 110	118 ± 100**	70 ± 56
IL-10										
serum	16 ± 13	32 ± 33	14 ± 10	37 ± 28	39 ± 27	17 ± 16	4± 3	13 ± 11	38 ± 24	73 ± 14
tumor	71 ± 36*	35 ± 27	18 ± 11	22 ± 12	50 ± 33	22 ± 16	20 ± 14*	32 ± 41	50 ± 23	45 ± 13*
IL-13										
serum	72 ± 39	75 ± 67	40 ± 37	25 ± 35	61 ± 60	48 ± 35	2 ± 2	45 ± 50	49 ± 37	180 ± 83
tumor	173 ± 44**	118 ± 47	90 ± 70	91 ± 50**	97 ± 57*	159 ± 48***	127 ± 61*	111 ± 73**	163 ± 39***	125 ± 35
IL-17										
serum	13 ± 8	27 ± 26	11 ± 9	13 ± 8	20 ± 26	29 ± 29	3 ± 2	34 ± 49	44 ± 59	30 ± 11
tumor	22 ± 13	34 ± 21	8 ± 6	10 ± 5	24 ± 21	24 ± 17	24 ± 7*	22 ± 25	26 ± 11	19 ± 5
TGF-β_1										
serum	624 ± 347	754 ± 680	696 ± 676	843 ± 541	833 ± 666	570 ± 264	484 ± 275	807 ± 694	868 ± 808	1,360 ± 355
tumor	174 ± 66**	275 ± 266**	760 ±667	61 ± 29**	436 ± 560**	210 ± 185**	83 ± 74*	278 ± 320**	662 ± 720	130 ± 48*

All values are expressed as mean ± standard deviation in pg/mL.
***p* < 0.05, ***p* < 0.01, *****p* < 0.001 (Wilcoxon test).**
SC-LC – small cell lung cancer; NSC-LC – non-small cell lung cancer; TNM – tumor, node, metastasis, IL – interleukin; TGF– transforming growth factor; Ad – adenocarcinoma; Sq – squamous cell carcinoma; Lc – large cell carcinoma.

Table 4

Comparison of cytokine data between groups of patients according to lung cancer histological type, presence of metastasis (M), and tumor size (T)

Sample	Group	/Group	IL-2	IL-12	IFN-γ	IL-4	IL-5	IL-6	IL-10	IL-13	IL-17	TGF-β_1
Serum	SC	/ Ad						*				
	SC	/ Sq	**	*								
	SC	/ Lc								*		
	Ad	/ Sq			*			**			*	
	Ad	/ Lc										
	Sq	/ Lc	*				*	*				
Tumor microcirculation	SC	/ Ad		*	*				*	*		
	SC	/ Sq		*	*	*	*		*	*		
	SC	/ Lc		*	**	*	*	***		**		
	Ad	/ Sq							*			
	Ad	/ Lc					*					
	Sq	/ Lc						***				
	M0	/ M1			*	**						
	M0	/ M1								**		
Serum	T1	/ T2										
	T1	/ T3							*			
	T1	/ T4		*		*	*		*	*		*
	T2	/ T3										
	T2	/ T4	*	**					**	*		*
	T3	/ T4		**		**	**		*	*	*	
Tumor microcirculation	T1	/ T2		*								
	T1	/ T3		*								
	T1	/ T4										
	T2	/ T3		*			*			*		
	T2	/ T4										
	T3	/ T4										

***p* < 0.05; ****p* < 0.01; *****p* < 0.001 (Mann-Whitney test).**

SC – small cell lung cancer; Ad – adenocarcinoma non-small cell cancer; Sq – squamous cell non small cell lung cancer; Lc – large cell non-small cell lung cancer; IL – interleukin;TGF – transforming growth factor.

Table 5

Concentrations of investigated cytokines in serum and lung tumor microcirculation samples according to the number of $CD14^+B7H4^+$ monocyte (Mo) in peripheral blood

Cytokine	Number of $CD14^+B7H4^+$ Mo in peripheral blood				Number of $CD14^+B7H4^+$ Mo in tumor microcirculation			
	basal	low	intermediate	extreme	basal	low	intermediate	extreme
IFN-γ	50 ± 57	71 ± 57	30 ± 79	28 ± 24	40 ± 12	99 ± 71	56 ± 39	41 ± 15
IL-2	29 ± 29	22 ± 25	26 ± 25	52 ± 72	19 ± 18	40 ± 7	27 ± 19	26 ± 22
IL-12	19 ± 8	5 ± 10	10 ± 9	3 ± 3	10 ± 7	15 ± 15	27 ± 33	66 ± 58
IL-4	29 ± 23	22 ± 17	22 ± 21	7 ± 8	21 ± 16	105 ± 59	31 ± 20	142 ± 76
IL-5	34 ± 31	50 ± 60	13 ± 22	0 ± 0	7 ± 13	38 ± 28	16 ± 13	28 ± 26
IL-6	11 ± 9	8 ± 8	17 ± 30	21 ± 26	126 ± 121	36 ± 35	134 ± 210	71 ± 65
IL-10	35 ± 36	26 ± 19	26 ± 19	30 ± 36	17 ± 18	36 ± 19	35 ± 22	42 ± 37
IL-13	38 ± 45	70 ± 52	114 ± 57	47 ± 60	94 ± 63	105 ± 59	80 ± 63	142 ± 76
IL-17	13 ± 11	17 ± 10	13 ± 10	21 ± 23	21 ± 23	18 ± 9	13 ± 9	20 ± 16
TGF-β_1	902 ± 608	1,325 ± 1,411	782 ± 564	446 ± 320	151 ± 43	122 ± 46	315 ± 343	363 ± 303

All values are expressed as mean ± standard deviation in pg/mL.

IFN – interferon; IL – interleukin;TGF – transforming growth factor.

The number of $CD14^+B7H4^+$ Mo that we detected in tumor microcirculation was significantly higher in all samples compared to blood values (Tables 5 and 6). Patients with basal/low number of the $CD14^+B7H4^+$ Mo demonstrated significant increment of IFN-γ, IL-2, and IL-6. Patients with extreme numbers of $CD14^+B7H4^+$ Mo in tumor microcirculation samples had significantly elevated levels of IL-12, IL-4, IL-10, IL-13, TGF-β_1 and TNF-α (Table 7).

Table 6

Significant differences in concentrations of investigated cytokines in serum and tumor microcirculation between groups with different $CD14^+B7H4^+$ monocyte (Mo) number

$CD14^+B7H4^+$ Mo number	Serum		Tumor microcirculation	
	cytokine	significance	cytokine	significance
Basal/low	IL-12	*	IFN-γ	*
Basal/low	IL-4	*	IL-2	*
Basal/low			IL-5	**
Basal/intermediate	IL-12	*	IL-10	*
Basal/intermediate	IL-4	*		
Basal/intermediate	IL-13	**		
Low/intermediate	IFN-γ	*		
Basal/extreme	IL-12	*	IL-12	*
Basal/extreme	IL-5	*	IL-10	*
Basal/extreme	TGF-β_1	*	TGF-β_1	*
Low / extreme	IFN-γ	*	IFN-γ	*
Low / extreme	IL-5	*	IL-5	*
Low / extreme	TGF-β_1	*		
Intermediate/ extreme	IL-12	*		
Intermediate/ extreme	IL-4	**	IL-4	*
Intermediate/ extreme	IL-13	*	IL-13	*

IFN – interferon; IL – interleukin;TGF – transforming growth factor.
***p* < 0.05; ****p* < 0.01 (Mann-Whitney test).**

Table 7

Cytokines with the highest average concentration according to the number of $CD14^+B7H4^+$ monocytes (Mo) in peripheral blood and tumor microcirculation samples

$CD14^+B7H4^+$ Mo number	Serum	Tumor microcirculation
Basal	IL-12, IL-4, IL-10, IL-1β, TNF-α	IL-9, IL-1β
Low	IFN- γ, IL-5, IL-17, IL-9	IFN- γ, IL-2, IL-5, IL-27
Intermediate	IL-6, IL-13,	IL-6, IL-22
Extreme	IL-2, IL-22, IL-27	IL-12, IL-4, IL-13, IL-10, TGF-β_1, TNF-α

IFN – interferon; IL – interleukin;TGF – transforming growth factor; TNF – tumor necrosis factor.

Discussion

Data from numerous experimental and clinical studies indicate that the cytokine network is an important factor that shapes the anti-tumor response. Although immune response – tumor is an extremely complex and dynamic relation that depends on individual tumor characteristics, disease stage, and immune response characteristics, the prevailing attitude is that Th_1 is associated with protective anti-tumor response while Th_2 characterize pro-tumor microenvironment. Numerous investigations stated that there is a domination of Th_2 over Th_1 profile in samples of LC patients. The presence of high IL-6, IL-10, GM-CSF, and IFN-γ with low IL-2 mRNA level [8], domination of IL-4/IL-10 over IL-2/ IL-12/IFN-γ in lung tumor biopsy and pleural effusion of NSC-LC patients [9, 13, 14], the prevalence of Th_2 in the peripheral blood [10] and depressed cytotoxicity paralleled with decreased IFN-γ production in NK/NKT cells [16] was demonstrated in tumor tissue from LC patients (NSC-LC). It was also shown that successful therapy reduced Th_2 and increased Th_1 profile [10, 11]. The first indication of the biological significance of Th_2 profile domination in lung cancer pathophysiology came from Zhang et al. [15]. They demonstrated that more than 75% of macrophages corresponded to M2 type, which was significantly associated with high IL4/IL10 and low IFN-γ/IL-12 found in the tumor tissue of NSC-LC patients but not benign lesion samples. Two studies from Ito et al. [23, 24] demonstrated the complexity and dynamic change of Th_1/Th_2 profile related to the different microenvironment and disease progression. Twenty years ago, they demonstrated that tumor-infiltrating lymphocytes of NSC-LC patients are dominantly Th_1, while T lymphocytes in peripheral blood of the same patients corresponded to Th_2 population [23]. They expanded their investigation at the cytotoxic population of T lymphocytes (Tc), and after 5 years of follow-up, they demonstrated a predomination of Th_2/Th_1 associated with Tc_2/Tc_1 [24]. Surprisingly, this high Th_2/Th_1 and Tc_2/Tc_1 ratio found in peripheral blood was significantly associated with better prognosis in NSC-LC patients with advanced disease (clinical stage II and III), but not in those with early disease (stage I). These studies showed a significant difference between lung tumor and peripheral blood in the profile of cytokine response and that there is an important change that follows or reflects disease progression.

Contrary to all studies performed mainly on samples from NSC-LC patients, our study included patients with both histology tumor types. When we analyzed the patient group without stratification to any of the parameters, we did not find any significant differences in the concentration of investigated cytokines. Our data underlined significant

differences in Th_1/Th_2 cytokines according to microenvironment (tumor/blood). Furthermore, we demonstrated that patients with SC-LC histology type, without metastasis, and with the smallest tumors had the highest local IL-12/IL-2, IFN-γ concentration.

New data indicate that IL-4/IL-13 could have an important immunosuppressive role in LC, based on effects on TAM and MDSC. IL-4 as a key Th_2 cytokine indirectly induces neoplastic proliferation of LC by stimulating TAM to gain M2 phenotype, produce insulin-like growth factor 1 (IGF1) locally [25], and secrete cathepsin, which increases tumor cells capacity to invade surrounding tissues [26]. Gocheva et al. [26] demonstrated that IL-4 producing cells in LC tumor tissue were both infiltrating T lymphocytes and tumor cells. Importantly, infiltrating T lymphocytes represented a minority, only 2% of total IL-4 producing cells, while up to 90% of the dominant IL-4 producing population are tumor cells themselves. These data indicate that tumor cells manipulate the intratumor monocyte population, transforming them into TAM or/and M2 macrophages due to the secretion of Th_2 cytokines. A study from Feng et al. [27] demonstrated a significant role of $CD11b^+CD14^+S100A^+$ suppressive monocytes isolated from peripheral blood samples of NSC-LC patients. NSC-LC patients, especially those with advanced disease and unsuccessful therapy response, had significantly more these cells compared to healthy controls. This monocyte population was highly immunosuppressive *in vitro*, potently inhibiting $CD8^+$ T cell activation, inhibited IFN-γ production, and secreted high amounts of iNOS, arginase, IL-8, IL-10, tumor necrosis factor (TNF)-α, hepatocyte growth factor (HGF), and IL-13. The authors concluded that $CD14^+$ $S100A9^+$ inflammatory monocytes in patients with NSC-LC represent a distinct MDSCs subset capable of suppressing T lymphocytes by the production of arginase, iNOS, and the IL-13/IL-4Rα axis. TAM and MDSC are not the only sources of IL-13 in tumors of LC patients, since in almost historic study Huang et al. [28] demonstrated that tumor lung cells are potent producers of IL-13. The importance of IL-13 in regulating tumor invasion and spreading is already described in ovarian [29] and pancreatic cancer [30].

Our patients with SC-LC had the highest average IL-13 value, particularly in tumor microcirculation. IL-13 was the only cytokine of all investigated whose concentration significantly increased in tumor samples of all patients compared to sera values, regardless of histologic type of the tumor. Additionally, our patients with the intermediate/extreme number of the $CD14^+B7H4^+$ monocytes in tumor microcirculation samples demonstrated significantly elevated levels of IL-4, IL-5, IL-10, IL-13, and $TGF\text{-}\beta_1$, which is in concordance with data from the previous studies. In our previous paper [22], we have demonstrated that lung cancer patients had significantly more $CD14^+$ $B7H4^+$ Mo than healthy people. The highest $CD14^+$ $B7H4^+$ Mo number was associated with N3 nodal status, with the largest tumors, but also with clinical stage III and patients without metastasis. In this research, we investigated values of key Th_1/Th_2 cytokines estimated in those LC patients and represented them in the context of $CD14^+$ $B7H4^+$ monocyte number, indicating a significant association of Th_2 profile with the highest number of this Mo population in tumor samples.

Conclusion

Samples of LC patients demonstrated polarization of cytokine response, which is significantly associated with microenvironment origin, tumor histology type, tumor size, and disease extent. Stratification of LC patients according to $CD14^+$ $B7H4^+$ monocyte number offers a new possibility for the interpretation of cytokine profiles. The highest number of $CD14^+$ $B7H4^+$ monocytes is significantly associated with a particular cytokine profile in tumor samples, dominated with Th_2 cytokines.

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Conflict of interest

The authors state no conflict of interest.

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Neoadjuvant chemotherapy followed by interval debulking surgery versus primary debulking surgery in the advanced epithelial ovarian cancer – a retrospective cohort study

Neoadjuvantna hemioterapija praćena intervalnom *debulking* hirurgijom nasuprot primarnoj *debulking* hirurgiji kod uznapredovalog epitelijalnog karcinoma jajnika – retrospektivna kohortna studija

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Abstract

Background/Aim. The gold standard in treating the advanced ovarian cancer (AOC) is primary debulking surgery (PDS) followed by platinum-based adjuvant chemotherapy. In the AOC, the extent of tumor resection (residual tumor volume) is the most important prognostic factor for overall survival (OS) and progression-free survival (PFS). Neoadjuvant chemotherapy (NACT) followed by interval debulking surgery (IDS) is an experimental treatment of the AOC, introduced in clinical practice in order to improve cytoreduction rate and prolong survival. The aim of this study was to compare the survival and cytoreduction rate of NACT+IDS and PDS in patients with the AOC. **Methods.** This retrospective cohort study included patients with the AOC, separated into two groups. The first group treated with PDS had 59 patients, while the second group, treated with NACT + IDS, had 33 patients. **Results.** A lower rate of suboptimal cytoreduction (39.39%) was found in the NACT + IDS group than in the PDS group (57.63%). The percentage of complete cytoreduction was higher in patients treated with NACT + IDS (51.52%) than in those treated with PDS (38.98%). Nevertheless, median OS and PFS were not significantly different between the groups ($p < 0.05$). OS was 35 months and 31 months in the PDS and NACT + IDS groups, respectively. PFS was 16 months in the PDS and 19 months in the NACT + IDS group. **Conclusion.** Despite the higher rate of optimal debulking surgery after NACT+ IDS, survival of patients treated with method was not better than those treated with PDS. The decision for either NACT+IDS or PDS should be tailored to the individual patient.

Key words:
cytoreduction surgical procedures; drug therapy; gynecologic surgical procedures; ovarian neoplasms; survival; prognosis.

Apstrakt

Uvod/Cilj. Zlatni standard u lečenju uznapredovalog karcinoma jajnika (AOC) je primarna citoreduktivna hirurgija (PDS) nakon koje sledi adjuvantna hemioterapija na bazi platine. Kod AOC, opseg resekcije tumora (rezidualni volumen tumora) je najvažniji prognostički faktor za ukupno preživljavanje (OS) i preživljavanje bez progresije bolesti (PFS). Neoadjuvantna hemioterapija (NACT) praćena intervalnom citoreduktivnom hirurgijom (IDS) je eksperimentalni tretman AOC, uveden u kliničku praksu kako bi se poboljšala citoredukcija i produžilo preživljavanje. Cilj rada je bio da se uporedi stopa preživljavanja i citoredukcije između NACT + IDS i PDS kod bolesnica sa AOC. **Metode.** Retrospektivnom kohortnom studijom bile su obuhvaćene bolesnice sa AOC, podeljene u dve grupe. U grupi lečenoj PDS bilo je 59 bolesnica, dok su u grupi lečenoj NACT + IDS bile 33 bolesnice. **Rezultati**. Utvrđena je niža stopa suboptimalne citoredukcije (39,39%) u NACT + IDS grupi u poređenju sa PDS grupom (57,63%). Procenat potpune citoredukcije bio je viši kod bolesnica lečenih NACT + IDS (51,52%) nego kod onih lečenih PDS (38,98%). Ipak, OS I PDS nisu se značajno razlikovali između grupa ($p < 0,05$). OS je bilo 35 meseci u PDS grupi i 31 mesec u NACT + IDS grupi; PFS je bilo 16 meseci u PDS i 19 meseci u NACT + IDS grupi bolesnica. **Zaključak**. Uprkos višoj stopi optimalne citoredukcije nakon NACT + IDS, preživljavanje bolesnica lečenih na ovaj način nije bilo bolje od preživljavanja bolesnica lečenih metodom PDS. Odluku za primenu NACT + IDS ili PDS treba prilagoditi svakoj bolesnici.

Ključne reči:
citoredukcija, hirurške procedure; lečenje lekovima; hirurgija, ginekološka, precedure; jajnik, neoplazme; preživljavanje; prognoza.

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Introduction

Ovarian cancer represents the second most common gynecological cancer and a leading cause of mortality among cancers in gynecology [1]. The lifetime risk for women to develop ovarian cancer is estimated to be 1 : 70 [2]. The advanced disease is present in 70% of the patients at the time of diagnosis due to ovarian cancer oncogenesis, lack of specific symptoms, and the fact that reliable prevention methods are still unavailable [3]. Therefore, the prognosis is poor, in general with an overall 5-year survival rate of 45%, and even lower in the advanced stages [2].

The gold standard in treating a newly diagnosed advanced ovarian cancer (AOC) is primary debulking surgery (PDS) followed by platinum-based adjuvant chemotherapy [4]. Patients who are not fit for surgery are candidates for primary chemotherapy or symptomatic treatment.

In the AOC, the extent of tumor debulking (cytoreduction) and a residual tumor volume are the most important prognostic factors for overall survival (OS) and progression-free survival (PFS) [4, 5]. Suboptimal cytoreductive surgery (residual disease > 1 cm) has no positive effect on survival [6]. Therefore, the AOC surgery is always made in order to achieve complete (no macroscopical residual disease) or at least optimal cytoreduction (residual disease < 1 cm) [7].

Neoadjuvant chemotherapy (NACT) in ovarian cancer is defined as 3–4 cycles of platinum-based chemotherapy, followed by interval debulking surgery (IDS) and adjuvant chemotherapy. NACT followed by IDS was introduced as a new treatment modality for the AOC with the hypothesis that the application of chemotherapy before surgery could shrink the tumor, make it more resectable, and thus increase the rate of cytoreduction with opposing the patient to a less extensive surgery at the same time. This would be of special importance in those patients where optimal cytoreduction is estimated to be unattainable by primary surgery. Moreover, the ones who are not fit to stand the extensive surgery at the time of diagnosis due to comorbidities or poor general condition can have the benefit of postponing the surgery with neoadjuvant chemotherapy.

Methods

We included NACT + IDS as an experimental treatment in our clinical protocol after the results of the EORTC55971 randomized trial that showed noninferiority of NACT + IDS to PDS in terms of PFS and OS, with less postoperative morbidity and a higher percent of optimal debulking in the experimental arm [8].

The results of treatment and survival of patients with the AOC operated after NACT were compared with the control group of patients treated with PDS in the same period. The main objective of this study was to compare these two treatments in terms of patients' survival and cytoreduction rate.

This retrospective cohort study included patients with advanced epithelial ovarian cancer. Subjects were separated into two groups based on a different treatment modality. The first group was treated with PDS, while the second one was treated with NACT + IDS.

All the analyzed data were gathered in a retrospective manner from our hospital information system. Medical records of the patients with the diagnosis of ovarian cancer operated in our institution from January 1st, 2013 until December 31st, 2017 were analyzed. We included patients with the newly diagnosed ovarian cancer in stages III and IV, as specified by the International Federation of Gynecology and Obstetrics (FIGO) staging criteria [9]. All included patients had histologically proven epithelial ovarian cancer. The protocol used for NACT consisted of paclitaxel in a dose of 175 mg/m^2 of the body surface area with carboplatin in a dose equal to the area under the curve (AUC) of 6, every three weeks.

Patients were considered to have undergone debulking (cytoreductive) surgery if any open surgery with the intention of performing the debulking procedure had been done. A surgical procedure after which no macroscopic disease was visible was defined as a complete debulking surgery. Patients were considered optimally debulked when residual lesions were present after the surgery and were less than 1cm in greatest diameter, while suboptimally debulked were those patients with residual disease bigger than 1 cm.

Estimation of the tumor resectability and the decision for NACT was made by the multidisciplinary Oncology Board for the gynecological tumors from our institution. The decision was based on a clinical examination, performance status, comorbidities, imaging results, CA125 levels, and previous diagnostic laparoscopy in individual cases. In all the cases where optimal cytoreduction seemed to be unachievable with primary surgery, NACT was advised.

Follow-up data were collected from patients' records and individual communication. The first day of follow-up corresponds to the date of the first cycle of chemotherapy in the NACT + IDS group, or the date of the operation in the PDS group. PFS was measured to the date of the first radiological progression of the disease. In cases where no progression was documented before, PFS was calculated to the date of the last contact and the date of death. OS was calculated to the time of death. Surviving patients were censored at the time of the last contact. Patients who were lost to follow-up were censored within the date of the last contact. CA125 values were expressed in U/mL.

This study was approved by the Ethical Committee of our institution and conducted in accordance with the Helsinki Declaration.

Statistical analysis

For the continuous variables, the correlation between investigated variables was represented with Pearson's correlation coefficient. Spearman's rank-order correlation was used for the analysis of ordinal variables. Student's *t*-test and χ^2 test were used for the comparison of variables between groups. Survival was analyzed using a Kaplan Maier method. Differences in survival were estimated by the

use of Log-rank (Mantel-Cox) and Gehan-Breslow-Wilcoxon tests. *P*-values at the level of 0.05 were considered statistically significant. Microsoft Excel 2007 with Statistica 13 software package (StatSoft Inc., Tulsa, OK, USA; University License University of Novi Sad) was used for statistical analysis.

Results

The control group of patients who underwent PDS had 59 patients (group 1), while the study group treated with NACT + IDS consisted of 33 patients (group 2).

In the NACT + IDS group, patients received 3.61 cycles of NACT on average. The decision for NACT was based on cytological findings from ascites or pleural effusion in 23/33 patients (69.70%), while 10/30 patients (30.30%) had histologically confirmed epithelial ovarian cancer by tumor biopsy prior to NACT. Resectability was determined mostly by imaging results in 30/33 (90.9%) of patients, and only 3/33 (9.1%) of patients had diagnostic laparoscopy to estimate the possibility of complete debulking. Imaging estimated complete response to NACT was obtained in 2/33 (6.06%) of the patients, partial response was obtained in 29/33 (87.88%), while 2/33 (6.06%) had stable disease after NACT. All the patients in this group had serous ovarian cancer confirmed after the operation, except two where the tumor tissue was not found in the surgical specimen (these were the same two patients with a complete response to NACT).

In the analysis of joint data from both cohorts, a moderate positive correlation was found between the level of cytoreduction and PFS ($r = 0.43$, $p < 0.05$), and the level of cytoreduction and OS ($r = 0.38$, $p < 0.05$) (Tables 1 and 2). This correlation was confirmed using a Kaplan Maier method, where a significant difference in OS was observed between each of the three groups of patients with separate levels of cytoreduction ($p < 0.05$). The group with complete cytoreduction had better OS than both groups with optimal and suboptimal cytoreduction, while the group with optimal cytoreduction had superior OS than the one with suboptimal cytoreduction (Figure 1). A negative correlation was found

Table 1
Pearson's correlation analysis of the examined parameters

Parameters	Pearson's correlation coefficient			
	Age	PFS	OS	CA125
Age		-0.1308	-0.0954	0.0552
PFS	-0.1308		0.8010*	-0.1558
OS	-0.0954	0.8010*		-0.1276
CA125	0.0552	-0.1558	-0.1276	

***Marked correlations are significant at $p = 0.05$ level.**
PFS – progression free survival; OS – overall survival.

Table 2
Spearman's rank-order correlation analysis of the examined parameters.

Parameters	Spearman's rank-order correlation coefficient					
	PFS	OS	Cytoreduction	FIGO stage	Age	CA125
PFS			0.435*	-0.221*		
OS			0.383*	-0.163		
Cytoreduction	0.435*	0.383*		-0.066	-0.086	-0.313*
FIGO stage	-0.221*	-0.163	-0.066		-0.080	0.197
Age			-0.086	-0.080		
CA125			-0.313*	0.197		

*** Marked correlations are significant at $p = 0.05$ level.**
PFS – progression free survival; OS – overall survival; FIGO – Federation of Gynecology and Obstetrics.

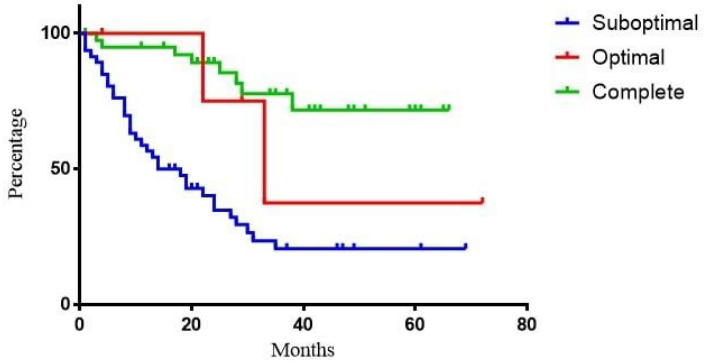

Fig. 1 – Kaplan-Meier curves of the overall survival (OS) for groups of patients with complete, optimal, and suboptimal cytoreduction.

to be significant between FIGO stage and PFS (r = -0.22, $p < 0.05$) and levels of CA125 and cytoreduction rate (r = -0.31, $p < 0.05$). (Tables 1 and 2). Higher levels of CA125 at the time of diagnosis were associated with a lower cytoreduction rate. Except above mentioned, other analyzed parameters were not in a significant correlation (Tables 1 and 2).

The significant difference between groups was found in CA125 levels before treatment (568 U/mL in the PDS vs. 1,129 U/mL in the NACT group; $p < 0.01$; Cohen's d = 0.64-medium effect size) (Figure 2). The median total number of chemotherapy cycles was 4.90 in the PDS group, significantly lower compared with 7.67 in the NACT + IDS group ($p < 0.001$; Cohen's d = 1.17 – large effect size) (Figure 2). Note that in the NACT + IDS group, the total number of cycles represents a sum of neoadjuvant and adjuvant treatment. Groups did not differ significantly in patients' age (Figure 2).

The distribution of FIGO stages in the two examined groups is shown in Figure 3. We observed the lower percentage of stage IIIb and the higher percentage of stage IVa in the NACT + IDS group. The difference between the two groups in the FIGO stage was found to be significant (χ^2 (4) = 2.97, $p = 0.56$).

We detected 39.39% of patients with suboptimal

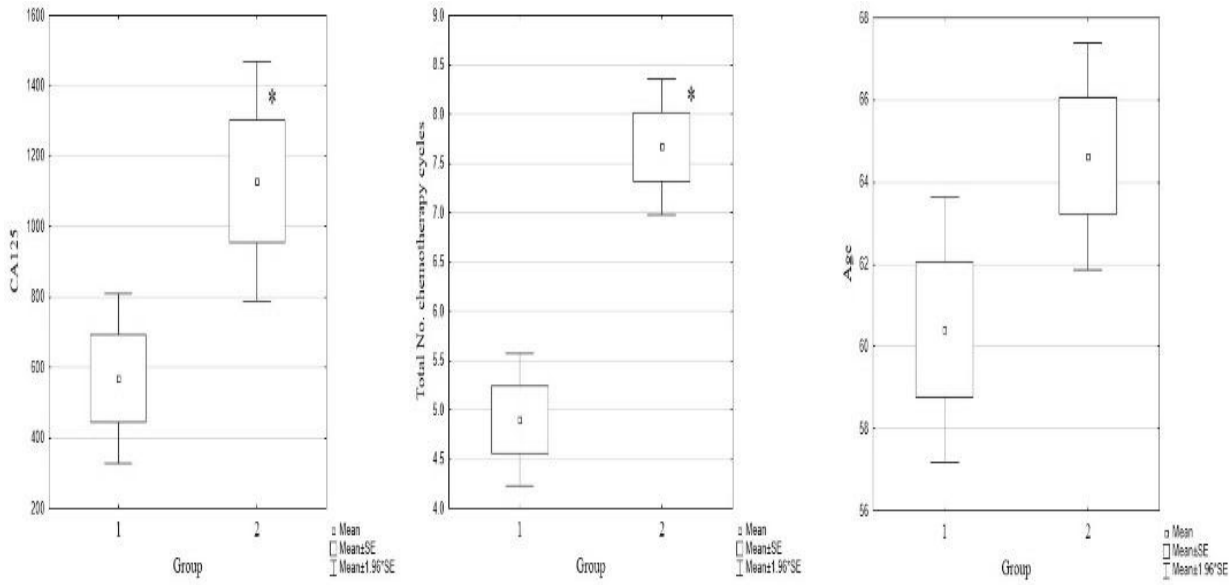

Fig. 2 – Box-plot graphs of the variables compared between the groups (* – statistically significant at $p = 0.05$ level).
Group 1 – patients treated by neoadjuvant chemotherapy + interval debulking surgery;
Group 2 – patient treated by primary debulking surgery.

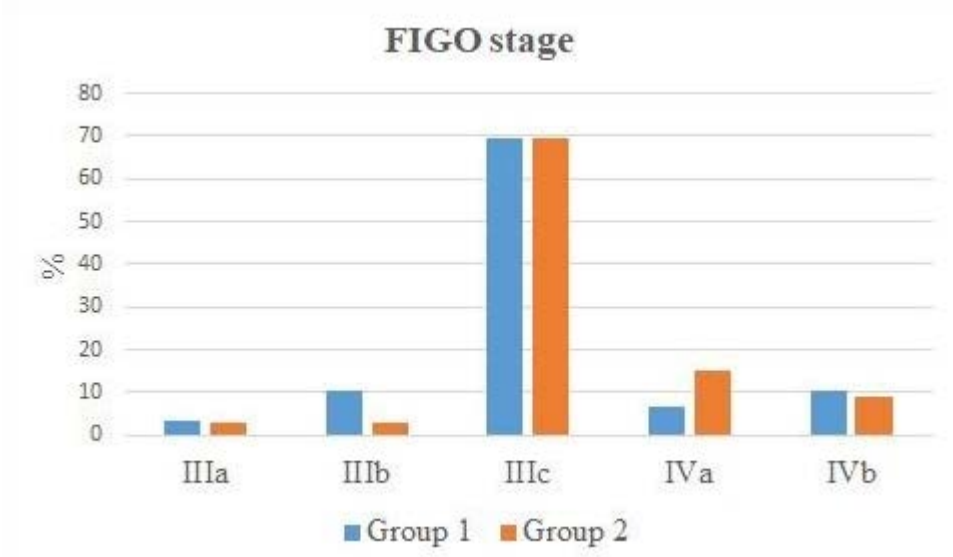

Fig. 3 – Difference in the FIGO – Federation of Gynecology and Obstetrics (FIGO) stage distribution between the examined groups.
For the explanation of the terms Group 1 and Group 2 see under Figure 2.

cytoreduction in the NACT + IDS group, which was lower than 57.63% observed in the PDS group. Furthermore, the rate of complete cytoreduction was higher in patients treated with NACT + IDS (51.52%) than in those treated with PDS (38.98%). This difference among examined groups in cytoreduction was significant (χ^2 (2) = 3.41, p = 0.18) (Figure 4).

Nevertheless, the median OS was not significantly different between the groups. This period was 35 months and 31 months in the PDS and the NACT + IDS group, respectively (p < 0.05) (Figure 5). Likewise, we did not observe a statistical difference in PFS, which was 16 months in the PDS and 19 months in the NACT + IDS group (p < 0.05) (Figure 6). The median follow-up time was 37 months in the PDS and 43 months in the NACT + IDS group.

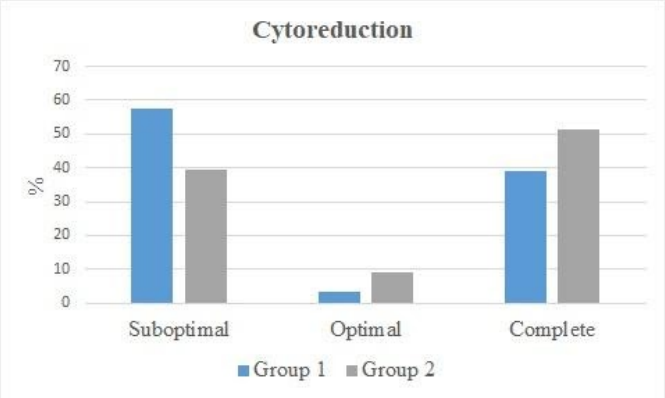

Fig. 4 – Difference in the cytoreduction rate distribution among the examined groups.
For the explanation of the terms Group 1 and Group 2 see under Figure 2.

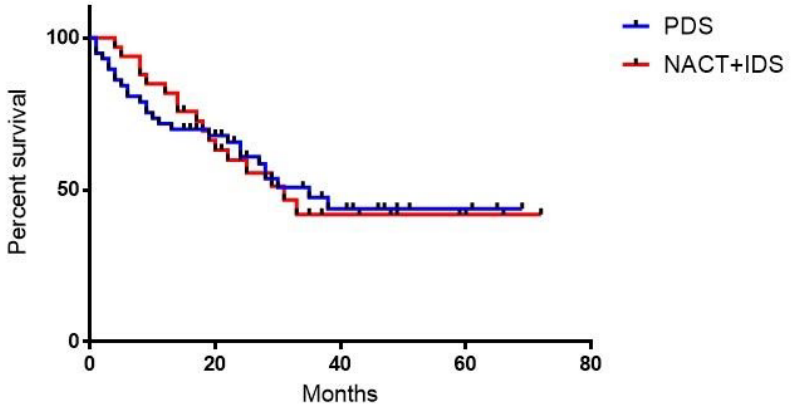

Fig. 5 – Kaplan-Meier curves of the overall survival (OS) for the examined groups of patients.
PDS – primary debulking surgery; NACT – neoadjuvant chemotherapy; IDS – interval debulking surgery.

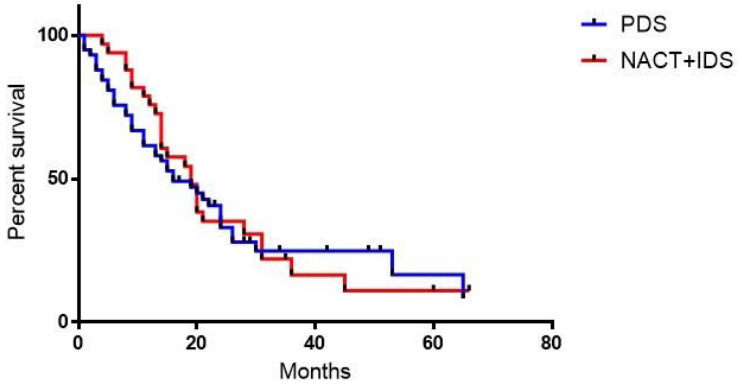

Fig. 6 – Kaplan-Meier curves of the progression-free survival (PFS) for the examined groups of patients.
For the abbreviations see under Figure 5.

Discussion

So far, it has been concluded in several studies that the primary goal of ovarian cancer surgery should be a complete cytoreduction with no residual disease since one improves the survival of patients with the AOC [5–8, 10–12]. Median survival is 1.5 months longer for every 10% increase of patients inside the cohort who are submitted to maximal cytoreductive surgery [5]. Still, patients with the residual disease up to 1 cm have better survival than those with the residual disease bigger than 1 cm [7, 13]. Therefore, optimal cytoreduction is appropriately defined as a residual disease < 1 cm and should continue to be the preferred surgical outcome in all the patients where the complete cytoreduction is unachievable. A positive correlation between the level of cytoreduction and survival of patients with the AOC was confirmed in our study, where better cytoreductive surgery with less residual disease corresponds to the longer OS.

We achieved optimal cytoreductive surgery (residual tumor < 1 cm) in 60.61% of patients in the NACT + IDS group, which was higher compared with 42.37% obtained in the PDS group. These results are similar to the ones from the randomized studies, where the optimal debulking rate was better after NACT + IDS than after the primary surgery [8, 10, 14]. The optimal debulking rate was 80.6% in the NACT + IDS group and 41.6% in the PDS group in EORTC55971 study [8], 73% vs. 41% in favour of NACT + IDS in CHORUS study [10], and 82% and 37%, in NACT + IDS and PDS group, respectively, in recently presented Japanese study [14].

Still, the higher rate of optimal debulking surgery observed in the NACT group did not mean better survival of patients treated with NACT + IDS compared with those treated with primary surgery. OS was 31 months in the NACT + IDS group and 35 months in the PDS group, without statistical difference. Additionally, the difference was not found in PFS, where we observed periods of 19 and 16 months in the NACT + IDS and the PDS group, respectively. Perspective, longer follow-up and a larger study population could alter the results and make the difference in survival between these groups statistically significant. Results of the three randomized trials published so far proved that the treatment with NACT + IDS does not yield longer survival than one with PDS [8, 10, 14]. On the other hand, two of these trials demonstrated noninferiority of NACT + IDS vs. PDS in terms of survival [8, 10].

It is questionable why the obvious difference in the extent of cytoreduction observed in this study and previous trials does not mirror in longer survival of patients treated with NACT + IDS. One of the explanations is that NACT can encourage the development of chemo-resistant clone cells, which reflects in lower survival than expected. A larger tumor mass at the start of NACT, due to its more numerous and heterogeneous cell population, has a higher potential for the selection of drug-resistant cells [15]. In Bristow's meta-analysis, median survival after NACT + IDS was lower than after PDS and was approximateve to that of suboptimally debulked patients after the primary surgery [16]. This can be explained by the selection bias of observational studies included in the meta-analysis where patients selected to NACT + IDS tend to be older, have worse performance status, have more comorbidities, and larger tumor burden. Partly, that was the case in our study, where the NACT + IDS group had higher FIGO stages than the PDS group. Also, significantly higher levels of CA125 at the time of diagnosis observed in the NACT + IDS group can reflect a larger tumor burden in this group. One of the potential confounders in our study could be the total number of chemotherapy cycles that patients received, which was higher in the NACT + IDS group and could give this group advantage over the PDS group in terms of survival. The average number of NACT cycles in our study was 3.61. It was observed that more than 4 cycles of NACT have a negative impact on median survival [16].

Should all patients with AOC have the same treatment, and should it be NACT + IDS or PDS? Subgroup analysis of the EORTC55971 trial demonstrated that the patients diagnosed in FIGO stage IIIc with the metastasis bigger than 5 cm and stage IV do better after NACT + IDS, while those in stage IIIc with the metastasis smaller than 5 cm had better survival if they underwent PDS [8]. The recent multicentric observational study showed the better survival of patients with ovarian cancer in FIGO stage IIIc if they underwent PDS, while there was no difference in survival between the NACT + IDS and PDS groups in stage IV disease [17]. Since the use of NACT + IDS for stage IIIa and IIIb ovarian cancer is unsupported with data from randomized studies, primary cytoreductive surgery remains the treatment of choice for those patients. Hence, some patients with AOC benefit more from NACT + IDS, some others from PDS. ESGO guidelines for ovarian cancer surgery [18] recommend that the primary surgery be the treatment of choice only when complete cytoreduction seems viable in patients fit for radical surgery. NACT is suggested in all other cases, and IDS is done only if the complete debulking appears achievable after a favorable response to NACT. Vergote et al. [19] suggested using certain criteria for the selection of patients for NACT based on the extent of the disease, tumor resectability, and a general condition of the patient.

As can be seen from the above recommendations, it is important to predict residual tumor volume before cytoreductive surgery in order to determine the best treatment for each patient and avoid interventions that are without benefit. Resectability can be predicted with certain accuracy using a CT scan, with sensibility 64–79% to presume suboptimal cytoreduction [20]. Same can be done with the help of several clinical and radiological criteria, all associated within a predictive model which has an accuracy of 73% [21]. Laparoscopy could be useful in prognosis of suboptimal cytoreduction, with a good sensitivity of 69–96% and 100% specificity [22]. In addition to conventional preoperative diagnostics, it can lower the percent of unsuccessful laparotomies from 39% to 10% [23]. Tumor marker CA125 can serve as a complement in decision-making since it lacks the accuracy to be used alone [24]. Higher levels of CA125 at the time of diagnosis lower the possibility

of optimal debulking, as observed in our and previous studies [24].

The data we have so far are inconclusive and motivate further research. Future trials with a different selection of patients and the use of bevacizumab in the neoadjuvant setting may elicit new evidence that can have implications in clinical practice and improve the survival of patients with ovarian cancer.

Conclusion

AOC treatment should be tailored to the individual patient and based on patients' age, performance status, comorbidities, histology, stage of the disease, and tumor resectability. PDS stays the standard of care in treating the AOC, while NACT + IDS should find its place in carefully chosen patients.

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CBCT analysis of bone density in bicortical defects after augmentation with alloplastic and xenogeneic bone substitutes – A study on domestic pigs

CBCT analiza gustine kosti u bikortikalnim defektima nakon pojačanja primenom aloplastičnog i ksenogenog koštanog zamenika – studija na domaćim svinjama

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Abstract

Background/Aim. A significant benefit in bicortical defects healing can be achieved by guided tissue and guided bone regeneration. The aim of this study was a cone-beam computed tomography (CBCT) radiographic bone density analysis of bicortical defects healing when treated with guided bone regeneration and two bone substitutes – bovine xenograft and alloplastic bone substitute. **Methods.** The research was performed on domestic pigs in two phases. In the first phase, extraction of all teeth in the intercanine sector was performed in the lower jaw and postextraction wounds were sutured. In the second phase, after the period required for healing, bicortical defects were formed – following the elevation of mucoperiosteal flaps from the vestibular and lingual side in the area of the previously extracted teeth, surgical removal of the cambium layer of periosteum was performed in the area of future defects with sharp surgical scissors and curette. Two defects, 10 mm in diameter, on the left and right side of the medial line were formed and filled with alloplastic bone substitute on the left and xenograft on the right side afterward. After augmentation, the defects were covered by a collagen resorptive membrane on both sides, and the flap was repositioned and sutured. After 12 weeks, experimental animals were sacrificed. The surrounding native bone was used as a control. **Results.** Analysis of bone tissue density showed a statistically significant difference between the examined bone substitutes ($p < 0.01$), with a better effect achieved by the use of alloplastic bone substitute. After applying Bonferroni correction, the difference was still statistically significant. **Conclusion.** Both bone substitutes used in the study showed good osteoconductive properties in the treatment of bicortical defects. Bone tissue density in defects filled with alloplastic bone graft was statistically significantly higher than that in the defects filled with xenograft.

Key words:
alveolar ridge augmentation; bone density; bone regeneration; bone transplantation; cone-beam computed tomography; dental implants; oral surgical procedures; swine.

Apstrakt

Uvod/Cilj. Vođena tkivna i vođena koštana regeneracija omogućuju značajnu korist u zarastanju bikortikalnih defekata. Cilj istraživanja bio je rendgenografska analiza koštanog zarastanja unutar bikortikalnih defekata primenom kompjuterizovane tomografije zasnovane na tehnologiji konusnog snopa (CBCT), kao i komparativna analiza gustine koštanog tkiva u bikortikalnim defektima tretiranim postupkom vođene koštane regeneracije uz korišćenje kolagene membrane i dva tipa koštanih zamenika: goveđeg ksenografta i aloplastičnog koštanog zamenika. **Metode.** U prvoj fazi istraživanja izvršena je ekstrakcija svih zuba interkaninog sektora donje vilice nakon čega su postekstrakcione rane zašivene. U drugoj fazi, nakon perioda zarastanja, pristupilo se formiranju bikortikalnih defekata. Nakon odizanja mukoperiostalnog režnja sa vestibularne i lingvalne strane oštrim makazicama i kiretom uklonjen je kambijalni sloj periosta u predelu budućih defekata. Zatim su sa leve i desne strane od medijalne linije formirana dva bikortikalna defekta promera 10 mm koji su popunjavani koštanim zamenicima i to aloplastičnim na levoj i goveđim ksenografton na desnoj strani. Nakon popunjavanja defekti su prekriveni kolagenim resorptivnim

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membranama obostrano, nakon čega je izvršena repozicija i ušivanje režnja. Nakon 12 nedelja eksperimentalne životinje su žrtvovane. Okolna nativna kost služila je kao kontrola. **Rezultati.** Analiza gustine koštanog tkiva pokazala je statistički značajnu razliku između testiranih zamenika kosti, pri čemu je bolji efekat postignut primenom aloplastičnog koštanog zamenika ($p < 0,01$). Nakon primene Bonferonijeve korekcije, razlika je i dalje bila statistički značajna grupe. **Zaključak.** Oba koštana zamenika korišćena u studiji pokazuju dobra osteokonduktivna svojstva u tretmanu bikortikalnih defekata. Gustina koštanog tkiva u defektima pojačanim aloplastičnim zamenikom je bila statistički značajno veća nego u defektima popunjavanim ksenograftom.

Ključne reči:
alveolarni greben, podizanja; kost, gustina; kost, regeneracija; transplantacija kosti; tomografija, kompjuterizovana, konusna; implantati, stomatološki; hirurgija, oralna, procedure; svinje.

Introduction

Bicortical, "tunnel" or transosseous defects are defined as defects characterized by lack of buccal and oral bone cortex or occurring when a surgical accessory bone window is formed on one side, with already present erosion on another cortical lamella. These defects typically have a 3-wall configuration with the mesial, distal, and craniocaudal walls, but without buccal or oral. In this type of defect, the rapid proliferation of the connective tissue can interfere with the bone structure ingrowth from the sidewalls of the defect, leading to so-called fibrous healing [1]. Authors who dealt with the problem of these defects consider that a significant benefit in their healing can be achieved by guided tissue, bone regeneration, primarily in the sense of preventing the onset of scarring fibrous healing [2–4].

The main element of guided bone regeneration is a barrier membrane, which is placed into the space between the mucoperiosteal flap and the bone defect, preventing the penetration of the gingival epithelial and connective tissue into a defect, and enabling regeneration, not reparation of the tissue [5]. In addition to the membrane, different types of bone substitutes are also used for the treatment of bone defects within the guided bone regeneration. Their role is not only to support the membrane and stabilize the blood clot but also in accelerating the healing period and contributing formation of a higher quality bone due to their osteoconductive, osteoinductive, and osteogenic properties [6].

The main method for assessing bone healing in augmented defects, which is still considered the gold standard, is histological analysis. However, as it is not adequate for analysis in clinical conditions, some radiographic noninvasive methods of bone tissue analysis are also suggested [7, 8]. In this context, microradiography is cited as a method that provides reliable data on trabecular architecture and density of bones without spatial deformations [9]. The introduction of the cone-beam computed tomography (CBCT) contributed to a significant reduction in the scanning time, radiation dose, costs, and also the possibility of its use in dental clinics as a piece of standard equipment. CBCT scanners can be used in various diagnostic procedures in dentistry, providing precise diagnostic data not only in the region of interest but also in the surrounding structures [10]. Soardi et al. [11] also stated that there is no statistically significant difference in determining bone tissue density between microradiography or CBCT devices.

The aim of this study was to analyze bone healing of bicortical defects treated with the guided bone regeneration and two bone substitutes (bovine xenograft and alloplastic bone substitute) using CBCT.

Methods

The study was approved by the Ethics Committee of the Faculty of Medical Science in Kosovska Mitrovica, with headquarters in Kosovska Mitrovica, Serbia (No. 09-423-1/26.03.2018). The research was carried out on ten domestic pigs with an average weight of 25 kg.

Surgical procedure

Surgical procedures were carried out in the ambulance of the domestic pigs' farm of the Agricultural High School in Lesak, Serbia. Before every surgical intervention, the animals were deprived of food and water for a period of 12 h. Premedication was performed by intramuscular (im.) injection of midazolam at a dose of 0.5 mg/kg (Midazolam® Panpharma 5 mg/5 mL, Rotexmedica GmbH Arzneimittelwerk, Germany). After fifteen min, the animals were introduced into anesthesia by i.m. administration of 1 mL/10 kg combined ketamine solution as an anesthetic, xylazine as a sedative, and atropine as an anticholinergic (100 mg/20 mg/1 mg *per* mL of solution, KET-A-XYLR Sens, Peru). To control pain and intraoperative bleeding in the area of the surgical field, local anesthesia was used (4 mL articaine chloride/adrenaline hydrochloride, 40 mg/mL/0.012/mL, Ubistesin Forte, 3M ESPE, Germany).

Surgical procedures were performed in two phases. In the first phase, after introducing the animals to short-term anesthesia and then with local anesthesia, the extraction of lower teeth in the intercanine sector was performed. The post-extraction wounds were sutured (Figure 1) with resorptive sutures (Polisorbtm 3–0, Covidien, Switzerland).

In the second phase, after 9 weeks required for healing of postextraction wounds [12], mucoperiosteal flaps from the vestibular and lingual side in the area of the previously extracted teeth were elevated, and bicortical defects were formed after removal of the cambium layer of periosteum with sharp surgical scissors and curette. Two defects on the left and right side of the medial line were formed with a round trepan bur with 10 mm diameter, with the use of a contra-angle handpiece with 700–800 rpm and with manda-

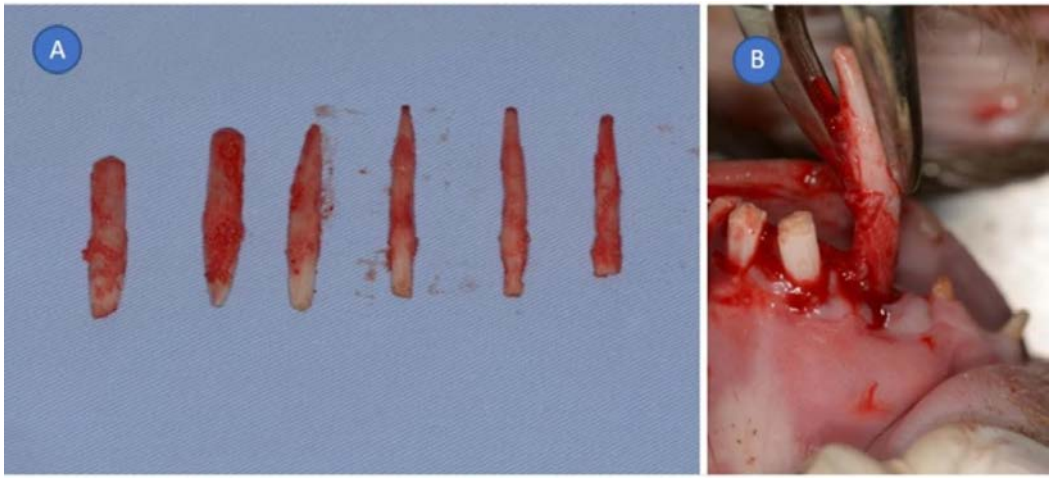

Fig. 1 – Extracted teeth (A) and extraction of the tooth in the inter-canine sector (B).

tory cooling with saline. Defects on the left side were filled with alloplastic bone substitute (Tixxu® graft, 60% hydroxyapatite – 40% tricalcium phosphate, Bredent, Germany) with granule sizes of 0.5–1.0 mm, while defects on the right side were filled with xenograft of bovine origins (D Bone®, Divinity Capital LLC) with granule sizes of 0.25–1 mm.

After augmentation, the defects were covered by placing the collagen resorptive membrane with a thickness of 0.6–0.8 mm (Angiopore, Bredent, Germany) from both vestibular and lingual sides of the defect; thus, the membrane passed over the edges of the defect for 5 mm or more, in order to prevent its collapse.

For easier identification of the augmentation site after a period of healing, fixing screws (Titan pin set, Botiss Biomaterials GmbH, Germany) were placed at a distance of 3 mm from the edges of the defect, thereby making the membrane additionally stabilized. After filling the defect and placing the membrane, the flap was repositioned and sutured with resorptive sutures (Figure 2).

The surrounding native bone was used as a control.

Postoperatively, for five days, antibiotic therapy with benzylpenicillin was ordered to experimental animals, with a dose of 12 000 IU/kg (Neo-penicillin 4 000 000 IU, FM Pharm d.o.o. Subotica).

CBCT analysis

After 12 weeks, experimental animals were sacrificed by intravenous (iv.) administration of 7.4% potassium chloride solution, which causes cardiac arrest. After sacrificing, the lower jaw was separated from the skull, and soft tissue was removed. Using a handsaw and performing a mandatory cooling with saline, the front segment of the mandible was detached and divided in the medial line area into two bone blocks. Each bone block contained an examined bone defect,

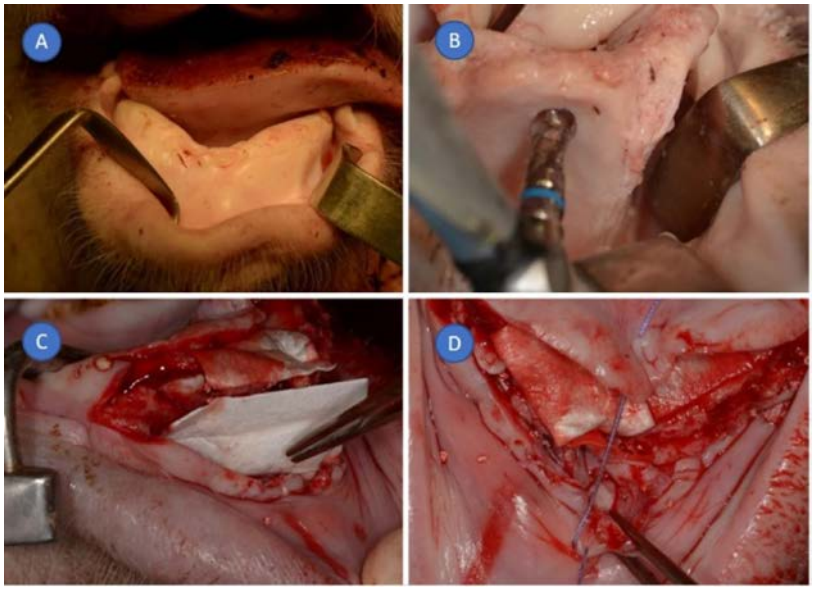

Fig. 2 –Surgical procedure: A) appearance of the toothless alveolar ridge 9 weeks after tooth extraction; B) preparation of bicortical defect after elevating the mucoperiosteal flap; C) filling a defects with a bone substitute and covering with a resorptive collagen membrane from the vestibular and lingual side; D) reposition and suturing of the flap.

clearly visible due to previously installed pins (Figure 3). Detached bone blocks were immersed in a 4% neutral formalin solution and sent for radiographic examination. The examination involved measuring the density of bone tissue in the region of examined defects, as well as in the region of surrounding native bone, expressed in the Hounsfield Unit (HU).

X-ray analysis was carried out in the V Dental Center in Podgorica (Montenegro), using the CBCT scanner PlanmecaProMax 3D and PlanmecaRomexis analysis software (Figure 4).

Statistical analysis

Descriptive methods and methods for testing statistical hypotheses were used for the analysis of data. Descriptive statistical methods included measures of central tendency (median) and measures of variability (range). Methods for testing statistical hypotheses included the Kruskal-Wallis test with Bonferroni correction. For statistical processing, the statistical software package SPSS 21 was used. Statistical hypotheses were tested at the statistical significance level of 0.05.

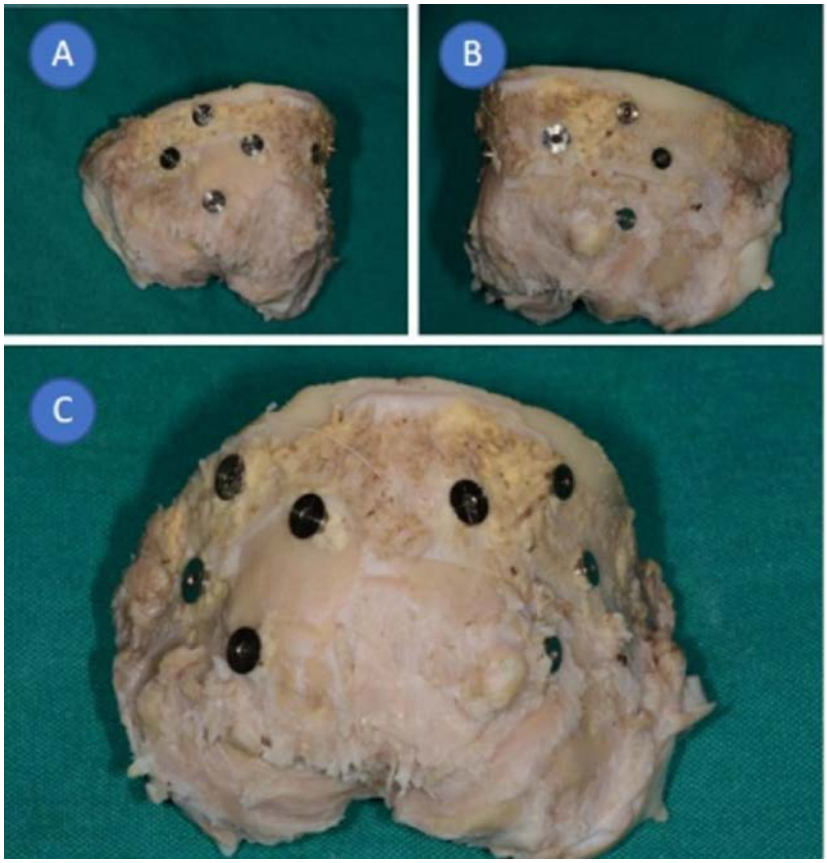

Fig. 3 – The bone defects marked with pins, prepared for cone-beam computed tomography analysis (A–C).

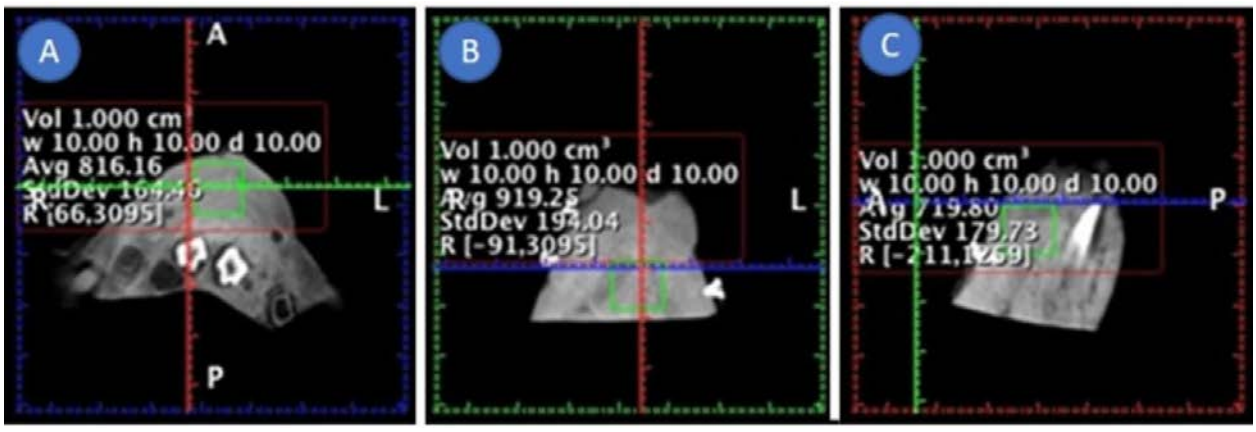

Fig. 4 – Analysis of bone density of defects filled with alloplastic substitute (A), xenogeneic substitutes (C) and bone density of surrounding native bone tissue (B).

Results

During the follow-up period, there were no postoperative complications in any animal.

Radiographic analysis of the examined defects revealed a full bone healing of all defects, whether it was filled with xenograft or with an alloplastic bone substitute.

Analysis of bone tissue density in the examined defects, as well as of surrounding native bone, expressed in Hounsfield units (HU), showed a statistically significant difference in all three examined samples (Table 1). The highest density was found in the area of the surrounding native bone (945.2 HU). The density of the defects treated with alloplastic bone substitute was substantially closer to the density values of the surrounding native bone than in the case of defects treated with xenograft, and it ranged from 773.5 to 895.8, with an average value of 829.7 HU. Values of the measured density in the defects treated with xenogeneic bone substituent were 649.7 HU.

Table 1

Bone density values of the examined defects

Bone substitute	Bone density (HU), median (range)	*p*
Allograft	829.7 (773.5–895.8)	
Xenograft	649.7 (586.4–723.5)	< 0.001
Native bone (control)	945.2 (925.7–963.5)	

HU – Hounsfield Unit.

After applying Bonferroni correction, the difference was statistically significant among all samples (Table 2).

Table 2

***p*-values after Bonferroni correction**

Difference in bone density	*p* (adjusted)
Xenogeneic substitute – alloplastic substitute	0.032
Xenogeneic substitute – native bone	< 0.001
Alloplastic substitute – native bone	0.032

Discussion

Tissue regeneration is defined as a reproduction of a lost, injured, or surgically removed part, so that the architecture and function of the lost, injured, or removed tissues could be completely restored [13]. Therefore, the bone tissue regeneration process in bicortical jaw defects can be significantly deranged as a result of periosteal damage. The periosteum is primarily a basic source of osteoprogenitor cells, necessary for the regeneration process. It also plays the role of a barrier that prevents penetration of gingival epithelium into the bone healing region. In the case of bicortical defects, the periosteum may be destroyed as a result of the infectious process that caused the appearance of bone defects. Its damage can also occur as a result of simple flap elevation, where the process in bone destroyed both cortexes completely [14]. Such damage can be the cause of incomplete bone healing or even fibrous healing, which makes possible future implant-prosthetic rehabilitation impossible in the defect area.

Numerous studies indicated a fact that guided tissue or bone regeneration can provide many benefits in terms of achieving a more predictable process of defect healing. Taschieri et al. [2, 3] emphasized that the use of guided bone regeneration increases the possibility of successful bone healing in bicortical defects. Similar results were pointed out by Pecora et al. [14]. The use of barrier membranes in such lesions improved possibility of regenerative processes, excluding the unwanted expansion of gingival connective tissue or migration of oral epithelium into the defect, which allowed the formation of the trabecular bone [15]. Moreover, concerning guided bone regeneration, many authors emphasize the importance of proper maintenance of the area below the membrane in terms of providing support for the membrane and stabilizing the blood clot by applying bone substitutes [16, 17].

In this study we determined the presence of bone healing of bicortical defects under resorptive membrane which covered two bone substitutes that augmented the defects. Biphasic calcium phosphate with a lower ratio of hydroxyapatite and β-tricalcium phosphate (60% : 40%), and bovine bone graft were used as materials for guided bone regeneration.

Xenograft is a bone substitute originating from different phenotypes, usually pigs, cattle, horses, or alternative natural sources, such as calcified corals [18] or algae [19, 20]. Although originating from living individuals, xenograft loses cells and bioactive molecules during the production process, so that it has only osteoconductive properties. Jensen et al. [21] and Buser et al. [22] indicate a slow rate of resorption of this graft, wherefore the newly formed bones in bone defects augmented with the xenograft show a slightly smaller volume than in the case of some more resorptive material or autotransplants; this is partly explained by the fact that limited resorption leaves less space for the formation of new bone.

The combination of hydroxyapatite and β-tricalcium phosphate enables the use of positive properties of each of them. Isolated β-tricalcium phosphate shows a high degree of resorption, creating a space for the ingrowth of blood vessels and the formation of new bone tissue in the region of resorbed granules [23]. Graft also releases calcium and phosphate ions, stimulating bone healing [24]. However, if accelerated resorption of this graft is not accompanied by simultaneous bone tissue formation, the ultimate effect may be a bone healing disorder [25]. A supplement of hydroxyapatite showing a low level of resorption [26, 27] provides good mechanical support and graft stability. Also, Jensen et al. [21, 28, 29] point out that such a low ratio of these bone substituents, as it was used in the study, allows a faster and larger formation of bone tissue compared to pure β-tricalcium phosphate, biphasic calcium phosphate with higher hydroxyapatitr/tricalcium phosphate (HA/TCP) ratios and demineralized bovine bone substitute. Although xenograft is widely used today and represents the best documented type of bone substitute, the study indicates that alloplastic material in the form of a combination of hydroxyapatite and β-tricalcium phosphate might serve as an adequate alternative, not only to xenograft but also to other types of bone substitutes. In the literature, results can be found which indicate that these materials, in addition to their osteoconductive effect, can promote osteoinduction,

which is another advantage [30]. Their use may also reduce the risk of transmissible diseases if allograft and xenograft were used, particularly the transmission of prion-induced infections after the use of a substitute with bovine origins [31, 32].

Analysis of the results obtained in our study indicates that both types of bone substitutes can be used for the treatment of bicortical bone defects in order to obtain predictable bone healing. The study presented a statistically significantly higher density of the newly formed bone in defects where an alloplastic material was applied compared to defects where xenograft was applied. Obtained density values were also closer to the density of the surrounding native bone. Having in mind the Misch classification [33], we estimated that the bone in the bicortical defects treated with alloplastic material had the D2 type density according to this classification, and in defects, with a bovine substitute, it had the D3 type.

Having in mind the requirements of dental implantation, bone density is a very important parameter of bone quality assessment, which can significantly affect the success of implantation itself. The D2 is a type of bone tissue built from a solid cortical bone on the surface and a dense trabecular bone in the central part. It is better vascularized than the D1 type, allowing bleeding during the preparation of an implant bed, which prevents overheating, and also has a high healing capacity after implantation and a very predictable osteointegration [34, 35]. The D3 type is composed of a thin, porous, compact, and loose spongy bone. It is characterized by good vascularization, but also with inferior mechanical properties in comparison to the D2 bone type. Assessing the mechanical properties of the trabecular bone of the mandible, according to the Misch bone density classification [33], Misch et al. [35] concluded that the D2 bone type has a 47% to 68% higher resistance to pressure than the bone with the D3 density, and a 50% higher hardness. This has a significant effect on the primary stability of implant built into such region, which is one of the basic predictive factors of implantation success. In terms of the elastic modulus, the bone type D2 has a much higher elastic modulus and is closer to the elastic modulus of titanium, compared to the D3 and D4 bone types, which enables a higher degree of implants survival. The study of Misch [35] further indicated that bone-implant contact, after the initial healing phase, was significantly higher in the bone type D2 than D3, which is another important factor for successful implantation.

Conclusion

We can conclude that both bone substitutes used in the study showed good osteoconductive properties in terms of the formation of new bone tissue and the treatment of bicortical defects by the process of guided bone regeneration. Bone tissue density in defects filled with alloplastic bone substitute was statistically significantly higher than that in defects filled with the xenogeneic substitute. Moreover, the density of the new bone in these defects was substantially closer to the density of the surrounding native bone.

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Resilience as a moderator in the relationship between burnout and subjective well-being among medical workers in Serbia during the COVID-19 pandemic

Rezilijentnost kao moderator povezanosti izgaranja na poslu i subjektivnog blagostanja kod medicinskih radnika u Srbiji u toku pandemije COVID-19

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Abstract

Background/Aim. During the ongoing COVID-19 pandemic, exhaustion and difficulties at work can seriously endanger the mental health of medical workers. The aim of this study was to examine whether resilience is a moderator of the association between burnout and subjective well-being among medical workers at the time of the pandemic. **Methods.** The research was conducted on a sample of 521 medical workers (354 female), among whom were 245 physicians and 276 medical technicians. The average age of the respondents was 38.66 years. Data were collected using online questionnaires comprising the Brief Resilience Scale, the Work Burnout Scale, the Short Subjective Well-being Scale, and the Sociodemographic Data Questionnaire. Regression and interaction analysis (by SPSS macro "PROCESS 3.5") was used for data analysis and processing. **Results.** The results showed that burnout was a significant negative predictor of subjective well-being of medical workers (ß = -0.19; $p < 0.01$) and a significant positive predictor of subjective well-being (ß = 0.40; $p < 0.01$), as well as that the interaction of resilience and burnout was a significant positive predictor of subjective well-being (ß = 0.09; $p < 0.01$). In subjects who had developed resilience at the level of +1 standard deviation (SD), the negative effect of burnout on subjective well-being was 2.8 times lower than in subjects who had resilience at the level of -1 SD. **Conclusion.** The study confirmed that resilience reduces the negative connection between burnout and subjective well-being, which is a significant argument that medical workers should be provided with resilience training programs to prevent burnout and preserve mental health during a pandemic.

Key words:
covid-19; resilience, psychological; burnout, professional; quality of life; medical staff; occupational exposure; surveys and questionnaires.

Apstrakt

Uvod/Cilj. U vreme pandemije COVID-19 iscrpljenost i teškoće na poslu mogu ozbiljno ugroziti mentalno zdravlje medicinskih radnika**.** Cilj ovog rada bio je ispitati da li je rezilijentnost moderator povezanosti izgaranja na poslu i subjektivnog blagostanja kod medicinskih radnika tokom pandemije. **Metode.** Istraživanje je sprovedeno na uzorku od 521 medicinskih radnika (354 ženskog pola), među kojima je bilo 245 lekara i 276 medicinskih tehničara. Prosečna starost ispitanika bila je 38,66 godina. Podaci su prikupljeni pomoću *online* upitnika koji su činili Kratka skala rezilijentnosti, Skala izgaranja na poslu, Kratka skala subjektivnog blagostanja i Upitnik sociodemografskih podataka. Za analizu i obradu podataka korišćena je analiza regresije i interakcije (SPSS macro "PROCESS 3.5"). **Rezultati.** Ustanovljeno je da je izgaranje na poslu značajan negativan prediktor subjektivnog blagostanja medicinskih radnika (ß = -0,19; $p < 0,01$), da je rezilijentnost značajan pozitivan prediktor subjektivnog blagostanja (ß = 0,40; $p < 0,01$) i da je interakcija rezilijentnosti i izgaranja na poslu značajan pozitivan prediktor subjektivnog blagostanja (ß = 0,09; $p < 0,01$). Kod ispitanika koji su imali razvijenu rezilijentnost na nivou +1 standardna devijacija (SD), negativan efekat sagorevanja na poslu na subjektivno blagostanje bio je 2,8 puta manji nego kod ispitanika koji imaju rezilijentnost na nivou od -1 SD. **Zaključak.** Istraživanje je potvrdilo da rezilijentnost umanjuje povezanost izgaranja na poslu i subjektivnog blagostanja što predstavlja značajan argument da medicinskim radnicima treba omogućiti programe obuke rezilijentnosti u cilju prevencije sagorevanja na poslu i očuvanja mentalnog zdravlja u uslovima pandemije.

Ključne reči:
covid-19; rezilijentnost; sagorevanje na radu; kvalitet života; kadar, medicinski; profesionalna izloženost; ankete i upitnici.

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Introduction

By the nature of their work, medical workers experience unpleasant and stressful situations. During the time of the COVID-19 pandemic, exhaustion and difficulties at work can seriously endanger the mental health of medical workers [1–6]. Even before the mentioned pandemic, high resilience was cited as a feature that enables medical workers to easily recover from various misfortunes at work which can be acquired through an appropriate training program [7–9]. Resilience is also cited as a trait that can reduce the association between burnout and mental health difficulties of health professionals [10–13].

As a personality trait, resilience refers to an individual's ability to return to a state of normal mental functioning after stressful or threatening events without lasting negative consequences [14]. As an individual's capacity, resilience can be defined as the sum of all protective factors that act in such a way that an individual maintains or improves his or her mental health after circumstances that may cause severe distress or mental trauma. Protective factors can be: 1) individual factors, such as ways of coping with stress, cognitive capacity, and strength of character of the individual; 2) factors arising from the social network of the individual, such as emotional or material support provided by family or close friends; 3) support from the wider community, such as support provided by state institutions, companies, and social organizations. Resilience is closely related to subjective well-being. Generally speaking, people with a higher degree of resilience also have a higher degree of subjective well-being and a lower degree of depression, anxiety, and negative self-evaluation [9, 15].

On the framework of the theory of subjective well-being, Diener [16] and Deiner et al. [17] emphasized the importance of happiness and life satisfaction for the mental health of an individual. The subjective well-being of an individual refers to his/her cognitive and affective evaluation of their own life. The concept of subjective well-being differs from the affective component, which includes a frequent experience of positive emotions and rare experience of negative emotions, and the cognitive component, which includes a positive evaluation of one's life, i.e., life satisfaction [18]. People's subjective well-being is positively correlated with their willingness to face different adjustment challenges. Resilience and subjective well-being can be treated as highly interdependent phenomena [9].

Burnout is chronic stress at work that adversely affects mental health and reduces employees' job satisfaction. Maslach et al. [19] defined the concept of burnout as a syndrome that encompasses the following dimensions: 1) emotional exhaustion related to the experience of lack of energy for work and loss of enthusiasm; 2) cynicism related to work (includes the experience of distancing from work and coworkers, as well as diminishing the importance of their work); 3) the experience of reduced professional efficiency [19]. Kristensen et al. [20] identify the following types of burnout: personal burnout, which refers to the experience of fatigue and exhaustion in general in life, work-related burnout, and client-related burnout. Physical and mental fatigue and exhaustion are the basis of each of these types of burnouts. Berat et al. [21] distinguish between two highly interrelated experiences within work-related burnout: work exhaustion and the experience of job frustration. Previous studies indicate that burnout of health workers increased during the COVID-19 pandemic compared to the time before that pandemic [22, 23].

Studies suggest that in physicians, resilience and burnout are interrelated phenomena, with greater resilience implying less burnout, just as greater burnout implies weaker resilience [11, 12]. Resilience acts as a factor that reduces anxiety in doctors at work, as well as their exhaustion at work [4]. Moreover, the studies indicate that a negative correlation between burnout and resilience also exists among medical technicians [13, 24]. A higher degree of resilience in medical technicians implies better coping skills at work, a higher level of self-efficacy and better social support at work, a lower level of exhaustion at work, as well as a lower level of anxiety and depression [25, 26].

Studies conducted by Yu et al. [25] and Wang et al. [27] indicate that burnout during the COVID-19 pandemic is negatively correlated with the subjective well-being and mental health of medical workers.

The aim of this study was to examine whether resilience is a moderator of the relationship between burnout and the subjective well-being of medical workers. The following hypotheses were set: 1) burnout is a negative and significant predictor of subjective well-being; 2) resilience is a positive and significant predictor of subjective well-being; 3) the interaction of resilience and burnout is a significant positive predictor of subjective well-being. A theoretical model was assumed in which the negative correlation between burnout and subjective well-being decreases with a higher degree of resilience.

Methods

Sample and procedures

We adopted a cross-sectional study design for this research. Inclusion criterion for the study sample was residents of Serbia aged 18 years or older being in a medical profession (medical doctors, medical technicians/nurses). Exclusion criteria were minors, residents of other countries, and members of any profession outside the medical field.

Since the research was conducted during the COVID-19 pandemic, the data were collected online using the Google Forms platform in the period from April 16, 2020, to May 2, 2020, in Serbia. The objectives of the research were explained to potential participants at the very beginning of the anonymous online questionnaire in Serbian. Participation in the research was voluntary and with informed consent, and respondents were guaranteed confidentiality and anonymity of the obtained data. All data was protected, only the research team had access. Duplicate and inappropriate survey responses were excluded with a manual review of gathered data.

The research was approved by the Institutional Review Board of the Department of Psychology, Faculty of Philosophy, University of Belgrade (Approval number: 2020-30). The procedures of this study were in accordance with the provisions of the Declaration of Helsinki on medical research involving human subjects [28].

Measures

Resilience. A version of the Brief Resilience Scale validated by Slišković and Burić [29] was used to test resilience, and the original version of this scale was created by Smith et al. [14]. According to the mentioned authors, the Brief Resilience Scale has very good reliability, the Cronbach's alpha coefficient was above 0.8 in previous research. The Brief Resilience Scale is one-dimensional and consists of 6 items. Items refer to resilience which is defined as the ability to recover from stressful or threatening events. Three items speak in favor of resilience (eg. Item 3: It does not take me long to recover from a stressful event), while three items speak against resilience and have the opposite scoring (eg. Item 4: It is hard for me to snap back when something bad happens). Respondents had an option to choose one answer on a five-point Likert-type scale, from 1 – strongly disagree to 5 – strongly agree. The total score on this scale is the arithmetic mean of all six items. Resilience was treated as a moderator variable.

Burnout. Burnout was examined by the version of the Work Burnout Scale given by Berat et al. [21] and based on the original scale constructed by Kristensen et al. [20]. The scale examines fatigue and exhaustion at work and has a total of 7 items. The items of the scale are divided into two dimensions: a) work exhaustion (eg. Item 1: Is your work emotionally exhausting?, and Item 7: Do you have enough energy for family and friends during leisure time?); b) work frustration (eg. Item 3: Does your work frustrate you?, and Item 5: Are you exhausted in the morning at the thought of another day at work?). Respondents had an option to choose one answer on a five-point Likert-type scale (from 1 – Almost never or To a very low degree, to 5 – Always or To a very high degree). Respondent's score on the Work Burnout Scale can range from 0 to 100, because after reverse scoring item 7 (Do you have enough energy for family and friends during leisure time?), all answers are recoded as follows: 1 into 0, 2 into 25, 3 into 50, 4 into 75, 5 into 100, and then calculate the arithmetic mean of all 7 items. The reliability of the Work Burnout Scale in previous research was over 0.8 [20, 21]. Burnout was assumed to be the main predictor variable (focal predictor).

Subjective Well-Being. The Short Subjective Well-Being Scale presented by Jovanović [18] was used to examine subjective well-being. The scale consists of 8 items divided into two dimensions: the positive affectivity and the positive attitude towards life. The positive affectivity includes 4 items that refer to the frequent experience of happiness and other positive emotions (eg. Item 4: I feel lively, and Item 7: I often feel happy and elated.). A positive attitude towards life includes 4 items that relate to the experience of life satisfaction (eg. Item 1: I feel that life is full of nice surprises, and Item 6: Life is full of good opportunities and possibilities). Respondents had an option to choose their answers on a Likert-type scale from 1 – I completely disagree to 5 – I completely agree. The score on the Short Subjective Well-Being Scale is the sum of all the answers. Subjective well-being was treated as a dependent variable.

Demographics. To collect data on gender, education, and age, a questionnaire of sociodemographic data was developed by the authors of this research. Gender, age, and occupation were selected as covariates, as has been done in previous research [1, 9, 10].

Data analysis

Mean values, standard deviations, minimum and maximum value, skewness, and kurtosis were used as measures of descriptive statistics. To check the reliability of the scales used, Cronbach's alpha coefficient was used as a measure of internal consistency. Regression analysis and a special moderation analysis procedure created by Hayes [30] were used to test the set hypotheses. Free SPSS macro PROCESS 3.5 was used, which performs regression analysis by examining the significance of the interaction of moderator and focal predictor, as a predictor of the dependent variable, with the assessment of the significance of statistics in the usual way (using the value of *p*) but also with a special procedure called bootstrapping. Bootstrapping includes regression analysis on a large number of random subsamples (resampling), and in this paper, the option is set to 5,000. This appendix allows the program to analyze the relationship between focal predictors and dependent variables at different levels of moderators (conditional effects of focal predictor at values of the moderator). Since macro PROCESS 3.5 produces a printout that gives non-standardized predictor regression coefficients before regression analysis, standardized variables that were used in the procedure to obtain standardized regression coefficients (ß) in the printout were calculated because they are commonly used in the display of results and for easier comparison of predictors.

Results

Participant characteristics

The sample consisted of 521 medical workers, among them were 245 physicians and 276 medical technicians. There were 354 female respondents (153 physicians and 201 medical technicians) and 167 male respondents (92 physicians and 75 medical technicians). The average age of the sample was [mean (M) ± standard deviation (SD)] 38.66 ± 9.46 years. The age ranges of medical technicians and physicians ranged from 19 to 62 years and 25 to 62 years, respectively.

Table 1 shows measures of descriptive statistics and scale reliability. All instruments used in this study had high reliability, which was expressed as the α coefficient of internal consistency (Cronbach's alpha), as was expected.

Table 1

Descriptive statistics for resilience, burnout, and subjective well-being

Scale	Min.	Max.	Mean	SD	Skew	Kurt	α
Resilience	1.00	5.00	3.19	0.87	-0.18	-0.17	0.82
Burnout	0.00	100.00	59.48	25.91	-0.38	-0.68	0.91
Subjective well-being	8.00	40.00	29.49	6.97	-0.68	0.15	0.92

SD – standard deviation.

Table 2

The predictors of subjective well-being

Model summary	R^2	MSE	F	df1	df2	*p*
	0.31	33.81	39.14	6	514	0.01
Predictors	ß	SE	T	*p*	LLCI	ULCI
burnout (FP)	-0.19	0.01	-4.28	0.01	-0.28	-0.10
resilience (M)	0.40	0.36	9.07	0.01	0.31	0.49
int. M*FP	0.09	0.01	2.53	0.01	0.02	0.16
age	-0.03	0.03	-1.02	0.31	-0.11	0.03
sex	0.06	0.56	1.84	0.07	-0.01	0.14
profession	0.04	0.53	1.20	0.23	-0.03	0.12
Test of Int. M*FP:	Change R^2		F	df1	df2	*p*
	0.01		6.38	6	514	0.01

Note: Int. M*FP – Interaction between Moderator (M) and Focal Predictor (FP); LLCI – Lower Limit of Confidence Interval (95%); ULCI – Upper Limit of Confidence Interval (95%).

Table 3

Effects of burnout on subjective well-being at different levels of resilience

Resilience (SD)	Effect	SE	T	*p*	LLCI	ULCI
-1.00	-0.28	0.06	-4.62	0.01	-0.40	-0.16
0.00	-0.19	0.04	-4.28	0.01	-0.28	-0.10
1.00	-0.10	0.05	-1.94	0.06	-0.20	0.00

Note: Levels of resilience were set on -1, 0, 1 standard deviation (SD) in PROCCESS 3.5 procedure; LLCI – Lower Limit of Confidence Interval (95%); ULCI – Upper Limit of Confidence Interval (95%).

Tables 2 and 3 show the results of regression and interaction analyses. These predictors explain 31% of the variance in subjective well-being.

Covariates – age, gender, and occupation (physician or medical technician) were not significant predictors of subjective well-being.

Table 3 shows the effects of burnout on subjective well-being at three different levels of resilience. At the low and medium level of resilience, burnout was a significant negative predictor of subjective well-being, while when resilience was expressed at a level of plus one standard deviation, the relationship between burnout and subjective well-being ceases was statistically significant [$p < 0.05$ and confidence interval (CI) includes zero]. It can be clearly seen that with the increase in the resilience of the respondents, the connection between burnout and subjective well-being decreased, as well as the magnitude of the negative effects of burnout on subjective well-being.

Discussion

The research findings confirmed the assumed model in which resilience is the moderator of the negative correlation between burnout and subjective well-being. Therefore, the negative effect of burnout on subjective well-being among medical workers decreases with greater resilience.

Our results showed that burnout is a significant negative predictor of the subjective well-being of medical workers (ß = -0.19; $p < 0.01$), which is in line with the results of previous research that indicated that burnout has a negative effect on mental health and the subjective well-being of medical workers [2, 22, 25, 27]. Some other studies also

showed that burnout is a significant negative predictor of subjective well-being [3, 10, 23, 27]. With more burnout, subjective well-being is lower. However, based on the confidence interval obtained and on the bootstrap procedure, it can be seen that the sample included respondents who differed greatly in the degree of connection between burnout and subjective well-being because the lower limit of confidence interval (LLCI) was 2.8 times lower than the upper limit of confidence interval (ULCI) (LLCI = -0.28 and ULCI = -0.10).

Exhaustion and frustration related to work are aspects of burnout [19–21], and these experiences are mutually conditioned with experiences of happiness and positive emotions in life, which are aspects of subjective well-being [16, 17]. This finding indicates that burnout has a negative effect on subjective well-being but also includes the possibility that the degree of subjective well-being affects the experience of burnout. Medical workers who experience their lives as difficult and unhappy find it easier to perceive their work as frustrating and exhausting.

In our study, resilience was a significant positive predictor of subjective well-being (ß = 0.40; $p < 0.01$), which is in line with previous research showing that resilience is a factor that has a positive effect on mental health and subjective well-being [9–12]. Greater resilience implies greater subjective well-being, and *vice versa* - with less resilience, subjective well-being is lower. Resilience is the result of all protective factors that act to keep an individual healthy in difficult circumstances [9, 14, 15] so that subjective well-being can be understood as a consequence of resilience but also as a factor of resilience. Developed resilience of medical workers implies personal skills and other opportunities to maintain a good mood, level-headedness, and correct judgment after stressful circumstances [7–9], which according to the findings of this research, preserves both the degree of their positive affectivity (the frequency of experiencing happiness and other positive emotions in life) and a positive attitude towards life, as to dimensions of subjective well-being. Preserved positive affectivity and a positive attitude towards life also represent an individual's ability to more easily endure various stressors and adversities in life, even at work, which is a factor of resilience when it is understood as an individual's potential.

In our study, the interaction of resilience and burnout was a significant positive predictor of subjective well-being (ß = 0.09; $p < 0.01$); with greater interaction, subjective well-being was better. The contribution of this interaction, although statistically significant, is not large – in the regression model, it contributes to the explanation of only 1% of the variance of subjective well-being. However, data on the confidence interval (CI), based on the bootstrap procedure, indicate that the sample includes very different respondents according to the degree of connection between this interaction and subjective well-being; the LLCI = 0.02 is as much as 8 times lower than the ULCI = 0.16.

Our study demonstrated that the negative association between burnout and subjective well-being among health care workers was significantly higher when they had low resilience. The negative effect of burnout on subjective well-being in subjects with a level of resilience at minus one standard deviation was 2.8 times higher than in subjects with a level of resilience at plus one standard deviation (see effect size in Table 3). This finding indicates that resilience prevents frustrations and work exhaustion from worsening the degree of subjective well-being of medical workers.

These scales have not been used so far for examinations of medical workers in Serbia. However, for the sake of insight into the bigger picture, these results will be compared with the results of previous research where the same instruments were used as in this research, without drawing solid conclusions. Smith et al. [14] examined the characteristics of the Brief Resilience Scale in the United States and indicated that the M value of resilience obtained in student samples was 3.53 (SD = 0.68, n = 128) and 3.57 (SD = 0.76, n = 64), while in the sample of heart patients undergoing rehabilitation it was 3.98 (SD = 0.68, n = 112). Slišković and Burić [29] showed that the M of resiliences in the sample of 3,010 teachers in Croatia was 3.20 (SD = 0.78). Bozdağ and Ergün [1] conducted the research during the pandemic on a sample of 214 medical workers in Turkey, received the M value of resilience of 18.43 (SD = 3.3) on a scale of 5 to 30, which is M = 3.68 (SD = 0.66) on a scale from 1 to 5. Jovanović [18], in the validation study of the short scale of subjective well-being, has reported that the M of subjective well-being is 33.43 (SD = 5.20; n = 226). The findings on the mean values of resilience and subjective well-being in this study do not deviate much from the findings of the mentioned studies, especially when standard deviations are taken into account. When it comes to burnout, things look much different. Berat et al. [21], in the research on a sample of 352 workers of different professions in Serbia, get M burnout of 44.99 (SD = 22.39). Kristensen et al. [20] examined a sample of 1,910 Danish workers in the auxiliary occupations sector and found that the work burnout M was 33.00 (SD = 17.70). The COVID-19 pandemic appears to have acted as a factor that increased the rate of burnout but did not particularly alter the degree of resilience and subjective well-being among the medical workers who made up the sample of this study. More research suggests an increased rate of burnout in health workers during the COVID-19 pandemic [3, 5, 6, 22, 23, 27]. The higher levels of burnout of nurses and doctors during the COVID-19 pandemic have also been reported in studies conducted in France, Italy, and Spain [31].

The design of this research does not allow the consideration of cause-and-effect relationships. The findings of this research do not exclude the possibility that there is an opposite direction of action in which favorable subjective well-being acts so that burnout is weaker and *vice versa* - that less favorable subjective well-being implies the experience of greater burnout. It has already been pointed out that subjective well-being can be understood both as a consequence and as a factor of resilience [9], as well as in those studies where it is suggested that burnout is negatively correlated with worker resilience [4, 12, 24–26]. It is possible that there is a circle in which the weakening of subjective well-being leads to weaker resilience, which leads to more

difficulties and unpleasant experiences related to work, and this then adversely affects subjective well-being and resilience. The analytical moderation procedure applied in this study best corresponds to the experimental designs [30]. Although the design of this study is not such, this procedure is used here in a creative and illustrative way only to show the correlation between the examined phenomena without concluding causation-consequential relationships.

The age of medical workers was not a significant predictor of subjective well-being. In addition to age, gender was chosen as a covariate variable because it was previously shown that gender could be a significant factor in the mental health of the health care workers [10], as well as in their subjective well-being [15]. Compared to men, women as respondents in the research perceived more often their mental health as a little less favorable and subjective well-being as somewhat lower. In a sample of this study, gender was not a significant predictor of subjective well-being. Furthermore, in this study, a covariant variable called profession with two possible indicators – physician or medical technician, did not prove to be a significant predictor of subjective well-being, indicating that the difference in job type is not significantly related to subjective well-being in this sample.

The sample of this research does not allow generalization of the results so that they are valid for the entire population of medical workers. In addition, findings on burnout indicate that the COVID-19 pandemic most likely contributed to a significant increase in burnout levels of health workers compared to the time before the pandemic, as indicated by other studies [3, 6, 23, 27].

Although the problem of this research arose on the basis of a very extensive scientific material on resilience, subjective well-being, and burnout, the assumed theoretical model is original in terms of variable positions, methods, and time of testing. This research, in addition to theoretical significance, could also have important practical significance when it comes to creating training that develops resilience among medical workers in order to prevent burnout and preserve subjective well-being during a pandemic.

Conclusion

The resilience of health workers is negatively associated with burnout, positively correlates with subjective well-being, and mitigates the negative correlation of burnout and subjective well-being. The findings of this study represent a significant argument that medical workers should be provided with resilience training programs in order to prevent burnout and maintain mental health in a pandemic.

Conflict of interest

The authors declare no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Expression of tumor necrosis factor-alpha receptor 2 and interleukin-1 in middle ear cholesteatoma

Ekspresija receptora 2 za faktor nekroze tumora alfa i interleukina-1 kod holesteatoma srednjeg uva

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Abstract

Background/Aim. Cholesteatoma is characterized by progressive growth with the erosion of surrounding bone due to pressure effects, enzymatic activity and activation of osteoclasts. The aim of this study was to examine the expression levels of tumor necrosis factor (TNF)-alpha receptor 2 (TNF R2) and interleukin-1 (IL-1) in chronic otitis media (COM) with and without acquired cholesteatoma and correlate them with the degree of bone destruction. **Methods.** The study included 178 patients of both sexes, aged 5–75 years, who underwent microsurgical treatment for COM, with and without cholesteatoma at the Ear, Nose and Throat Department, University Clinical Center of Republika Srpska (UCC RS), Banja Luka from 2015 to 2018. Based on cholesteatoma presence, the patients with COM were divided into two groups: with cholesteatoma (CCOM) (n = 97) and without cholesteatoma (COMWC) (n = 81). Samples of cholesteatoma perimatrix in the CCOM group and tympanic cavity inflamed mucosa in the COMWC group were collected intraoperatively. Intraoperative exploration of the middle ear included the status of the ossicular chain, individual ossicles, osseous walls of the external auditory canal (EAC) and tympanic cavity. Expression levels of TNF R2 and IL-1 were investigated by immunohistochemical analysis of tissue samples obtained during ear surgery. **Results.** The correlation between the level of osteodestruction and the presence of cholesteatoma was significant ($p < 0.01$). Elevated expression levels of TNF R2 and IL-1 were most frequent in CCOM patients with osteodestruction. The probability of osteodestruction of EAC and tympanic cavity walls was significantly higher in patients with high TNF R2 expression ($p < 0.05$). With respect to IL-1 expression levels, no significant correlation with the described pathomorphological changes was observed. Correlation between TNF R2 and IL-1 expressions and ossicular chain destruction was significant ($p < 0.01$). **Conclusion.** Cholesteatoma presence and elevated expression levels of TNF R2 and IL-1 in COM patients are significantly correlated. Expression levels of TNF R2 and IL-1 in acquired cholesteatoma tissue have a potential clinical significance for the occurrence of bone destruction compared to expression levels in inflamed mucosa of the tympanic cavity.

Key words:
interleukin-1; cholesteatoma; chronic disease; otitis media; bone resorption; receptor, tumor necrosis factor-alpha.

Apstrakt

Uvod/Cilj rada. Holesteatom karakteriše progresivni rast sa erozijom okolnih koštanih struktura usled efekta pritiska, enzimske aktivnosti i aktivacije osteoklasta. Cilj rada bio je da se utvrde nivoi ekspresije receptora 2 za faktor nekroze tumora alfa (TNF R2) i interleukina-1 (IL-1) kod hroničnog *otitis media* (HOM), sa i bez stečenog holesteatoma, i njihova korelacija sa stepenom koštane destrukcije. **Metode.** U studiju je bilo uključeno 178 bolesnika oba pola, starosti od 5 do 75 godina, koji su bili podvrgnuti mikrohirurškom lečenju HOM sa i bez holesteatoma, u Klinici za bolesti uva, grla i nosa Univerzitetskog kliničkog centra Republike Srpske (UKC RS), Banja Luka od 2015. do 2018. godine. Na osnovu prisustva ili odsustva holesteatoma, bolesnici su podeljeni u 2 grupe: sa holesteatomom (n = 97) i bez holesteatoma (n = 81). Uzorci perimatriksa holesteatoma (grupa sa holesteatomom) i inflamirane sluznice *cavum tympani* (grupa bez holesteatoma) su uzeti intraoperativno. Intraoperativna eksploracija srednjeg uva je uključivala proveru stanja osikularnog lanca, pojedinih osikula, koštanih zidova spoljašnjeg slušnog kanala i *cavum tympani*, kao i susednih anatomskih

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struktura. Nivoi ekspresije TNF R2 i IL-1 određeni su imunohistohemijskom analizom intraoperativno dobijenih tkivnih uzoraka. **Rezultati.** Korelacija između stepena osteodestrukcije i prisustva holesteatoma bila je statistički značajna. Visoki nivoi ekspresije TNF R2 i IL-1 bili su učestaliji u grupi bolesnika s holesteatomom koji su imali osteodestrukciju u odnosu na grupu bolesnika bez holesteatoma. Verovatnoća osteodestrukcije zidova spoljašnjeg slušnog kanala i *cavum tympani* bila je značajno veća kod bolesnika sa većom ekspresijom TNF R2 ($p < 0,05$). U odnosu na nivoe ekspresije IL-1, nije uočena značajna korelacija s opisanom patomorfološkom promenom. Korelacija između ekspresije TNF R2 i IL-1 sa destrukcijom osikularnog lanca bila je značajna ($p < 0,01$). **Zaključak.** Prisustvo holesteatoma i povišen nivo ekspresije TNF R2 i IL-1 kod bolesnika sa HOM značajno su povezani. Nivoi ekspresije TNF R2 i IL-1 u tkivu stečenog holesteatoma imaju potencijalan klinički značaj kod nastanka koštane destrukcije.

Ključne reči:
interleukin-1; holesteatom; hronična bolest; otitis medija; kost, resorpcija; receptor, faktor nekroze tumora-alfa.

Introduction

Chronic *otitis media* (COM) is a chronic inflammatory disease of the middle ear and mastoid that often results in partial or total loss of the tympanic membrane and ossicles, leading to conductive hearing loss that can range in severity up to 60–70 dB [1]. COM is characterized by insidious and asymptomatic onset, slow and long duration as well as by potentially substantial destructive effects, especially when cholesteatoma is present (CCOM). COM may occur either with cholesteatoma (CCOM) or without cholesteatoma (COMWC). Most intracranial complications (95.8%) are caused by CCOM that occur with greater frequency during the first three decades of life, with a greater incidence in males [2].

Cholesteatoma is a cystic lesion composed of epithelium and stroma, surrounded by an inflammatory reaction. The main characteristic of cholesteatoma is progressive growth with erosion of surrounding osseous structures due to the pressure effect and osteoclast activation [3]. Cholesteatoma are classified as either congenital, which occurs in 2%–4% of cases, or acquired. An annual incidence of acquired cholesteatoma is 3 in 100,000 in childhood and 9.2 in 100,000 in the adult population, predominantly in males [4–6]. Cholesteatoma causes destruction of the temporal bone due to mechanical pressure, enzymatically mediated bone resorption and the promotion of acute and chronic infections [7, 8]. Molecular and cellular processes that result in clinical characteristics of cholesteatoma (migration, uncoordinated proliferation, altered differentiation and aggressiveness) have not been entirely explained so far [9].

Proinflammatory mediators released during the inflammatory process in the perimatrix of cholesteatoma could be responsible for bone destruction. Tumor necrosis factor (TNF)-α stands out as one of the major cytokines that, together with the receptor activator of nuclear kappa-B ligand (RANKL), interleukin (IL)-1, IL-2 and IL-6 participate in the process of bone destruction and remodelling [10, 11]. Two TNF-α receptors, TNF R1 and TNF R2, have similar activities. They are involved in the inflammatory process linked with bone erosion, and they also may cause apoptosis (programmed cell death) that is particularly related to the expression level of TNF R2 [12]. IL-1 is produced by cholesteatoma's epithelial cells and by the inflammatory cells of the surrounding granulation tissue. The expression levels of IL-1α and IL-1β receptors are considerably higher in cholesteatoma than in normal squamous epithelium. IL-1 is involved in the bone resorption process, and it has been proven that it stimulates the proliferation of keratinocytes [13–15].

The aim of this study was to examine the expression levels of the TNF R2 and IL-1 in COM with and without cholesteatoma and correlate them to the degree of bone destruction.

Methods

This cross-sectional study included 178 patients of both sexes, aged 5–75 years, who underwent microsurgical treatment for COM, with and without cholesteatoma at the Ear, Nose and Throat Department, University Clinical Center of Republika Srpska (UCC RS), Banja Luka from 2015 to 2018. The research was approved by the UCC RS Ethics Review Committee (No.: 01-10566-2/13). Written informed consent was obtained from all patients included in the study. The diagnosis of COM was established based on the clinical history and physical examination of the patients. Exclusion criteria were the following: the presence of congenital cholesteatoma, the malignant middle ear tumor, *otitis externa* or a previous history of ear surgery.

The study also excluded the cholesteatoma samples that lacked perimatrix. Based on the presence of cholesteatoma the patients were divided into two groups: 1) patients with cholesteatoma (CCOM) (n = 97) and 2) patients without cholesteatoma (COMWC, control group) (n = 81). Medical records were the source of personal data and included information about the duration, symptoms and previous treatment of COM. The detailed clinical examinations and preoperative preparations were performed routinely by the same examiner.

Depending on the type and degree of the chronic middle ear inflammatory process, a standard, closed and open tympanoplasty technique with modifications was applied. During the microsurgical procedures, samples of the perimatrix of acquired cholesteatoma in the CCOM group and of the inflamed mucosa of the tympanic cavity in the COMWC group were collected. Intraoperative examination

of the middle ear included checking the status of the ossicular chain and individual ossicles to determine the ossicular chain destruction degree (OCDD). The degree of destruction in the ossicular chain was scored according to the Mills and Padgham scoring system [16]. The OCDD was categorized as follows: O_0-intact ossicular chain, O_1-incus eroded with a discontinuity of the ossicular chain, O_2-incus and the superstructure of the stapes eroded, O_3-the manubrium of the malleus and incus are missing, the superstructure of the stapes eroded. Intraoperative examination of the middle ear also included checking the status of the osseous walls of the EAC and tympanic cavity, and of nearby anatomic structures.

The samples obtained during microsurgical procedures were fixed in 10% formaldehyde and subsequently assembled into paraffin blocks from which 4 μm thick semiserial sections were obtained using a rotatory microtome (microTec CUT 4055, SLEE medical GmbH, Mainz, Germany). After deparaffinization, the samples were stained using the routine hematoxylin-eosin (HE) method, and then examined under a light microscope (DM2500 Leica Microsystems GmbH, Wetzlar, Germany). The samples were treated with citrate buffer by heating in a microwave oven for 20 min in the PT module (PreTreatment Module™ Thermo Scientific, Fermont, USA) to unmask antigens. After the blockade of endogenous peroxidase with hydrogen peroxide (H_2O_2) in methanol, the samples were rinsed in Tris buffered saline (TBS) solution, pH 7.4.

For the immunohistochemical (IHC) analysis of TNF R2 in the samples of cholesteatoma and inflamed mucosa from the tympanic cavity, primary polyclonal TNF R2 antibody synthesized from rabbit (TNFR2 Polyclonal Antibody, Product # PA1-21148, Thermo Fisher Scientific, Fermont, USA) was used, and for IL-1 analysis, a polyclonal antibody IL-1A (IL1A Polyclonal Antibody, Product # PA5-25921, Thermo Fisher Scientific, Fermont, USA) was used. Antibodies were used in a dilution of 1 : 50. The IHC identification of the investigated mediators was performed with the application of the UltraVision LP Detection system: HRP polymer & DAB Plus Chromogen (Product # TL-125-HD, Thermo Scientific™ Lab Vision™ UltraVision™, Fermont, USA). The 3.3'-diaminobenzidine (DAB) (Thermo Fisher Scientific, Fermont, USA) was used as a chromogenic substrate, and contrasting was performed with hematoxylin. The IHC analyses were performed manually in the authorized laboratory of the Clinical Pathology Department of the UCC RS. Original reagents were used.

IHC analysis was based on the quantitative (0-absent, 1-present) and semiquantitative determination of the intensity of expression using a light microscope and a 0-3 grading system (0-absent, 1-weak, 2-moderate, 3-high intensity). Samples were divided into 4 categories based on the percentage of stained cells. The results of the IHC analysis were considered negative if there was no staining, and labeled with 0; weak positive at ≤ 25% of positive cells and labeled with 1; moderate positive at ≥ 25–50% of positive cells and labeled with 2; high positive and labeled with 3 if ≥ 50% of positive cells were present. The total outcome of the IHC reaction was calculated from the product of the results of the expression intensity and the percentage of stained cells. The overall results of the analysis were deemed negative for result ≤ 1 and labeled 0, weak positive if result was ≥ 2 ≤ 3 (label 1+), moderate positive for result ≥ 4 ≤ 6 (label 2+) and high positive when result was 9, with the label 3+.

Statistical analysis

The data was analyzed by descriptive statistics, with the calculation of absolute and relative distributions of the patients in the CCOM and COMWC groups, according to the levels of expression of tested mediators, and with the calculation of arithmetic mean and standard deviation for the patients' age. Pearson's χ^2 test was used to calculate the difference in the distribution between the COMWC patients and the CCOM patients, and Mann-Whitney *U* non-parametric test was used to calculate the patients' age difference between the groups. Logistic regression was used to establish the link between independent factors, tested mediators, with the presence of the bone wall and ossicular chain destruction. The results were deemed significant if $p < 0.05$. Statistical data processing was performed by using the Statistical Package for the Social Sciences for Windows version 21.0 data processing software (IBM corp., Armonk, USA).

Results

This study included 178 patients of both sexes, with an average age of 49.11 ± 17.06. The youngest patient was 5, and the oldest was 75 years old. The average age of the CCOM and COMWC groups were 45.01 ± 18.72 and 54 ± 13.38 years, respectively. There was a statistically significant difference between the mean average age in the groups (Mann-Whitney $U = 2{,}910.00$, $Z = -2.97$, $p = 0.003$; $p < 0.01$). Of all patients, 95 (53.4%) were male and 83 (46%) were female. The results of the χ^2 test revealed a statistically significant difference between the two groups in terms of gender distribution (χ^2 (1) = 7.75, $p = 0.005$; $p < 0.01$), with males being more represented in the CCOM group.

Based on the history taking and clinical examination, it was found that 35 (19.7%) of patients had bilateral COM, among them 17 (17.5%) were in the CCOM group, and 18 (22.2%) were in the COMWC group. The time interval between the onset of the first symptoms and the diagnosis varied between 15 days and 50 years, with an average value of 12.39 ± 12.15 years, while the time interval between the diagnosis and admission for surgery varied between 10 days and 30 years, with an average value of 8.69 ± 12.15 months. Regarding the symptoms reported by patients upon admission to the clinic, 158 (88.8%) listed otalgia, 169 (94.9%) otorrhea, 158 (88.8%) hearing loss, 153 (85.9%) tinnitus, 47 (26.4%) headache and 41 (23%) dizziness.

Destruction of the bone walls of the EAC and tympanic cavity was found in 70 (72.2%) of patients in the CCOM

group and only in 5 (6.2%) of patients in the COMWC group. Logistic regression established a statistically significant correlation between the destruction of the bone walls of the EAC and tympanic cavity with the presence of cholesteatoma (Wald = 46.23; $p = 0.000$). The probability that the patients with CCOM would have the described pathomorphological change was 45 times greater than those in the COMWC group (95% CI: 15.202-137.804) (Table 1).

The high levels of TNF R2 expression were most represented in the CCOM group patients who exhibited destruction of the bone walls of the EAC and tympanic cavity. Out of 70 (72.2%) patients who exhibited the described pathomorphological change, the highest number of them - 33 (80.5%) were with moderate, and 22 (81.5%) with high TNF R2 expression. All 5 (6.2%) patients with the described pathomorphological change in the COMWC group had moderate TNF R2 expression. It was established that the statistical probability of the EAC and tympanic cavity bone walls destruction onset was significantly higher (Wald = 12.04; $p = 0.007$) in the category of patients with high expression of TNF R2. It was also determined that the statistical probability of the onset of the described pathomorphological change (95% CI: 0.056-0.719) was significantly lower (Wald = 6.09, $p = 0.014$) in the patient category with weakly expression of TNF R2 (Table 1).

The predictor model that encompassed IL-1 expression categories relative to inflamed mucosa of the tympanic cavity and cholesteatoma tissue established a statistically significant correlation between the presence of cholesteatoma and the EAC and tympanic cavity bone walls destruction (Wald = 50.43, $p < 0.01$). The probability that the described pathomorphological change would be found in patients with CCOM was 48 times greater than in patients in the COMWC group (95% CI:16.509-140.001). In the CCOM group, the patients with high levels of IL-1 expression were most represented, of whom 29 (76.3%) exhibited high, and 26 (72.2%) moderate IL-1 expression. Of the 14 patients in the COMWC group with described pathomorphological change, 5 patients (11.1%) had high IL-1 expression. Relative to the levels of IL-1 expression, no statistically significant correlation with the EAC and tympanic cavity bone walls destruction was observed (95% CI: 0.16-0.94) (Table 2).

The destruction of the ossicular chain was found in 78 (80.4%) of patients in the CCOM and in 17 (21.0%) of patients in the COMWC group. In the CCOM group of patients with OCDD O_3, high and moderate expression of TNF R2 had 5 (55.6%) and 18 (43.9%) patients, respectively. In the OCDD O_1 group of patients, high and moderate expression of TNF R2 had 7 (25.9%) and 11 (26.8%) patients, respectively. The predictor model that included the level of TNF R2 expression relative to inflamed mucosa of the tympanic cavity and cholesteatoma tissue demonstrated that cholesteatoma may be considered a statistically significant predictor (Wald = 56.49; $p < 0.01$) of a greater degree of ossicular chain destruction. A statistically significant correlation was established between the TNF R2 expression and the destruction of the ossicular chain, so in patients with lack of TNF R2 expression (Wald = 3.62; $p < 0.05$), lower degrees of ossicular chain destruction may be expected (Table 3).

In the CCOM group, high IL-1 expression had 22 (57.9%) and 8 (21.1%) patients with OCDD O_3 and OCDD O_1, respectively. Moderate IL-1 expression was recorded in 14 (38.9%) and 10 (27.8%) patients with OCDD O_3 and OCDD O_2, respectively. The predictor model that included

Table 1

Correlation of the expression of tumor necrosis factor receptor 2 (TNF R2) and bone destruction of external auditory canal and tympanic cavity in the chronic otitis media

Factors/predictors	B	S.E.	Wald	Exp(B)	95% CI for EXP(B)	*p*
Cholesteatoma presence (CCOM group)	3.824	0.562	46.231	45.770	15.202–137.804	0.000
TNF R2 expression level						
total			12.038			0.007
≤ 1; negative (0)	-1.099	0.726	2.290	0.333	0.080–1.383	0.130
≥ 2 ≤ 3; weakly positive (1+)	-1.604	0.650	6.092	0.201	0.056–0.719	0.014
≥ 4 ≤ 6; moderate positive (2+)	0.240	0.586	0.167	1.271	0.403–4.008	0.683
Constant	-2.455	0.685	12.831	0.086		0.000

B – coefficient for the constant; S.E. – standard error; Exp (B) – exponentiation of the B coefficient; CI – confidence interval.

Table 2

Correlation of the expression of interleukin-1 (IL-1) and bone destruction of external auditory canal and tympanic cavity in the chronic otitis media

Factors/predictors	B	S.E.	Wald	Exp(B)	95% CI for EXP(B)	*p*
Cholesteatoma presence (CCOM group)	3.873	0.545	48.076	48.076	16.509–140.0	0.000
IL-1 expression level						
total			2.659			0.447
≤ 1; negative (0)	-0.723	0.714	0.485	0.485	0.120–1.965	0.311
≥ 2 ≤ 3; weakly positive (1+)	-0.930	0.696	0.395	0.395	0.101–1.544	0.182
≥ 4 ≤ 6; moderate positive (2+)	-0.611	0.484	0.543	0.543	0.210–1.403	0.207
Constant	-2.471	0.484	26.097	0.085		0.000

B – coefficient for the constant; S.E. – standard error; Exp (B) – exponentiation of the B coefficient; CI – confidence interval.

a degree of IL-1 expression relative to the inflamed mucosa of the tympanic cavity and cholesteatoma tissue revealed that cholesteatoma may be considered a statistically significant predictor (Wald = 67.3; $p < 0.01$) of a greater degree of ossicular chain destruction. A statistically significant correlation was established between IL-1 expression and the ossicular chain destruction, with lower degrees of ossicular chain destruction expected in the patients with negative (Wald = 6.82; $p < 0.01$) and weak positive IL-1 expression (Table 4).

Regarding the other pathomorphological changes in the overall sample, facial nerve canal dehiscence was found in 32 (18%) of patients, five patients (2.8%) had lateral semicircular canal fistula, one (0.6%) patient had a fistula on the promontorium, 19 (10.7%) patients had sigmoid sinus dehiscence, 24 (13.5%) patients had denuded sigmoid sinus and 16 (9%) patients had denuded middle cranial fossa dural plate. ICH findings of TNF R2 and IL-1 expressions in the CCOM and COMWC groups of patients are shown in Figures 1–4.

Table 3

Correlation of the expression of tumor necrosis factor receptor 2 (TNF R2) and ossicular chain destruction in the chronic otitis media

Factors/predictors	E	Wald	95% CI	*p*
Cholesteatoma presence				
CCOM group	2.788	56.490	2.06–3.52	0.000
COMWC group	-	-	-	-
TNF R2 expression level				
≤ 1; negative (0)	-1.115	3.624	-2.26–0.03	0.050
≥ 2 ≤ 3; weakly positive (1+)	-0.644	1.801	-1.58–0.29	0.180
≥ 4 ≤ 6; moderate positive (2+)	-0.175	0.181	-0.98–0.63	0.671
9; strong positive (3+)	-	-	-	-

E – coefficient for the constant; CI – confidence interval; CCOM – chronic otitis media with cholesteatoma; COMWC – chronic otitis media without cholesteatoma.

Table 4

Correlation of the expression of interleukin-1 (IL-1) and ossicular chain destruction in the chronic otitis media

Factors/predictors	E	Wald	95% CI	*p*
Cholesteatoma presence				
CCOM group	3.165	67.315	2.41–3.92	0.000
COMWC group	-	-	-	-
IL-1 expression level				
≤ 1; negative (0)	-1.550	6.820	-2.71– -0.387	0.009
≥ 2 ≤ 3; weakly positive (1+)	-1.084	3.769	-2.18–0.01	0.050
≥ 4 ≤ 6; moderate positive (2+)	-0.611	2.953	-1.31–0.09	0.086
9; strong positive (3+)	-	-	-	-

E – coefficient for the constant; CI – confidence interval; CCOM – chronic otitis media with cholesteatoma; COMWC – chronic otitis media without cholesteatoma.

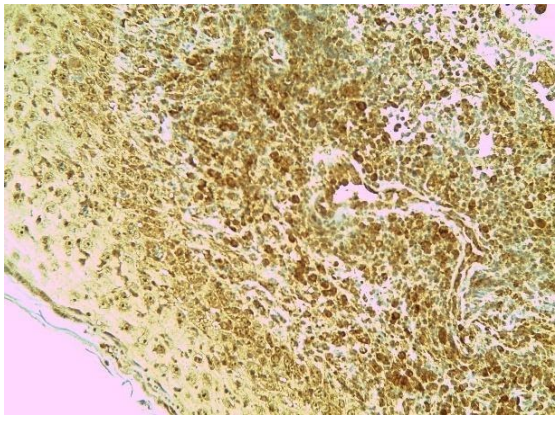

Fig. 1 – Immunohistochemical staining of stromal and inflammatory cells in cholesteatoma: high expression of tumor necrosis factor receptor 2 (TNF R2) (x400).

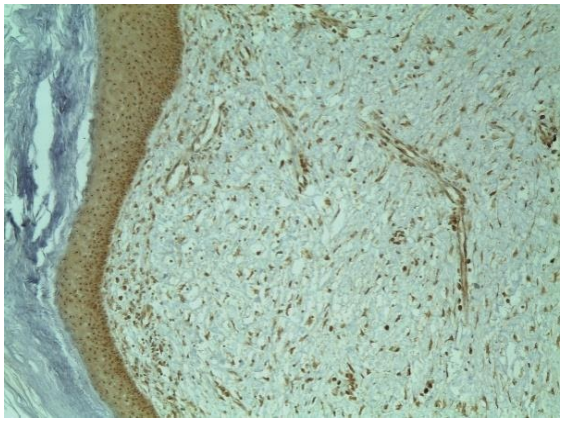

Fig. 2 – Immunohistochemical staining of stromal and inflammatory cells in cholesteatoma:weak expression of tumor necrosis factor receptor 2 (TNF R2) (x400).

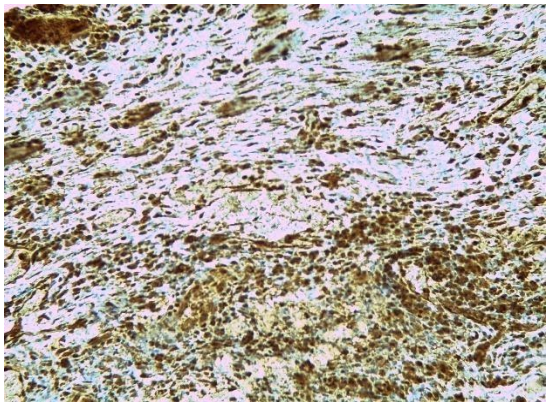

Fig. 3 – Immunohistochemical staining of stromal and inflammatory cells in cholesteatoma: high expression of interleukin-1 (x400).

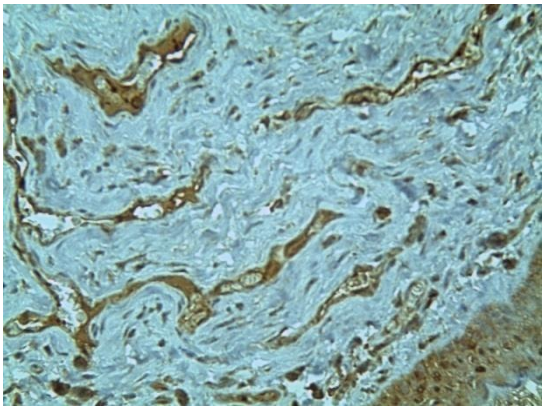

Fig. 4 – Immunohistochemical staining of stromal and inflammatory cells in cholesteatoma: weak expression of interleukin-1 (x400).

Discussion

This study included 95 male and 83 female patients, aged 5–75 years, who underwent surgical treatment for COM, with and without cholesteatoma. The levels of expression of TNF R2 and IL-1 in intraoperative samples of the perimatrix of acquired cholesteatoma and in the tissue of inflamed mucosa of tympanic cavity of COMWC group were tested using IHC, and compared with the degree of bone destruction. Hamed et al. [17] reported that various molecules were examined in terms of their relation to the severity of bone resorption and the incidence of complications, and a positive correlation was established between their expression in the cholesteatoma tissue and the degree of bone erosion.

Osteoclasts are the central cells involved in bone resorption. They originate from monocytes and macrophages and act directly on the bone matrix, causing bone erosion and remodeling. TNF-α, IL-6, IL-1 α, IL-1 β and the macrophage colony stimulating factor (MCSF) cause differentiation and regulation of osteoclasts and have direct impact on bone resorption [12, 18, 19]. Research also revealed that RANKL and matrix metalloproteinases (MMPs) have a key role in the bone tissue destruction in cholesteatoma [20–23]. Bone erosion of the ossicular chain and otic capsule may cause hearing damage, vestibular dysfunction, facial palsy and intracranial complications [20, 23].

A positive and statistically significant correlation was established between the occurrence of cholesteatoma and the bone destruction of the EAC and tympanic cavity. The levels of TNF R2 and IL-1 expression in the perimatrix of cholesteatoma were significantly higher in comparison with the control group , which allows the conclusion that there is a correlation of the higher levels of expression of the mediators under observation with the bone destruction of the above mentioned anatomical structures. It was also proven that the presence of cholesteatoma had statistically predictive significance in the emergence of more severe degrees of ossicular chain destruction, with the patients with high levels of TNF R2 and IL-1 expression being most represented in the same group.

The results of the present study are in line with the data from the literature. Yetiser et al. [24] found significantly higher levels of TNF-α, IL-1 and epidermal growth factor (EGF) in 23 CCOM patients relative to 16 COMWC patients, and they concluded that bone destruction was mediated by these cytokines. A similar study was also conducted by Akimoto et al. [25], who found significantly higher levels of TNF-α and IL-1 in the tissue of inflamed congenital and acquired cholesteatoma in comparison with the skin of the EAC. Li et al. [26] reported that TNF-α in cholesteatoma tissue acts both directly, causing bone erosion as an autocrine growth factor, and indirectly as an important mediator that stimulates the release of enzymes causing bone destruction. Welkoborsky [27] reported that different cytokines and lytic enzymes, such as intracellular adhesion molecule (ICAM), RANKL, IL-1, IL-2, IL-6, MMP-2, and MMP-9, cause activation and maturing of osteoclasts, which results in degradation of extracellular bone matrix and hyperproliferation, bone erosion and, eventually progression of the disease.

We found that a high expression of TNF R2 has a statistically significant effect on higher probability of bone destruction of the EAC and tympanic cavity. We also observed that a weak TNF R2 expression has a statistically significant effect on lower probability of incidence of bone destruction of the EAC and tympanic cavity, which we did not prove in the case of IL-1 expression. Regarding the ossicular chain destruction, we also found that negative and weak expression of TNF R2 and IL-1 indicated statistically significantly lower probability of occurrence of this pathomorphological change. This leads to the conclusion that the levels of expression of the mediators under observation, proven in the perimatrix of cholesteatoma, may have predictive significance for the occurrence of bone destruction and, potentially, for otogenic complications. Kuczkowski et al. [28] found that heightened expression of TNF-α, IL-1α and IL-6 in COM and a highly positive correlation between the levels of these cytokines and the degree of bone destruction point to the destructive character of cholesteatoma and granulation tissue.

Although high levels of TNF R2 and IL-1 expressions were found in the CCOM group of patients (in whom we found bone destruction on the surrounding anatomical

structures of the middle ear), due to the small size and nonrepresentative character of the sample, we were not in the position to conduct statistical analysis. However, the research has proven that elevated levels of TNF-α and lysosomal enzymes in the epithelial and subepithelial tissue of cholesteatoma reflect clinical severity of the disease, which is seen in their significant increase in the cases of erosion of two or three ossicles, dural plate, sigmoid sinus and facial nerve canal and extended cholesteatoma. Increased activity of inflammatory cytokines in patients with CCOM may indicate the need for a "wall-down" tympanoplasty technique and a "second look" surgery after a few months [28, 29].

Based on the study results we concluded that elevated expression levels of TNF R2 and IL-1 in cholesteatoma of the middle ear indicate its destructive characteristics. Inflammatory mediators have predictive significance in terms of the manifestation of aggressive characteristics of the acquired cholesteatoma of the middle ear and the occurrence of bone destruction with potential otogenic complications. This implies that inflammatory mediators might be helpful in monitoring and treating COM and cholesteatoma, but further research is necessary to explain all mechanisms and aspects of that relationship.

Conclusion

The cholesteatoma presence and the elevated expression level of TNF R2 and IL-1 in COM patients are significantly correlated. Expression levels of TNF R2 and IL-1 in acquired cholesteatoma tissue have a potential clinical significance for the bone destruction occurrence.

Conflict of interest

The authors declare that they have no conflicts of interest associated with the publication of this article. The authors bear the sole responsibility for the content and writing of this article.

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Roles of sulfur-containing amino acids in gastrointestinal physiology and pathophysiology

Uloge sumporovitih aminokiselina u gastrointestinalnoj fiziologiji i patofiziologiji

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Key words:
amino acids, sulfur; cysteine; gastrointestinal tract; homocysteine; metabolism; methionine; physiology; taurine; pathology.

Ključne reči:
amino kiseline, sumporne; cistein; gastrointestinalni sistem; homocistein; metabolizam; metionin; fiziologija; taurin; patologija.

Introduction

Sulfur-containing amino acids (SAA) are methionine (Met), cysteine (Cys), homocysteine (Hcy), and taurine (Tau). Only Met and Cys are included in protein synthesis. Amino acids (AA), apart from being incorporated in proteins, are now recognized to have other significant roles in metabolism, such as being precursors of essential molecules, acting as mediators or signal molecules, and affecting numerous functions.

Essential AA must be provided by feed and are limiting for growth as they are the building blocks for protein synthesis. For a better understanding of the physiological consequences of an insufficient intake of these AA, their nonproteinogenic functions must be also considered. Methylation processes of SAA can affect metabolism and cell functions by their participation in the control of oxidative stress [1].

There is a lot of evidence indicating that SAA metabolism in gastrointestinal tissue is linked to human health and gut diseases. Met and Cys play a metabolically and functionally important role in the gastrointestinal system [2]. They maintain many gut functions, including the digestion, absorption, and metabolism of nutrients, the immunity of intestinal mucosal epithelial cells.

Historically, it is assumed that dietary AA are absorbed from the lumen into the portal blood without degradation. Recent results support the view that absorbed AA are captured, transformed, and degraded in tissues of the intestine before they enter portal circulation; 30% of dietary Met is metabolized by the intestine in the first pass [3]. Studies in rats [4] and piglets [5] demonstrated that the gastrointestinal tissues possess the significant activities of enzymes necessary to transform Met to Cys, and further utilization of Met by the intestine [2, 6]. Met is necessary for normal growth and development [7]. In every cell, Met is used for protein synthesis and the methylation cycle, where it is converted to S-adenosylmethionine (SAM), the principal methyl donor.

In the methylation process of DNA or proteins, SAM is transformed to S-adenosylhomocysteine (SAH), which is then hydrolyzed to Hcy [8]. Low Met intake or folate deficiency will reduce SAM concentrations, which can further induce deregulation in DNA methylation in various cancers, including colorectal cancer [9].

Hcy is a sulfur-containing nonproteinogenic AA derived in Met metabolism by transmethylation. Hyperhomocysteinemia (HHcy), increased plasma Hcy level, is recognized as a risk factor for cardiovascular and cerebrovascular diseases [10] and gastrointestinal diseases [11], including constipation, Crohn's disease, inflammatory bowel disease (IBD),

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and colorectal cancer [12, 13]. The connection between inflammatory remodeling of the digestive tract and HHcy has been shown, resulting in higher production of reactive oxygen species (ROS). HHcy was also recognized as one of the risk factors for colorectal cancer, mesenteric venous thrombosis, and subsequent bowel infarction [14].

Cys is an AA incorporated in the tripeptide glutathione (GSH) (Glu-Cys-Gly) and plays a key role in its cellular antioxidant function, and its availability is dependent upon Met intake [15]. GSH has an important role in intestinal gut redox status [16]. The concentration of Cys, which is the limiting AA in GSH synthesis, is very important for the maintenance of epithelial cell GSH concentration and regulation of intestinal cell redox status [17]. Cys plays a key role in cellular redox function and susceptibility to oxidant stress in the intestine [18, 19].

Tau is involved in numerous physiological functions. It regulates bile conjugation, osmolarity regulation, calcium modulation, and cytoprotective effects such as antioxidative properties, membrane stabilization, and immunomodulation [20–22]. Tau is found in high concentrations in mammalian cells, and it has endogenous antioxidant and a membrane-stabilizing function [23]. Tau is a protective agent against oxidative stress-induced disorders such as gastrointestinal damage [24] and can inhibit oxidative stress-induced apoptosis in epithelial cells [25].

Methionine

Met is an essential AA that takes part in many metabolic processes such as protein synthesis, methylation of DNA, and polyamine synthesis. Met absorption from the gastrointestinal tract is highly efficient and it is rapidly removed by tissues. In particular, the liver clears great amounts of Met from blood plasma [26]. Among the SAA, Met is the most valuable because it can serve as a sulfur donor to generate the other two SAA, Cys, and cystine, but reversed reaction is not possible. Met makes thus a precious and versatile contribution to the daily requirement for SAA. The estimated SAA intake for adult humans ranges between 13 and 16 mg/kg *per* day (17–27 mg/g protein) [27].

Apart from being a sulfur donor for Cys biosynthesis, Met represents the main cellular donor of methyl groups after conversion to SAM [28]. SAM is included in many metabolic pathways like the synthesis of norepinephrine, dopamine, and serotonin. Moreover, it has been proposed as a potential treatment for depression [29]. Furthermore, by serving as a methyl donor for DNA methylation, SAM has key control over the whole cellular transcriptome [30].

Met is a proteinogenic AA responsible for the initiation of protein translation and plays a structural role in the hydrophobic cores of proteins. Apart from being incorporated in polypeptide chains, Met also has important functions as a single molecule as a redox sensor and ROS scavenger. In cell membranes, Met often attacks the lipid bilayer, which is susceptible to oxidation. Met, together with tryptophan and Cys, is one of the most susceptible AA to oxidation by ROS [31]. It is oxidized to Met-sulfoxide, which can be reduced back to Met by Met-sulfoxide reductase. Reversible Met oxidation/reduction in proteins might act as a regulatory mechanism. Sulfoxide residues of Met are more hydrophilic compared to Met, which can lead to unfolding and progressive loss of protein function.

Met requirements are 30% lower in parenterally fed than in enterally fed piglets because in the first-pass the splanchnic tissues significantly reduced the level of Met [32]. Recent studies recognized that gastrointestinal tissues of rats [7] and piglets [5] possess significant activities of enzymes necessary to utilize Met and convert it to Cys [2, 6]. These studies have also shown that developing gut is a significant site of Met conversion to Cys and Hcy. SAA deficiency preferentially reduces mucosal growth and antioxidant function in neonatal pigs.

In SAA-free pigs compared with control plasma levels of all SSA, total erythrocyte GSH concentration and body weight were significantly decreased. Whole-body Met and Cys fluxes were reduced, although Met utilization for protein synthesis and its remethylation were preserved, in response to SAA deficiency. Met and Cys concentrations were also reduced in intestinal tissue [5]. The activity of Met metabolic enzymes: Met adenosyltransferase, Met synthase and cystathionine-synthase, and SAM concentration in the jejunum were increased by SAA deficiency. Dietary SAA deficiency-induced small intestinal villous atrophy, small intestine weight, and protein and DNA mass were lower, lower goblet cell numbers, and Ki-67 positive proliferative crypt cells in association with lower tissue GSH, especially in the jejunum. SAA deficiency suppresses epithelial growth and upregulates intestinal Met cycle activity [5]. Met requirements in the neonatal pigs are higher than in the infant pigs, not only for protein synthesis, but also for the synthesis of SAM, the methyl donor in cells and a precursor for polyamine synthesis [33].

Transsulfuration and synthesis of Cys is another important function of Met [34]. Cys is the only precursor for Tau synthesis and the limiting AA in the GSH synthesis [35]. Cys also plays an important role as an extracellular reducing agent [1]. SAA-free pigs had lower blood concentrations of GSH and Tau than control pigs, thus the lower transsulfuration rate and Cys flux.

Dietary restriction of Met also renders benefits. One-month dietary restriction of Met had an impact on the tight junction (TJ) barrier in rat gastrointestinal tissue. Increased transepithelial electrical resistance with lower paracellular diffusion of 14C-D-mannitol was registered in the rat colonic mucosa of experimental rats (Met restriction diet) compared to control, suggesting improved barrier function. Improved barrier function was accomplished by the modification of TJ structure proteins that could have resulted from the DNA methylation in colon epithelial cells. Therefore, Met restriction could be useful in various IBD, such as Crohn's disease [36].

High consumption of red meat is considered a risk factor for developing colorectal cancer. Met, a component of animal proteins, and folic acid are included in the one carbon cycle and play an important role in DNA methylation and cancer development. That is the reason why dietary modifi-

cations involving lower levels of Met and folic acid might inhibit colon cancer development. Carcinogenesis is associated with inflammation by inhibiting apoptosis, inducing gene mutations, and stimulating angiogenesis [37]. Met has been recognized as a contributing factor in inflammation-induced colon cancer and in the inhibition of several pathways important in colon carcinogenesis [38]. Therefore, it is notable that dietary Met intake might have a protective effect on colorectal cancer risk. The connection between the risk of colorectal cancer and dietary Met intake has been shown, however, the findings are conflicting, which has been proven by several epidemiological studies [39].

A new strategy in cancer growth control, especially for cancers dependent on Met for survival and proliferation, could be the restriction of Met. The reason for Met dependence in these cancers may be deletions, polymorphisms, or alterations in the expression of genes included in Met salvage pathways. Defects in the metabolism of folate may also lead to the Met dependence in cancer. Met-dependent cancer cells have been killed using culture media deficient in Met [40]. Several studies on animals that were on Met restricted diet have reported inhibition of cancer growth and extension of a healthy life-span. Diets low in Met, such as vegan diets, could be a useful nutritional method to control cancer growth in humans [40].

Homocysteine

There are more and more research results suggesting that Hcy is an important factor for human health status. Hcy is metabolized through two major pathways: methylation and transsulfuration. In most tissues, in physiological conditions, approximately 50% of Hcy is remethylated via enzyme Met synthase (5-methyltetrahydrofolate-homocysteine methyltransferase) forming Met. In the liver, this conversion from Hcy to Met is mostly done via betaine-Hcy S-methyltransferase [41]. In the transsulfuration pathway, Hcy is metabolized to form cystathionine, which is the immediate precursor to Cys.

In HHcy plasma, levels of Hcy are higher than 15 μmol/L. HHcy induces oxidative stress in vascular endothelial cells, which increases cardiovascular risk [42]. The incidence of HHcy is 5–7% in the general population and 25% among people that already have some vascular diseases [43, 44]. Although there is a clear association between plasma homocysteine concentration and cardiovascular diseases, folic acid therapy was not useful in the prevention of myocardial infarction and stroke in the majority of trials [45–47]. HHcy is also associated with kidney disorders in general and diabetic populations, apart from its important role in cardiovascular and cerebrovascular diseases [48, 49].

There are data from the literature showing that sulfur AA can impact bowel motility. DL-Hcy thiolactone (Hct) has shown to be a potential prokinetic agent by increasing the contractile activity of isolated duodenum in rats [50]. Inhibition of nitric oxide synthesis caused by N-nitro-L-arginine methyl ester (L-NAME) caused a significant increase in tone, amplitude, and frequency of the contractions in the presence of Hct. Hct has also decreased the nonadrenergic-noncholinergic relaxation of bowel smooth muscle caused by stimulation with low-frequency electrical field [51].

HHcy was reported as a risk factor for many gastrointestinal diseases such as constipation, Crohn's disease, IBD, and colorectal cancer [12, 13]. HHcy occurs in inflammatory remodeling of the gastrointestinal tract which could lead to increased oxidative stress. The reason for this state could be the disease itself because sulfur AA are metabolized and transported in the gastrointestinal tract. Moreover, HHcy has been correlated to mesenteric venous thrombosis, bowel infarction, and colorectal cancer [14]. HHcy can cause upregulation of inducible nitric oxide (NO) synthase, resulting in inflammatory changes during hemorrhagic shock and leading to functional and morphological injury of intestine [52]. Hcy has an important role in the pathophysiology of many inflammatory disorders of the intestine by affecting the activity of matrix metalloproteinases (MMPs). MMP-2 was reported as an enzyme that has a protective function during intestinal inflammation. However, MMP-9 can cause mucosal damages during inflammatory processes [53]. These findings suggest that inhibition of MMPs could have a therapeutic potential in intestinal inflammatory diseases.

Hcy was suggested to be a prooxidant agent. Hcy significantly increased thiobarbituric acid-reactive substances – a marker or lipid peroxidation – in rat duodenum, ileum, colon, and liver. Likewise, the activity of catalase, an antioxidative enzyme, was significantly decreased in these tissues by acute administration of Hct [54]. Acute administration of Hcy decreased activities of superoxide dismutase and glutathione peroxidase [55].

A high growth rate is one of the most characteristic features of malignant cells, thus they require more Met because the synthesis of proteins is increased, while normal cells can cover their Met consumption from Hcy remethylation. However, malignant cells in the colon cannot convert Hcy to Met, they accumulate Hcy and are Met dependent. A higher level of Hcy is related to the concentration of folate. Folate cofactors play an important role as intermediators in Hcy remethylation to Met, in the synthesis of SAM, and also in the production of nucleotides for DNA/RNA synthesis. SAM/SAH ratio is significantly lower in Met-dependent cells comparing to normal cells. Reduction of intracellular SAM levels can cause repression of tumor suppressor genes and activation of protooncogenes by altering cytosine methylation in CpG islands of DNA which induces malignant transformation [56]. A high level of SAH increases Hcy level as long as Hcy is not converted to Cys by transsulfuration pathways. Higher Hcy level and normal plasma level of Cys were detected in patients with cancer [57].

HHcy is found in about 5% of the general population and it is considered an important risk factor for arterial and venous thrombosis [35, 58]. The presence of Hcy in the IBD, patients' mucosa has been demonstrated, which can be, at least partially, brought into a relationship with the inflammation of the IBD endothelium [59] and recent meta-analysis by Oussalah et al. [60] suggested that HHcy was four times more frequent in IBD patients. Vascular complications in patients

with IBD are very common and appear earlier than in the general population. Although reports have been mainly focused on venous thromboembolism, there are also series that have documented arterial thromboembolism in IBD patients.

HHcy takes place in atherosclerosis pathophysiology. It increases oxidative stress and decreases NO production, which results in impaired endothelial function, and finally, in aberrant remodeling and atherosclerotic plaques [61]. Few reasons are explaining this phenomenon. Nutritional deficiencies of vitamins B6, B12, and folate are related to poor intake and/or malabsorption. The use of drugs that reduce folate absorption (sulfasalazine) or inhibit its metabolism (methotrexate) reduces intracellular folate stores [62]. Third, folate activity can be compromised by genetic factors such as a mutation in the methylenetetrahydrofolate reductase gene [60, 63]. The choline status of patients with IBD has been minimally explored, except for two studies in patients with active ulcerative colitis (UC). The results of those studies demonstrated the reduction of choline and betaine concentrations in colonic mucosa and serum [64, 65].

Cysteine

L-Cys is a semi-essential AA that can be absorbed from diets or got from the transsulfuration pathway from Met degradation and catabolism of endogenous proteins. L-cystine is the main form of Cys because it is more stable when oxidized in physiological conditions. Cys is included in many metabolic reactions like protein synthesis, the generation of GSH, Tau, pyruvate, and inorganic sulfur [66]. The metabolic pathways of Cys catabolism to H_2S, Tau, and especially GSH show important therapeutic and nutritional implications in the improvement of human and animal health. A recent study recognized that a three-week-long i.p. application of Cys and Met can lead to significantly lower concentrations of cholesterol, urea, and creatinine in rats compared to control [67]. Biochemical evaluation of liver and pancreatic function in the condition of high Met intake showed lower concentrations of aspartate aminotransferase and alkaline phosphatase and higher serum amylase levels compared to control [67]. The ratio between Cys and L-cystine (its oxidized form) is very important in controlling oxidative stress and inflammatory response [68-70]. Dietary intake of SAA affects cell signaling via modulation of postprandial Cys and L-cystine concentrations and Cys/L-cystine redox ratio [71, 72]. There is a growing interest in the use of Cys for improving health in animals and humans. Maintaining normal redox status is particularly important in intestinal epithelial cells, which are exposed to high levels of oxidative stress because of intensive metabolism and exposure to luminal toxins and oxidants derived from diets [73, 74]. These findings may be important for the treatment of diseases related to oxidant injury in the digestive organs.

The intestinal barrier is important as a selective barrier against endogenous and exogenous noxious antigens and pathogens [75]. Disruption of the intestinal barrier promotes luminal antigens to penetrate subepithelial tissues, inducing a mucosal and systemic inflammatory response, which is the major pathogenic mechanism for most intestinal diseases [76]. Multiple factors, including inflammation and oxidative stress, can give rise to intestinal barrier damage [76, 78]. Overproduction of pro-inflammatory cytokines and ROS can disrupt the intestinal epithelial integrity and function [77–79]. Therefore, inhibition of intestinal inflammation and oxidative stress may exert beneficially prevent the greater intestinal disruption. Cys could alleviate oxidative stress via GSH synthesis in IBD [77], and it has been established that Cys supplementation ameliorated local inflammation and improved intestinal barrier restoration in induced porcine colitis model [80]. These data demonstrated that Cys was effective in suppressing inflammation and oxidative stress and recognize Cys as a promising nutritional agent for intestinal integrity protection.

N-acetylcysteine (NAC) is acting as a precursor for the substrate Cys in the synthesis of GSH [81]. NAC is an antioxidant and has gastroprotective (antiulcerative), anti-inflammatory effects in rat models [82].

Taurine

Tau is SAA that is not incorporated into the cellular proteins nor metabolized [83, 84]. Studies have demonstrated that a high level of extracellular Tau can protect cells against damaging stimuli such as ROS, toxic xenobiotics, cellular excitotoxicity, and osmotic derangements [85]. Tau is involved in many biological and physiological functions: conjugation of bile salt, membrane stabilization, calcium modulation, osmoregulation, anti-oxidation, and immunomodulation.

Sukhotnik et al. [86] showed a protective effect of Tau on intestinal recovery following intestinal ischemia-reperfusion injury in rats. It is well known that during reperfusion of ischemic tissue ROS and reactive nitrogen species are generated in excess. Tau was found to be a protective agent against oxidative stress in developed atherosclerosis [87], complications of diabetes mellitus [88, 89], hepatic [90, 91], and gastrointestinal damage [91]. In addition, taurine was also reported to have anti-apoptotic properties [91–93]. Tau inhibits oxidative stress-induced apoptosis in several cells, such as hepatocytes [91], cardiomyocytes [92], and epithelial cells [93].

Myeloperoxidase activity generates high levels of hypochlorous acid in the inflamed colon tissue and produces Tau-chloramine in the reaction with Tau. Administration of Tau reduces inflammation and activity of colonic MPO in rats with induced colitis [94]. The effects of Tau treatment may be partly through Tau-chloramines [95]. However, Tau can also have an anti-inflammatory effect not only through Tau-chloramines, but also directly by inhibition of IL-8 secretion induced by TNF-a [96]. Moreover, Tau in synergy with 5-aminosalicylic acid also shows an anti-inflammatory effect by decreasing the level of interleukin-1b [95].

Recent findings indicate that Tau can have a significant inhibiting effect on cell proliferation [97]. It is also shown that Tau induced apoptosis in human colon cancer cells and that this effect is based upon regulation of p53-upregulated modulator of apoptosis.

Conclusion

Further investigations should examine the real advantages and disadvantages of high sulfur-containing AA diets and the restriction of these AA in particular digestive diseases such as inflammatory bowel disease and colorectal cancer. Advanced studies are needed to understand: the role of new preventative dietary supplements or medicaments, which will decrease plasma Hcy level, the molecular background of Hcy interaction with its target molecules in gastrointestinal tissues, and the epigenetic alteration of DNA methylation profiles in correlation with the pathogenesis of digestive diseases. Therapeutic lowering of Hcy level and supplementation of Cys and N-acetylcysteine in the prevention of gastrointestinal disorders are promising tools. Future studies should test some of the medical applications of Met restriction and its ability to enhance epithelial Tjs and barrier function in various diseases whose etiology likely involves changes in Tj proteins, for example, inflammatory bowel diseases such as Crohn's disease. Diets low in Met, such as vegan diets, could be a useful nutritional method for cancer growth control in Met-dependent tumors.

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CASE REPORTS

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Balloon angioplasty of cardiac vein in a patient treated by cardiac resynchronization therapy

Balon angioplastika srčane vene kod bolesnika lečenog primenom kardioresinhronizacione terapije

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Abstract

Introduction. Cardiac resynchronization therapy (CRT) reduces mortality and hospitalization in patients with symptomatic heart failure and left bundle branch block (LBBB) on optimal drug therapy. Among all, the reasons for "non-response" to CRT pacemaker could be the failure to achieve optimal left ventricular (LV) lead position due to severe curve or stenosis/occlusion of the target vein. **Case report.** We presented a 79-old-male patient, New York Heart Association (NYHA) class III, with atrial fibrillation, chronic coronary syndrome (CCS), and prior myocardial infarction. The patient underwent coronary artery bypass surgery and mechanical prosthetic aortic valve implantation. He was indicated for CRT. The patient's venogram revealed ostial/proximal severe curve and stenosis of the posterolateral vein, the only vein of coronary sinus that led to anatomically optimal LV segment for stimulation. After the balloon angioplasty of the curved and stenotic portion of the target vein with compliant balloon 4.0 × 30 mm, a satisfactory and stable position of quadripolar LV lead was achieved. **Conclusion.** Compliant balloon angioplasty could be a safe and efficient way to override severe coronary vein stenosis in some CRT cases.

Key words:
angioplasty, balloon; cardiac resynchronization therapy; coronary sinus; heart failure.

Apstrakt

Uvod. Kardioresinhronizaciona terapija (CRT) smanjuje smrtnost i broj hospitalizacija kod bolesnika sa simptomatskom srčanom insuficijencijom i blokom leve grane (LBBB) koji su na optimalnoj terapiji lekovima. Jedan od razloga za „nereagovanje" (*non-response*) posle ugradnje CRT pejsmekera može biti neuspeh u postizanju optimalnog položaja elektrode koja stimuliše levu komoru zbog izražene krivine ili stenoze/okluzije ciljne vene**. Prikaz bolesnika**. Prikazan je muškarac, starosti 79 godina, *New York Heart Association* (NYHA) klase III, sa atrijalnom fibrilacijom, hroničnim koronarnim sindromom (CSS) i preležanim infarktom miokarda koji je hirurški revaskularizovan sa implantacijom veštačke mehaničke aortne valvule. Bolesniku je indikovana ugradnja CRT pejsmejkera a venogramom mu je detektovana ostijalno/proksimalna teška krivina i stenoza posterolateralne vene koronarnog sinusa, anatomski jedine vene za optimalnu stimulaciju leve komore. Posle balon-angioplastike zakrivljenog i stenotičnog dela ciljne vene sa komplijantnim balonom 4.0 × 30 mm, postignuta je zadovoljavajuća i stabilna pozicija kvadriplolarne elektrode za stimulaciju leve komore. **Zaključak**. U nekim slučajevima CRT, komplijantna balon angioplastika bi mogla biti siguran i efikasan način za prevazilaženje teške stenoze vene koronarnog sinusa.

Ključne reči:
angioplastika, balonska; srce, terapija, resinhronizaciona; koronarni sinus; srce, insuficijencija.

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Introduction

Cardiac resynchronization therapy (CRT) is a Class I indication for treatment of patients with symptomatic systolic heart failure (HFrEF) and QRS > 150 ms and left bundle branch block (LBBB) QRS morphology, on optimal drug therapy [1].

One of the main reasons for "non-response" on CRT is suboptimal lead placement due to impassable target vein because of vein anatomy, i.e. severe curve or/and thrombosis/stenosis/occlusion, which occurs in 1–4% of the cases [2]. One of the non-routine options is to use percutaneous transluminal coronary angioplasty (PTCA) balloon.

Case report

We presented a case of a 79-year-old male patient, New York Heart Association (NYHA) class III, with a history of hypertension, chronic coronary syndrome (CCS), prior myocardial infarction. The patient underwent coronary artery bypass surgery together with implantation of a mechanical prosthetic aortic valve six years ago.

The electrocardiogram (ECG) showed permanent atrial fibrillation (AF) and complete LBBB, with a QRS duration of 180 ms and ECG signs of the antero-apical scar. Echocardiography revealed reduced left ventricular (LV) ejection fraction (LVEF) 30%, dilated LV end-diastolic diameter (LVEDD) 61 mm, LV end-systolic diameter (LVESD) 45 mm with mechanical dyssynchrony, and normal function of the mechanical prosthetic aortic valve. Since the patient had no history of ventricular tachycardia or ventricular fibrillation and on ambulatory 24-hours HOLTER monitoring only isolated ventricular premature beats (VPBs) were recorded, a CRT pacemaker was intended to be implanted.

Right ventricular lead (Tendril™ STS, St. Jude Medical, 58 cm) was implanted via a cephalic vein and placed on the mid interventricular septum with satisfactory electrical parameters. On coronary sinus (CS) balloon-assisted venography (introducing sheath: Acuity™ Pro, Boston Scientific, 9F, 54 cm, plus Balloon catheter, model 6747, Boston Scientific), posterolateral branch was identified with a good distal diameter reaching the posterolateral LV segment of the latest activation. Unfortunately, the proximal tortuosity and sharp curve (Figure 1) made the vein unpassable with quadripolar (QUARTET™ 1458Q-86, St. Jude Medical), or also with bipolar lead (QUICKFLEX™ 1258T-86, St. Jude Medical). The vein was made passable only with coronary wire 0.014 Fr (ASAHI SION, 180 cm). Subselecting introducing sheath (Attain Select II™ 90^0, Medtronic) with two different curves also failed to achieve the passage of the lead. Finally, we solved the problem with the compliant percutaneous transluminal angioplasty (PTCA) balloon (Sprinter Legend™ Medtronic) 4.0 × 30 mm, which was introduced and placed into the proximal part of the posterolateral vein with gradual expansion up to 12 atmospheres (Figure 2).

During the inflation of the PTCA balloon, vein stenosis (probably due to local thrombus) was confirmed, and after the dilatation, the quadripolar LV lead (QUARTET™ 1458Q-86, St. Jude Medical) easily reached the desired destination (Figure 3). The electrical parameters for LV lead on the anatomically optimal position were acceptable (P4/M3 threshold 0.625V/ 0.4 ms, without phrenic nerve stimulation).

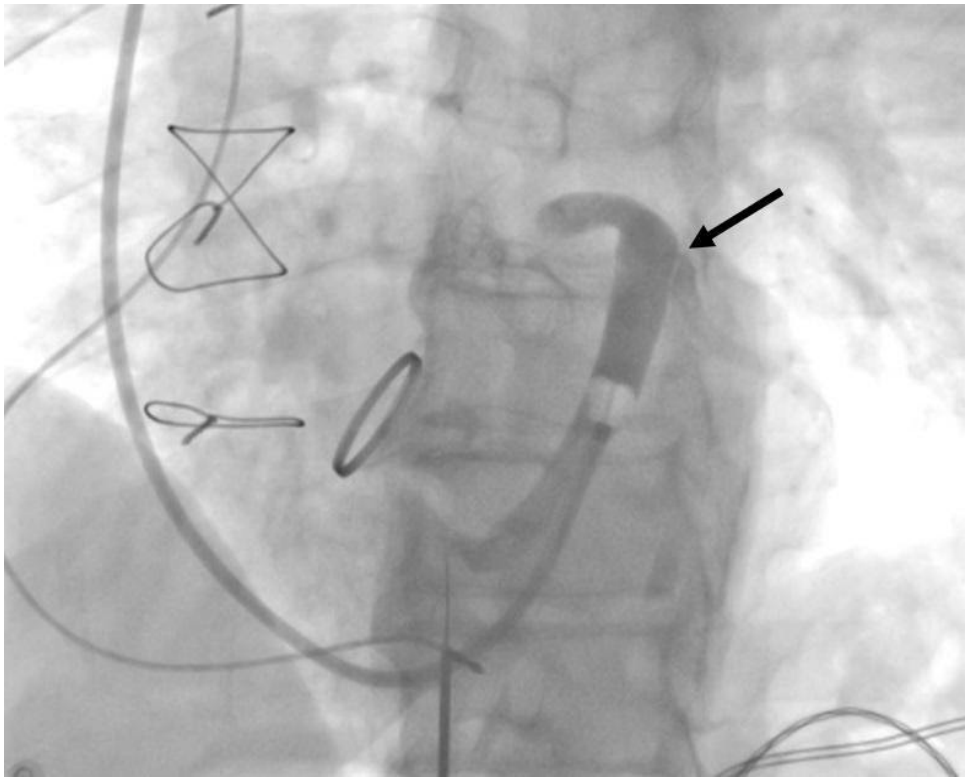

Fig. 1 – Venogram of the coronary sinus. Note the stenosis of the proximal segment of the posterolateral vein.

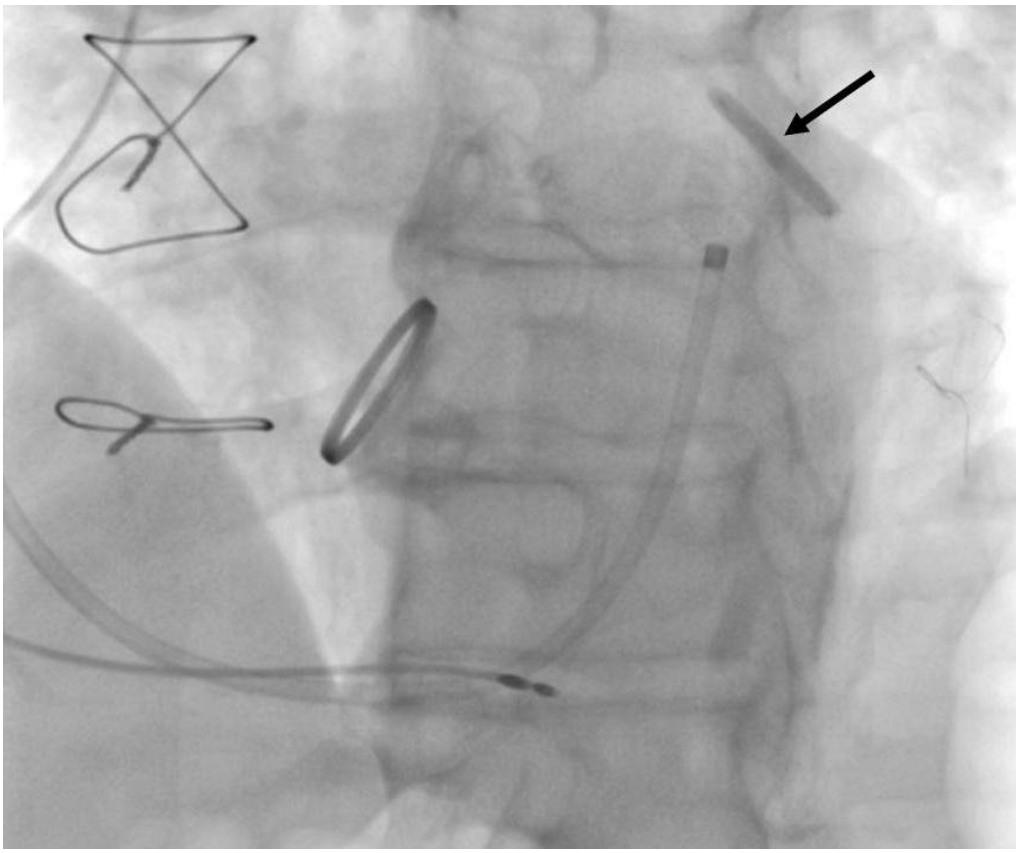

Fig. 2 – Venous angioplasty into the ostial and proximal part of posterolateral vein with compliant percutaneous transluminal coronary angioplasty balloon (Sprinter Legend™ Medtronic) 4.0 × 30 mm.

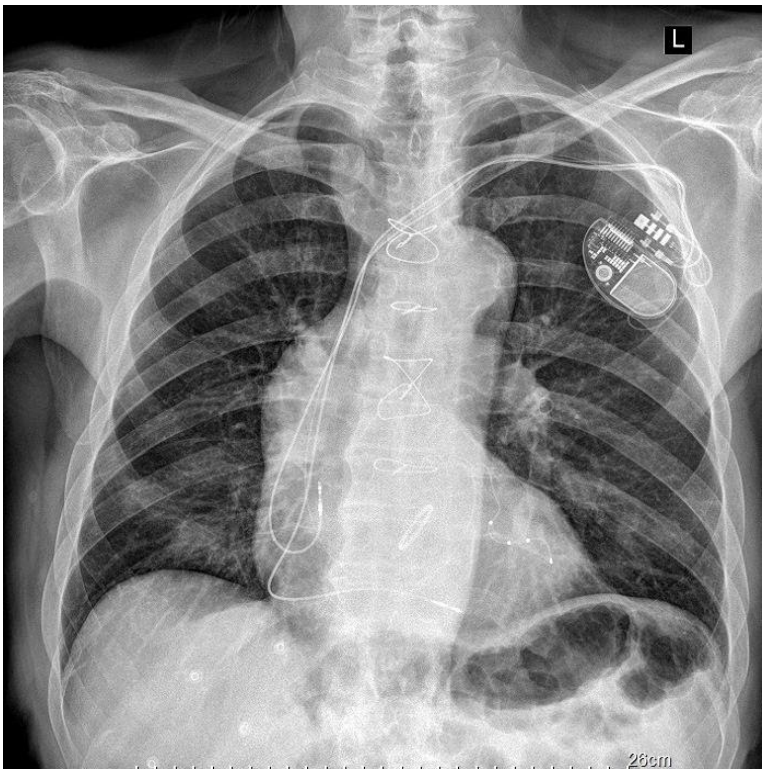

Fig. 3 – Successful implantation of quadripolar left ventricular electrode (QUARTET™ 1458Q-86, St. Jude Medical) after balloon dilatation of proximal segment of posterolateral vein.

Finally, the atrial lead (Tendril™ STS, St. Jude Medical, 52 cm) was implanted since the realistic chance of sinus rhythm in the follow-up period was expected due to a left atrial diameter of less than 50 mm, and a pulse generator (Quadra Allure MP™ St. Jude Medical) was placed in standard subclavicular subcutaneous pocket.

The patient was discharged from the hospital the next day without complication and with QRS narrowed for 40 ms, with normal biventricular capture (according to the lead V1/2). After four months of follow-up, the patient was referred as a clinical "responder" (LVEF improved up to 45%), with evident signs of reverse remodeling, having one NYHA class less and better tolerance of effort.

Discussion

Although CRT is clearly indicated [3–5] in patients with symptomatic HFrEF and optimal drug treatment, having complete LBBB with a QRS duration > 150 ms, in sinus rhythm (Class IA) or atrial fibrillation (Class IIA), randomized controlled trials [6, 7] reported 7.5–10% of unsuccessful implantation due to failure of pacing the LV lead on optimal and stable position.

We presented a case of a 79-year old male patient with permanent atrial fibrillation, a mechanical prosthetic aortic valve with an indication for CRT pacemaker, with the proximal stenosis of the posterolateral coronary vein, which aggravated optimal LV lead positioning. After venous angioplasty with a compliant PTCA balloon, optimal and stable position of quadripolar LV lead was achieved.

Multiple studies have reported the utility of applying interventional principles and equipment in coronary venous circulation to accomplish optimal biventricular stimulation [8, 9]. Luedorff et al. [10] showed on retrospective analysis in a single-center with 705 CRT cases that in 3.5% of the cases, venous angioplasty (balloon 3.0 mm in size usually) was needed and successfully performed. Moreover, the collateral veins could be also dilated in case of occlusion of big coronary vein branches, using small diameter guidewires and balloons [11] to retrogradely approach the target zone of the latest activation of LV, but which is a risky, delicate, and prolonged procedure.

Some authors propose placing the stent on the stenotic portion of the vein (after the dilatation), but this could lead to post-procedural vein occlusion in a short follow-up period with further deterioration of LV ventricle function, which would diminish the positive effect of CRT. Such a case was described in a case report by Jachec´ et al. [12], making these options obscure and potentially dangerous.

On the other hand, some implanters perform stent implantation in the veins parallel to the LV lead, but for other reasons than stenosis [13] (to prevent lead dislodgement). In that case, the potential huge technical and clinical problem would arise in case an extraction was needed. Therefore, one should carefully analyze cost-benefit before the decision.

Finally, since the importance of reaching the optimal LV segment for LV stimulation was well established and recently again emphasized (in 69% of lateral or posterolateral segments) [14], the present case report, which is an example of a non-routine approach and has not been published up to now in domestic journals, showed a relatively easy and safe way to solve the problem of unpassable target vein by using only simple PTCA balloon. In our opinion, a compliant balloon is better than a non-compliant or semi-compliant balloon primarily because of safety issues with polyolefin copolymer material.

Conclusion

In the case of vein stenosis, which precludes placement of LV lead, balloon angioplasty is a relatively easy and safe approach to facilitate the CRT procedure.

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CASE REPORT

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Leiomyosarcoma of the upper respiratory tract: A case report and review of literature

Lejomiosarkom gornjih delova respiratornog trakta

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Abstract

Introduction. Leiomyosarcoma is a malignant smooth muscle tumor that is most often found in the gastrointestinal tract, uterus, and retroperitoneum. It is uncommon in the upper respiratory tract. **Case report.** A 53-year-old woman sought treatment for a 3-month history of nasal obstruction and facial pain. An endoscopic examination revealed a polypoid mass involving the right nasal cavity. The patient was operated on. During surgery, a bulky tumor mass was found, which involved the posterior part of the right inferior concha projecting into the lumen of the epipharynx and upper part of the oropharynx. The lesion was completely excised with adequate margins of uninvolved tissue. Histologic slides stained with hematoxylin and eosin showed interlacing fascicles of atypical spindle-shaped cells. Mitotic figures and bizarre giant cells were frequently observed. Immunohistochemical staining revealed that the tumor was positive for smooth muscle actin and h-caldesmon and negative for S-100. Histological and immunohistochemical features were consistent with leiomyosarcoma. The follow-up was regularly performed by nasal endoscopy and computed tomography, and the disease-free period has been 53 months so far. **Conclusion.** To the best of our knowledge, this is the longest disease-free period in a patient with leiomyosarcoma of the upper respiratory tract since 1996. This tumor has obscure clinical behavior and prognosis, thus we believe it is necessary to have more published data to determine the best combination of existing therapies, as well as potential new therapies, and enable a longer survival period.

Key words:
diagnosis, differential; leiomyosarcoma; histological techniques; otorhinolaryngologic surgical procedures; pharyngeal neoplasms; respiratory system; survival.

Apstrakt

Uvod. Lejomiosarkom je maligni tumor glatko-mišićnog tkiva i uglavnom je prisutan u gastrointestinalnom sistemu, materici i retroperitoneumu. Gornji delovi respiratornog trakta nisu uobičajena lokalizacija ovog tumora. **Prikaz bolesnika.** Bolesnica stara 53 godine je potražila pomoć lekara zbog zapušenog nosa i bola lica, koji su bili prisutni u prethodna tri meseca. Endoskopskim pregledom je otkrivena polipoidna masa koja je ispunjavala desnu nosnu šupljinu. Bolesnica je operisana. Tokom operacije je nađena velika tumorska masa koja je zauzimala zadnji deo desne nosne šupljine i pružala se u lumen epifarinksa i gornji deo orofarinksa. Promena je u potpunosti odstranjena, sa odgovarajućim zaštitnim rubom okolnog zdravog tkiva. Na histološkim presecima obojenim hematoksilin-eozinom uočeni su snopovi atipičnih vretenastih ćelija, sa prisutnim čestim mitozama i ćelijama bizarnog izgleda. Imunohistohemijskom analizom je dokazana pozitivnost na aktin glatkih mišićnih ćelija i h-kaldezmon i negativnost na S-100. Histološke i imunohistohemijske karakteristike tumora su odgovarale lejomiosarkomu. Bolesnica je redovno kontrolisana endoskopskim pregledom i kompjuterizovanom tomografijom i do sada je 53 meseca bila bez simptoma. **Zaključak.** Prema našim podacima, ovo je najduži period preživljavanja kod bolesnika sa lejomiosarkomom gornjih partija respiratornog trakta (od 1996. godine). Taj tumor nema jasan klinički tok i prognozu. Potrebna su dodatna istraživanja da bi se odredila najbolja kombinacija postojećih terapija, kao i potencijalne nove terapije, u cilju dužeg preživljavanja obolelih.

Ključne reči:
dijagnoza, diferencijalna; lejomiosarkom; histološke tehnike; hirurgija, otorinolaringološka, procedure; farinks, neoplazme; respiratorni sistem; preživljavanje.

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Introduction

Leiomyosarcoma is a malignant smooth muscle tumor that is most often found in the gastrointestinal tract, uterus, and retroperitoneum [1]. It is uncommon in the upper respiratory tract. Only 1.5–2.3% of soft tissue tumors in the nasal cavity, paranasal sinuses, and nasopharynx are diagnosed as leiomyosarcomas [1, 2]. Compared to uterine and gastrointestinal variants, tumors involving this localization are much more aggressive and have a worse outcome [3].

Case report

A 53-year-old woman sought treatment for a 3-month history of nasal obstruction and facial pain. Rhinoscopy was performed, and a reddish polypoid mass involving the right nasal cavity was discovered. The patient was referred to a computed tomography scan, which confirmed a lesion with smooth contours obliterating the nasopharynx and upper portion of the oropharynx.

Therefore, endoscopic excision of this lesion was performed. During the endoscopic procedure, after aspiration of abundant thick mucus from the left nasal cavity, further examination through inferior meatus revealed a soft grayish mass in the epipharynx. Superior and middle meatus were unreachable due to deviation of the nasal septum. After resection of the septum, access to the right meatus was enabled and a bulky polypoid tumor mass was revealed, which involved the posterior part of the right inferior concha projecting into the lumen of the nasopharynx and upper part of the oropharynx without infiltration of the adjacent structure. Figure 1 shows endoscopic picture: 1-tumor, 2-choana, 3-middle nasal concha. Other significant pathologic changes were not found.

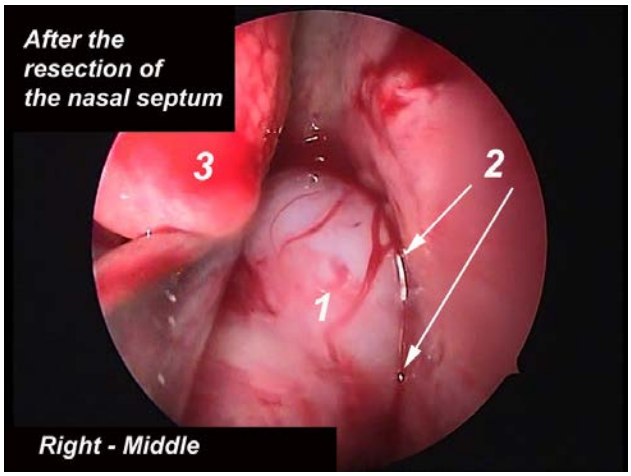

Fig. 1 – Endoscopic finding of a bulky polypoid tumor mass: 1 – tumor, 2 – choana, 3 – middle nasal concha.

The tumor was completely excised with adequate margins of uninvolved tissue and extracted through the oral cavity.

Macroscopically, the surgical specimen consisted of a polypoid pale grey tissue with a smooth surface measuring 38 × 40 × 20 mm. Figure 2 shows a tumor surgical specimen. Cross sectioning revealed a solid, structureless tissue with gelatinous areas. The tissue was entirely sampled for histological analysis.

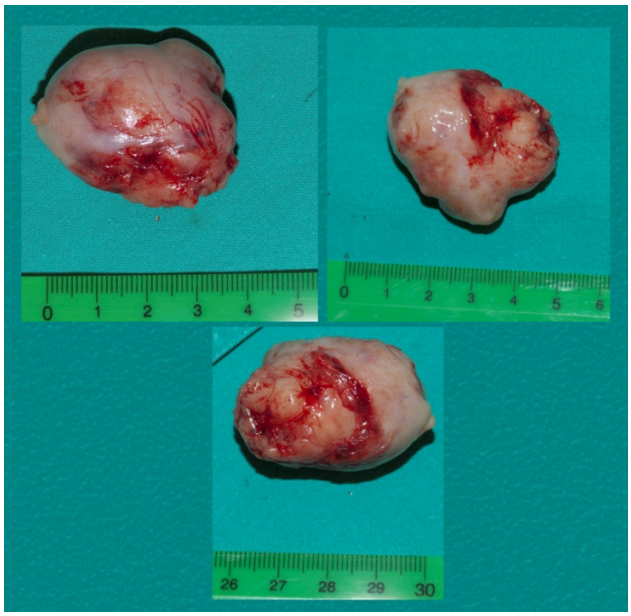

Fig. 2 – Tumor surgical specimen.

Histological slides, stained with hematoxylin and eosin (H&E), showed interlacing fascicles of atypical spindle-shaped cells with elongated blunt-ended nuclei and eosinophilic cytoplasm. Mitotic figures were frequently observed (15 mitoses per 10 high power fields), and bizarre giant cells were often present. Figure 3 shows H&E stained sections of respiratory mucosa with tumor. No necrosis was found. Immunohistochemical staining revealed that the tumor was positive for smooth muscle actin and h-caldesmon and negative for S-100. Immunohistochemistry staining of smooth muscle actin corresponding to the diffuse form of positivity is shown in Figure 4. Histological and immunohistochemical features were consistent with leiomyosarcoma. According to the French Federation of Cancer Centers Sarcoma Group [4], the histologic grade was 2. The lymphovascular invasion was not identified.

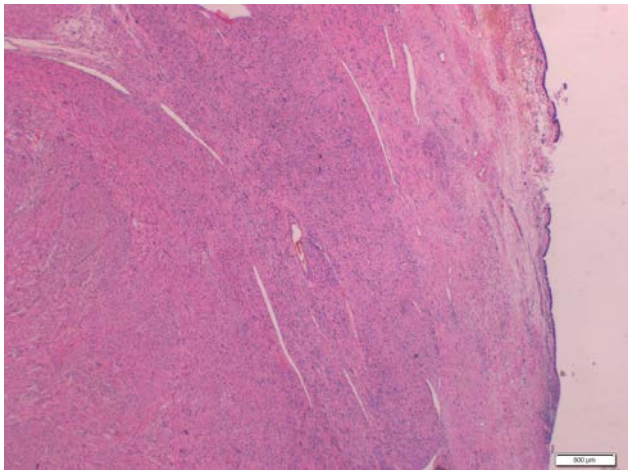

Fig. 3 – Respiratory mucosa with tumor observed (hematoxylin and eosin, ×20).

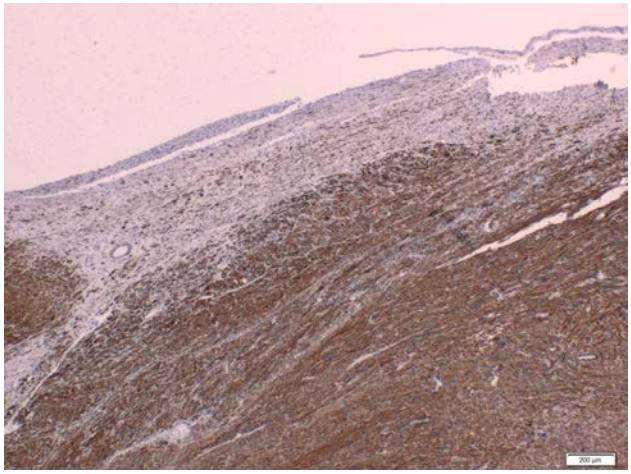

Fig. 4 – Immuhistochemical staining of smooth muscle actin shows a diffuse positivity (x40).

The patient did not receive any adjuvant therapy. The follow-up was regularly performed by nasal endoscopy and computed tomography, and the disease-free period has been 53 months so far.

Discussion

As mentioned earlier, leiomyosarcoma of the upper respiratory tract is very rare. The first case of leiomyosarcoma of the nasal cavity was documented by Dobben [5] in 1958. He reported a 69-year-old woman with leiomyosarcoma in the posterior portion of the nasal cavity. However, to verify pathohistological findings, he used only special stain Gomori. To the best of our knowledge, there are approximately 50 published case reports so far [6], but a significant number of these cases have not been confirmed by immunohistochemistry.

There are several theories about the origin of leiomyosarcoma in the upper respiratory tract. Mindell et al. [7] believe that leiomyosarcoma probably develops from the smooth muscles in the *tunica media* of the vessel walls. Mindell's theory is supported by a higher incidence of this tumor in the posterior part of the nasal cavity, which is more richly vascularised [2, 8]. The primary site of the tumor in our patient was the posterior area of the nasal cavity, too. Other authors speculated that leiomyosarcoma might originate from the dispersed undifferentiated mesenchymal cells [9,10] or myoepithelial cells of submucosal glands [11], or that they are metastases of a primary tumor located elsewhere [12]. The latter scenario is highly unlikely in our case, considering the long disease-free period of more than four years.

The exact risk factors for developing leiomyosarcoma are not known. Some authors proposed possible risk factors like prior exposure to radiation with a latency period from 6 to 35 years [13]. Moreover, there is evidence concerning the development of leiomyosarcoma in paranasal sinuses six years after irradiation and chemotherapy with cyclophosphamide due to prior Wegener's granulomatosis in a 66-year-old man [14]. Immunodeficiency is one of the predisposing risk factors: immunocompromised conditions like human immunodeficiency virus (HIV) or Epstein-Barr virus (EBV) infection are associated with the development of this tumor in any site, especially unusual locations like the central nervous system and endocrine glands [15]. Furthermore, leiomyosarcoma can develop in patients with a history of hereditary bilateral and nonhereditary unilateral retinoblastoma [16, 17]. In support of this theory, Stratton et al. [18] found a connection between Rb1 mutation and leiomyosarcoma development.

Our patient did not have any of the listed risk factors.

Prognosis is variable, and it depends on location, tumor size, the status of surgical margins, and histologic grade [2, 14]. Kuruvilla et al. [2] reported that prognosis is poorer for cases extending to ethmoid sinus rather than those localized in the nasal cavity. Also, tumor diameter greater than 5 cm is considered a poor prognostic factor [19]. However, small lesions of the paranasal sinuses could behave more aggressively and have a higher risk of local recurrence than larger tumors in the nasal cavity because of their closer anatomic proximity to vital structures [2]. The treatment of choice is radical resection with tumor-free surgical margins. In many cases, complete resection with negative margins is very hard to achieve, and in these cases, the mortality rate is much higher [3]. In our case, the tumor-free surgical margin was successfully obtained. Histologic grade is an important prognostic factor and strongly correlates with prognosis [20]. It is recommended that histologic grade is determined by the French Federation of Cancer Centers Sarcoma Group, which is the most used soft tissue grading system. It has three grades, and it is based on three parameters: differentiation, mitotic activity, and necrosis [4].

Pathohistological evaluation is necessary for a definitive diagnosis of leiomyosarcoma. Histologically, leiomyosarcoma is a spindle cell tumor with moderate to marked nuclear atypia, frequent necrosis, and brisk mitotic activity. Immunohistochemistry is essential to differentiate leiomyosarcoma from other neoplasms with spindle cell morphology such as sarcomatoid carcinoma, spindle cell melanoma, malignant peripheral nerve sheath tumor and rhabdomyosarcoma. Positive smooth muscle markers and negative S100, and cytokeratin AE1/AE3 are crucial for diagnosis [3, 21].

Leiomyosarcoma has a high rate of local recurrence of 50–75% within the first year [22, 23]. According to the survey, the 5-year survival rate for leiomyosarcoma of the nasal and paranasal sinuses is 20% [8, 10, 23]. It tends to spread hematogenously, mainly to the lungs, liver, and bones [8]. Regional lymph nodes involvement is rare, but there are few reports of sinonasal leiomyosarcoma with cervical lymph node metastasis [24, 25]. The treatment of choice is surgery. Chemotherapy with surgical treatment is recommended for inoperable cases and patients with distant metastasis. Ulrich et al. [26] reported significant tumor shrinkage after etoposide and a high dose of ifosfamide. Also, Fusconi et al. [27] documented a remarkable reduction of the tumor after ifosfamide, epirubicin, dacarbazine, and adriamycin application while Kudo and Suzaki [28] reported a significant decrease in tumor size and withdrawal of clinical symptoms following cyberknife radiotherapy of an upper respiratory tract leiomyosarcoma.

Conclusion

The presented patient did not have any of the risk factor for leiomyosarcoma. The tumor had good prognostic factors like localization in the nasal cavity, total tumor size less than 5 cm, and tumor-free surgical margins. To the best of our knowledge, this is the longest disease-free period in a patient with leiomyosarcoma of the upper respiratory tract since 1996. This tumor has obscure clinical behavior and prognosis. Therefore, we believe it is necessary to have more published data to determine the best combination of existing therapies, as well as potential new therapies, which enable a longer survival period.

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Based on the decision of the Editorial Board of the journal "Vojnosanitetski pregled", made at the meeting of the members of the Editorial Board on November 09, 2021, the following article is retracted from the Journal because it was published earlier in almost identical form ("duplicate publication"):

Nevenka Pavlović, Ljiljana Marković Denić, Katarina Vojvodić. Application of a geographic information system in the study of spatial aspects of cervical cancer incidence in Belgrade Vojnosanit Pregl 2020; 77(4):373-381 (DOI: 10.2298/VSP180412095P)

Previously published article:

Pavlović Nevenka, Rakić U, Ristić J, Vojvodić K. Application of geographic information system in perceiving spatial distribution of incidence of cervical cancer in Belgrade. In: Pavlović N, editor, Proccedings of the 25th Conference "Modern approach in the prevention of infectious and non-infectious diseases". Belgrade: Institute of Public Health of Belgrade; pp. 79-96. (ISBN 978-86-83069-41-5) (Serbian).

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d) Zaključak može da bude posebno poglavlje ili se iznosi u poslednjem pasusu diskusije.

e) Podaci o autoru za korespodenciju.

2. ***Apstrakt i ključne reči***

Na drugoj stranici nalazi se strukturisani apstrakt (250-300 reči za originalne članke i meta-analize) sa naslovom rada. Kratkim rečenicama na srpskom i engleskom jeziku iznosi se **Uvod/Cilj** rada, osnovne procedure **– Metode** (izbor ispitanika ili laboratorijskih životinja; metode posmatranja i analize), glavni nalazi **– Rezultati** (konkretni podaci i njihova statistička značajnost) i glavni **Zaključak**. Naglasiti nove i značajne aspekte studije ili zapažanja. Strukturisani apstrakt za kazuistiku (do 250 reči), sadrži podnaslove **Uvod, Prikaz bolesnika** i **Zaključak**). Ispod apstrakta, „Ključne reči“ sadrže 3–10 ključnih reči ili kratkih izraza koje ukazuju na sadržinu članka.

3. ***Tekst članka***

Tekst sadrži sledeća poglavlja: **uvod**, **metode**, **rezultate** i **diskusiju**. **Uvod.** Posle uvodnih napomena, navesti cilj rada. Ukratko izneti razloge za studiju ili posmatranje. Navesti samo važne podatke iz literature a ne opširna razmatranja o predmetu rada, kao ni podatke ili zaključke iz rada o kome se izveštava.

Metode. Jasno opisati izbor metoda posmatranja ili eksperimentnih metoda (ispitanici ili eksperimentne životinje, uključujući kontrolne). Identifikovati metode, aparaturu (ime i adresa proizvođača u zagradi) i proceduru, dovoljno detaljno da se drugim autorima omogući reprodukcija rezultata. Navesti podatke iz literature za uhodane metode, uključujući i statističke. Tačno identifikovati sve primenjene lekove i hemikalije, uključujući generičko ime, doze i načine davanja. Za ispitivanja na ljudima i životinjama navesti saglasnost nadležnog etičkog komiteta.

Rezultate prikazati logičkim redosledom u tekstu, tabelama i ilustracijama. U tekstu naglasiti ili sumirati samo značajna zapažanja.

U **diskusiji** naglasiti nove i značajne aspekte studije i izvedene zaključke. Posmatranja dovesti u vezu sa drugim relevantnim studijama, u načelu iz poslednje tri godine, a samo izuzetno i starijim. Povezati zaključke sa ciljevima rada, ali izbegavati nesumnjive tvrdnje i one zaključke koje podaci iz rada ne podržavaju u potpunosti.

Literatura

U radu literatura se citira kao superskript, a popisuje rednim brojevima pod kojima se citat pojavljuje u tekstu. Navode se svi autori, ali ako broj prelazi šest, navodi se prvih šest i *et al*. Svi podaci o citiranoj literaturi moraju biti tačni. Literatura se u celini citira na engleskom jeziku, a iza naslova se navodi jezik članka u zagradi. Ne prihvata se citiranje apstrakata, sekundarnih publikacija, usmenih saopštenja, neobjavljenih radova, službenih i poverljivih dokumenata. Radovi koji su prihvaćeni za štampu, ali još nisu objavljeni, navode se uz dodatak „u štampi“. Rukopisi koji su predati, ali još nisu prihvaćeni za štampu, u tekstu se citiraju kao „neobjavljeni podaci“ (u zagradi). Podaci sa interneta citiraju se uz navođenje datuma pristupa tim podacima.

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Tabele

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

Ilustracije

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

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

Skraćenice i akronimi

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

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

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