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Unknown author: The Sun in the Hand (color photo).
Nepoznati autor: Sunce na dlanu (fotografija u boji).

The experts have been pointing out to the risks of overexposure to Sun's rays for a long period, particularly emphasising, yet, the risk of developing skin cancer, including malignant melanoma for which there is still no efficient drug but its early diagnosis and removing. In the spring/summer time this problem is actual again and again.

In this issue of the *Vojnosanitetski pregled* there are two papers on melanoma: by Kandolf-Sekulović et. al. (p. 312–316), discussing on clinicopathological characteristics, diagnostics and treatment of melanoma in Serbia, while the one by Stanojević et. al (p. 342–349) considers the impact of interferon alpha on suppressor cells of myeloid origin in patients with melanoma.

Već duže vreme stručnjaci upozoravaju na opasnost od preteranog izlaganja sunčevim zracima. S tim u vezi posebno se ističe rizik od razvoja karcinoma kože, uključujući maligni melanom, za koji još uvek, osim ranog otkrivanja i uklanjanja, nema efiksanog leka. Svaki put, na početku prolećno-letnje sezone, ovaj problem iznova se aktuelizuje.

U ovom broju „Vojnosanitetskog pregleda" melanom je tema dva članka. U članku Kandolf-Sekulović i sar. (str. 312–316) govori se o kliničkopatološkim karakteristikama, dijagnostici i lečenju melanoma kod bolesnika iz Srbije, dok se u članku Stanojevića i sar. (str. 342–349) razmatra uticaj interferona alfa na supresorke ćelije mijeloidnog porekla kod obolelih od melanoma.

ORIGINAL ARTICLES

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Oral health-related quality of life of edentulous patients after complete dentures relining

Oralno zdravlje i kvalitet života bezubih pacijenata nakon podlaganja totalnih zubnih proteza

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Abstract

Background/Aim. Tooth loss affects oral health-related life quality. More than a third of edentulous patients are not fully satisfied with their complete dentures and mainly complain of insufficient stability, retention, and pain during mastication. Solving the problem may include relining by materials that are based on silicone or acrylic. The aim of this study was to determine the level of patients' satisfaction before and after relining upper dentures with soft and rigid liners. **Methods.** The patients (n = 24) were divided into two study groups. Maxillary denture relining of the first group of patients was performed with hard acrylic based resins while in the second group of patients complete denture was relined with a silicone-based soft liner. They were asked the questions from the specifically adapted the Oral Health Impact Profile Questionnaire for edentulous patients before and three months after relining dentures. **Results**. After relining the patients showed a higher degree of satisfaction with their dentures in all the tested domains (masticatory function, psychological discomfort, social disability and retention and hygiene). The patients with soft denture relines were more satisfied. **Conclusion.** Relining of maxillary complete dentures significantly positively impacts the quality of life of patients in all the tested domains (masticatory function, psychological discomfort, social disability, pain and oral hygiene). Better results were achieved using a silicone-based soft liner, which recommends it as the material of choice for relining dentures.

Key words:
denture rebasing; acrylates; silicons; quality of life.

Apstrakt

Uvod/Cilj. Gubitak zuba utiče na oralno zdravlje i na kvalitet života. Više od trećine bezubih pacijenata nije zadovoljno svojim totalnim protezama, a većina njih se žali na nedovoljnu retenciju i stabilizaciju, kao i bol prilikom žvakanja. Rešenje njihovog problema moglo bi biti podlaganje proteza materijalima na bazi silikona ili akrilata. Cilj ovog istraživanja bio je utvrđivanje stepena zadovoljstva pacijenata pre i posle podlaganja proteze mekim i tvrdima materijalima. **Metode**. Pacijenti (n = 24) bili su podeljeni u dve studijske grupe. Kod prve grupe pacijenata podlaganje gornje totalne proteze izvršeno je akrilatnim materijalima, dok su u drugoj grupi proteze podlagane silikonskim materijalom. Pacijentima su postavljena pitanja iz specijalno prilagođenog the *Oral Health Impact Profile* upitnika pre i tri meseca nakon podlaganja proteza. **Rezultati.** Pacijenti su pokazali veći stepen zadovoljstva u svim ispitivanim oblastima (mastikatorna funkcija, psihološka i socijalna nelagodnost, retencija i higijena) nakon podlaganja. Pacijenti su bili zadovoljniji nakon podlaganja proteze mekim materijalom. **Zaključak.** Podlaganje maksilarnih totalnih proteza značajno doprinosi poboljšanju kvaliteta života bezubih pacijenata u svim ispitivanim domenima. Bolji rezultati postižu se silikonskim podlogama, što ih čini materijalima izbora za podlaganje totalnih proteza.

Ključne reči:
zubna proteza, podlaganje; akrilati; silikoni; kvalitet života.

Introduction

Edentulism is a handicap that affects the quality of life and nutrition [1]. Tooth loss leads to functional impairment at the level of the orofacial system, significantly interfering with chewing, swallowing and speech, as well as to temperomandibular joint dysfunction, disorders of intermaxillary relation and facial physiognomy [2,3]. In addition, a less attractive facial appearance, difficulty with speech and avoidance of social contacts may result in psychosocial problems [4].

Well-designed denture may improve the oral health of patients and consequently their quality of life [5,6]. The success of prosthetic rehabilitation by conventional complete dentures is variable and depends on capacity of adaptation to treatment limitations [7]. In addition to training of dentists, a very important factor for the acceptance of prosthesis by pa-

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tients is the degree of their satisfaction [8, 9]. From the patient's point of view, denture satisfaction appears to be primarily related to aesthetics, retention and function [4]. The level of acceptance of complete denture depends on gender, education level, socioeconomic status, and the type of personality [10–12].

More than a third of edentulous patients are not fully satisfied with their complete dentures, and mainly complain of insufficient stability, retention and pain during mastication [13]. Continuous residual alveolar ridge resorption leads to the insufficient support of complete dentures. Liner should be added to the inside of the denture to allow equal force distribution, reduce localized pressure as well as improve denture retention and stability [14, 15].

Relining can be made with soft materials, which maintain resilience for a long time, or cold-cured hard poly (methacrylate), which are chemically identical to the material denture base is made of.

The Oral Health Impact Profile (OHIP) is a commonly used questionnaire for assessing oral health related quality of life [16]. The original English version of the OHIP was presented by Slade and Spenser in Australia in 1994 [17]. The OHIP consists of 49 questions divided into seven constitutive domains: functional limitations, physical discomfort, phychological discomfort, physical disability, phycholosical disability, social disability and handicap. Both original and modified OHIP versions were used in different language areas respecting linguistic and cultural differences existing in these regions [18–21]. This questionnaire demonstrated satisfactory validity, reliability and responsiveness in different cultural areas and surrounding countries [22–25]. In 2009 Stančić et al. [26] presented the Serbian version of OHIP-14 as a part of assessing oral health-related quality of life of elderly patients. Pisani et al. [27] proposed version of the questionnaire related to the edentulous patients, and their version was used in this study (Appendix 1). The OHIP-EDENT was adapted as a specific instrument for edentulous subjects, which was aimed at detecting changes as influenced by clinical aspects of edentulism and treatments [28, 29].

The aim of this study was to determine the level of satisfaction of edentulous patients before and after relining maxillary dentures with soft and rigid denture liners.

Methods

Study sample and procedure

The study was conducted at the Dental Clinic, Faculty of Medicine, University of Niš, with the approval of the Ethics Committee (No. 01-2113-2). The sample of patients was formed after taking anamnesis and clinical examination. The sample consisted of 24 patients of both sexes, aged 50–70 years, with a minimum of one and maximum of five years maxillar denture usage time. The patients wore upper total and lower partial dentures. The study included the patients who had indication for maxillary complete denture relining, because of pain or lack of retention and stability. The study did not include patients with inflammatory changes in the oral cavity, candidiasis, hyperplasia, neurological disorders and malignancies. For the sake of uniformity of understanding the questionnaire all the patients included in the study had secondary degree of education.

The sample (n = 24) was randomly divided into two study groups (n = 12). Maxillary denture relining of the first group of patients was performed with cold curing acrylic-based rigid liner (Triplex Cold, Ivoclar Vivadent, Lichtenstein). In the second group of patients dentures are relied with silicone-based resilient liner (GC Reline Soft, USA). Denture relining was performed according to chair side procedures prescribed by the manufacturer's instructions. Denture relining with silicone-based soft liner implied the use of an appropriate primer.

The patients were asked the questions from specifically adapted questionnaire in the time baseline and three months after relining dentures.

Translation of OHIP-EDENT

The original version of OHIP-EDENT was translated into Serbian by the accredited translator and revised by the prosthodontist, accompanied by back-translation into English after the Serbian version was revised (Appendix 2). The translation of the questions was adjusted to the questionnaire version provided by Stančić et al. [26] Three possible answers to these questions and their scores were: never – 0, sometimes – 1 and almost always – 2 (Likret scale) [27].

The results were statistically analysed using the Wilcoxon test (SPSS version 16.0, USA, $p < 0.05$).

Results

Table 1 presents the mean score values and standard deviations for the questionnaire, as well as comparisons between periods.

Table 1
The mean score values before and after relining of dentures (Wilcoxon test)

Material	Treatment	Score ($\bar{x} \pm SD$)	*p*
Rigid denture liner			
Masticatory related complains	before	3.33 ± 1.44	0.004*
	after	1.17 ± 1.03	
Psychological discomfort	before	4.58 ± 1.38	0.002*
	after	2.08 ± 1.31	
Social disability	before	2.08 ± 1.83	0.133
	after	1.08 ± 0.67	
Retention and hygiene	before	7.67 ± 1.97	0.005*
	after	4.75 ± 1.14	
Sum	before	17.67 ± 4.60	0.003*
	after	9.08 ± 1.93	
Silicone-based soft liner			
Masticatory related complains	before	2.92 ± 1.38	0.007*
	after	0.83 ± 1.34	
Psychological discomfort	before	4.50 ± 1.93	0.009*
	after	1.33 ± 1.72	
Social disability	before	2.33 ± 2.10	0.026*
	after	0.67 ± 1.15	
Retention and hygiene	before	8.50 ± 1.38	0.002*
	after	4.75 ± 0.96	
Sum	before	18.25 ± 3.72	0.002*
	after	7.58 ± 2.81	

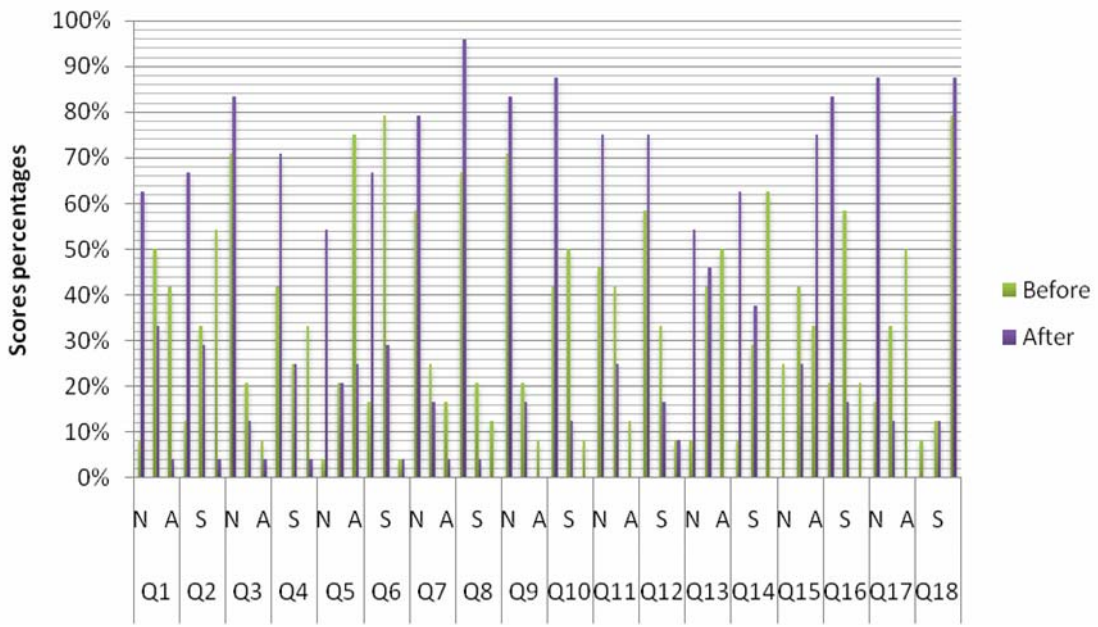
Score: 'Never'– 0, 'Sometimes' – 1, 'Almost always' – 2.
*** Statistically significant difference ($p < 0.05$).**

All four domains indicated a statistically significant difference ($p < 0.05$) between the reference period, except the social disability for rigid denture relining. Statistically significant reduction in mean scores indicates the improvement in all the domains after relining dentures. Larger decrease in mean scores was observed in soft liner which indicates the advantage in the use of silicone liners relative to the rigid acrylic ones.

The percentage score for responses N (never), S (sometimes) and A (almost always) in the time baseline and after 3 months of relining is shown in Figure 1. After relining in all the tested domains a higher response rate of 'never' (N) was observed. The answers 'sometimes' (S), and 'almost always' (A) were more common in the period before relining of dentures, which proved positive effect of relining of dentures.

prosthetic appliance to which he/she is accustomed, while improving its functionality at the same time.

The study included a specific group of patients wearing upper complete and lower partial dentures, which represents the most frequent clinical situation in prosthodontic patients between 50 and 70 years of age. The uniformity of clinical finding and period of wearing dentures significantly affect the results of patients' satisfaction. Empirically, it was concluded that the patients were more satisfied with retention and stabilisation of upper complete dentures compared to lower total dentures. Thus, the starting assumption of the study was that comparison of relining effects of these two dentures on oral health related quality of life would be irrelevant. In addition, construction of lower complete denture and upper partial denture is rarely indicated. The patients with both complete dentures were excluded from the



Q – the number of a question.

Fig. 1 – Percentage of scores: N (never), S (sometimes) and A (almost always).

Discussion

Tooth loss affects masticatory function, disabling normal nutrition of the patient. Wearing complete dentures is associated with their many functional limitations, to which the patients adapt over time and for which neuromuscular mechanism is the most responsible [8]. To be able to use it, patients must be confident with their dentures and satisfied with them.

With respect to changes that constantly occur in the orofacial system, primarily concerning alveolar ridge resorption, retention and stability of dentures gradually decreases, and security in their use also decreases. Creating new dentures is not always the solution to the problem, and represents considerable financial problem. Having the existing dentures relined, the patient continues to use a study due to absolute loss of tooth propriceptor and rare need for simultaneous relining of both complete dentures. For the sake of uniformity, of understanding the questions, and since OHIP-EDENT has not been used in Serbian speaking region so far, the subjects used in the study were of similar educational status.

The used questionnaire contained all the necessary parameters related to functionality of dentures, possible occurrence of pain and the psychological and social aspect of their use. The answers to these questions are a clear indication of how patients feel about their dentures. The obtained results clearly showed that patients were satisfied after relining dentures. There was an improvement of comfort and chewing ability, eating enjoyment, food choices, security and speech after denture modification. Relining of dentures improved the quality of patients' life, reducing their

anxiety and concern. The results positively correlated with the results of several studies [4, 27, 29].

The results of this study suggest the advantage of using soft liners in comparison with cold curing acrylic resin. The use of soft liners became a reality in dentistry because they have many clinical advantages. Soft materials have the ability to repair the inflamed mucous membrane, uniformly distribute load functional area in support of the dentures and improve their retention and stability [27]. Kimoto et al. [30] also concluded a higher degree of satisfaction of patients whose dentures were lined with resilient material compared to conventional heat curing acrylate. Contrary to the results, the study of Mohamed [4] proved better chewing ability and biting force after relining dentures with conventional heat curing acrylate compared to resilient liners. There results showed a significant decrease in masticatory function six months after resilient relining.

Conclusion

Relining of maxillary complete dentures significantly positively impacts the quality of life of patients in all the tested domains (masticatory function, psychological discomfort, social disability, pain and oral hygiene). Better results were achieved using a silicone-based soft liner, which recommends it as the material of choice for relining dentures.

Taking into account the study limitations on the patients with upper total denture, further research should be directed towards more complete understanding of different prosthodontic treatment effects on oral health related quality of life in patients with lower complete dentures and edentulous patients.

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Appendix 1

The Oral Health Impact Profile (OHIP) questionnaire for edentulous patients (OHIP-EDEN)
Please circle the number next to the answer of the question: 0-never; 1-sometimes; 2-almost always.

Masticatory related complains
Do your braces interfere during chewing?
Do you avoid certain foods because of problems with your dentures?
Do you feel pain and cracking in the joints?
Psychological discomfort
Are you worried and anxious due to the problems with denture?
Are you constantly aware of the presence of dentures
Have you ever had to end a meal due to problems with denture?
Are you worried by the thought that you will forever serve denture?
Are you embarrassed because you wear denture?
Social disability
Do you avoid social contacts due to problems with denture?
Are you irritable toward others because of problems with denture?
Do you have a feeling that because of denture you can't relax in the society?
Do you feel that your life has less quality because you wear denture?
Retention and hygiene
Did you notice that on your denture retains food?
Can you see food particles under the denture?
Are your denture stable fit in your mouth?
Does denture make wounds in your mouth?
Do you feel pain while your denture is in your mouth?
Are you able to properly clean your denture?

Appendix 2
The Oral Health Impact Profile (OHIP) questionnaire for edentulous patients (OHIP-EDEN) translated into Serbian
Molim Vas zaokružite broj odgovora pored postavljenog pitanja: 0-nikad, 1-ponekad, 2-skoro uvek.

Smetnje u žvakanju
Da li imate smetnje pri žvakanju hrane?
Da li teže jedete određenu hranu zbog problema sa protezama?
Da li dok jedete osećate bol i pucketanje u zglobovima?
Psihološke smetnje
Da li ste zabrinuti ili uznemireni zbog problema sa protezom?
Da li ste stalno svesni činjenice da Vam je proteza u ustima?
Da li ste nekada morali da prekinete obrok zbog problema sa protezom?
Da li ste prihvatili činjenicu da ćete od sada stalno nositi protezu?
Društvene smetnje
Da li zbog problema sa protezom izbegavate kontakte sa ljudima?
Da li ste neprijatni sa ljudima iz okoline zbog problema sa protezom?
Da li se zbog problema sa protezom ne možete opustiti u društvu?
Da li mislite da je Vaš život manje kvalitetan od kada nosite protezu?
Retencija i higijena proteze
Da li ste primetili da Vam se na protezi zadržava hrana?
Da li ste primetili ostatke hrane ispod proteze?
Da li Vam se proteza pomera u ustima?
Da li dok koristite proteze osećate bol?
Da li lako čistite Vaše proteze?

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Clinicopathological characteristics, diagnosis and treatment of melanoma in Serbia – the Melanoma Focus Study

Kliničkopatološke karakteristike, dijagnoza i lečenje melanoma u Srbiji – The *Melanoma Focus* studija

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Abstract

Background/Aim. Treatment options for metastatic melanoma in Serbia are limited due to the lack of newly approved biologic agents and the lack of clinical studies. Also, there is a paucity of data regarding the treatment approaches in different tertiary centers and efficacy of available chemotherapy protocols. The aim of this study was to obtain more detailed data about treatment protocols in Serbia based on structured survey in tertiary oncology centers. **Methods.** Data about the melanoma patients treated in 2011 were analyzed from hospital databases in 6 referent oncology centers in Serbia, based on the structured survey, with the focus on metastatic melanoma patients (unresectable stage IIIC and IV). **Results.** A total of 986 (79–315 in different centers) patients were treated, with 320 (32.45%) newly diagnosed patients. There were 317 patients in stage IIIC/IV, 77/317 aged < 50 years. At the time of diagnosis 47.3% of patients were < 60 years of age (24.2% < 40 years, 23% 50–59 years, 52.6% > 60 years). At initial diagnosis 12.5% of patients were in stage III and 4.5% in stage IV. The most common type was superficial spreading melanoma (50–66%), followed by nodular melanoma (23.5–50%). Apart from the regional and distant lymph node metastases, the most frequent organs involved in stage IV disease were distant skin and soft tissues (12–55%), lungs (19–55.5%), liver (10–60%), and bones (3–10%). The first line therapy in stage IV metastatic melanoma was dacarbazine (DTIC) dimethyl-triazeno-imidozole-carboxamide in 61–93% of the patients, while the second line varied between the centers. Disease control (complete response + partial response + stable disease) was achieved in 25.7% of the patients treated with the first line chemotherapy and 23.1% of the patients treated with the second line therapy, but the duration of response was short, in first-line therapy 6.66 ± 3.36 months (median 6.75 months). More than 90% of patients were treated outside the clinical trials. **Conclusion.** Based on this survey, there is a large unmet need for the new treatment options for metastatic melanoma in Serbia. The development of national guidelines, and greater involvement in international clinical studies could lead to widening of treatment options for this chemotherapy resistant disease.

Key words:
melanoma; diagnosis; neoplasm staging; neoplasm metastasis; therapeutics; clinical protocols.

Apstrakt

Uvod/Cilj. Terapijske mogućnosti za metastatski melanom u Srbiji su ograničene zbog nedostupnosti novoodobrenih bioloških lekova i veoma malog broja multicentričnih internacionalnih kliničkih studija. Takođe, postoji mali broj podataka o terapijskom pristupu metastatskom melanomu u različitim tercijarnim centrima i efikasnosti raspoloživih protokola hemioterapije. Cilj ove studije bio je da se dobiju detaljniji podaci o protokolima lečenja u Srbiji, na osnovu strukturisane ankete u tercijarnim onkološkim centrima. **Metode.** Podaci o obolelima od melanoma, lečenih u 2011. godini u Srbiji, dobi-

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jeni su i analizirani iz bolničkih baza šest referentnih onkoloških centara u Srbiji, na osnovu strukturisane ankete, sa fokusom na metastatski melanom (inoperabilni stadijum IIIC i IV). **Rezultati.** Ukupno je lečeno 986 (79–315 u različitim centrima) bolesnika, od čega je 320 (32,45%) bilo novodijagnostikovanih. Bilo je 317 bolesnika u stadijumu inoperabilnog melanoma IIIC/IV, 77/317 (24,29%) starosti < 50 godina. U vreme postavljanja dijagnoze 47,3% bolesnika bili su < 60 godina starosti (24,2% < 40 godina, 38% 40–60 godina, 46% > 60 godina). Kod 12,5% bolesnika dijagnoza je postavljena u stadijumu III, a kod 4,5% u stadijumu IV bolesti. Najčešći kliničkopatološki tip bio je površnošireći (50–66%) i nodularni melanom (23,5–50%). Osim regionalnih i udaljenih metastaza limfnih čvorova, najčešće zahvaćeni organi u IV stadijumu bolesti bili su; udaljene metastaze kože i mekih tkiva (12–55%), pluća (19–55,5%), jetra (10–60%) i kosti (3–10%). Prva linija terapije u inoperabilnom stadijumu III i stadijumu IV metastatskog melanoma bio je dakarbazin (dimetril-triazeno-imidazol-karboksamid – DTIC) kod 61–93% bolesnika, dok je druga linija varirala između centara. Kontrola bolesti (kompletan odgovor + parcijalan odgovor + stabilna bolest) ostvarena je kod 25,7% bolesnika lečenih prvom linijom hemioterapije i 23,1% bolesnika sa drugom linijom terapije. Trajanje odgovora bilo je kratko: u prvoj liniji terapije 6,66 ± 3,36 meseci (medijana 6,75 meseci). Više od 90% bolesnika lečeni su van kliničkih studija. **Zaključak.** Ovo istraživanje ukazuje da postoji velika potreba za novim terapijskim opcijama za lečenje metastatskog melanoma u Srbiji. Razvoj nacionalnih smernica i veće učešće u međunarodnim kliničkim studijama može dovesti do proširenja opcije za lečenje ove bolesti otporne na hemioterapiju.

Ključne reči:
melanom; dijagnoza; neoplazme, određivanje stadijuma; neoplazme, metastaze; lečenje; protokoli, klinički.

Introduction

The data about the epidemiology and clinicopathological characteristics of melanoma in South East Europe are scarce, with majority of information obtained from the cancer registries. Based on the Cancer Registry of Central Serbia, crude incidence of melanoma in 2009 was 9.3 in males and 7.7 in females, while age-standardized rate was 5.5 in males and 4.5 *per* 100,000 population [1]. In total, around 500 new cases of melanoma are diagnosed in Serbia annually and the incidence is rising reflecting the same trends noted in the rest of the Europe and the world [1]. However, due to underreporting, the epidemiological registries often contain a lot of missing data, especially on clinicopathological characteristics. Also, there are no data about the standard procedures in melanoma diagnosis and treatment in Serbia so far.

The aim of this study was to obtain more detailed data about demographics, clinicopathological features, diagnostic and treatment protocols in Serbia based on structured survey in tertiary oncology centers.

Methods

Data about the patients with melanoma treated in 2011 were analyzed from hospital databases in 6 referent oncology centers in Serbia, based on the structured survey, with the focus on metastatic melanoma patients (unresectable stage IIIC and IV). The structured survey was approved by Ethics Committees in all the participating centers. The participants answered the survey based on the data from the hospital registries. Survey retrieved data about the number of treated patients, newly diagnosed melanoma cases, age and sex distribution, localization, clinicopathological type, clinical stage at diagnosis. For inoperable stage III and stage IV metastatic disease, treatment protocols, disease control rates [complete response (CR) + partial response (PR) + stable disease (SD)] for 1st or 2nd line therapy and duration of response were analyzed. The standard surgical treatment consisted of excision of melanoma with the margins recommended by European guidelines [2, 3]. Collected data were presented using descriptive statistical analysis.

Results

Based on the data from the structured survey, a total of 986 (79–315 in participating centers) patients were treated, with 320 (32.45%) newly diagnosed patients. There were 877 (92.41%) cutaneous melanoma, 13 (1.37%) mucosal, 24 (2.53%) ocular, 24 (2.53%) unknown primary melanoma, and other and unspecified location, 11 (1.11%) and 37 (3.75%), respectively, because of the unavailability of data in some centers.

The distribution of the patients between the centers is presented in Table 1.

Out of 965 patients treated in 2011 with available data on age, 319 (33%) were < 50 years of age. There were 317 patients in stage IIIC/IV and in this group 77/317 (24.29%) patients were < 50 years of age. At the time of diagnosis 47.3% of the patients were < 60 years of age (24.2% < 40

Table 1

The distribution of the patients diagnosed with melanoma in Serbian tertiary oncology centers

Oncology center	Number of patients (%)
Institute of Oncology and Radiology of Serbia	315 (32)
Military Medical Academy	273 (28)
Clinical Center Nis	129 (13)
Institute of Oncology of Vojvodina	96 (10)
Clinical Center „Bezanijska Kosa“	94 (9.5)
Clinical Center of Vojvodina	79 (8)
Total	986 (100)

years, 23% 50–59 years, 52.6% > 60 years). The age distribution of all the patients and in patients with unresectable stage III and stage IV disease is presented in Figure 1.

The most common type was superficial spreading melanoma (50–66%), followed by nodular melanoma (23.5–50%). The wide range of distribution between clinicopathological types is due to the restrospective nature of the study and lack of predefined criteria for primary and secondary nodular melanoma.

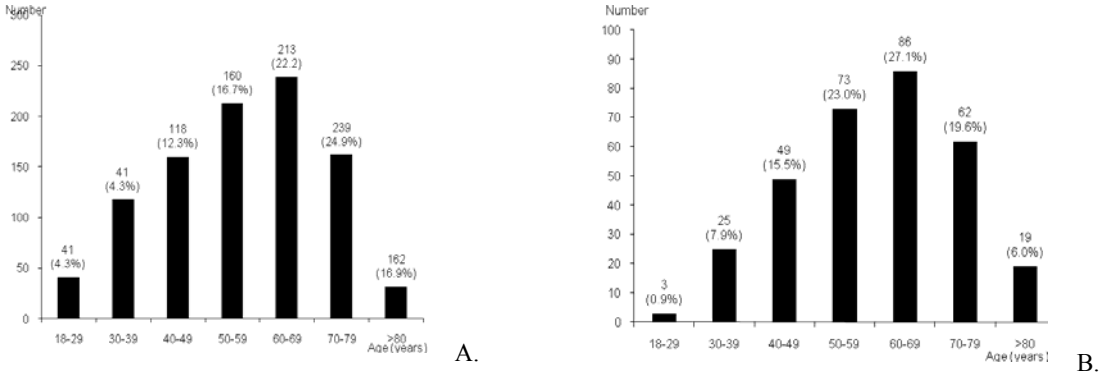

Fig. 1 – Age distribution in: A) All the treated patients; B) The patients with unresectable stage III and stage IV melanoma (Serbia, 2011).

Clinical stage at diagnosis is presented in Figure 2. At initial diagnosis, 12.5% of the patients were in stage III and 4.9% in stage IV. In the patients with localized disease and complete histopathological reports, 232/548 (42.3%) presented with high-risk melanoma, of which 131 (50%) were thicker than 4 mm. Only 17 (3.1%) patients were diagnosed with *in situ* melanoma, and in 217/548 (39.59%) melanoma with Breslow thickness of < 1 mm were noted.

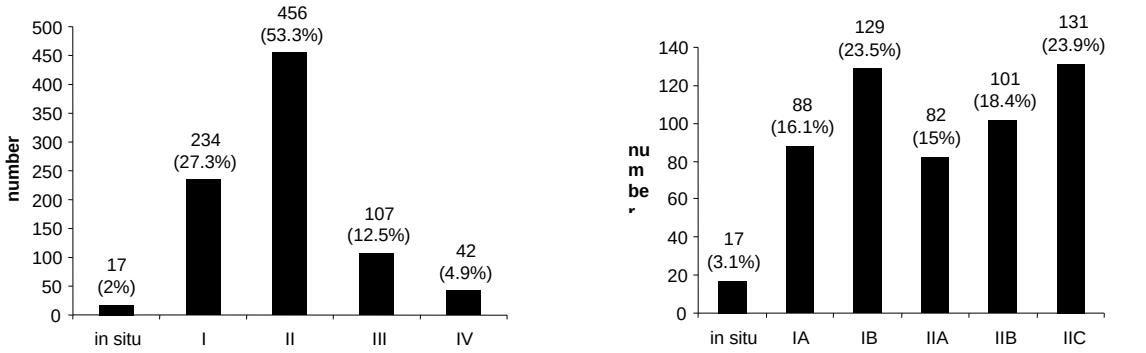

Fig. 2 – Clinical stage at the time of the diagnosis in Serbian melanoma patients treated in 2011.

Apart from the regional and distant lymph node metastases, the most frequent organ involved in stage IV disease were distant skin and soft tissues (12–55%), lungs (19–55.5%), liver (10–60%), and bones (3–10%). Brain metastases were detected in an average of 15.48% of the patients (3.5% at the Military Medical Academy, Belgrade, Serbia, to 25% in the Clinical Center Niš, Niš, Serbia).

The median time to diagnose melanoma from patient referral to the hospital to histopathological analysis was 22 days (21–35 days), and the median time for histopathological analysis from the time of surgery was 8.5 days (7–15 days).

Regarding to surgical treatment, in 4 of 6 centers sentinel lymph node biopsy was available, but is still not the standard surgical treatment for all the patients with indication for this procedure. After complete surgical resection in stage II and stage III melanoma the standard of care was regular follow-up of the patients at 3 month interval. Adjuvant treatment with interferon-α is not reimbursed by the national healthcare provider. The systemic treatment of melanoma in Serbia is based on chemotherapy regimens outside the clinical trials in more than 95% of patients. In 37/317 (11.67%) patients with stage IV disease surgery was performed – in cases with solitary metastases or for palliation.

The first line therapy in unresectable stage III or stage IV metastatic melanoma was administered in 271/312 (86.8%) patients, and complete data about the treatment were available for 171 patients. Dacarbazine (dimethyl-triazeno-imidazole-carboxamide – DTIC) monochemotherapy was most frequently used, in 60.97–93% of the patients in different centers. The second most common first-line chemotherapy regimen was cisplatin-vinblastin-dacarbazine (CVD) in 5–39.1%. In ≤ 5% of the patients cisplatin-dacarbazine was administered as the first line option, and in 2% carboplatin-paclitaxel in only one center. Disease control (CR + PR +

SD) was achieved in 25.7% of the patients treated with the first line chemotherapy; complete response was noted in 3.5%, partial response in 5.3%, stable disease in 17%, and disease progression in 74.3% of patients. Also, the duration of response to chemotherapy was short: in first-line therapy 6.66 ± 3.36 months (median 6.75 months) (Table 2).

Table 2

Systemic treatment of melanoma patients in Serbia, 2011

Parameters	First-line therapy	Second-line therapy
Treated patients, n (%)	271/312 (89.7%)	50/132 (37.8%)
Regimens (%)	DTIC 60.97–93% CVD 5–39.1%. DTIC-CDDP 5% in 2 centers Carboplatin-paclitaxel 2% in 1 center	CVD (5–39.1%) DTIC-CDDP 5% in 2 centers Carboplatin-paclitaxel 30%
Response rates (CR + PR + SD)	ORR 25.7% CR 3.5% PR 5.3% SD 17% PD 74.3%	ORR (CR + PR + SD) 23.1% PD 76.9%
Duration of response (months), $\bar{x} \pm SD$	6.66 ± 3.36	6.75 ± 0.75

DTIC – dimethyl-triazeno-imidazole-carboxamide; CVD – cisplatin, vinblastin, dacarbazine; CDDP – cis-diaminedichloroplatinum; CR – complete response; PR – partial response; SD – stable disease; PD – progresion of disease; ORR – overall response rate.

The second-line therapy was employed in 50 of 132 (37.8%) patients with available data with variable regimens among the centers: dacarbazine based regimens (CVD) 20%, DTIC-cis-diaminedichloroplatinum (CDDP) 80%, vinblastin-bleomycin-cisplatin in 100% of patients in one center and carboplatin-paclitaxel in 30% of patients in another center. Second-line therapy achieved disease control (CR + PR + SD) in 23.1% of patients and this response was also short-lived (6.75 ± 0.75 months) (Table 2).

Discussion

Data about the clinicopathological characteristics and treatment patterns of metastatic melanoma in Serbia are scarce. As far as we know, this survey was the first attempt to form national melanoma data register and to colect data about therapeutical approach to these patients. In this study the basic clinicopathological characteristics and treatment patterns were analyzed based on the data from the structured survey from six tertiary oncology centers in Serbia. In 2011, 986 patients with melanoma were treated, 317 with unresectable stage III and stage IV metastatic disease. The most common type of melanoma was superficial spreading type and nodular melanoma, which is in line with other studies from Europe and US [4–7]. In newly diagnosed patients with localized disease, 234/548 (42.7%) of the patients were presented with high-risk melanoma, of which 131 (50%) were of ≥ 4 mm Breslow thickness (stage IIC). Only 17 (3.1%) patients were diagnosed with *in situ* melanoma, and in 217/548 (39.59%) melanoma with Breslow thickness of < 1 mm was noted. It is in line with previously published data from the single tertiary institution in Serbia, and also the main difference compared with data from Western Europe, USA and Australia, where up to 70% of patients are diagnosed as in situ or thin melanoma [4–7]. This points out to the late diagnosis, and an urgent need for efficient primary and secondary prevention measures. Also, there is a possibility that in this survey a substantial proportion of thin and *in situ* melanomas were not recorded since they were treated in regional centers and private practice. Thus, the central melanoma registry is needed to cover all (or at least the majority) of diagnosed melanoma cases.

The standard surgical treatment of melanoma in all centers consists of excision with the margins up to 2 cm, based on the European guidelines [2, 3]. In 4 of 6 centers sentinel lymph node biopsy is available, but in some institutions it is still not the standard surgical treatment for all patients with indication for this procedure.

In stage IV of the disease, the patients most frequent had distant skin and soft tissues metastases (12–55%), followed by metastatic disease in lungs (19–55.5%), liver (10–60%), brain (3.5–25%) and bones (3–10%). The large differences between centers are attributed to the different structure of the patients in different centers. More relevant data will be available with the establisment of the Central registry of melanoma of Serbia.

In 2011 and until the submission of this article, the only reimbursed treatment for metastatic melanoma by National Insurance Fond in Serbia was dacarbazine (DTIC). It was used as the first-line treatment in 60.97–93% of the patients in the participating centers. In 5 centers monochemotherapy was employed in up to 93% of patients while in one CVD polychemotherapy was used in 39.3% of patients as the first-line option. This points out to the need for the national guidelines and establishment of standard-of-care treatment for metastatic melanoma in Serbia. Concerning the overall systemic treatment patterns in unresectable stage III and stage IV disease, > 95% of patients are treated outside the clinical trials. Having in mind that even now in the era of approved and registered biologic drugs for metastatic melanoma like vemurafenib and ipilimumab, clinical trials remain as one of the first treatment options, large inaccessibility of patients to clinical trials with the new more promising treatments is evident.

Based on the previous studies [8, 9], dacarbazine has an overall response rate of ≤ 20% and complete response rate of 2–3% and this is in line with our data. Even in patients with complete responses, these are rarely durable, 6.6 months in our survey. Similar figures were found in previous studies [8, 9]. Based on the European and US treatment guidelines vindesine, fotemustine, paclitaxel are also indicated as monochemotherapy in first or second-line treatment with similar efficacy [2, 3, 10]. However, vindesine and fotemustine are not registered and paclitaxel is registered but not reimbursed in Serbia. The only second line options is polychemotherapy with dacarbazine and platinum compounds ± vinblastine (CVD), although CVD regimen was found in the previous study not to be effective in second-line setting in patients previosly treated with dacarbazine [11]. This is the reason why in the modern treatment of metastatic melanoma, cytotoxic chemotherapy is largely abandoned as the first-line option, except in much selected cases (BRAF wild-type tumors, ipilimumab-resistant disease and unavailability of clinical studies). In Serbia, it is still the standard of care.

Conclusion

This study revealed a large unmet need for the new diagnostic and treatment options for melanoma, especially for metastatic melanoma in Serbia. The development of the national guidelines, access to novel treatment options and greater participation in international clinical studies could lead to widening of treatment options for this agressive malignant disease.

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Significance of microvessel density in prostate cancer core biopsy

Značaj gustine krvnih sudova u biopsijama karcinoma prostate

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Abstract

Background/Aim. In prostate tumors, angiogenesis, measured as microvessel density, is associated with tumor stage and Gleason score. The aim of this study was determine neovascularization of prostatic adenocarcinomas in core biopsies and corresponding prostatectomies. **Methods.** The study population included 61 patients who underwent radical prostatectomy (RP) for localized prostate carcinoma patients and did not receive chemohormonal, or radiation therapy before surgery. Tumor blocks were immunostained using the endothelial-specific antibody CD31 and subsequently evaluated at × 400 magnification in both biopsies and corresponding prostatectomies. **Results.** When comparing microvessel density in core biopsies and corresponding prostatectomies, no statistically significant difference was found ($p > 0.1$). A statistically significant positive correlation was found when determining correlation between microvessel density (as linear and categorical variable, i.e. with the cut-off value of 48) that was associated with the Gleason score ($p < 0.05$) and tumor stage ($p < 0.0001$). There was no correlation between microvessel density and preoperative values of serum prostate-specific antigen (PSA) ($p > 0.1$). **Conclusion.** Microvessel density can be reliably applied to needle prostate biopsy specimens. Quantification of the microvascular density in biopsies is an accurate pre-operative predictor of tumor stage, discriminating between organ-confined and organ-extending neoplasms.

Key words:
prostatic neoplasms; biopsy, fine-needle; prostatectomy; immunohistochemistry; neovascularization, pathologic.

Apstrakt

Uvod/Cilj. U karcinomima prostate, angiogeneza određena merenjem gustine krvnih sudova, povezana je sa stadijumom tumora i Gleason skorom. Cilj ovog istraživanja bio je proučavanje neovaskularizacije adenokarcinoma prostate u uzorcima biopsije iglom i odgovarajućih prostatektomija. **Metode.** U naše istraživanje bio je uključen 61 bolesnik kojima je urađena radikalna prostatektomija (RP) na osnovu kliničke procene da se radi o lokalno ograničenom karcinomu prostate, koji preoperativno nisu primili nikakvu hemio-, hormonalnu ili zračnu terapiju. Tumorsko tkivo je analizirano primenom imunohistohemijskog markera, endotel-specifičnog antitela CD31, koje je zatim procenjivano na mikroskopskom uveličanju × 400 u uzorcima biopsija iglom i tkiva dobijenih nakon RP. **Rezultati.** Prilikom poređenja vrednosti gustine krvnih sudova određenih na biopsijama prostate uzetih iglom sa gustinom krvnih sudova odgovarajućih RP nije nađena statistički značajna razlika ($p > 0,1$). Statistički značajna pozitivna korelacija nađena je prilikom određivanja povezanosti gustine krvnih sudova (kao linearne i kategorijalne varijable sa ograničenom vrednošću 48) i Gleason skora ($p < 0,05$), kao i stadijuma bolesti ($p < 0,0001$). Statistički značajna povezanost nije utvrđena između gustine krvnih sudova i predoperativnih vrednosti serumskog prostatičnog specifičnog antigena (PSA) ($p > 0,1$). **Zaključak.** Određivanje gustine krvnih sudova može se pouzdano koristiti za uzorke prostate dobijene biopsijom iglom. Kvantifikacija gustine krvnih sudova u biopsijama iglom tačan je i nezavisan predoperativni prediktor stadijuma tumora (lokalno ograničen karcinom prostate u odnosu na lokalno proširenu bolest).

Ključne reči:
prostata, neoplazme; biopsija tankom iglom; prostatektomija; imunohistohemija; neovaskularizacija, patološka.

Introduction

The incidence of prostate cancer (PC) is on an exceptional increase in the whole world, thanks to early detection programs that include digital rectal examination, determining of serum prostate-specific antigen (sPSA), transrectal ultrasonography and needle biopsy of the prostate [1, 2]. This is a heterogeneous disease with unpredictable clinical flow from a relatively indolent disease to an aggressive form with rapid metastatic spreading of the disease and fatal outcome. Unfor-

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tunately, there are still no parameters that can be safely used to foresee whether it is a locally non-invasive prostate cancer (pT2) or invasive and metastasis expanded neoplasm (pT3). According to data from different researches published so far, PC in 24–60% patients was clinically under-staged before surgery, whereas in 8–45% of cases it was over-staged [3–9]. Potential biomarkers are still being researched as well as different methods of diagnostics, which could improve detection, preoperative grading and staging systems for prostate cancer in order to get a clearer picture about possibilities and risk of the application of adequate therapy procedures for each individual patient. Angiogenesis (neovascularization) is the process of creation of new functional capillary microvessels from the already existing vascular network. Vascularization of the primary tumor results in an expanded growth and the tumor then gets metastatic potential, whereas development of microvessels is necessary for growth of distant metastatic tumor hotspot [10].

Tumor microvessels are not the same as the microvessels of normal tissue, they are heterogeneous in terms of organization, function and structure [11]. Angiogenesis is present in all the tumors, but with characteristic and significant differences between different types of tumors (the biggest intensity of angiogenesis was found with glioblastoma, followed by renal cell carcinoma, colorectal cancer, breast cancer, lung cancer, PC), but inside every individual type of tumor in different patients – individual tumors are well vascularized, while the others are poorly vascularlized [12]. The prognostic value of microvessel density (MVD), as a measure of tumor angiogenesis, is still unclear in the PC, particularly on samples of needle prostate biopsies [13–15]. Thus, the aim of this study was to determine prognostic significance of MVD, as a stage predictor in prostatic carcinomas in core biopsies (CB) and corresponding prostatectomies.

Methods

This retrospective study included 61 previously untreated patients with prostatic adenocarcinoma admitted with elevated serum PSA levels at the Clinic of Urology, Clinical Center of Vojvodina, in the period 2005–2006. All the patients underwent systematic sextant transrectal ultrasonography-guided core biopsies performed with an 18-gauge automated spring-loaded biopsy gun. The diagnosis of prostatic carcinoma in needle biopsies was followed by radical retropubic prostatectomy (RP) with bilateral pelvic lymphadenectomy. All tumors were primary diagnosed without previous therapy and none of the patients had clinical evidence of metastasis prior to surgery. Tumor grading on needle biopsies and prostatectomy specimens were undertaken according to Gleason. The final pathological stage on the whole mount prostatectomy specimens was determined according to the tumor-nodus-metastasis (TNM) system.

From these 61 patients, a total of 366 core biopsies were available, out of which 254 contained carcinomatous tissue (median 2; range 1–6 *per* case). These 254 biopsies were analyzed and only those containing at least two microscopic fields of neoplastic glands at x400 magnification were selected for determination of MVD. Insufficient tumor tissue was found in 55 core biopsies, and these biopsies were excluded. Finally, analyses were performed on 199 core biopsies. All RP specimens from 61 patients were evaluated in a standard fashion. Surgical margin (SM) sections from the apex and base were taken as shaved margins. Extraprostatic extension (EPE) was diagnosed if tumor was seen in the periprostatic soft tissue or was seen penetrating through a fibromuscular capsule and coming out on the other side. The seminal vesicles (SVs) were evaluated at the junction where they enter the prostate gland. All pelvic lymph nodes were evaluated for the presence of metastatic disease. All the cases were assigned with a Gleason sum (GS). After review of each case, the blocks with the highest GS and greatest density of tumor and those containing at least two microscopic fields of neoplastic glands at ×400 magnification were selected for immunohistochemical staining. The study excluded the patients who had received prostate-related therapy before RP, including androgen deprivation therapy, chemotherapy, radiation therapy, or other therapy. It also excluded the patients in whom there was no cancer remaining in the needle biopsy tissue to perform MVD analysis, as well as matched totally embedded RP specimens.

Serum PSA concentration was determined before RP and analyzed as a continuous and categorical variable with the cut-off value 10 ng/mL and 20 ng/mL.

Immunohistochemistry

Tumors from 61 patients – 199 core biopsies with sufficient tumor areas, as well as sections from 61 selected tissue blocks of corresponding RP were analyzed by immunohistochemistry. Routine formalin-fixed, paraffin-embedded 3–4-µm-thick sections from each patient were attached to silanized slides, sequentially deparaffinized and rehydrated. Access to tissue antigen sites for antibody attachment was enhanced by microwaving slides which were treated by citrate buffer for 20 minutes. Detection of microvessels was performed using a monoclonal antibody against the CD31 antigen (clone JC/70A; Dako, Glostrup, Denmark). Dilution of the primary antibody was 1 : 40 in Tris buffered saline (TBS) / 1% BSA / 1% human serum and were incubated for 30 minutes. The EnVision technique and development with the chromogen 3,3´-diaminobenzidine tetrachloride (DAB) was used for visualization. Sections were lightly counterstained with hematoxilyn. Intense cytoplasmic immunoreactivity was observed in endothelial cells of small, medium-sized and large blood vessels in all study specimens. Normal prostate biopsy tissue served as a negative control after deletion of the primary antibody step and substitution of buffer during each run to suppress microvessel staining.

Determination of microvessel density

MVD was determined by light microscopy analysis for the areas of the tumor containing the most capillaries and small venules (microvessels, neovascular "hot spots") using the counting method introduced by Weidner and modified by Rogatsch [13, 16–18]. Prostate cancers are multifocal and heterogeneous in their MVD. The tumor area in CB and RP specimens containing the maximum number of descrete (brown) microvessels staining for CD31 was identified by scanning at low power (×40

and ×100) [13, 16, 17]. These areas were most frequent at the margins of carcinoma. After identification of the three areas of highest neovascularization, individual microvessels were counted at ×400 magnification, where one field is equivalent to 0.19 mm² representative ×400 high power fields (Olympus BH-2 microscope, Olympus Optical Co. Ltd., Japan) [18]. Both isolated immunoreactive brown-staining endothelial cells and endothelial cell clusters, separate from adjacent microvessels clearly, tumor cells and connective-tissue elements, were considered countable vessels. Vessel lumens do not need to be considered as a microvessel and red blood cells were not used to define a vessel lumen. Exclusion of occasional immunoreactive macrophages and plasma cells was based on their morphological appearance [13, 16, 17]. The highest readings in CB and corresponding prostatectomy were expressed as the highest number of microvessels identified within any single ×400 field. An average of multiple fields was not used. Assessment of MVD was done without knowledge of any clinicopathological data. MVD within normal prostate tissue and hyperplastic nodules served as internal control.

Statistics

The correlation of MVD (in a categorical and continuous fashion) in biopsies and corresponding prostatectomies was calculated using the MANOVA. Microvascular counts in organ confined (pT2) *versus* organ-extending tumors (pT3) were compared with MANOVA and $\chi 2$ test. To determine the relationship between tumor grade, final pathological stage and MVD in biopsies and prostatectomies, the median value, i.e. 48 of microvessel counts of 61 tumors was set as the cut-off point. The $\chi 2$ test, MANOVA, discriminative analysis, Pearson coefficient (χ) multiple correlation coefficient (R) were applied to identify the associations between MVD counts of 48 and less of 48 and more than 48 and final pathologic results, using a significance level of 0.05, and level of 0.001 for a very high statistical significance.

Results

Clinical findings

The mean age was 66 years (SD ± 5.28; range from 52 to 78) at the time of surgery. The pretreatment serum PSA ranged from 2.8 to 73.3 ng/mL (mean 14.73 ± 12.75 ng/mL) (Table 1). PSA (continuous variable) was examined for the association with pT (MANOVA: $p = 0.817$, that is χ^2 test: $p = 0.602$) and GS (MANOVA: $p = 0.901$, that is $\chi 2$ test: $p = 0.949$) both as a continuous variable and in a categorical fashion (< 10 ng/mL *vs* 10–20 ng/mL *vs* > 20 ng/mL). No statistically significant association was seen (Tables 2 and 3).

Table 1

Clinical and pathological parameters in prostate carcinoma in 61 patients with prostate cancer

Variables	Patients (n = 61)	
	n	%
Tumor stage		
T2	37	60.66
T3	24	39.34
Metastasis in regional lymph nodes		
N0	55	90.16
N1	6	9.84
Gleason score (GS)		
< 7	21	34.43
≥ 7	40	65.57

Table 2

Tumor stage (pT) and clinical and pathological results in prostate carcinoma

Variables	pT2 (n = 37)	pT3 (n = 24)	Significance
Age at surgery (years), mean ± SD, (range)	66.03 ± 5.84 (52–75)	66.92 (SD ± 4.35) (54–78)	$p = 0.525$ MANOVA; $p > 0.1$
Preoperative sPSA, (ng/mL) mean ± SD, (range)	16.13 ± 13.98 (2.8–73.3)	15.27 (SD ± 14.49) (6.1–70.0)	$p = 0.817$ MANOVA; $p > 0.1$
< 10, n (%)	12 (32.4)	10 (41.7)	$p = 0.602$ χ^2–test; $p > 0.1$
10–20, n (%)	17 (45.9)	11 (45.8)	
> 20 n, (%)	8 (21.6)	3 (12.5)	
Gleason score (GS), n (%)			
< 7	20 (54.1)	17 (45.9)	$p = 0.000$ χ^2–test; $p < 0.001$
≥ 7	1 (4.2)	23 (95.8)	
Metastasis in regional lymph nodes pN, n (%)			
N0	37 (60.65)	18 (29.50)	$p = 0.001$ χ^2– test; $p = 0.001$
N1	0	6 (9.84)	

PSA – prostate specific antigen.

Table 3

Gleason score (GS) and clinical and pathological results in prostate carcinoma

Variables	Gleason score		Significance
	< 7	≥ 7	
Age at surgery (years), mean ± SD (range)	66.19 ± 6.10) (52–75)	66.47 ± 4.88 (54–78)	$p = 0.844$ MANOVA; $p > 0.1$
Preoperative sPSA (ng/mL) mean ± SD (range)	15.48 ± 11.11 (4.1–47.8)	15.95 ± 15.52 (2.8–73.3)	$p = 0.901$ MANOVA; $p > 0.1$
< 10, n (%)	7 (31.8)	15 (68.2)	$p = 0.949$ χ^2– test; $p > 0.1$
10–20, n (%)	10 (35.7)	18 (64.3)	
> 20, n (%)	4 (36.4)	7 (63.6)	
Tumor stage (pT), n (%)			
T2	20 (54.1)	17 (45.9)	$p = 0.000$ χ^2– test; $p < 0.001$
T3	1 (4.2)	23 (95.8)	
Metastasis in regional lymph nodes (pN), n (%)			
N0	21 (38.2)	34 (61.8)	$p = 0.062$ χ^2– test; $p < 0.1$
N1	0	6 (100)	

Pathological results

Final pathological staging in RP of 61 tumors fulfilling the selection criteria recorded 37 (61%) as pT2, and 24 (39%) as pT3 (organ-extended) (Table 1). The 24 pT3 cases demonstrated EPE in all the cases of which seminal vesicles invasion in 19 (31%). These scores correlated significantly with tumor stage when analyzed by the χ^2 test ($p < 0.0001$ for biopsies and prostatectomies, respectively) as shown in Table 2. Regional lymph node metastases were present in 6 (10%) cases, all of them were pT3 and GS ≥ 7 (GS 7–2 cases, GS 8–3 patients and GS 9–1 case). A statistically significant correlation was found between metatases in regional lymph nodes on one side and pT ($p = 0.001$; χ^2 test), and GS ($p = 0.062$ χ^2 test) (Tables 2 and 3) on the other side. The median Gleason score for all tumors was 6 (range 4–8) in core biopsies and 7 (range 4–9) in prostatectomies. There was a significant discordance between biopsy and matched prostatectomy grades. Needle core biopsy underestimated tumor grade in 39% of cases (15 cases: GS6→GS7; 4 cases: GS7→GS8; 2 patients: GS6→GS8; 2 cases: GS5→GS6; and 1 patient in GS5→GS7, GS4→GS6, GS 7→GS 9) and overestimated in 1% (1 case: GS 8→GS 7). pT2 tumors scored 6 (range 4–8) in biopsies and 7 (range 4–9) in RP; the median score in carcinomas staged as pT3 was 7 (range 4–8) in core biopsies and in RP (range 4–9). These scores correlated significantly with the tumor stage when analyzed by the χ^2 test ($p < 0.0001$ for biopsies and prostatectomies, respectively) as shown in Tables 2 and 3. The median number of biopsies *per* case involved by cancer was 3 (range 1–5). Immunostaining for CD31 exhibited intense and homogeneous staining of the endothelial cells of blood microvessels on all the 61 examined cases, as evidenced by positive staining of non-tumor-associated vessels (Figures 1 and 2). It did not react with lymphatic endothelium or fibroblasts. Immunoreactivity was also observed in a small number of macrophages and plasma cells. MVD ranged from 22 to 89 (mean 49.84 ± 13.36) in core biopsies and 46.85 ± 14.47 in prostatectomies). When comparing these values, no statistical significance was found (MANOVA: $p = 0.239$). MVD (continuous variable) was examined for the association with pT both as a continuous variable and in a categorical fashion (pT2 *vs* pT3).

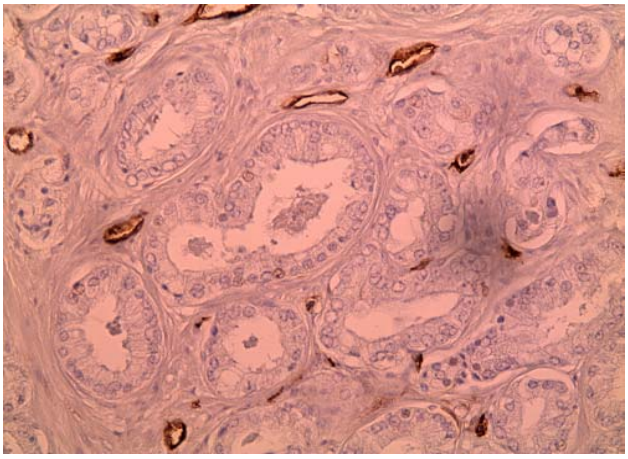

Fig. 1 – Prostate carcinoma showing low vascularization, Gleason score 6 (CD31; × 200).

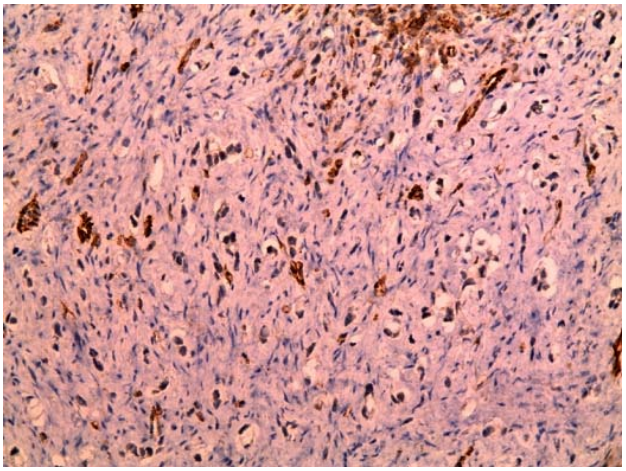

Fig. 2 – Prostate carcinoma showing high vascularization, Gleason score 9 (CD31; × 200).

A very high statistical significance was evident when the microvessel density in prostatectomies and the final pathological stage were compared (MANOVA: $p = 0.001$) (Table 4, Figure 3).

Table 4

Microvessel density (MVD) and clinical and pathological results in prostate carcinoma

Parameters	Patients (n)	MVD ± SD	MVD ≤ 48 (n = 24)	MVD > 48 (n = 37)	*p* value
Age at surgery (years), mean ± SD (range)			66.96 ± 5.34 (52.0–74.0)	66.00 ± 5.29 (54.0–78.0)	$p = 0.493$ MANOVA; $p > 0.1$
Preoperative sPSA (ng/mL), mean ± SD (range)			18.55 ± 15.59 (3.4–73.3)	14.00 ± 12.89 (2.8–70.0)	$p = 0.220$ MANOVA. $p > 0.1$
< 10	22	53.64 ± 12.51 (30–78) (NB) 47.14 ± 14.82 (30–81) (RP)	6 (25.0%)	16 (43.2%)	
10–20	28	48.29 ± 13.52 (25–72) (NB) 45.71 ± 13.28 (28–89) (RP)	12 (50.0%)	16 (43.2%)	$p = 0.279$ χ^2-test; $p > 0.1$
> 20	11	46.18 ± 13.97 (22–72) (NB) 49.18 ± 17.59 (29–88) (RP)	6 (25.0%)	5 (13.5%)	
p-value (MANOVA)		$p = 0.393$; $p > 0.1$			
Gleason score. GS mean ± SD (range)					
< 7	21	46.11 ± 13.51 (22–73) (NB) 38.79 ± 9.54 (28–60) (RP)	12 (50.0%)	9 (24.3%)	$p = 0.039$ χ^2-test; $p < 0.05$
≥ 7	40	53.00 ± 12.57 (27–78) (NB) 53.70 ± 14.51 (29–89) (RP)	12 (50.0%)	28 (75.7%)	
p-value (MANOVA)		$p = 0.044$; $p < 0.1$			
Tumor stage. pT mean ± SD (range)					
T2	37	46.24 ± 13.10 (22–73) (NB) 42.24 ± 11.64 (28–70) (RP)	19 (79.2%)	18 (48.6%)	$p = 0.017$ χ^2-test; $p < 0.05$
T3	24	55.38 ± 11.99 (27–78)(NB) 53.96 ± 15.73 (32–89) (RP)	5 (20.8%)	19 (51.4%)	
p value (MANOVA)		$p = 0.001$; $p = 0.001$			
Metastasis in regional lymph nodes. (pN) mean ± SD (range)					
N0	55	48.85 ± 13.63 (22–78) (NB) 45.60 ± 13.79 (28–89) (RP)	24 (100%)	31 (83.8%)	$p = 0.038$ χ^2-test; $p < 0.05$
N1	6	58.83 ± 5.19 (50–65) (NB) 58.33 ± 16.83 (39–88) (RP)	0 (0%)	6 (16.2%)	
p-value (MANOVA)		$p = 0.071$; $p = 0.082$ (NB). $p = 0.040$ (RP); $p < 0.1$			

NB – needle biopsy; RP – radical prostatectomy.

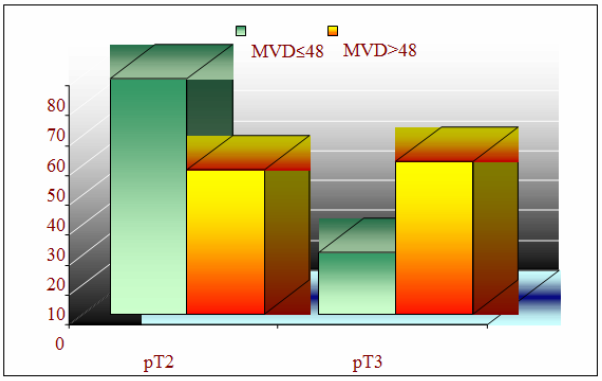

Fig. 3 – Correlation between pathological stages (pT2 *vs* pT3) and microvessel density (MVD).

This difference in MVD between pT2 and pT3 tumors was confirmed by univariate analysis when the mean microvessel count of all tumors, *ie*, 48, was set as a cut-off value (χ^2: $p = 0.017$) (Figure 3, Table 4). A statistically significant correlation was found between metatases in regional lymph nodes (pN0 *vs* pN1) and MVD (MANOVA; $p = 0.071$ for continous and χ^2: $p = 0.038$ for categorical variable) (Table 4). MVD was examined for association with GS both as a continuous variable and in a categorical fashion (≤ 48 *vs* > 48). Although a statistically significant discrimination between low- and high-grade tumors was found (MANOVA: $p = 0.044$ for continuous and χ^2 test: $p = 0.039$ for categorical variable), it did not reach the level of final pathological stage. An increase in MVD was not associated with pretreatment PSA as a continous variable (MANOVA: $p = 0.393$) or as a categorical variable (χ^2 test : $p = 0.279$) (Table 4). All those variables previously analyzed (clinical and pathological characteristics) were statistically significantly associated with final pathological stage (pT). Additionally, univariate analyses (Pearson test, multiple correlation coefficient) and multivariate analyses (MANOVA; discriminative analysis) were done in order to determine the most significant factors in prediction organ-confined (pT2) *versus* organ-extending prostate cancer (pT3). The Gleason score ($p < 0.001$) and metastases in regional lymph nodes ($p = 0.001$) showed a strong statistical significance. MVD also showed statistical significance ($p = 0.017$), but not so strong as previously mentioned (Table 5). Preoperative serum PSA alone was not significant in predicting final pathological stage ($p = 0.322$) (Table 6).

Table 5

Microvessel density (MVD) studies: correlation with pathological results

Study	Specimen	IHC/Method	Correlation/Yes	Correlation/ No
Weidner et al. [35]	74 RP	F 8/Weidner	GS, pN1	
Brawer et al. [24]	32 RP and 5 TURP	F 8/computer	pT	
Silberman et al. [41]	109 RP (GS5-7)	CD31/Weidner modified x 400		GS, EPE
Rubin et al. [25]	87 RP	CD31/Weidner		GS, pT
Rogatsch et al. [18]	36 NB and RP	CD31/Weidner modified × 400	pT	
Offersen et al. [36]	64 TURP	CD31, F8/ -	Survival	
Borre et al. [38]	221 NB TURP	F8/ -	GS, clinical stage	
Bostwick et al. [21]	186 NB and RP	F8/computer	pT	
Di Lorenzo et al. [4]	72 RP	CD31/Weidner	pT, GS, sPSA	
Tretiakova et al. [48]	169 RP	CD31/computer		GS
Taverna et al. [49]	27 RP	CD34/computer	sPSA	pT, GS
Erbersdobler et al. [43]	3261 RP	CD31/Weidner	pT, GS	sPSA
van Niekerk et al. [47]	28 RP	CD31/computer		GS
Steiner et al. [15]	69 RP	CD31,CD34 /Weidner × 400	pT, GS	
Jiang et al. [9]	73 RP	CD31/Weidner × 400	GS	sPSA

IHC – immnunohistochemistry; RP – radical prostatectomy; TURP – transurethral *resectio prostatae*; NB – neobladder; SPSA – serum prostate-specific antigen; GS – Gleason score; EPE – extraprostatic extension.

Table 6

Correlation between pathological stages (pT2 vs pT3) and clinical and pathological variables and microvessel density (MVD)

Variables	χ	R	F	*p*	c.dsc
Preoperative sPSA (<10 *vs* 10–20 *vs* > 20 ng/mL)	0.128	0.129	0.999	0.322	0.027
MVD (≤ 48 *vs* > 48)	0.292	0.305	6.059	0.017	0.012
Gleason score (< 7 *vs* ≥ 7)	0.456	0.513	21.065	0.000	0.237
pN (pN0 *vs* pN1)	0.379	0.410	11.929	0.001	0.183

Pearson's contigency coefficient (χ); multiple correlation coefficient (R); discrimination coefficient (c.dsc).

Discussion

If prostate cancer is diagnosed on time, whether primary or recurrent, it may be curatively treated. The process of the very diagnostics, screening and staging of the disease is controversial, due to limitation in its disclosure [14]. As a result of widespread testing of patients for sPSA over the past decade, most patients with prostate cancer now present with the clinically localized disease, and their tumors are rarely graded with Gleason scores < 6 [19]. In general, serum PSA levels correlate with a larger tumor volume, advanced pathologic stage and higher grade [20–22]. Although higher grade cancer produces less PSA per cell, when compared to lower grade tumors, overall, poorly differentiated tumors are associated with higher PSA levels as these tumors tend to be larger and of a more advanced stage [23]. There are exceptions with very high grade prostate cancers which are so poorly differentiated that associated serum PSA levels are disproportionally low [20]. In our study, a statistically significant correlation was not found between serum PSA levels (as continuous and categorical variables) and stage of disease (pT2 *vs* pT3), and also between sPSA and tumor grade (Gleason score) (<7 *vs* ≥ 7). Preoperative serum PSA in our investigation, as in some other studies, showed that it could not give useful pathologic correlations on individual basis, for each patient [5, 9, 22, 24–26]. A significant overlap of sPSA values between different tumor stages (pT2 *vs* pT3) did not enable clear distinction between these values (regarding organ-confined prostate carcinoma *versus* organ-extending carcinoma) in relation to locally limited PC, namely locally invasive PC.

Clinical doctors classify patients with newly diagnosed PC by stage and grade. This classification is important because of the extraordinary variability in the potential for disease progression. Tumor grade, stage, and the presence of competing medical hazards are the most powerful predictors of survival [19]. There is a significant discordance between biopsy and matched prostatectomy grades. Needle core biopsy underestimates tumor grade in 33–45% of cases and overestimates in 4–32% [27–29]. Grading errors are common in biopsies with small amounts of tumor and low-grade tumor [30]. In our study, Gleason score values before and after the surgery differed in 40.98% of the patients. Needle core biopsy underestimated tumor grade in 39.34% of the cases, with the greatest discordance in distinguishing GS 6 from GS 7. Needle core biopsy overestimated tumor grade in one patient 81.63% (GS 8 *versus* GS 7 on matched prostatectomy). Judging by univariate and multivariate studies, tumor grade is one of strongest and most useful prognostic parameters which forecast tumor stage [20, 27, 31]. This possibility of forecasting can be applied to almost every determination of pathologic tumor stage, including EPE (extraprostatic extension), SVI (seminal vesicle invasion), regional node metastasis, and bone metastasis. In research, patients are usually grouped according to the Gleason score as low risk (GS < 7), medium-risk (GS 7) and high-risk groups (GS 8–10). In some studies, patients with the Gleason score 7 have the same disease outcome and behavior of PC as the ones with GS 8–10, so some researchers put them in the same group [18]. According to some researchers, GS ≥ 8 determined on needle core biopsy as a strong prognostic factor which indicates the possibility of the existence of regional lymph node metastasis [32]. Both sPSA and GS can provide significant prognostic data when their values are at either high (sPSA < 20 ng/mL, GS 8–10), or low (sPSA < 4 ng/mL, GS 2–4) level. However, the majority of patients are exactly in the middle, namely with GS 7 and intermediary level sPSA [22]. Our study shows a statistically high correlation between tumor stage and grade, namely 95.8% of patients with GS ≥ 7 had locally invasive PC (pT3). Also, all the patients with regional lymph nodes metastases (pN1) had poorly differentiated PC (GS ≥ 7). A need for correct preoperative tumor stage determination is essential, especially after studies on massive tumor series clinically diagnosed as organ-confined prostate cancer (T2), out of which approximately 24–60% after RP had pathologic confirmation of locally invasive and metastatically spread disease (pT3, pN1) (1.3–8). In 8–45% patients PC were preoperatively clinically over-staged [9]. In our research, after radical prostatectomy, 61% PC were in tumor stage pT2, and 39% were in a stage pT3, while as 10% patients had metastatically spread disease.

Since 1971, when Folkman determined that tumor growth and dissemination depended on angiogenesis and also that tumors, along with inflammatory cells and related vasculature, created a complex ecosystem which communicates through chemical signals, many studies have been made in order to support this theory [33]. MVD varies widely depending on tumor type; all tumors, including the ones with smallest MVD, depend on angiogenesis [34].

Reference publications contain numerous conflicting studies which relate to the possibility of angiogenesis to predict pathological stadium for patients with clinically organ-confined prostate carcinoma. Many studies proved the connection between MVD and GS, disease stage, as well as possibilities of metastatic spread of carcinoma in future, while as in other studies MVD in relation to stage pT has not shown superior predictive value (Table 6). Still, most researchers estimate that MVD increased values have a prognostic significance in estimation of biological behavior of PC. In a Weidner et al. [35] research, patients with metastasis had double higher values of MVD in relation to locally invasive PC, and higher values of MVD were related to higher Gleason score, but only in poorly differentiated PC. Offersen et al. [36] points to the fact that the maximum value, and not the median one MVD, is significantly associated with survival estimation for patients with CP. In a Bostwick-lead multi-institutional study, logOMVD (optimized microvessel density) was statistically significantly correlated with GS and pre-operative sPSA values, as well as with pT3. However, disease outcome forecasted by OMVD did not relate to patients who had organ-confined disease (pT2) and GS 6–9 [21]. In our research, higher MVD continual and categorical variables were correlated with poorly differentiated prostate carcinoma, namely GS ≥ 7, with higher tumor stage (pT3), namely they were higher in metastatic spread disease (pN1). Such results have been confirmed in studies of many au-

thors [4, 6, 18, 37–41]. Our study does not show a statistically significant correlation with pre-operative values of sPSA, which is in line with certain findings listed in the literature [5, 9, 25, 42]. In a tissue microarray study (TMA), used on the largest number of samples so far (3261 RP), Erbersdobler et al. [43] prove using univariate analyses, a significant correlation between an increased MVD and advanced stadium of PC, pT3 ($p < 0.001$), as well as a higher GS, (GS $\geq$ 7) ($p < 0.001$), but also points out to the existence of significant differences between tumors, taking into account their localization, that is, that the transitional zone tumors (TZ) have a lower MVD, compared to the peripheral zone (PZ) tumors. However, MVD has not been proved to be an independent prognostic parameter in multivariant analyses, instead it is closely connected to the other factors contributing to tumor aggression. The authors point out that if the antiangiogenic therapy for prostate cancers has not been established yet and if it starts being applied, knowing the differences in MVD between individual tumors and tumor locations (TZ *versus* PZ tumors) would become significant [43]. Steiner et al. [15] has established a mild correlation between mRNA of individual endothelial factors (CD31, CD 34) in prostate cancer tissue compared to the histologically determined MVD, even though higher values of histologically determined MVD were statistically significantly related to higher GS and stadium, pT3 ($p < 0.001$).

Contrary to the previously reported research results, certain authors have reached completely opposite conclusions in their studies [41, 44]. Silberman et al. [41] determined a correlation between MVD and tumor progression after RP, but not with pathologic stage in patients who had GS 5–7. A correlation between MVD and disease stage, as well as metastasis, was not proved in the work of Matsushima et al. [45], while a correlation between MVD and Gleason grade was statistically almost significant. Rubin et al. [25] also did not find a correlation between MVD and GS, tumor stage, positive surgical margins or seminal vesicle invasion, but also not with increased postoperative sPSA values as a sign of disease recurrence. By using multivariate analysis (using estimation p53, retinoblastoma, chromogranin A and MVD), Krupski et al. [46] has determined that MVD values showed no prognostic significance of importance in comparison to p53 and retinoblastoma in estimation of patient survival. Gettman et al. [5] found no correlation between OMVD and DNA ploidy, Gleason grade, pathologic stage, or with sPSA (preoperative serum PSA), neither the application of univariate and multivariate analysis proved OMVD as a predictor of clinical or biochemical disease recurrence. By using the image analysis system for determination MVD, van Niekerk et al. [47] determined no consistent increase of MVD in TZ tumors in terms of the surrounding unchanged tissue of prostate and benign tissue hyperplasia, unlike PZ prostate cancer, which had almost double increase of MVD value, explaining this with intrinsic biological differences between these two zonal types of tumors (such as heterogeneous of microvasculature of TZ tumor), which, at least partly, condition their different biological behavior. In this study, no correlation was found between MVD and Gleason score of TZ and PZ tumor. Unlike van Niekerk et al, Tretiakova et al. [48] in their research using computer analysis of MVD conclude that MVD is not statistically significantly increased in PC compared to the normal surrounding tissue of the prostate, as well as neither in low grade PC (GS $\leq$ 3 + 4) compared to high grade PC (GS $\geq$ 4 + 3), as well as that MVD cannot be considered a useful prognostic parameter. Taverna et al. [49, 50], examining two-dimensional geometrical complexity of vasculature of PC, divided the patients in two groups, taking into account increase/decrease of fractal dimension of tumorous vascular surface and surrounding non-tumorous tissue, establishing that the patients with a lower tumorous vascular surface had a worse clinical outcome, that is, that the tumor progression was not dependant on angiogenesis. At the end, Taverna et al. [51] leave an open question as to whether angiogenesis is a "canonic hallmark" of PC and point out that there are no powerful methods of quantifying the reversal of neovascularity.

The majority of these studies, using different antibodies, methods of counting and selection, show some significant correlations between MVD and poorly differentiated PC and shorter patient survival, suggesting that MVD is a strong measurer of tumor angiogenic activity. The controversy of results is a consequence of practical problems which limit the usage of MVD measuring on surgical material, namely there is no consensus neither on vessels counting nor on "cut-off"" value which would differentiate/separate high- and low-grade neoplasms. Different methodologic problems occur while counting blood vessels, such are different observations by different pathologists, even the same pathologist, during the first count/selection of areas with most intensive neovascularisation ('hot-spot'), as well as heterogeneity of tumor, which remain unresolved and can therefore influence on results of immunohistochemistry analysis. The number of published results on MVD up to now is 14–300, and along with that 'cut-off', which varies between 23–160 [35, 39, 43, 52, 53]. In our research the mean value of MVD on samples of needle core biopsies was 49.84 ± 13.36 (22–78), and on samples of matched RP 46.85 ± 14.47 (28–89), and after their comparison there was no statistically significant difference, while as 'cut–off' value was 48. If one neglects the variations in patient selection (for example, Tretiakova et al. [48] divide 67% of patients with GS 7 PC to low grade PC group ($\leq$ 3 + 4) and high grade PC group ($\geq$ 4 + 3), one finds that those different values are mostly conditioned by different techniques of tumor blood vessels counting. These differences can be a consequence of endothelial antibodies choice, selection of vascular parameters, choice of tumor field in which measurement is done, vessel counting method, determination of 'cut-off' values which is used in correlation analysis along with other clinic and pathologic variables and survival, as well as wrong statistical methods which are used. Selection of 'cut-off' values was based on personally estimated median value of MVD, bellow which the prognosis was good, and above it bad, and therefore it had to be seen arbitrary until valid values were identified. Such differences can depend on the fact whether MVD is estimated on periphery, or in the center of tumor [39]. As it was shown that there was a strong correlation in MVD values gained by using different antibod-

ies, where CD-31, which we used in our study, was shown to be more sensitive, showing 18–33% higher results of microvessel counting, MVD, than some other antibodies which are usually used (for example, CD34 is detectable, except in endothelial cells, in mesenchymal and inflammatory cells and lymphatic vessels), the biggest discrepancies related to other of the listed reasons [18, 45]. Studies that could not confirm the prognostic significance of MVD were the ones which mostly differed from the methodology described by Weidner et al. [16, 17, 35]. Each of the previously mentioned authors used numerous modifications of this method (e.g., determination of the so-called 'hot spots', namely areas with the highest number of blood vessels, how many fields are counted and where, whether focuses overlap). This study also used a modification of the Weidner [13] method of microvessel counting on needle biopsy and matched radical prostatectomy specimens on a high-power microscopic field, ×400. This method selection in our research has a foundation in certain studies on different tumors with good correlations between results of blood vessels calculation at ×400 magnification in relation to ×200 magnification [16]. The approach chosen in this investigation has also beensupported by several studies performed on other human tumor types showing good correlations between vascular counts performed in ×400 *versus* ×200 magnifications [13, 18]. MVD values in our study are higher in relation to previous studies [35, 53]. A possible explanation of such results is, as Rogatsch et al. [18] stressed, that higher resolution ×400 results in MVD value increase by 11–33% when it is compared to ×200 magnification as shown breast carcinoma [16, 17]. Application of image analysis system in histologically determined MVD, as was suggested by a few groups of researchers is more expensive, more demanding, unsuitable for routine application, and is not more accurate in comparison to calculation done by other researchers in person [5, 6, 21, 25, 47–49]. Precisely, these differences in the manner of determination of MVD in many studies, disable their adequate interpretation and comparison, thus a consensus-agreed methodology for determination of MVD could be used to provide more proper comparison and interpretation of MVD values when compared to clinical and pathological parameters.

Having in mind the values of GS and MVD, non-invasive imaging technique that can reflect both GS and MVD to be able to provide timely diagnostics and determination of PC characteristics [14]. Histological heterogeneity and multifocality of PC limit use of needle biopsy in determination of all carcinomas grades and sites [9]. What would be valuable for choosing targets for prostate biopsies would be an imaging method, which could indicate increasing in MVD and it could also provide a foreseeable Gleason score. This should result in a change of biopsy strategy, the outcome of which would be a higher detection rate of prostate cancer and a more accurate Gleason grading, meaning, a more adequate therapeutic strategy [9]. In line with this, the most used conventional imaging methods today are ultrasonography with moleculary targeted constrast microbubbles (CEUS) and magnetic resonance imaging (MRI), amended with moleculary, metabolic and functional imaging techniques [14]. Some researchers correlate the results obtained by imaging methods with histologically determined MVD. Lee et al. [54] evaluated tumorous angiogenesis using the mouse xenograft model injected with human PC-3 prostate cancer cells, using contrast-enhanced sonography, establishing a statistically significant correlation of the US maximum intensity and CD31-positive microvesel count. Jiang et al. [9] established on samples of needle prostate biopsies, that the peak intensity of prostate cancer at CEUS was statistically significantly increased with a higher GS and histologically determined MVD. Osimani et al. [55] showed that in PC blood volume and permeability surface area product measurements obtained with perfusion computed tomography had the highest correlation with immunohistochemical markers of angiogenesis, MVD [55]. Unlike them, Franiel et al. [56], using MRI perfusion and blood volume hotspots with histological MVD, determined no significant correlation, explaining this with heterogeneous vascularization of the normal and tumorous prostate tissues, as well as different thickness of MRI slices, that is, histological paraffin blocks, but also with technical limitations of MRI, suggesting that the computer-based 3D prostate model could be used in the future to provide a more accurate correlation of histological and MRI imaging findings. Even though the prognostic value of microvessel density in prostate cancer is contradictory and microvessel density is not recommended for routine application by the World Health Organization, it is still the subject of research, particularly in the samples of needle prostate biopsies, where its prognostic significance is still unclear [57].

Conclusion

Although the number of patients in this study was small, the obtained results indicate that quantification of microvascular density in biopsies is an accurate pre-operative predictor of tumor stage, discriminating between organ-confined and organ-extending neoplasms.

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Characteristics of norovirus infection in Serbia

Karakteristike norovirusne infekcije u Srbiji

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Abstract

Background/Aim. Norovirus (NoV), formerly Norwalk-like virus is the most common cause of acute gastroenteritis in humans of all ages. It is known that 90% of viral gastroenteritis and about 60–85% of all outbreaks of gastroenteritis, especially in the territory of United States of America, Europe and Japan are caused by this virus. For the countries of the northern hemisphere, individual cases and outbreaks of acute NoV gastroenteritis appear in seasonal pattern, mainly during the winter months. The aim of this study was to describe characteristics of acute gastroenteritis with the established NoV etiology in Serbia. **Methods.** The study group included 88 patients with the symptoms of acute gastroenteritis, throughout the year 2010 and 2011. From all the patients, stool samples were taken less than three days from the onset of symptoms. Detection of NoV in stool samples was performed by commercial qualitative immunochromatography assay. Statistical analysis included application of χ^2 test, Mann–Whitney *U*-test, Kruskal-Wallis's test, Spearman's rank correlation test and logistic regression analysis. **Results.** Outbreaks of acute gastroenteritis caused by NoV were recorded to be the most common in children with the incidence of infection of 50% in the age group 0–15 years. Analysis of individual symptoms in the NoV proven infection, showed that diarrhea was the most common symptom, followed by vomiting especially in small children, while abdominal pain was most common in elderly (≥ 65 years). The presence of frequent vomiting, more than 4 times/day, indicated NoV infection in the women, while for men the infection was always presented with diarrhea. **Conclusion.** The obtained results confirmed that small children and elderly are the most susceptible to NoV infection and that outbreaks are more frequent in the winter months. Those who consumed food in restaurants and other public facilities were not at higher risk for NoV infection.

Key words:
norovirus; gastroenteritis; incidence; risk assessment; disease outbreaks; serbia.

Apstrakt

Uvod/Cilj. Norovirus (NoV), raniji naziv virus koji je sličan Norwalk virusu, najčešći je uzročnik akutnog gastroenteritisa kod ljudi svih starosnih doba. Poznato je da je 90% virusnih gastroenteritisa i oko 60–85% svih epidemija gastroenteritisa uzrokovano ovim virusom, posebno na teritoriji Sjedinjenih Američkih Država, Evrope i Japana**.** Za zemlje severne hemisfere, norovirusne epidemije i pojedinačni slučajevi akutnog gastroenteritisa pokazuju sezonski obrazac pojavljivanja, uglavnom tokom zimskih meseci. Cilj ovog rada bio je da se opišu karakteristike akutnog gastroenteritisa sa utvrđenom norovirusnom etiologijom kod bolesnika u Srbiji. **Metode.** Studijskom grupom bilo je obuhvaćeno 88 bolesnika sa simptomima akutnog gastroenteritisa tokom 2010. i 2011. godine. Od svih bolesnika, uzimani su uzorci stolice najviše tri dana od pojave simptoma infekcije. Za otkrivanje NoV u uzorcima stolice korišćen je komercijalni kvalitativni imunohromatografski test. Za statističku obradu podataka korišćeni su χ^2 test, Mann–Whitney *U*-test, Kruskal-Wallis test, Spirmanov test korelacije ranga i metode logističke regresije **Rezultati.** Slučajevi akutnog gastroenteritisa uzrokovanog NoV najčešće su zabaleženi kod dece, sa incidencijom od 50% u grupi od 0–15 godina starosti. Analizom simptoma kod bolesnika sa dokazanom norovirusnom infekcijom zaključeno je da je dijareja bila najčešći simptom, zatim, po učestalosti, sledilo je povraćanje, posebno kod manje dece, dok je abdominalni bol bio karakterističan za ispitanike starije od 65 godina. Prisustvo učestalog povraćanja, preko četiri puta dnevno, ukazivalo je na NoV infekciju kod žena, dok je kod muškaraca infekcija bila uvek manifestovana dijarejom. **Zaključak.** Rezultati istraživanja su potvrdili da su manja deca i starije osobe najosetljivije grupe za NoV infekciju i da su epidemije češće u zimskim mesecima. Bolesnici koji su se hranili u restoranima ili ustanovama za javnu ishranu nisu imali veći rizik od razvoja NoV infekcije.

Ključne reči:
norovirus; gastroenteritis; incidenca; rizik, procena; epidemije; srbija.

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Introduction

Norovirus (NoV), formerly Norwalk-like virus is the most common cause of acute gastroenteritis in humans of all ages [1, 2]. It is known that 90% of viral gastroenteritis and about 60–85% of all outbreaks of gastroenteritis especially in the territory of the United States of America (USA), Europe and Japan is caused by this virus [3, 4]. For the countries of the northern hemisphere, individual cases and outbreaks of acute NoV gastroenteritis appear in a seasonal pattern, mainly during the winter months [4–6]. Outbreaks usually occur in closed collectives such as hotels, hospitals [7], homes for the elderly [8], kindergartens, schools and cruise ships [4].

Humans are the only reservoir of infection and common routes of transmission including fecal contamination of food and water, direct contact with an infected person, spreading the virus in the form of aerosol and contact with contaminated surfaces in the environment. High resistance to environmental factors, the low infectious dose (10–100 viral particles enough to develop symptoms of disease) and the lack of long-term immunity are important factors contributing to the rapid spread of NoV [1].

The disease occurs after an incubation period of 12–48 h accompanied by diarrhea, nausea, vomiting and abdominal cramps. In most cases the infection ends with complete recovery after 4–6 days. In 30% of cases, it passes as asymptomatic infection [9, 10], while in 10% of infected it is manifested as severe form of disease that requires hospitalization [11, 12]. Fatal outcome was recorded more frequently in the group of preterm born children, and in persons older than 65 years. On the United States territory, 90% of annuall death outcomes associated with NoV were recorded in patients older than 65 years [13].

The virus is shedded by feces or vomits where it can be detected during 4 weeks after infection, with a peak of shedding in 2–5 days following the onset of symptoms [9]. Most microbiology laboratories in industrialized countries use molecular techniques for routine and confirmatory detection of norovirus, but for the rapid screening of NoV infections, it is faster and cheaper to use immunochromatography tests for the detection of NoV antigen in stool samples. These tests enable identification of the virus with the specificity of 87.5% compared to polymirase chain reaction (PCR) [14]. In Serbia, NoV infection has not so far been considered as causative agent of acute gastroenteritis, so there are no previously published data on the prevalence of these infections in outbreaks or in sporadic cases of acute gastroenteritis. The Public Health Institute of Belgrade was the first to investigate NoV as a cause of acute gastroenteritis according to epidemiological indications. The aim of this study was to describe the characteristics of acute gastroenteritis with the established NoV etiology in Serbia.

Methods

The study group included 88 patients with the symptoms of acute gastroenteritis, throughout the year 2010 and 2011. A total of 88 stool samples was conducted in the Microbiology Laboratory of Public Health Institute of Belgrade. They were divided in subgroups defined on the basis of epidemiological data.

Testing for NoV infection was conducted according to indications set by The Epidemiological Control Department of Public Health Institute of Belgrade. The study included testing for NoV infection in: 15 sporadic cases (13 cases from the general city population during summer months, 2 from the gerontology center); and in 6 suspected outbreaks as follows – two in the nursery (14 cases), three in restaurants (31 cases) and one in a medical institution (28 cases).

Acute onset of diarrhea, vomiting, nausea and abdominal pain, were considered typical symptoms of acute gastroenteritis, while malaise, fever, chills, muscle pain, headache and dizziness, were considered nonspecific. The information about water and food supply during 48 h before the onset of acute gastroenteritis was also gathered.

The study included only samples that met the following criteria: stool samples taken less than three days from the onset of symptoms; stools with no visible traces of blood, without fixatives or preservatives; samples available in sufficient quantity volume of 2 mL or 2 g; samples transported and stored in accordance with the Good Laboratory Practice.

Detection of NoV in stool samples was performed by commercial qualitative immunochromatography assay RIDA®QUICK Norovirus (R-Biopharm, Darmstadt, Germany), that utilizes specific monoclonal antibodies to norovirus antigens GG I and GG II.

Statistical analysis

Data statistical analysis were performed using the SPSS software package 20.0 (IBM SPSS Statistic for Windows, Amonk, NY, USA). Individual characteristics between the groups were compared using Mann Whitney and Kruskal-Wallis's test for continuous variables and χ^2 test for categorical data. The receiver operating characteristic (ROC) curve was used for determination of NoV markers. Spearman's rank correlation test was utilized to evaluate the relationship between variables. Logistic regression analysis was used for determination of odds ratio (OR) for diarrhea in men and risk estimation parameters for NoV infection in childhood. Statistical significance was defined by the value of $p \leq 0.05$.

Results

NoV antigen was detected in stool samples of 37 (42%) out of 88 patients suffering from acute gastroenteritis. The majority of NoV positive cases was discovered in sporadic cases of gastroenteritis (73.3%, n = 11/15) and among the patients in healthcare facilities (42.9%, n = 12/28). The incidence in outbreaks of NoV infection was 35.6%.

In the group of patients with proven NoV infection, 44 (50%) were aged 0–15 years, approximately 1/3 [28 (31.8%)] were aged ≥ 65 years, while those aged 15–64 years (16 patients) accounted for only 18.2%.

Among the patients with proven NoV infection, the recorded symptoms were: diarrhea (35), followed by vomiting

(29), nausea (19), abdominal pain (19), malaise (19), fever (11), chills/shivering (4), dizzines (1). None of the patiens with the proven NoV infection had headache or muscle pain. The frequency (%) of specific and nonspecific symptoms of NoV infection was shown in Figure 1.

Analysis of individual symptoms showed that diarrhea was the only symptom that was significantly more present in NoV proven infection compared to acute gastroenteritis without proven NoV infection (94.6%, n = 35/37 *vs* 78.4%, n = 40/51, respectively; $p = 0.035$). All the children aged 0–7 with the proven NoV infection had vomiting (100%, n = 13/13), while vomiting was present in only 50% of children with non-NoV acute gastroenteritis [χ^2 (19.1) = 7.72, p = 0.021; OD 5.33, CI 95% 1.92–14.7].

In the group of patients aged ≥ 65 years, abdominal pain was significantly more frequent in proven NoV infection (84.6%, n = 11/13) than in patients with no NoV present (40%, n = 6/15), [χ^2 (28.1) = 5.81, p = 0.020; OR 8.25, CI 95% (1.33–51.26)]. The most severe clinical manifestations with ≥ 10 stools/24 h and /or ≥ 10 vomits/24 h and t ≥ 39 ºC were encountered in 28 patients (15 NoV positive and 13 NoV negative), and the majority (57.1% , n = 16/28) of them were children aged 0–15 years (NoV positive 56.3%, n = 9/16, NoV negative 43.7%, n = 7/16).

There was no statistically significant difference in the incidence of proven NoV infection by gender (women 33.3%, n = 12/36 *vs* men 48.1%, n = 25/52; χ^2 (88.1) = 1.9, p = 0.168). In the group of NoV-positive patients aged ≥ 65 years, men were more present (84.6%, n = 11/13) than women. The women were more often infected in the age group 16–65 years (66.7%, n = 4/6). In childhood NoV infection was equally present in both sexes.

The women with proven NoV infection were shown to have more severe vomiting. In fact, more than 4 vomiting *per* day in women was significantly correlated with the presence of NoV antigens (sensitivity 75% and specificity of 81.2%, p = 0.030, area 0.777).

The men with NoV infection suffered from diarrhea in all the cases (100%, n = 25/25), while the men with NoV negative acute gastroenteritis had this symptom in 77.8% (n = 21/27), [χ^2 (52.1) = 6.28, p = 0.012]. The number of unformed stools in 24 h was not significantly more present in the patients with NoV infection than in those without it.

NoV infection was more often detected in winter than summer (Figure 2).

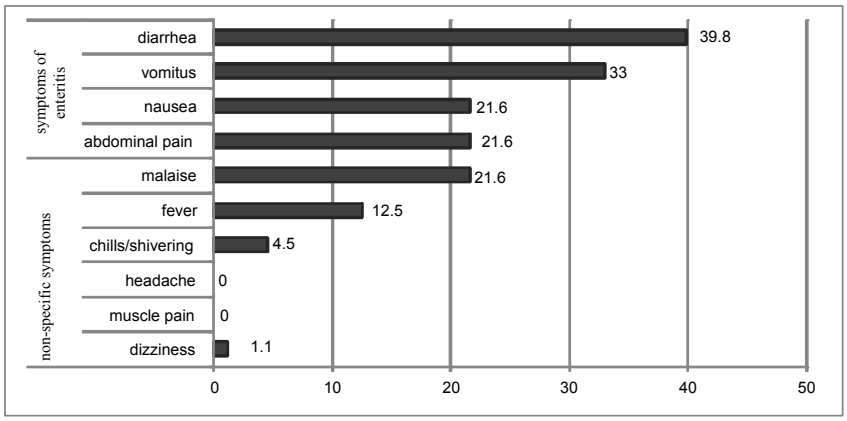

Fig. 1 – The frequency of specific and nonspecific symptoms of acute gastroenteritis in the norvirus positive patients

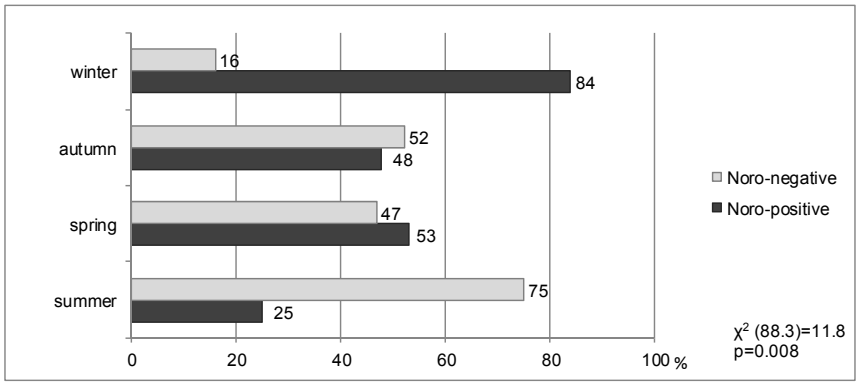

Fig. 2 – The percent of patients diseased in different seasons.

Water and food in the last 48 h before symptoms

None of the patients used well water, bottled water was used by one, local water supply by 14 and urban water supply by 73 patients. The frequency of NoV infection among the patients who used local water supply was 50% (n = 7/14), urban water supply 38.7% (n = 29/73) and there was no statistically significant difference between these two groups [χ^2 (87.1) = 0.511, p = 0.475].

The analysis of food taken 48 h before the onset of symptoms revealed that the NoV positive result was most

frequently detected in the patients who ate at home and in kindergarten, while the restaurants were the least common places connected with the infection (Figure 3).

Totally 73.7% of the the children aged 0–7 years had meals in kindergarten 48 h before the onset of acute gastroenteritis 21.1% of the children ate only at home, while 5.3% ate in restaurants. Most patients aged ≥ 65 years ate in a medical institution (92.9% , n = 26/28), while only 7.1% at home (n = 2/28).

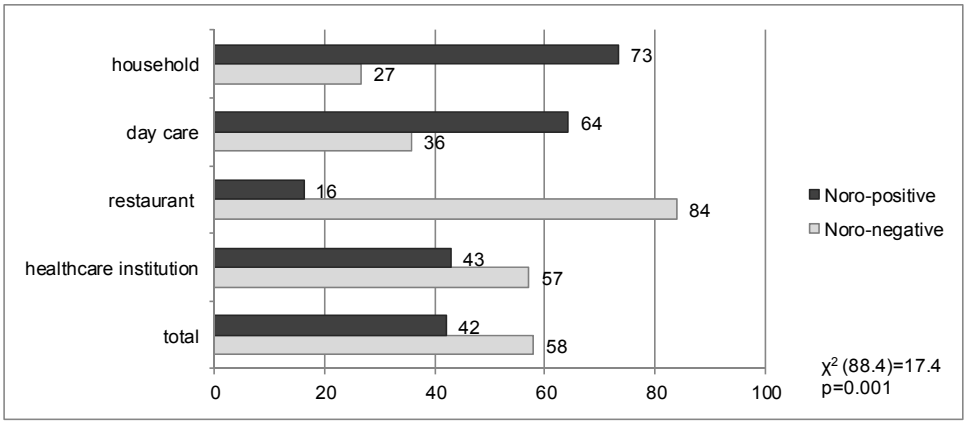

Fig. 3 – The percentage of patients regarding the places of having meal in 48 h before the onset of symptoms.

Discussion

NoV infect persons of all ages, often causing epidemic outbreaks of acute gastroenteritis as well as sporadic cases. The frequency of NoV infection in outbreaks and sporadic cases of acute gastroenteritis in the South Eastern Europe region ranges from 15% in Bulgaria [15] to 57% in Greece [4]. The results of this study show that overall incidence of NoV infection was 42%; in sporadic cases it was 75%, while in the outbreaks 35.6%.

Outbreaks of acute gastroenteritis caused by NoV were recorded to be the most common in children (two in kindergarten and two in school-aged children in the recreational classes). A high incidence of NoV infection in children aged 5–11 years was also shown in Spain [16]. Similarly, in Greece 57% of patients was NoV positive in the age less than 15 years [4]. This is in agreement with the results of this study where the incidence of infection was 50% in the age group 0–15 years. However, most of NoV-positive children were of even younger age (4–7 years) in outbreaks that have occurred in kindergartens. Sanitary and hygienic conditions in the kindergarten were good, but the characteristics of NoV (resistance in the environment, low infectious dose) probably contributed to the spread of infection by direct contact between the children. In a survey conducted by Bernard et al. [17], most cases of disease were observed in hospitals (32%), elderly care centers (28%), households (24%) and centers for the care of children (10%).

The other age group at risk for developing NoV infection is people older than ≥ 65 years [16]. The outbreak in the medical institution in this study was manifested by acute NoV gastroenteritis mostly in elderly people, mean age of 72 years, who were hospitalized because of exacerbation of their chronic disease. The highest percentage of NoV-positive patients in this outbreak was found in patients with chronic cardiovascular (83.3%, n = 5/6), and neurological diseases (62.5%, n = 5/8). It was pointed out by different authors [18–20] that viral contamination of the environment, the existence of asymptomatic cases (approximately 12% [7]) and prolonged excretion of the virus (up to 10 days) are the most common factors that contribute to infection of elderly patients in health institutions and elderly care centers.

In this study group there were 39.8% of NoV positive patients with diarrhea, 33% with vomiting, and only 12.5% patients with fever. These symptoms are consistent with previously reported clinical manifestations of NoV infection [16]. The clinical presentation of NoV infection varied in relation to age group. In children under the age of 7 (100%) vomiting was a major symptom of NoV infection. If acute gastroenteritis in children, under the age of 7, was manifested by vomiting, it was 5.3 times more likely to identify NoV in the stool. On the other hand, the occurrence of abdominal pain in the elderly represented 8.2 times greater risk for NoV positivity.

Usually, severe disease occurs in young children, the elderly, and persons with chronic illness [21]. Some authors report that disease can be very serious and sometimes fatal, particularly for the vulnerable population of children under 5 years of age and people ≥ 65 [22, 23]. The risk for hospitalization and death of patients of ≥ 65 years is increased by 20–30% during epidemic outbreaks of NoV, while for the users of centers for care of the elderly, the risk is increased by another 10% [24]. In this study, no deaths from NoV infection were recorded, although difficult cases of acute gastroenteritis were more prevalent in the age group of 0–15 years (with similar frequency of NoV positive and negative cases). These results suggest that the severity of the clinical presentation of viral gastroenteritis is the result of vulnerability of children and elderly for intestinal infections in general.

Although NoV infection occurs with a similar frequency in males and females, present study shows some distinctions associated with gender. The presence of frequent vomiting, more than 4 times, indicated NoV infection in

women with a specificity of 81.4%, while for men the infection was always presented with diarrhea. Similarly, Arias et al. [16] report that abdominal pain and fever were more common in men than women. When anlysed according to age groups, men were more often infected in older age (≥ 65 years) and women in the age group including reproductive period (16–65 years). In childhood NoV infection was equally present in both sexes. Since the differences in the number of infected patients according to gender were observed only in adults, the influence of hormonal status on the sensitivity could be expected and might be the subject of future investigation.

The previously published results showed that sporadic cases of NoV infections were more common in the summer months. On the other hand, the season for outbreaks of NoV infection was winter in adults, while in children the outbreaks were reported throughout the year [4, 5, 25, 26]. The occasional amplification of viral activity in the population is explained by the emergence of new viral genotype [27]. In this study, the overall prevalence (sporadic cases and outbreaks) of NoV infection was shown to be higher in winter months.

The present study shows no difference in the incidence of infection in relation to water supply, despite the known fact that hygienic conditions and intense precipitation precede the occurrence of epidemics, especially in summer [4]. A lower recorded incidence of NoV infection after consuming food in restaurants can be explained by the fact that older children and adults who frequently visit these facilities were less sensitive to NoV in relation to pre-school children and the elderly.

Conclusion

The results of this study confirm that young children and elderly are the most susceptible to norovirus infection and that infection is more frequent in winter. Diarrhea was the most common symptom followed by vomiting especially in young children. Those who consumed food in restaurants and other public facilities were not at higher risk for norovirus infection.

These results indicate the need for improvement of acute viral gastroenteritis prevention, as well as the need to introduce a better system of surveillance that would include screening for norovirus infection in asymptomatic employees of facilities of collective shelters, and institutions for feeding and medical treatment of children and the elderly. Also, a major contribution to the prevention of viral gastroenteritis outbreaks, can be made through education of physicians in primary health care about vulnerable groups, phenotypic characteristics of norovirus infection in different age groups, as well as the indications and possibilities for rapid testing of this infection.

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End-of-life costs of medical care for advanced stage cancer patients

Medicinski troškovi palijativne nege bolesnika sa karcinomom

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Abstract

Background/Aim. Cancer, one of the leading causes of mortality in the world, imposes a substantial economic burden on each society, including Serbia. The aim of this study was to evaluate the major cancer cost drivers in Serbia. **Methods.** A retrospective, in-depth, bottom-up analysis of two combined databases was performed in order to quantify relevant costs. End-of-life data were obtained from patients with cancer, who deceased within the first year of the established diagnose, including basic demographics, diagnosis, tumour histology, medical resource use and related costs, time and cause of death. All costs were allocated to one of the three categories of cancer health care services: primary care (included home care), hospital outpatient and hospital inpatient care. **Results.** Exactly 114 patients were analyzed, out of whom a high percent (48.25%) had distant metastases at the moment of establishing the diagnosis. Malignant neoplasms of respiratory and intrathoracic organs were leading causes of morbidity. The average costs *per* patient were significantly different according to the diagnosis, with the highest (13,114.10 EUR) and the lowest (4.00 EUR) ones observed in the breast cancer and melanoma, respectively. The greatest impact on total costs was observed concerning pharmaceuticals, with 42% of share (monoclonal antibodies amounted to 34% of all medicines and 14% of total costs), followed by oncology medical care (21%), radiation therapy and interventional radiology (11%), surgery (9%), imaging diagnostics (9%) and laboratory costs (8%). **Conclusion.** Cancer treatment incurs high costs, especially for end-of-life pharmaceutical expenses, ensued from medical personnel tendency to improve such patients' quality of life in spite of nearing the end of life. Reimbursement policy on monoclonal antibodies, in particular at end-stage disease, should rely on cost-effectiveness evidence as well as documented clinical efficiency.

Key words:
health care costs; serbia; carcinoma; drug therapy; antibodies, monoclonal; terminal care.

Apstrakt

Uvod/Cilj. Maligna oboljenja jedan su od vodećih uzroka smrtnosti u svetu, čije lečenje ima veliki finansijski uticaj na budžete zdravstvenih sistema svakog društva, uključujući Srbiju. Cilj ovog rada bio je da se odredi strukutura najvećih troškova tokom lečenja obolelih od karcinoma u Srbiji. **Metode.** Retrospektivna analiza baze podataka tipa "odozdonagore" sprovedena je da bi se kvantifikovali relevantni troškovi. Analizirani su podaci bolesnika koji su preminuli tokom prve godine nakon postavljanja dijagnoze: demografski podaci, dijagnoza, histologija tumora, troškovi upotrebe svih medicinskih usluga, vreme i uzrok smrti. Svi troškovi su pridodati jednoj od tri kategorije lečenja: primarna, vanbolnička i bolnička nega. **Rezultati.** Analizirana su 114 bolesnika, pri čemu su najveći deo činili bolesnici sa prisutnim udaljenim metastazama u trenutku postavljanja dijagnoze (48,25%). Vodeći uzrok smrtnosti bili su maligniteti respiratornih organa. Prosečni troškovi po bolesniku bili su značajno različiti u odnosu na vrstu karcinoma, pri čemu su najveći troškovi zabeleženi kod bolesnica sa tumorom dojke (13 114,10 EUR), a najniži kod bolesnika sa melanomom (4,00 EUR). Najveći finansijski uticaj na ukupne troškove odnosio se na lekove, 42% (među njima, troškovi za monoklonska antitela iznosili su 34% sredstava, ili 14% u odnosu na ukupne troškove), zatim troškovi za medicinsku negu u onkologiji (21%), terapiju zračenjem i intervencijsku radiologiju (11%), hirurgiju (9%), dijagnostiku snimanjem (9%) i laboratorijske troškove (8%).

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Zaključak. Lečenje obolelih od karcinoma uključuje velike troškove, posebno za lekove za bolesnike u terminalnom stadijumu bolesti, uz nepredvidiv ishod lečenja. Unapređivanjem i finansiranjem programa za ranu detekciju bolesti i odgovarajućom politikom refundiranja na nacionalnom nivou (npr. troškova lečenja monoklonskim antitelima) moglo bi se očekivati više koristi i manje ekonomskog opterećenja društva troškovima za lečenje malignih oboljenja.

Ključne reči:
zdravstvena zaštita, troškovi; srbija; karcinomi; lečenje lekovima; antitela, monoklonska; nega, terminalna.

Introduction

Cancer is one of the most important world public health concerns and the leading cause of mortality in the developed world [1]. It is estimated that the number of global cancer deaths will increase from 7.4 million in 2004 to 11.8 million in 2030 [2]. In the European region, mortality of cancer amounted for 166 *per* 100,000 inhabitants, in the year 2008, and the number of cancer cases will probably increase for 40% in most of these countries, in 2015 [3, 4]. High mortality rates were also observed in the rest of the world, with the exceptions of South-East Asia and South-Mediterranean Region, where mortality rates of cancer in the year 2008 amounted for 125 and 127 *per* 100,000 inhabitants, respectively [3].

Recent Serbian history and its geographical location determine its, to some extent, peculiar situation at the Continent. In the last three decades, several ecological accidents occurred in the region, starting with Chernobyl disaster. Military conflicts, involving problems of depleted uranium during NATO bombing campaign, post-war syndrome and problems of post-communist society, which led to elevated levels of anxiety and mood disorders in general population [5], probably significantly contributed to rising cancer incidence rates in some malignant neoplasms [6, 7]. In the nation-wide population-based cancer data for Serbia, a significant increase in overall cancer incidence and mortality within the observed 10-year period (1999–2009) was found, as well as the alarmingly high mortality rates in Serbia compared to the rest of Europe [7]. In the 2009, cancer mortality rate amounted 181.1 for men and 113.8 for women, *per* 100,000 inhabitants. In the same period, lung cancer showed the highest incidence rate among men, achieving 70.8/100,000 among male population, and the most common cancer among women was breast cancer, with the same incidence rate among female population [7]. Furthermore, lung cancer is the fourth of ten most common causes of death by disease, gender and age among the Serbian population in 2012 [8].

Malignant diseases impose a substantial economic burden on each society. According to the US National Institute of Health, Americans spent 77.4 billion dollars for direct medical costs for cancer care, in the year 2008 [9]. There are published estimates that one third of all aforementioned costs of cancer treatment incur in the final year of disease and almost 80% of that amount is spent in the last month [10]. In the European Union (EU), health cancer costs reached 51 billion euro, with health care accounting for 40% in 2009 [11].

Serbian health system practice and its financing are predominantly hospital-oriented [12]. Therefore, there is necessity to evaluate major cancer cost drivers in order to achieve more efficient health policy strategies.

The aim of this study was to assess major cancer cost drivers in Serbia. Two major research questions were discussed, namely whether there is a cost difference among the patients at primary care, hospital inpatient and hospital outpatient care, as well as on the major cost drivers.

Methods

Study design and patients selection

A retrospective data base analysis was utilized in order to answer to the relevant research questions [13]. An in-depth bottom-up analysis of consumption patterns and service provision expenses related to cancer diagnosis, treatment and related issues was conducted from the third party payer's perspective, i.e. from the national Republic Fund of Health Insurance (RFHI). The wide proportions of costs in Serbia also comprise out-of-pocket patients' expenditure [14], but these and indirect, loss productivity related costs, remain out of the scope of this study.

Two national cancer databases were reviewed, concerning newly diagnosed patients from the Central Serbia region, which can be regarded representative of national cancer incidence and prevalence rates [15]. They were treated in the university tertiary health care hospital, in a 2-year period (January 1, 2010–Decembar 31, 2011). Those were RFHI database and Oncology registry of morbidity and mortality, provided by the Institute of Public Health of Serbia "Dr Milan Jovanović - Batut", Belgrade. Out of the 1st database were collected information on medical costs and consumption, and the 2nd database supplemented the clinical and epidemiological evidence for the same patients. In total, for 1,222 patients completed data emerged from combining both databases. Out of them, patients were selected, who were newly diagnosed and deceased within the observed period (i.e. all of them deceased within the first year since the diagnosis was established). For some of them, discrepancy of two databases occurred, i.e. cancer incidence date was closely approaching the date of death, which was clarified by the clerks in charge by data handling procedure. Due to these reasons and other minor lacking data, 37 cases out of 151 deceased were eliminated. In total, 114 complete patient files remained to be analysed [15].

Structure and pricing of the used recourses

Each patient received initial chemotherapy, surgical and/or radiation treatment, according to the attending oncolo-

gist's recommendations following confirmed diagnosis, malignancy stage and grade determination [16]. Basic demographics, diagnosis, tumour histology and clinical stage at diagnosis, medical recourse use, related costs, time and cause of death were obtained for each patient. The structure of calculated costs is shown in Figure 1.

The official RFHI pricelist was applied at the time of the service provision. Exchange rates were calculated according to average official exchange rates of the National Bank of Serbia during the observed period (1 EUR = 100.60 RSD).

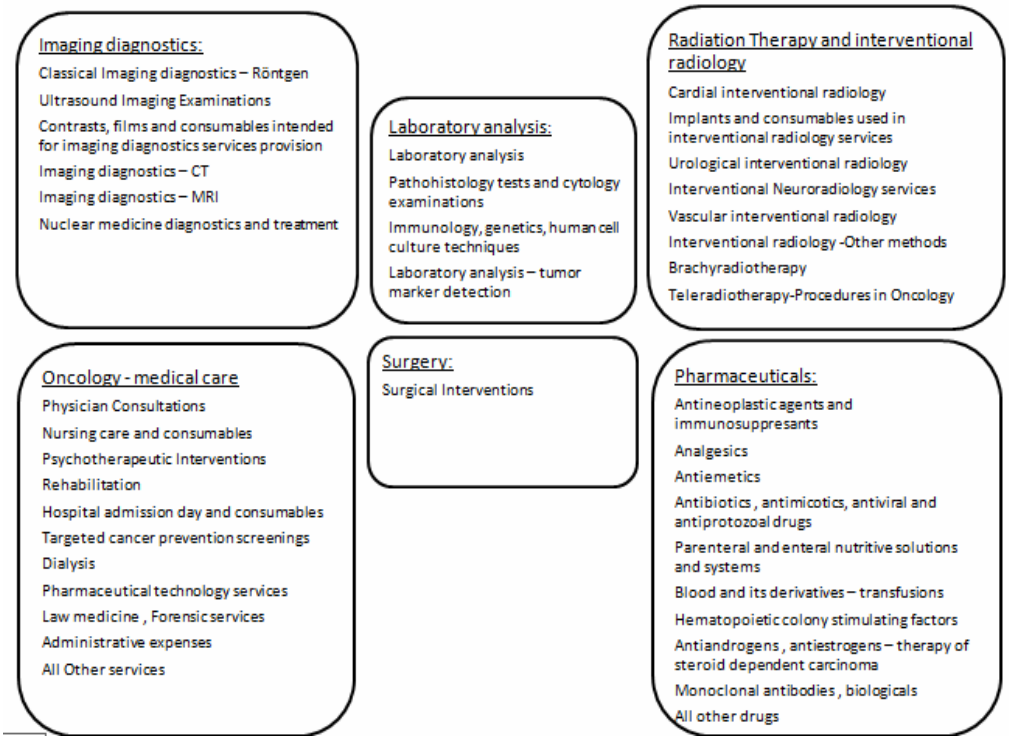


Fig. 1 – Structure of calculated costs for the study subjects

The total health care cost of end of life care was calculated and analysed for the one of the three categories of cancer health care services: primary care (outside the hospital), hospital outpatient and hospital inpatient costs for the observed patients.

Statistical analysis

Categorical variables were presented as frequencies of certain categories, while continuous variables were summarized as mean, standard deviation and 95% confidence interval (CI). The significance of the difference between continual variables was tested by Kruskal-Wallis analysis. The relationship between survival days, total costs and the International Classification of Diseases (ICD-10) diagnosis was tested by Spearman's coefficient of correlation; thereafter, impact of ICD-10 diagnosis and costs on survival was analysed by multiple regression analysis. Statistical analyses were performed using Microsoft Office Excel 2007 and Statistical Software PASW Statistics 18.

The study was conducted in line with The Declaration of Helsinki and has been approved by the regional Ethics Committee of the University Clinical Center Kragujevac, Serbia. Decision number 01-5978 issued on May 28, 2013.

Results

A total number of observed patients, who deceased within the first year from the moment of establishing the diagnosis, were 114 (77 male and 37 female), with the mean age of 67 ± 9 years (mean ± standard deviation). The greatest proportions of them were patients with present distant metastasis at the moment of establishing the diagnosis and the unstaged malignancies (48.25% and 38.60%, respectively) (Table 1).

Table 1

Patients' distribution according to the stage of the disease at the time of establishing the diagnosis of cancer

Cancer stage at the diagnosis	Patients, n (%)
Carcinoma *in situ*	–
Cancer localized within primary tissue/organ of origin	2 (1.75)
Locally advanced malignancy	6 (5.26)
Locally advanced malignancy spreading to the nearby lymph nodes	7 (6.14)
Presence of distant metastasis	55 (48.25)
Unstaged malignancies	44 (38.60)

Most frequent morbidity causes classified according to ICD-10 are shown in Table 2. Malignant neoplasms of respiratory and intrathoracic organs were leading causes of morbidity, followed by malignant neoplasms of digestive organs and breast, accounting for 73% of all the cancer cases.

Table 2
Most frequent types of malignant neoplasms (MNs) causing morbidity in patients

Diagnosis (Diagnosis codes of ICD-10)	Patients, n (%)
MNs of respiratory and intrathoracic organs (C30-C39)	45 (39.47)
MNs of digestive organs (C15-C26)	31 (27.19)
MNs of breast (C50-C50)	7 (6.14)
MNs of female genital organs (C51-C58)	7 (6.14)
MNs of urinary tract (C64-C68)	7 (6.14)
MNs of ill-defined, other secondary and unspecified sites (C76-C80)	6 (5.26)
MNs of male genital organs (C60-C63)	5 (4.39)
MNs, stated or presumed to be primary, of lymphoid, hematopoietic and related tissue (C81-C96)	3 (2.63)
MNs of lip, oral cavity and pharynx (C00-C14)	1 (0.88)
Melanoma and other MNs of skin (C43-C44)	1 (0.88)
MNs of eye, brain and other parts of central nervous system (C69-C72)	1 (0.88)

ICD – International Classfication of Diseases.

Average medical costs *per* patient according to the ICD-10 diagnosis, including CI 95% limits, are shown in Figure 2. They are significantly different among the diagnosis groups (C^2 = 19.307, p = 0.037). The highest cost *per* patient (13,114.10 EUR) was observed in breast cancer (C50-C50), and the lowest cost in melanoma and other malignant neoplasms of the skin (C43-C44) (4.00 EUR).

Average medical costs *per* patient with regards to service groups and according to ICD-10 diagnosis groups were presented in Figure 3. These costs are significantly different according to different kind of services (for primary care C^2 = 33.533, $p < 0.001$; hospital outpatient care C^2 = 51.231, $p < 0.001$; hospital inpatient care C^2 = 23.006, p = 0.011).

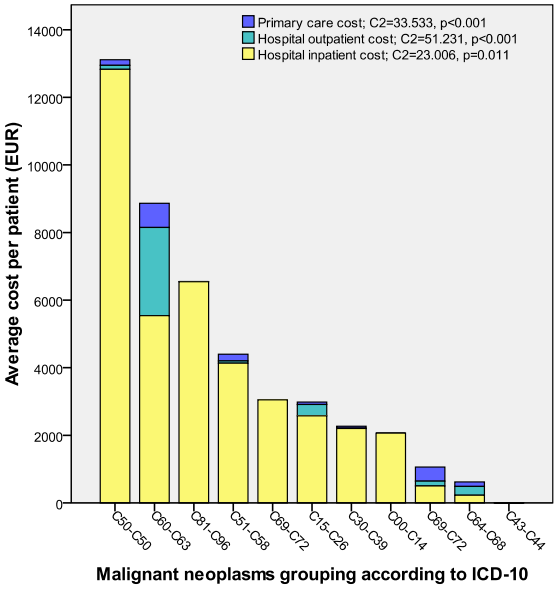
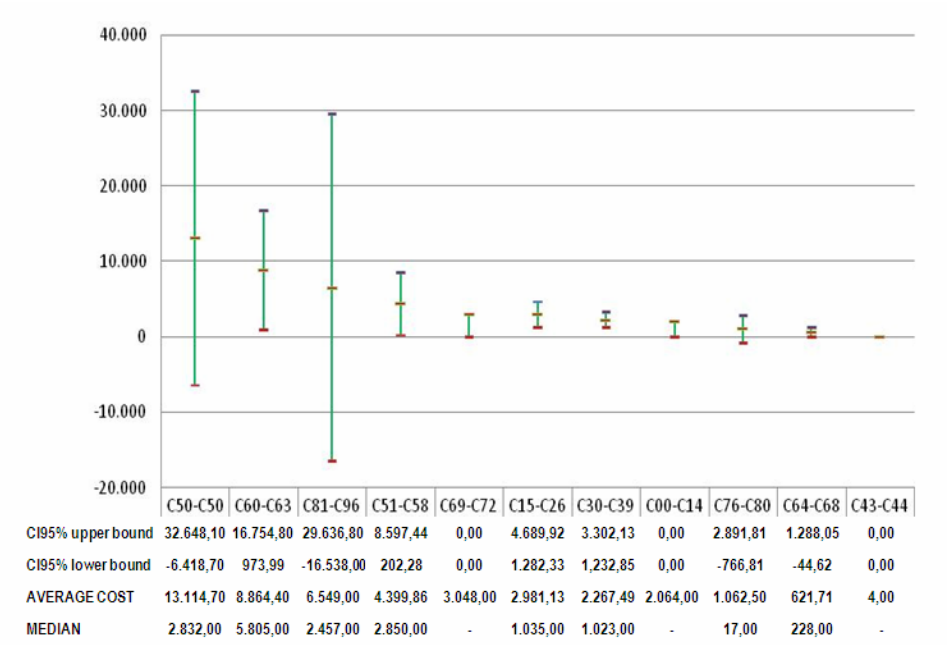


Fig. 3 – Average medical cost (EUR) *per* patient according to International Classification of Diseases (ICD)-10 and the groups of services (primary care, hospital outpatient and hospital inpatient care).
The ICD-10 code groups are explained in Table 2.

The greatest impact on total medical costs was observed concerning pharmacotherapy cost. Share of pharmaceuticals cost was 42.37% [monoclonal antibodies (MABs) amounted



	C50-C50	C60-C63	C81-C96	C51-C58	C69-C72	C15-C26	C30-C39	C00-C14	C76-C80	C64-C68	C43-C44
CI95% upper bound	32.648,10	16.754,80	29.636,80	8.597,44	0,00	4.689,92	3.302,13	0,00	2.891,81	1.288,05	0,00
CI95% lower bound	-6.418,70	973,99	-16.538,00	202,28	0,00	1.282,33	1,232,85	0,00	-766,81	-44,62	0,00
AVERAGE COST	13.114,70	8.864,40	6.549,00	4.399,86	3.048,00	2.981,13	2.267,49	2.064,00	1.062,50	621,71	4,00
MEDIAN	2.832,00	5.805,00	2.457,00	2.850,00	-	1.035,00	1.023,00	-	17,00	228,00	-

Fig. 2 – Average medical costs *per* patient (Eur) according to the International Classification of Diseases (ICD)-10 malignancy group. The ICD-10 code groups are explained in Table 2.

14,52% of total costs (and 34% of all medicines cost], as shown in Table 3.

Oncology–medical care cost amounted to 21%, followed by radiation therapy cost and interventional radiology, surgery, imaging diagnostics and laboratory tests costs (Figure 4).

Average survival *per* diagnosis group expressed in months is presented in Figure 5. The longest survival was observed among the patients with malignant neoplasm of male genital organs (C60-C63) with observed average survival of 8.31 months. The shortest survival, which amounted 0.13 months, was recorded in patients with melanoma and

Table 3

Average drug acquisition cost *per* patient

Cost domain (EUR *per* patient)	Average (CI 95% lower – upper limit) (EUR)	Proportion of total costs – %
Total cost	3,481.77 (2,220.45–4,743.09)	100.00
Costs of all medicines (MABs included)	1,475.08 (628.45–2,321.70)	42.37
Costs of MABs	505.47 (-223.44–1,234.39)	14.52

MABs – monoclonal antibodies; CI – confidence interval.

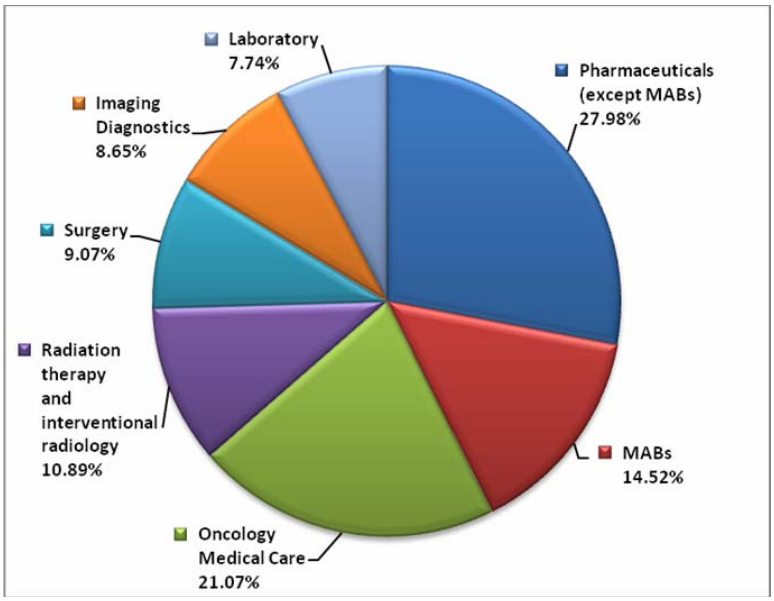


Fig. 4 – Structure and percentage ratio of average medical costs *per* patient with advanced stage of carcinoma.
MABs – monoclonal antibodies.

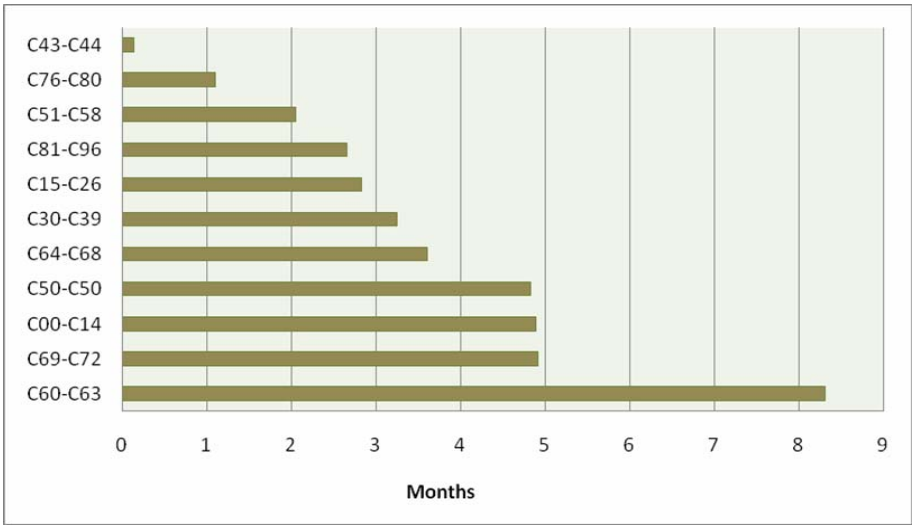


Fig. 5 – Average survival of deceased patients according to the International Classification of Diseases (ICD)-10 malignancy group (expressed in months)
The ICD-10 code groups are explained in Table 2.

other malignant neoplasms of the skin (C43-C44). Correlation between survival and ICD 10 diagnosis and costs showed low degree of correlation (r = 0.087, r = 0.134, respectively). Thereafter, multiple regression analysis was performed and no significant influence of costs and ICD 10 diagnosis, as constants, on survival (F = 2.066, p = 0.132) was observed.

From our patients' database, average end-of-life costs of medicines for each patient were calculated (approximately 1,475.08 EUR). The results showed that about 4.4 % of the total costs for medicines was needed for cancer related end-of-life pharmaceutical costs during the observed period.

Discussion

Our results are in accordance with official ones, i.e. cancer mortality in Serbia among patients with malignant disease was highest at the age of 60 to 69 among ones with lung cancer [17].

Total costs for medicines in Serbia in 2010 and 2011 amounted approximately 710 million Euro *per* year [18, 19]. In these two years, 21,139 and 21,007 people died due to all kinds of malignant diseases in Serbia [20, 21].

There is an upward trend of more aggressive end-of-life cancer treatment in the world, according to the study performed among US patients older than 65 years [22], which is consistent with our study results. One of the observed occurrences is a more frequent administration of chemotherapy within the two final weeks of life, as well. Our results correspond to these data, i.e. the most of the expenses of patients in terminal stages of malignancies resulted from hospital inpatient care, where received chemotherapy contributed to the highest costs [10]. Besides expensive treatment options, some of the diagnostic imaging procedures as well as invasive radiology procedures have been clearly described as major cost drivers in the recent findings from the region [12, 23].

Pharmacotherapy cost included in our analysis accounted for 42.37% of all cancer related health costs *per* patient. Overall, health care spending for cancer care in EU reaches 4% of total health care expenditure, with the highest inpatient care costs accounting for 56% of cancer related costs. Medicines expenditure varies substantially between countries, from 15% in Lithuania to 61% in Cyprus [11]. With regards to chemotherapy cost within the final weeks of life, our data is in accordance with the observed worldwide trend, resulting in a medicines share of 42% of total direct medical costs. MABS influenced total expenditure for pharmacotherapy with one third of value (14.52%; or 34% of all medicines costs).

Limitations of the study

There is an apparent lack of education and awareness contributed to underreporting of all newly cancer cases in Serbia [15]. In spite of all efforts to improve such practice, such as decentralization, active data collection promotion, informatics support, etc., there is still a large probability of time delay for registering these patients, which might lead to shorter survival periods of official records compared to the actual survival periods.

Cost data were related only to the direct medical costs which have accrued since the diagnosis date. Although Payer's perspective has been adopted, substantial improvement in future would present inclusion of lost productivity related indirect costs of cancer. In case of late diagnosed terminal stage population, calculation of premature death cost to the society, based on Grossman's human capital approach [24] would be particularly helpful. Nevertheless such analysis remains out of scope or the budget for this study.

Policy interventions needed in future

In spite of currently cutting edge medical technologies, cancer treatment incurs high costs and its outcomes remain unpredictable [25]. The growing need of today is implementation of efficient measures in order to cut down such costs, but to preserve an appropriate and satisfactory health care system, in order to handle malignant diseases successfully. One of the appropriate options is to promote and finance cancer-screening programs. Costs of cancer treatment rise with the disease progression and therefore allocation of certain financial recourses to cancer screening programs would result in lower costs and more affordable health care.

At the European Cancer Congress 2013, data from a large European survey was presented suggesting that colorectal cancer mortality fell by 73% in men and 82% in women in 11 European countries [26]. The reduction was greater considering the population where screening rate was higher. The same promising results were obtained from cervical cancer screening. Unfortunately, breast and prostate cancer screening has not shown such promising results. A possible reason for those findings could be the fact that when colorectal and cervical cancers are considered, the screening precursor lesion is the target; therefore its removal will not allow them to turn into cancer. This is not the case for breast and prostate cancers, where screening programs simply result in early detection of such diseases and their higher incidence. The results of our study show that among the most frequent morbidity causes are malignant neoplasms of digestive organs (27.19%) and female genital organs (6.14%). Therefore, more aggressive implementation of screening programs for those diseases would bring benefits.

On the other hand, most frequent morbidity causes were malignant neoplasms of respiratory and intrathoracic organs and there is an urge to find another approach to reduce mortality and cancer costs. One of the proposals come from the Florida Society of Clinical Oncology [27]. They suggest some measures, in order to attempt to reduce health care costs without restricting access and reducing payments for such care. Proposed measures consisted of implementing patient management through overall good clinical practice, including organized and established path of disease management, as well as enlargement of palliative hospi-

ce care for patients in the final stage of the disease. As Marsland et al. [27] further suggested, significant savings could be achieved through evidence based therapy options, but adjusted for individual patient needs, trained personnel to evaluate patients for treatment related toxicities and prevent symptoms escalation. Therefore, it could be possible to avoid some unnecessary hospital admissions. Discussion between physicians and terminally ill patients concerning less aggressive medical care would bring some more benefits in terms of better quality of life outcomes and fewer costs [28].

Each particular malignant entity should be reconsidered in order to bring adequate measures for its prevention, treatment and cutting costs. Serbia belongs to eastern European middle income countries with limited public budget and recourses for conducting its own health technology assessments. Therefore policy makers have a greater need to rely on predominantly foreign health technology assessments recommendations such as National Institute for Health Excellence, while making their own decisions. However, transferability of a health technology assessment has limitations, especially in oncology. Local adjustment of such data is necessary, particularly due to services pricing, labour wages and budget differences compared to mature economies [29].

Conclusion

In a series of 114 patients who deceased within the first year from the moment of established cancer diagnosis, the greatest impact on total treatment costs was achieved by medicines, with largest share of monoclonal antibodies. It emerged mostly from medical personnel tendency to improve such patients` quality of life, in spite of nearing the end of life. It is expected that more benefit would be brought from regulatory rationalized reimbursement policy, especially for monoclonal antibodies therapy at the end-stage disease.

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ORIGINAL ARTICLE

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Interferon alpha-induced reduction in the values of myeloid-derived suppressor cells in melanoma patients

Sniženje vrednosti supresorskih ćelija mijeloidnog porekla kod bolesnika sa melanomom indukovano interferonom alfa

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Abstract

Background/Aim. Interaction between tumor cells and host's immunoregulatory cells in creation of microenvironment that supports tumor progression is the focus of numerous investigations in recent years. Myeloid-derived suppressor cells (MDSCs) are heterogeneous population of immature dendritic cells, macrophages and granulocytes. In cancer patients, these cells accumulate in tumor microenvironment, tumor-draining lymph nodes, peripheral blood and the liver and their numbers correlate with the stage of the disease and the metastatic disease. The aim of the study was to investigate the effect of interferon alpha on MDSCs percentage in peripheral blood of melanoma patients. **Methods.** The interferon treated melanoma patients were given subcutaneously interferon alpha, in optimal dose, for a period of at least 6 months before the analysis. Blood samples were collected from the melanoma patients (n = 91) and the age/sex matched healthy controls (n = 8). The following anti-human monoclonal antibodies were used for immunostaining: anti-CD15-FITC, anti-CD33-PE, anti-CD45-ECD, anti-HLA-DR PE/Cy5, anti-CD14-FITC, anti-CD16-PE and anti-CD11b-PE. **Results.** Comparison of myeloid-derived suppressor cells values in the stage 2 melanoma patients with and without interferon alpha therapy did not show a significant difference. When we compared the MDSCs values in the patients within stage 3 melanoma, we found a significant difference in granulocytic subset values between the interferon alpha-treated and the untreated group. Comparison of values of all suppressor cells populations between the interferon alpha-treated patients and healthy controls showed a significant increase in suppressor cells percentage in the melanoma patients. The granulocytic and total MDSCs values were significantly lower in the interferon alpha treated melanoma patients with progression in comparison with untreated patients with stable disease. **Conclusion.** We confirmed that interferon alpha effect in stage 3 melanoma patients was reduction in MDSCs percentage. We also found an unexpected bounce back of these suppressor cells levels, many months after the discontinuation of interferon alpha therapy.

Key words:
melanoma; myeloid cells; interferon-alpha.

Apstrakt

Uvod/Cilj. Interakcija između tumorskih ćelija i imunoregulatornih ćelija domaćina u stvaranju mikrookruženja koje pospešuje progresiju tumora nalazi se u žiži brojnih istraživanja poslednjih godina. Supresorske ćelije mijeloidnog porekla predstavljaju heterogenu populaciju nezrelih dendritičnih ćelija, makrofaga i granulocita. Kod bolesnika sa tumorom ove ćelije akumuliraju se u tumorskom mikrookruženju, drenažnim limfnim čvorovima, perifernoj krvi i jetri i njihov broj koreliše sa stadijumom bolesti i metastatskom bolešću. Cilj rada bio je ispitivanje efekata interferona alfa na procentualnu zastupljenost supresorskih ćelija mijeloidnog porekla u perifernoj krvi bolesnika sa melanomom. **Metode.** Bolesnici lečeni interferonom dobijali su interferon alfa potkožno, u optimalnim dozama, najmanje šest meseci pre izvođenja analize. Uzorci krvi uzimani su od bolesnika sa melanomom (n = 91) i zdravih kontrola (n = 8) sličnog uzrasta i pola. Sledeća antihumana monoklonska antitela korišćena su za imunofenotipizaciju: anti-CD15-FITC, anti-CD33-PE, anti-CD45-ECD, anti-HLA-DR PE/Cy5, anti-CD14-FITC, anti-CD16-PE i anti-CD11b-PE. **Rezultati.** Poređenjem vred-

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nosti supresorskih ćelija mijeloidnog porekla između bolesnika u 2. stadijumu melanoma koji jesu i bolesnika koji nisu lečeni interferonom alfa, nije utvrđena statistički značajna razlika. Kada smo uporedili vrednosti supresorskih ćelija mijeloidnog porekla kod bolesnika u 3. stadijumu melanoma pronašli smo značajnu razliku u vrednostima granulocitne podgrupe ovih ćelija između grupe lečenih i grupe nelečenih interferonom alfa. Poređenjem vrednosti svih populacija ovih supresorskih ćelija između bolesnika lečenih interferonom alfa i zdravih osoba utvrđene su značajno više vrednosti supresorskih ćelija kod bolesnika sa melanomom. Granulocitne i ukupne supresorske ćelije mijeloidnog porekla bile su značajno niže kod bolesnika sa progresijom melanoma koji su lečeni interferonom alfa nego kod bolesnika sa stabilnom bolešću koji nisu lečeni interferonom alfa. **Zaključak.** Interferon alfa dovodi do sniženja vrednosti supresorskih ćelija mijeloidnog porekla kod bolesnika u 3. stadijumu melanoma. Takođe, utvrdili smo povratak visokih vrednosti ovih supresorskih ćelija nakon mnogo meseci od prestanka terapije interferonom alfa.

Ključne reči:
melanom; ćelije, mijeloidne; interferon-alfa.

Introduction

Although malignant melanoma comprises < 5% of all malignant skin tumors it is responsible for almost 60% of lethal skin neoplastic diseases [1]. In the World Health Organisation (WHO) classification there are 4 common types of melanomas (superficial spreading, nodular, lentigo maligna and acral lentiginous) and 6 less frequent types (desmoplastic, melanoma arising from a blue nevus, melanoma arising in a congenital nevus, melanoma of childhood, nevoid melanoma and persistent melanoma) [2]. A typical patient is a Caucasian in the 4th decade of life and the most common locations are on the back in males and the leg in females. Risk factors for developing melanoma are pale skin, blond or red hair, numerous freckles and tendency to burn and tan poorly, the presence of more than 50 acquired nevi, > five dysplastic nevi, chemical exposures, immunosuppression, scars, genetic factors etc. Intermittent sun exposure is recognized as the most important factor [1].

The risk of recurrence after surgical removal of primary tumor, for stage IIB and stage III melanoma patients is reported to be approximately 60% and 75%, respectively [3], so the need for adjuvant therapy is obvious. Malignant melanoma is an immunogenic tumor, susceptible to attack by the host's immune system [4] and, therefore, a broad spectrum of immunotherapies was developed. Unfortunately, many of the tested agents (nonspecific immunostimulants, vaccine and cytokine therapies) failed to demonstrate significant clinical impact. Malignant melanoma is known for its aggressive behavior that is caused by various factors including certain immunosuppressive and immunomodulating molecules released by host cells and melanoma cells [interleukin-10 (IL-10), transforming growth factor-beta (TGF-β), NO, matrix metalloproteinases (MMPs)], tumor editing and other escape mechanisms [5]. Interaction between tumor cells and host's immunoregulatory cells in creation of microenvironment that supports tumor progression is the focus of numerous investigations in recent years. Beside a well-known regulatory T lymphocytes (Tregs), myeloid-derived suppressor cells (MDSCs) function as suppressors of an anti-tumor immunity. Both cell types are involved in development of malignant melanoma [6, 7].

MDSCs are a heterogeneous population of immature dendritic cells, macrophages and granulocytes. In mice, they are identified by CD11b+, IL-4Rα+ and Gr1+ expression. The same cell population is less well defined in humans, but in general MDSCs are myeloid derived (CD33+), CD11b+, lineage not determined (Lin-: CD3-, CD19-, CD56-, CD14-), suppressive and with poor antigen presenting function (HLA-DR-/low). In healthy people they are rare or absent, but under some circumstances (trauma, sepsis) may accumulate in order to temper immune response. In cancer patients, MDSCs accumulate in the tumor microenvironment, tumor-draining lymph nodes, peripheral blood and the liver. Their number correlates with the stage of the disease and the metastatic disease [8]. The influence of MDSCs on anti-tumor immune response is strong and comprehensive, hence these cells are an excellent target in fighting strategies against tumors such as: stimulation of differentiation MDSCs into mature non-suppressive phenotype, decreasing numbers of MDSCs, and inhibition of suppressive function of MDSCs on anti-tumor immunity [9–11]. MDSCs play an important role in melanoma progression and/or as a predictive test for the response to immune-therapy. Finkelstein et al. [12] showed that melanoma and renal cell carcinoma patients with low MDSCs values and a high dendritic cells/MDSCs ratio significantly better responded to high dose IL-2 therapy [12]. The evidence of significant role of MDSCs in melanoma development is accumulating [13].

Interferons demonstrate diverse effects on tumor cells and, between others, interferon alpha (IFNα) showed the highest degree of activity against melanoma cells. Although the precise mechanisms of action are not well understood, antitumor effects of IFNα could include direct anti-proliferative effects, the enhancement of natural killer (NK) cells activity and the up-regulation of tumor antigens and/or major histocompability complex (MHC) class I and class II molecules expression [14]. Early trials with adjuvant IFNα therapy showed significantly longer relapse-free and overall survival rates in melanoma patients [15]. Based on the study of Kirkwood et al. [15], the U.S. Food and Drug Administration (FDA) approved the use of postsurgical adjuvant therapy of high-risk melanomas and this was widely adopted in the community as the best standard of care [16]. Subsequent trials with IFNα showed controversial results [17].

The IFNα effects on MDSCs could be a consequence of induction of maturation in these immature suppressive cells. In addition to lowering the number of MDSCs, IFNα therapy also leads to inhibition of their suppressive activity *in vitro*, as shown in the study of Zoglmeier et al. [18]. Lower suppressive activity of MDSCs under the influence of IFNα therapy could be the consequence of reduced arginase activity and reduced production of reactive oxygen species by MDSCs.

The correlation of IFNα therapy with MDSCs and Tregs levels in peripheral blood of melanoma patients was examined in more detail by Tarhini et al. [19] in 2012 who showed a significant decrease of MDSCs percent in peripheral blood of melanoma patients on day 29 from the beginning of IFNα therapy (after completion of the induction phase of IFN) and day 85 (after completion of one course of IFNα therapy in combination with anti-CTLA-4 antibody).

The IFNα therapy effects on MDSC amount in peripheral blood are noted during therapies of some other diseases, particularly in chronic hepatitis C virus (HCV) infection. Mohamed et al. [20] showed significantly lower MDSC values in patients with chronic HCV infection who had good response to IFNα therapy when compared with patients who had poor response to IFNα.

The aim of this study was to investigate the effect of IFNα on MDSCs percentage in peripheral blood of melanoma patients.

Methods

Patients and healthy controls

Malignant melanoma patients were recruited for this study from the Clinic for Dermatovenerology and Clinic for Plastic and Reconstructive Surgery of the Military Medical Academy (MMA) in Belgrade. Healthy controls were recruited from periodical systematic examinations of apparently healthy persons, with no prior history of cancer. All patients and healthy controls were consented and this study was approved by the local Research Ethics Committee. Melanoma patients were classified according to the 7th edition of the American Joint Committee on Cancer (AJCC) classification for melanoma [21, 22].

IFNα dosage and recorded parameters

All IFNα treated melanoma patients were given subcutaneously 10×10^6 IU five times per week for one month (induction), followed by maintenance regime in optimal dose according to age and stage of the disease (range 3 to 6 × 106 IU) three times *per* week. The patients were on treatment for at least 6 months before the analysis was carried out. Follow-up examinations were repeated every three-months. The parameters were obtained by clinical and dermoscopic examination, laboratory analyses: complete and differential blood count, general biochemical analyses, lactate dehydrogenase (LDH) and S100A protein, ultrasound examination of regional lymph nodes, radiographic and periodic MSCT imaging.

Samples

Three to six milliliters of venous blood were collected from 91 melanoma patients whose age/sex was matched with 8 healthy controls in the period between October 2012 and December 2012. Blood samples were drawn into 3 milliliters vacuettes with Na-EDTA. Erythrocytes were removed with lysing buffer (EDTA, NH_4Cl, $KHCO_3$) for 10 minutes with constant mixing. Remaining nucleated cells were washed twice in RPMI 640 medium with 5% of normal human serum, by standard centrifuge and resuspension processes. The cells were counted both manually, in improved Neubauer chamber, and automatically on Beckman Coulter ACT differ blood counter, and 1×10^6 cells/100 μL of suspension was aliquoted in 12 × 75 mm test tubes for further immunostaining.

Immunophenotypic analysis of cells

The following anti-human monoclonal antibodies were used for immunostaining of fresh peripheral blood samples: anti-CD15-FITC, anti-CD33-PE, anti-CD45-ECD, anti-HLA-DR PE/Cy5, anti-CD14-FITC, anti-CD16-PE, anti-CD11b-PE, anti-CD3-FITC, anti-CD19-FITC and anti-CD56-FITC (Beckman Coulter), in a different combination for multicolor analysis. Stained cells were analyzed using Beckman Coulter FC 500 flow cytometer with CXP analysis software. MDSCs were defined as lineage negative (CD3-, CD19-, CD56-, CD14-), HLA-DR-/low, CD11b+ and CD33+ cells. They were primarily gated on CD11b Vs. HLA-DR plot. The cells with negative/low expression of HLA-DR and positive for CD11b, were further analyzed for lineage markers, CD15 and CD45 expression. Detection of granulocytic and monocytic subsets was made on the basis of CD15 and CD14 expression, respectively. MDSCs percentages were expressed as percent of all nucleated cells.

Statistical analysis

Data analysis was performed using GraphPad Prism 5 software using unpaired, two tailed Student *t*-test for analysis of two groups, and one-way ANOVA test for analysis of multiple groups

Results

MDSCs values in the IFNα treated and untreated melanoma patients

The values of MDSCs were determined in 91 melanoma patients grouped according to the AJCC classification for melanoma. Eleven out of these 91 patients were at active IFNα therapy at the time of MDSCs analysis, and all of them were in the AJCC stage 2 or stage 3. The AJCC subclassification (2a, 2b, 2c, 3a, 3b, 3c) could not be used for statistical analysis because of the small number of patients within each sub-stage.

Comparison of two MDSCs populations, both granulocytic subset of MDSCs (GrMDSCs) and total MDSCs between IFNα treated and untreated melanoma patients did not bring any significant difference, regardless of the AJCC stage (Figure 1). Comparison of GrMDSCs and total MDSCs values in stage 2 melanoma patients with and without IFNα therapy did not show any significant difference (data not shown). However when we compared the MDSCs values in the patients within AJCC stage 3 melanoma, we found a significant difference in GrMDSCs values between the IFNα treated and untreated group. Yet, there was no real significance observed in the total MDSCs values in patients within the AJCC stage 3 (Figure 2).

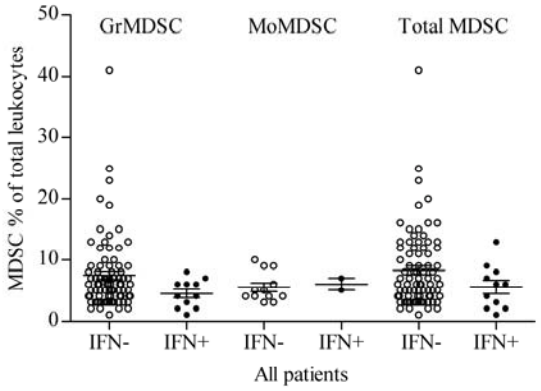

Fig. 1 – Myeloid-derived suppressor cells (MDSCs) values of the American Joint Committee on Cancer (AJCC) stage III melanoma patients, interferon (IFNα)-treated (IFN+) and untreated (IFN-).

The frequency of granulocytic subset of MDSCs (GrMDSCs), monocytic subset of MDSCs (MoMDSCs) and total MDSCs was compared between all the IFN+ (n = 11) and all IFN- (n = 80) melanoma patients regardless of the AJCC classification, using unpaired two-tailed Student's *t*-test, and there was no significant differences. The values are given as mean ± standard error of the mean (SEM).

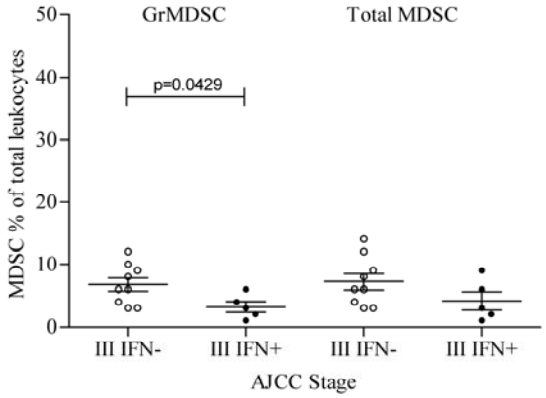

Fig. 2 – Myeloid-derived suppressor cells (MDSCs) values (Gr, Mo and total MDSCs populations) in the interferon alfa (IFNα)-treated (IFN+) and untreated (IFN-) melanoma patients, regardless of the American Joint Committee on Cancer (AJCC) classification.

The frequency of granulocytic subset of MDSCs (GrMDSCs) and the total MDSCs was compared between IFN+ melanoma pateints in the AJCC stage III (n = 6) and IFN- (n = 9) melanoma patients in the AJCC stage III, using unpaired two tailed Student's *t*-test, and difference in frequency of GrMDSCs was significant ($p = 0.049$). The values are given as mean ± standard error of mean (SEM).

Examination of monocytic subset of MDSCs (MoMDSCs) between patients in different AJCC stages was not possible because of the small number of patients with detectable levels of this subset within single stages of melanoma. GrMDSC values in peripheral blood of stage 3 melanoma patients at IFNα therapy were significantly lower than GrMDSC values of stage 3 melanoma patients without IFNα therapy.

MDSCs values in IFNα treated melanoma patients and healthy controls

Comparison of values of all MDSCs populations between IFNα treated patients and healthy controls showed a significant increase in GrMDSCs, MoMDSCs and total MDSCs numbers in melanoma patients samples (Figure 3).

Disease progression and MDSCs values in the IFNα treated and untreated melanoma patients

The 22 out of 91 melanoma patients showed progression of the disease (advance to the next stage, local reccurrence of melanoma within the same stage). The 22 patients with melanoma progression were further classified in two groups: the group under IFNα therapy (n = 6) and without IFNα therapy (n = 16).

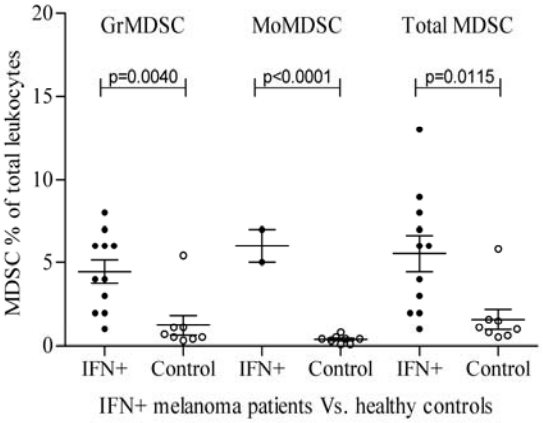

Fig. 3 – Myeloid-derived suppressor cells (MDSCs) values of the interferon (IFNα)-treated (IFN+) melanoma patients and the healthy controls.

The frequency of granulocytic subset of MDSCs (GrMDSCs), monocytic subset of MDSCs (MoMDSCs) and the total MDSCs was compared between all the IFN+ melanoma patients (n = 11) and the healthy controls (n = 8), using unpaired two-tailed Student's *t*-test, and differences in frequency of GrMDSCs, MoMDSCs and the total MDSCs were significant ($p = 0.0040$, $p < 0.0001$, $p = 0.0115$, respectively). The values are given as mean ± standard error of the mean (SEM).

Both groups of patients were compared for all MDSCs values with the following results. There was no statistically significant difference in GrMDSCs and total MDSCs (data not shown). When we excluded extreme values, we found a significant difference in GrMDSCs percentage between IFNα trea-

ted and untreated melanoma patients with progressive disease (Figure 4). Again, the total MDSCs number did not differ significantly between the two examined groups even after exclusion of extreme values. Examination of the MoMDSCs subset was not possible because of the small number of patients with detectable levels of this subset. The most important findings were significantly lower values of GrMDSCs in the patients with melanoma progression who were on IFNα therapy *versus* those with melanoma progression without IFNα therapy.

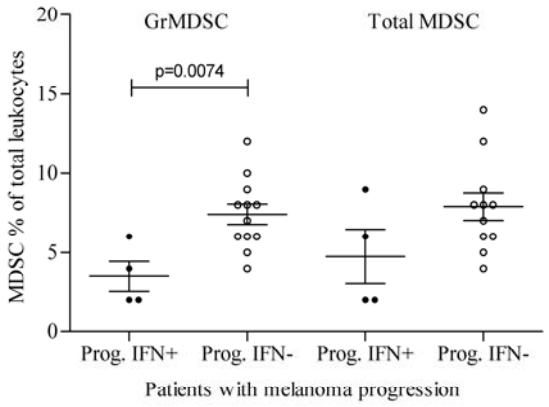

Fig. 4 – Disease progression and myeloid-derived suppressor cells (MDSCs) values in the interferon (IFNα)-treated (IFN+) and untreated (IFN-) melanoma patients.
The frequency of GrMDSCs and the total MDSCs was compard between the IFNα treated melanoma patients with progressive disease (Prog.IFN+, n = 6) and the IFNα untreated melanoma patients with progressive disease (Prog.IFN-, n = 16), using unpaired two tailed Student's *t*-test, and difference in frequency of GrMDSC was significant (p = 0.0074). The values are given as mean ± standard error of the mean (SEM).

MDSCs values in the IFNα untreated patients, with and without melanoma progression

On the basis of two criteria, advancing to the next stage of the disease and local reccurrence of melanoma within the same stage 22 of 91 patient were classified in the group of those with melanoma progression, 55 patients comprised the group of patients with stable disease, while for the 4 of 91 patients there was no sufficient clinical data to determine progression status and they were excluded from the analysis. This classification was made regardless of clinical and pathohistological stage at the time of diagnosis. Within the group of patients with melanoma progression, 15 of 22 patients were IFNα untreated, while in the group of patients without progression, 50 of the 55 patients were IFNα untreated, and the MDSC values were compared between these two groups. We found that the patients with melanoma progression had significantly higher GrMDSCs values (p = 0.0475) than the patients without melanoma progression (Figure 5). With additional statistical processing, by exclusion of extreme values, we found statistically highly significant differences in GrMDSC (p = 0.0034) and total MDSC (0.0051) values between the two groups (Figure 6). The MoMDSCs subset was detectable in 11 patients with stable disease and 3 patients with melanoma progression, and we did not find any statistically significant difference between the two groups in the values of this MDSCs subset (Figure 5).

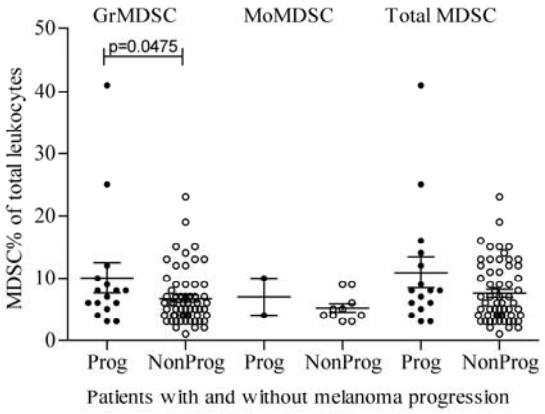

Fig. 5 – Myeloid-derived suppressor cells (MDSCs) values in the interferon (IFNα)-untreated patients with (Prog) and without (NonProg) melanoma progression.
The frequency of granulocytic subset of MDSCs (GrMDSCs), monocytic subset of MDSCs (MoMDSCs) and the total MDSCs was compared between the IFNα-untreated melanoma pateints with progressive disease (Prog, n = 15) and the IFNα-untreated melanoma pateints without disease progression (NonProg, n = 50), using unpaired two tailed Student's *t*-test, and the difference in frequency of GrMDSC was significant (p = 0.0475). The values are given as mean ± standard error of the mean (SEM).

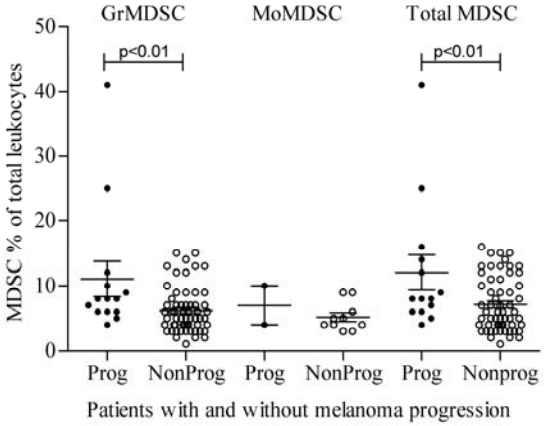

Fig. 6 – Myeloid-derived suppressor cells (MDSCs) values in the interferon (IFNα)-untreated patients with (Prog) and without (NonProg) melanoma progression (extreme values excluded).
The frequency of granulocytic subset of MDSCs (GrMDSCs), monocytic subset of MDSCs (MoMDSCs) and total MDSCs was compared between the IFNα untreated melanoma pateints with progressive disease (Prog, n = 15) and IFNα untreated melanoma pateints without disease progression (NonProg, n = 50) regardless of the American Joint Committee on Cancer (AJCC) classification, using unpaired two-tailed Student's *t*-test, and the differences in frequency of GrMDSC and total MDSCs were significant (p = 0.0034 and p = 0.0051, respectively). The following extreme values were excluded: ID876 = 1%, ID964 = 19% and ID973 = 23% within the group of patients without progression, and ID949 = 3% within the group of patients with melanoma progression. Values are given as mean ± standard error of the mean (SEM).

The values of GrMDSCs and total MDSCs were significantly higher in the group of patients with melanoma progression when compared with the group of patients with

stable disease, while the values of MoMDSCs did not show any statistically significant difference.

MDSCs values in the IFNα treated patients with disease progression and the IFNα untreated patients without melanoma progression

On the basis of two criteria, disease progression and application of IFNα therapy, our melanoma patients were classified in two groups. The group I comprised of patients without melanoma progression and without IFNα therapy (n = 61), while the group II comprised of patients with progressive melanoma disease who were on IFNα therapy at the time of analysis (n = 6). Comparision of these two groups showed a significantly lower GrMDSCs and total MDSCs values in the patients with melanoma progression and IFNα therapy, *versus* the group of patients without melanoma progression and without IFNα therapy (Figure 7). Examination of the MoMDSCs subset was not possible because of a small number of patients with detectable levels of this subset.

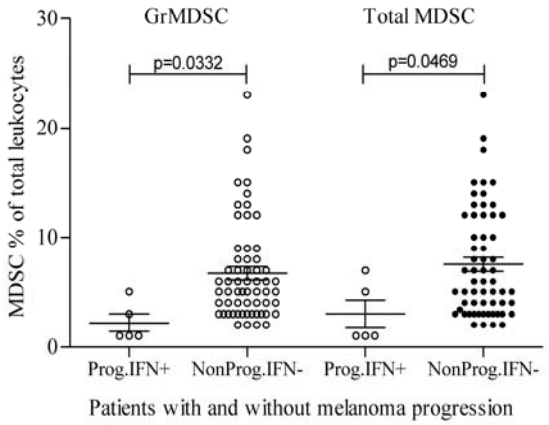

Fig. 7 – Myeloid-derived suppressor cells (MDSCs) values in the patients without melanoma progression and without IFNα therapy (NonProg.IFN-) *versus* the patients with melanoma progression and with IFNα therapy (Prog.IFN+).
The frequency of granulocytic subset of MDSCs (GrMDSCs), monocytic subset of MDSCs (MoMDSCs) and total MDSCs was compared between the IFNα-untreated melanoma patients with stable disease (NonProg.IFN-, n = 61) and IFNα-treated melanoma patients with progressive disease (Prog.IFN+, n = 6) regardless of the American Joint Committee on Cancer (AJCC) classification, using unpaired two tailed Student's *t*-test, and differences in frequency of GrMDSCs and total MDSCs were significant ($p = 0.0332$ and $p = 0.0469$, respectively). The values are given as mean ± standard error of the mean (SEM).

The obtained results show that GrMDSCs and the total MDSCs values were significantly lower in the melanoma patients with progression and at IFNα therapy than in the melanoma patients without disease progression and without IFNα therapy.

Discussion

We compared MDSC values in the two groups of melanoma patients irrespective of the stage. One group was treated with IFNα and the other was not. We found that the MDSCs values for these two groups did not show a significant difference. When we analyzed MDSCs values in all melanoma patients separated in groups by melanoma stage, we found a trend of increase in MDSCs numbers with stage progression. MDSCs values in the stage IV melanoma patients were significantly higher compared to all other stages, however there was no statistical significance between the successive melanoma stages (I-III) (data not shown).

Comparison of MDSCs values in the IFNα treated and untreated groups for each stage showed significant differences for the stage III melanoma patients. The melanoma patients with IFNα therapy had significantly lower GrMDSCs values. IFNα therapy has already been implemented into national guidelines for the treatment of stage III melanoma patients in many European countries [23, 24]. In a large study which comprised 1,256 patients with resected stage III melanoma, Eggermont et al. [25] showed that adjuvant pegylated interferon alfa-2b had a significant, sustained effect on recurrence-free survival. Sondak and Flaherty [26] emphasized that in the Eggermont's study, patients with micrometastases in sentinel lymph node, had the strongest benefit from IFNα therapy.

In our study, 4 of 6 (67%) patients within stage III melanoma at IFNα therapy, had micrometastases in sentinel lymph nodes. This finding implies comparison of MDSCs values in IFNα-treated patients with micrometastases *versus* IFNα-treated patients with macrometastases, in order to investigate eventual correlation of the above mentioned therapy benefit with the reduction of MDSCs levels.

Kimberly et al. [27] showed that MDSCs levels correlate with the disease progression in melanoma patients. Our patients with progressive disease without IFNα therapy had higher MDSCs values in peripheral blood in comparison with the group of patients with stable disease, also without IFNα therapy. We showed that IFNα-treated melanoma patients with progressive disease had significantly lower values of MDSCs than those with no IFNα therapy. In IFNα treated patients with progressive disease MDSCs reduction was very marked, the average MDSCs number was lower than a corresponding value in the patients with stable disease. Again, comparison of MDSCs values from patients with progressive disease at IFNα therapy with those with stable melanoma disease who were without IFNα therapy, showed significantly lower MDSCs values in patients with progressive disease at IFNα therapy at the time of analysis.

Unexpectedly, 2 of our melanoma patients (IDs 956 and 958), had the history of IFNα therapy prior to entering the study, with their therapy being finished more than 24 months before MDSCs measurements hence they were classified as patients without IFNα therapy. In these 2 patients MDSCs values were extremely high, 14% and 20% of total leukocytes, respectively, raising the question on long-term effects after IFNα therapy cesation. Also, the time from discontinuation of IFNα to MDSCs level measurement is 4 times longer in our study than in the study of Mohamed et al. [20] who showed that 4–6 months after IFNα treatment MDSCs values in hepatitis C patients with good response to IFNα therapy were

significantly lower than the values obtained during active treatment in the same patients. Finally, Mohamed et al. [20] measured MDSCs values in HCV patients, so the studies could not be directly compared. Our findings show that long-term effects, after discontinuation of IFNα therapy, on MDSCs levels in peripheral blood may be the opposite from expected and this deserves further investigations. Essentially there could be a significant bounce back of MDSCs levels, many months after discontinuation of IFNα, resulting in greater numbers than would normally be found.

When we compared MDSCs values in all the melanoma patients at IFNα therapy at the time of the analysis with MDSCs values in the healthy controls not subjected to INFα, we found significantly higher values of GrMDSCs, MoMDSCs and the total MDSCs in the IFNα-treated group. So, although IFNα therapy showed significant effects on MDSCs levels in peripheral blood of melanoma patients, MDSCs levels in patients receiving IFNα therapy could not be decreased to the levels of MDSCs in healthy controls.

Conclusion

This study confirmed that the effect of IFNα in stage III melanoma patients was the reduction in MDSCs percentage. IFN therapy must be considered when analyzing MDSCs values in peripheral blood. We also found an unexpected bounce back of MDSCs levels, many months after the discontinuation of IFNα therapy in melanoma patients.

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Levels of interleukin-6 in tears before and after excimer laser treatment

Nivoi interleukina-6 u suzama pre i posle tretmana *excimer* laserom

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Abstract

Background/Aim. Immune response and consequent inflammatory process which originate on ocular surface after a trauma are mediated by cytokines. Photoablation of corneal stroma performed by excimer laser causes surgically induced trauma. Interleukin-6 (IL-6) is mostly known as a proinflammatory cytokine. However, it also has regenerative and anti-inflammatory effects. It is supposed that this cytokine is likely to play a significant role in the process of corneal wound healing response after photoablation of stroma carried out by laser *in situ* keratomileusis (LASIK) or photorefractive keratectomy (PRK) methods. The aim of this study was to determine and compare the levels of IL-6 in tears before and after treatment with LASIK and PRK methods. **Methods.** The study included 68 shortsighted eyes up to -3.0 diopter sphere, i.e. 198 samples of tears (*per* three samples taken from each of the eyes), divided into two groups according to the kind of excimer laser intervention performed: the group 1 – eyes treated by LASIK method (n = 31), and the group 2 – eyes treated by the PRK method (n = 37). The samples of tears were taken from each eye at the following time points: before excimer laser treatment (0 h, the control group), 1 h after the treatment (1 h) and 24 h after the treatment (24 h). The patients did not use anti-inflammatory therapy 24 h after the intervention. Tear samples were collected using microsurgical sponge. Level of IL-6 in tear fluid was determined by the flow cytometry method, applying a commercial test kit which allowed cytokine detection from a small sample volume. **Results.** The values of IL-6 were detectable in 16% of samples before LASIK treatment and in 30% of samples before PRK treatment. One h after the treatment IL-6 was detectable in 29% of samples for the LASIK group and 43% of samples for the PRK group, and 24 h after the treatment it was detectable in 19% of samples for the LASIK group and in 57% of samples for the PRK group. When we analyzed the dynamics of IL-6 production in particular groups, we noticed that both in the LASIK and PRK group the number of samples with increased values of IL-6 after 1 h, and after 24 h, was considerably larger than the number of samples with decreased values of IL-6 after the intervention. Analyzing the dynamics of IL-6 concentration changes in the 1 h samples *vs* 24 h samples there was a statistically significant increase in the number of samples with IL-6 concentration decline in the LASIK group, while at the same time no considerable changes occurred in the PRK group. Comparing average IL-6 values between the two treatment groups in all tear samples at 0 h, 1 h and 24 h after intervention a significantly higher level in the PRK group 24 h after procedure ($p = 0.0031$) was detected. **Conclusion.** IL-6 level in tears increases 1 h and 24 h after LASIK and PRK treatments. This increment is significantly larger 24 h after the treatment with the PRK method than with the LASIK method. Changes of IL-6 production levels in tears after excimer laser treatment indicate that this cytokine takes part in the corneal recovery process after stromal photoablation.

Key words:
keratomileusis, laser in situ; photorefractive keratectomy; interleukin-6; tears; laser therapy; treatment outcome.

Apstrakt

Uvod/Cilj. Imunski odgovor i posledični inflamacijski proces koji nastaju na okularnoj površini nakon dejstva traume, pod uticajem su citokina. Fotoablacija strome rožnjače dejstvom *excimer* lasera dovodi do hirurški nastale traume. Interleukin-6 (IL-6) poznat je kao proinflamacijski citokin, ali on ispoljava i regenerativna i antiinflamacijska dejstva. Pretpostavlja se da bi ovaj citokin mogao imati značajnu ulogu u procesu zarastanja rane rožnjače nakon izvršene fotoablacije strome LASIK (laser *in situ* keratomilensis) i PRK (fotorefraktivna keratektomija) metodom. Cilj ovog rada bio je

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određivanje i upoređivanje nivoa IL-6 u suzama pre i posle lečenja LASIK i PRK metodama. **Metode.** U studiju je bilo uključeno 68 kratkovidih očiju do -3,0 dioptrija sfere, tj. 198 uzoraka suza (iz svakog oka po 3 uzorka suza), podeljenih u 2 grupe, zavisno od vrste izvedene *excimer* laser intervencije: grupa 1 – oči lečene LASIK metodom (n = 31) i grupa 2 – oči lečene PRK metodom (n = 37). Uzorci suza su uzeti iz svakog oka i to: pre izvođenja lečenja *excimer* laserom (0 h, kontrolna grupa), 1 h posle i 24 h posle lečenja *excimer* laserom. Pacijenti nisu koristili antiinflamacijsku terapiju 24 h nakon intervencije. Uzorci suza su prikupljani mikrohirurškim sunđerom. Nivo IL-6 u suznoj tečnosti određivan je metodom protočne citometrije, primenom komercijalnog kompleta za testiranje koji omogućuje detekciju citokina iz malog volumena. **Rezultati.** Vrednosti IL-6 bilo je moguće otkriti u 16% uzoraka pre LASIK i u 30% uzoraka pre PRK lečenja. Jedan sat nakon lečenja IL-6 je bilo moguće otkriti u 29% uzoraka LASIK grupe i 43% uzoraka PRK grupe, dok je 24 h nakon lečenja bilo moguće otkriti u 19% uzoraka LASIK grupe i 57% uzoraka PRK grupe. Kada je analizirana dinamika promene vrednosti koncentracije IL-6 u pojedinim grupama, zapaženo je da je i u LASIK grupi i u PRK grupi broj uzoraka u kojima je došlo do porasta vrednosti IL-6 posle 1 h, odnosno posle 24 h, bio značajno veći nego broj uzoraka u kojima su vrednosti IL-6 bile snižene. Analizirajući dinamiku promene koncentracije IL-6 u uzorcima 1 h prema 24 h, u LASIK grupi došlo je do statistički značajnog porasta broja uzoraka u kojima je registrovan pad koncentracije IL-6, dok u PRK grupi nije bilo značajnih promena. Poređenjem srednjih vrednosti IL-6, u svim uzorcima suza u okviru termina 0 h, 1 h, i 24 h, između LASIK i PRK grupe nađen je značajno viši nivo ovog citokina samo 24 h posle tretmana u PRK grupi (p = 0,0031). **Zaključak.** Nivo IL-6 u suzama raste 1 h i 24 h nakon LASIK i PRK tretmana. Ovaj porast je značajno veći 24 h nakon PRK tretmana u poređenju sa LASIK tretmanom. Promene u nivoima produkcije IL-6 u suzama nakon *excimer* laser lečenja ukazuju na učešće ovog citokina u procesu oporavka rožnjače nakon fotoablacije strome.

Ključne reči:
keratomileusis, laser in situ; fotorefraktivna keratektomija; interleukin-6; suze; lečenje laserom; lečenje, ishod.

Introduction

Excimer laser keratectomy implies a remodelling of cornea by photoablation, thus removing its stroma [1]. The photoablative process removes tissue material of corneal stroma with great precision, leaving the surface behind perfectly smooth [2]. Laser *in situ* keratomileusis (LASIK) and photorefractive keratectomy (PRK) are the two most frequently performed refractive surgical procedures using excimer laser. Corneal wound healing response after photoablation of stroma by excimer laser is the determinant of efficiency and safety of these procedures. Clinical outcomes as well as numerous complications of the procedures (hypercorrection, hypocorrection, regression, stromal haze) are directly related to the processes of reparation and the complex nature of corneal cell response. Both methods apart from their positive effect in terms of correction of existing ametropia, lead to surgically induced trauma. Trauma response consists of a complex cascade of cellular interactions mediated by cytokines, growth factors and chemokines. Corneal trauma response is intertwined by interactions of epithelial, stromal, neural, lacrimal cells and immune system cells. Interactions between these cells determine corneal wound healing response and they help regenerate and maintain anatomy and normal physiology of cornea [3, 4].

When responding to wound healing, in the PRK method cornea may more frequently react in subepithelial haze than in the LASIK method. The main cause of the haze is the interaction between epithelium and stromal keratocytes mediated by cytokines, which activates keratocytes and causes degradation of stromal extracellular matrix. Compared to the PRK method, corneal wound healing response after the LASIK method is featured with a weaker interaction between the epithelium and stromal keratocytes since the epithelial surface remains generally intact in the LASIK method [5].

Immune response and the consequent inflammatory process occurring on the ocular surface after the effect of trauma are mediated by cytokines. Interleukin-6 (IL-6) is produced by the following cells from the ocular surface: macrophages, mast cells, epithelial cells of conjunctiva and cornea, keratocytes, fibroblasts and vascular endothelial cells [6]. This cytokine is mostly known as proinflammatory cytokine. However, it also has regenerative and anti-inflammatory effects [7]. For these reasons it is interesting to examine the role of IL-6 in corneal wound healing response after stromal photoablation. The aim of this study was to determine and compare the levels of IL-6 in tears before and after the treatment with LASIK and PRK methods.

Methods

This clinical randomized prospective cohort study was carried out, with the permission given by the Ethical Board of Military Medical Academy in Belgrade. With a notified consent from each of the participants the study included 68 shortsighted eyes up to -3.0 diopter sphere, i.e. 198 samples of tears (*per* 3 samples taken from each eye) divided into two groups based on the kind of excimer laser intervention performed: the group 1 – eyes treated with the LASIK method (n = 31), and the group 2 – eyes treated with the PRK method (n = 37).

Each group was then divided into three subgroups based on the time of observations, i.e. time of tears sampling. Tear samples were taken from each eye as follows: before excimer laser treatment (0 h, the control group), 1 h after the treatment (1 h) and 24 h after the treatment (24 h).

The inclusion criteria were that patients did not use anti-inflammatory therapy 24 h after the intervention and had no presence of general or eye disorders. The exclusion criterion was a dioptric error greater than -3.0 diopter sphere.

There were 35 participants included in the study, that is 68 eyes were treated (two patients had one eye operated). There was a total of 16 patients in the LASIK group (9 men and 7 women), and a total of 19 patients in the PRK group (14 men and 5 women).

The LASIK and PRK methods were performed by a Wavelight Allegretto (400 Hz) excimer laser. In the PRK method the energy of excimer laser is applied directly onto deepithelialized corneal stroma (Figure 1). In the LASIK method the energy of excimer laser is applied on deeper layers of stroma, i.e. at a larger distance from corneal epithelium (Figure 2). To create a flap we used a Moria microkeratome during the LASIK method procedure, and when we applied the PRK method we removed corneal epithelium with an Amoils rotational brush.

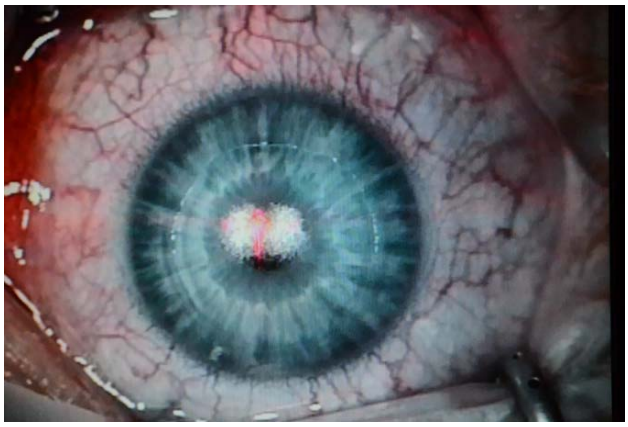

Fig. 1 – Corneal stroma after photoablation during photorefractive keratectomy method.

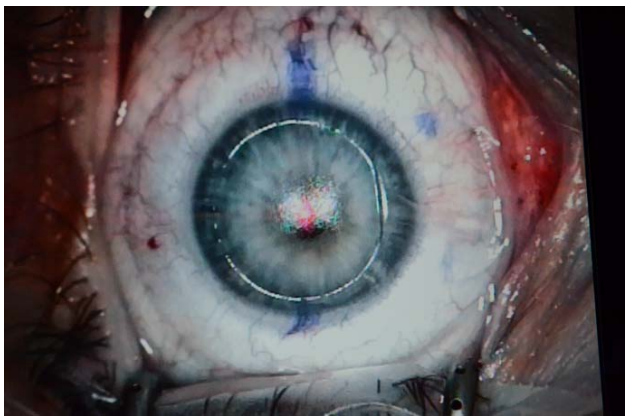

Fig. 2 – Corneal stroma after photoablation during laser *in situ* keratomileusis method.

A procedure described in the study by Acera et al. [8] was applied to collect the samples of tears. The tear fluid was sampled from lower lateral tear meniscus with minimal irritation of ocular surface and the edge of eyelids, with no use of anesthetics (Figure 3). Each tear sample was taken by using a cellulose microsurgical sponge (Alcon, USA). After sampling, the tear fluid was separated by centrifugation of the sponge in a 0.5 mL volume of phosphate buffered saline (PBS). The samples were centrifugated at 13,000 rpm for 15 min at 4°C (MPW-350 r, Med. Instruments, Poland). Collected samples were kept at -80°C until the final examination.

IL-6 level in tear fluid was determined by the flow cytometry method, and we used the commercial test kit (Human Th1/Th2 11 plex FlowCytomix Multiplex) intended for cytokine detection from a small sample volume.

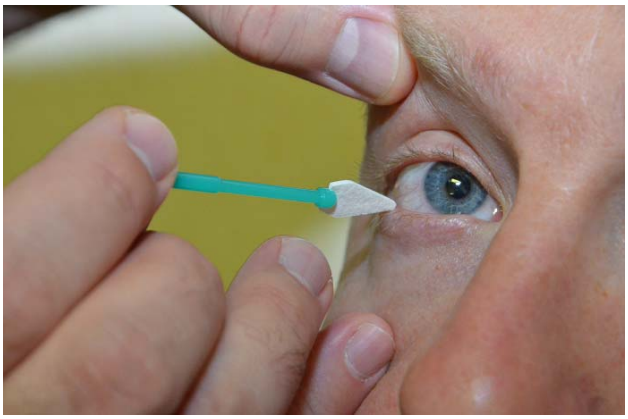

Fig. 3 – Tear fluid sampling from lower lateral tear meniscus using a cellulose microsurgical sponge with minimal irritation of ocular surface.

Methods of descriptive and inferential statistics were used to process statistical data: mean, standard deviation, maximum and minimum values, mode and median for descriptive statistics, and Wilcoxon signed-rank test, ANOVA-Bonferroni test, Mann-Whitney test and Chi square test for inferential statistics.

Results

The average age of the patients in the LASIK group was 34 (33.81 ± 6.52) years, and in the PRK group it was 33 (33.05 ± 6.11) years.

IL-6 concentration was estimated in samples obtained from 31 eyes treated with the LASIK method and 37 eyes treated with the PRK method, at the following time intervals: before the intervention (0 h), 1 h after and 24 h after the intervention (Table 1).

IL-6 was detectable in 16% of samples before LASIK treatment and 30% of samples before the PRK treatment. One h after the treatment IL-6 was detectable in 29% of the LASIK group samples and in 43% of the PRK group samples, and 24 h after the treatment IL-6 was detectable in 19% of the LASIK group samples and 57% of the PRK group samples (Figure 4).

The analysis of tear samples with a detectable IL-6 concentration showed a significant increase in the PRK treated group when we compared posttreatment levels (1 h and 24 h) to the control (pre-treatment level, 0 h). Namely, IL-6 concentration changes showed a significant increment in the PRK treated patients in both time intervals after the treatment compared to pretreatment (0 h/1 h, $p = 0.0031$; 0 h/24 h, $p = 0.0059$). IL-6 concentration average value ratios were 2.365 *vs* 13.01 pg/mL in 0 h/1 h samples and 2.365 *vs* 19.09

pg/mL in 0 h/24 h samples. There was no significant difference between IL-6 levels in 1h and 24 h samples. In the LASIK treated group there was no significant increase in concentration of IL-6 in serial tear samples (Table 2).

Table 1

The levels of IL-6 (pg/mL) in tear samples from the participants treated with the LASIK and PRK methods

ID	LASIK (n = 31)			ID	PRK (n = 37)		
	0 h	1 h	24 h		0 h	1 h	24 h
34	0.00	0.00	0.00	1	0.00	0.00	12.61
35	0.00	0.00	0.00	2	0.00	0.00	18.90
40	0.00	17.13	0.00	3	0.00	0.00	0.00
41	0.00	15.20	0.00	4	0.00	3.85	2.63
42	0.00	9.32	0.00	5	11.12	18.31	27.44
43	0.00	0.00	0.00	6	4.52	25.71	21.38
44	0.63	0.00	0.00	7	0.00	3.52	91.95
45	0.00	0.00	0.00	8	0.00	0.00	0.00
46	0.00	0.00	0.00	9	1.90	0.00	0.00
47	0.00	0.00	0.00	10	0.00	0.00	4.66
48	0.00	0.00	0.00	11	0.00	0.00	116.82
49	0.00	10.75	0.00	12	0.00	0.00	86.10
50	0.00	0.63	0.00	13	1.90	0.00	0.00
51	0.00	0.00	0.00	14	0.00	0.00	0.00
52	0.00	0.00	0.00	15	0.00	0.00	0.00
53	14.07	27.44	14.92	16	26.57	2.55	0.00
54	0.00	0.00	0.00	17	1.90	86.55	0.00
55	0.00	0.00	87.12	18	0.00	6.54	0.00
56	18.31	0.00	0.00	19	0.00	0.00	2.96
57	0.00	0.00	0.70	20	0.00	0.00	0.00
58	0.00	0.00	0.00	21	0.00	0.00	0.00
59	0.00	0.00	3.64	22	0.00	0.00	4.66
60	0.00	0.00	0.00	23	0.00	0.00	94.49
61	87.59	111.79	99.01	24	0.00	0.00	0.00
62	7.23	22.36	17.76	25	0.00	7.92	0.00
63	0.00	17.52	0.00	26	6.88	86.09	0.00
64	0.00	0.00	0.00	27	7.70	17.52	0.00
65	0.00	0.00	0.00	28	0.00	0.00	22.22
66	0.00	0.00	0.00	29	0.00	16.74	85.90
67	0.00	0.00	0.00	30	0.00	18.31	8.18
68	0.00	0.00	0.00	31	0.00	86.89	0.00
				32	0.00	7.23	1.63
				33	0.00	0.00	8.18
				36	3.20	5.87	3.98
				37	19.90	87.88	2.96
				38	0.00	0.00	2.63
				39	1.90	0.00	86.03

LASIK – laser *in situ* keratomileusis; PRK – photorefractive keratectomy; IL-6 – interleukin 6.

Table 2

Statistical significance of differences in production of IL-6 in tear samples with detectable IL-6 values collected 1 h and 24 h after treatment by LASIK and PRK methods, compared to the control (0 h)

Colection time of tears samples	LASIK *(p)*	PRK *(p)*
0 h/1 h	> 0.05	0.0031*
0 h/24 h	> 0.05	0.0059*
1 h/24 h	> 0.05	> 0.05

***Statistically significant difference (Wilcoxon test); LASIK – laser *in situ* keratomileusis; PRK – photorefractive keratectomy; IL-6 – interleukin 6.**

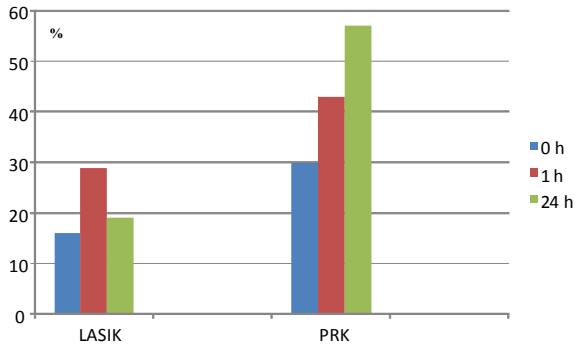

Fig. 4 – Percentages of samples of detectable concentration of IL-6. Tear samples were collected before (0 h) and after (1 h and 24 h) LASIK and PRK treatments.

LASIK – laser *in situ* keratomileusis; PRK – photorefractive keratectomy; IL-6 – interleukin 6.

When we analyzed all tear samples (including samples with undetectable concentration of IL-6) we found a significant difference in the production of IL-6 in tear samples collected 24 h after the treatment compared to the control (0 h) only in the PRK group (Table 3).

values for all the shortsighted eyes included in the study were up to -3.0 diopter sphere in order to expose corneas of every eye to a similar photoablative trauma. Efficiency and safety of the LASIK and PRK method in shortsightedness correction are shown in the studies of Vukosavljević et al. [9] and Resan et al. [10].

Table 3

Statistical significance of differences in production of IL-6 in all analyzed tear samples collected 1 h and 24 h after treatment by LASIK and PRK methods, compared to the control (0 h)

Colection time of tears samples	LASIK (*p*)	PRK (*p*)
0 h/1 h	> 0.05	> 0.05
0 h/24 h	> 0.05	< 0.05*
1 h/24 h	> 0.05	> 0.05

***Statistically significant (ANOVA, Bonferroni test)**
LASIK – laser *in situ* keratomileusis; PRK – photorefractive keratectomy; IL-6 – interleukin 6.

Comparison of average IL-6 values in all tested samples between the two treatment groups showed a significantly higher level of IL-6 in the PRK group 24 h after procedure ($p = 0.0031$) (Table 4).

Table 4

Statistical significance of differences in average IL-6 values in all tested samples between the treatment groups, LASIK and PRK, at the different points of time

Colection time of tears samples	LASIK *vs* PRK (*p*)
0 h	> 0.05
1 h	> 0.05
24 h	0.0031*

***Statistically significant difference (Mann-Whitney test).**
LASIK – laser *in situ* keratomileusis; PRK – photorefractive keratectomy; IL-6 – interleukin 6.

Frequency of IL-6 value change showed similar characteristics both in the LASIK and PRK group when we analyzed posttreatment (1 h and 24 h) to pretreatment (0 h) numbers of increments. Almost 80% and 70% of all samples collected 1h and 24h after treatments, respectively in both groups had an increase in IL-6 concentration compared to 0 h (before the treatment). When we analyzed 1 h *vs* 24 h numbers of increments, we found a significantly lower number of samples with increased IL-6 concentration in the LASIK group in comparison to the PRK group (Table 5).

In our study values of IL-6 were detectable in 16% of tear samples before the LASIK treatment and in 30% of tear samples before PRK treatment. One h after the treatment IL-6 was detectable in 29% of the LASIK group samples and 43% of the PRK group samples, while 24 h after the treatment IL-6 was detectable in 19% of the LASIK group samples and 57% of the PRK group samples. When we analyzed dynamics of IL-6 production in separate groups we noticed that the number of samples with increased values of IL-6, 1 h and 24 h after the treatments, was larger than the number of samples with decreased values of IL-6 in both groups (LASIK and PRK) compared to the control (0 h). Analyzing the dynamics of IL-6 production in samples with detectable level of cytokine, collected 1 h and 24 h after the treatments, we found a significantly higher number of samples of lower concentration of IL-6 in the LASIK group 24 h after the treatment compared to 1 h. No significant changes between the number of samples with decreased or increased concentration of IL-6 were observed in the PRK group at the same time points.

Leonardi et al.[11] in their study examine levels of different cytokines and chemokines in tears from shortsighted eyes before and after LASIK intervention as well as in corneal fibroblast cultures before and after excimer laser treatment. Tears were sampled by a glass capillary micropipette from the eyes of 15 shortsighted patients before, 1 h after and 24 h

Table 5

Dynamics of IL-6 production in tear samples from the patients treated with the LASIK and PRK methods (only samples with detectable IL-6)

Colection time of tears samples	LASIK (%)		PRK (%)	
	▲	▼	▲	▼
0 h/1 h	82	18	79	21
0 h/24 h	75	25	74	26
1 h/24 h	25	75	54	46

▲ – number (%) of samples with increased IL-6 level;
▼ – number (%) of samples with decreased IL-6 level.

Discussion

Our study included shortsighted eyes because shortsightedness is the most common ametropia and at the same time the most common indication for laser diopter removal. Diopter

after LASIK intervention. The levels of cytokine in tears were determined by the multiplex bead analysis system. In this study IL-6 was not detected in patients' tears before LASIK treatment. Postoperatively, 24 h after LASIK treatment, the level of IL-6 in tear samples rosen in 9 out of 15

patients (60%). The mean tear IL-6 value was in a significant correlation with the mean symptom score value 1 h after LASIK treatment. Corneal fibroblast culture had an elevated IL-6 level before excimer laser treatment. One hour after the culture was exposed to excimer laser the IL-6 level decreased. At 24 h after excimer laser treatment IL-6 level was significantly increased as compared with the baseline level and 1 h value. After the surgery, the symptom score was only in correlation with tear sample IL-6 values which showed direct involvement of this cytokine in postsurgical inflammation development and in the corneal wound healing processes [12]. In our study, compared with the Leonardi et al. [11], IL-6 was detectable 24 h after the treatment in 19% of tear samples of the LASIK treated patients.

Malecaze et al.[13] studied the role of IL-6 in corneal wound healing after PRK treatment. Similarly to our results, they obtained the increase in IL-6 level in tear samples 24 h after treatment and stated that IL-6 is probably produced by epithelial cells and keratocytes.

Prada et al. [14] analyzed the gene expression for TNF-α and IL-6 in corneas of rats after phototherapeutic keratectomy (PTK) treatment. Regarding IL-6, there was a significant rise of gene expression 1 h after PTK treatment compared to the control one. Twelve hours after the treatment there was an even larger elevation of IL-6 gene expression, only to decline 24 h after treatment. Still, gene expression remained significantly elevated compared to the control one. The IL-6 expression was detected not only in epithelial and endothelial cells but also in keratocytes of corneal stroma. In our study, there is a similar dynamism only in IL-6 detectability change in tear samples of the LASIK treated patients. The PTK treatment procedure is more similar to the PRK method than the LASIK method. However, it is to be mentioned that the Prada et al. [14] study was carried out on an animal model.

The results of our study indicate that the local production of IL-6 was of higher magnitude after the PRK comparing to the LASIK method. Higher local bioavailability of this cytokine after the PRK treatment could be the consequence of more intense injury of corneal epithelium influenced by the method itself.

Conclusion

IL-6 level in tears increases 1 h and 24 h after laser *in situ* keratomileusis and photorefractive keratectomy treatments. This increment is larger 24 h after the treatment in photorefractive keratectomy method than in laser *in situ* keratomileusis method. Changes of IL-6 production levels in tears after excimer laser treatment indicate that this cytokine takes part in the corneal recovery process after stromal photoablation.

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Analysis of the symmetric configuration of the circle of Willis in a series of autopsied corpses

Analiza simetričnosti konfiguracije Willis-ovog prstena na seriji obdukovanih tela umrlih osoba

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Abstract

Introduction. The forming of the blood vessels network configuration at the base of the brain and interconnecting of blood vessels during the embryogenesis is directly related to the phylogenetic development of the brain and brain structures. A blood vessel configuration at the brain base, in the form of a ring or a hexagon, stands in direct relation to the perfusion needs of certain parts of the brain during its primary differentiation. The aim of this paper was to determine the incidence of certain blood vessel configurations at the base of the brain and understanding their symmetry or asymmetry. **Methods.** Analysis of the blood vessels at the base of the brain was performed on the autopsied subjects. The object of observation was the anterior segment of the circle of Willis consisting of C1- *a. carotis interna* (ICA), above *a. communicaus posterior* (PcoA), the segment A1 *a. cerebri anterior* (ACA) from *a. carotis interna* bifurcation to the *a. communicans anterior* (AcoA) and *a. communicans anterior* itself, as well as the posterior segment consisting of PcoA and the segment P1 – *a. cerebri posterior* (PCA) from the *a. basilaris* bifurcation to the PcoA. For the purpose of grouping the findings, the four basic configuration types of the circle of Willis were identified based on its symmetry or asymmetry. Type-A (symmetric circle of Willis), type-B (asymmetric circle of Willis' due to the unilateral hypoplastic A1-ACA); type-C (symmetric circle of Willis with bilateral symmetric changes on PcoA) and type-D (asymmetric circle of Willis due to the asymmetric changes on PcoA). **Results.** Autopsy was performed on 56 corpses. A total of 41 (73.2%) subjects were recorded with a symmetric configuration of the circle of Willis', of which 27 (48.2%) subjects had type A and 14 (25%) type C. The asymmetric configuration was present in 15 (26.8%) subjects, of whom 9 (16%) had type B and 6 (10.8%) type D. The symmetric Willis group (73.2%) did not have a homogeneous finding that would fit into the schematic presentation of the symmetric type A and type C. A total of 17 (30.4%) findings were classified in this group of the so-called conditionally symmetric configurations. In all the cases, type B (16%) had unilaterally reduced diameter A1 and hyperplastic AcoA. **Conclusion.** The presence of asymmetric Willis configuration in 26.8% of the cases, which makes up more than one fourth, indicates that the asymmetric configurations do not represent a pathological form of connecting the blood vessels at the base of the brain, but rather one aspect of its adaptation. The forming of the basic types of configurations of the circle of Willis is associated with a tendency toward certain types of hemodynamic disorders and more frequent pathological changes in places of reduced resistance.

Key words:
circle of willis; cerebrovascular circulation; neuroanatomy.

Apstrakt

Uvod/Cilj. Formiranje konfiguracije mreže krvnih sudova u bazi mozga i međusobno povezivanje krvnih sudova tokom embriogeneze direktno je povezano sa poligenetskim razvojem mozga i njegovih struktura. Konfiguracija krvnog suda u bazi mozga u obliku prstena ili šestougla ima direktnu vezu sa potrebama perfuzije određenih delova mozga tokom njegove primarne diferencijacije. Cilj rada bio je utvrđivanje učestalosti odgovarajućih konfiguracija krvnih sudova na bazi mozga i sagledavanje prisustva njihove simetričnosti ili asimetričnosti. **Metode.** Analiza krvnih sudova na bazi mozga vršena je na obdukovanim ispitanicima. Posmatran je prednji segment Willis-ovog prstena koji su činili *a. carotis interna* (ICA) (C1-ICA) iznad *a. communicans posterior* (PcoA), A1-ACA (ACA/*a. cerebri ant.*/ od račve *a. carotis interna* do *a. communicans anterior*) i sama *a. communicans interna* (AcoA), i zadnji segment koji su činile *a. communicans posterior* (PcoA) (PCA)/ *a. cererbi posterior*/ i (P1-PCA) od račve *a. basilaris* (AB) do *a. communicans posterior* (PcoA). Radi grupisanja dobijenih nalaza, formirano je četiri osnovna tipa konfiguracija Willis-ovog prstena, na osnovu prisustva njegove simetričnosti ili asimetričnosti: tip A – simetričan Willis-ov prsten, tip B – asimetričan Willis-ov prsten zbog hipoplazije A1-ACA jedne strane; tip C – simetričan

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Willis-ov prsten sa bilateralnim simetričnim promenama na PcoA i tip D – asimetrični Willis-ov prsten zbog asimetričnih promena na PcoA. **Rezultati.** Obdukcija je izvršena na 56 umrlih osoba. Kod 41 (73,2%) ustanovljena je simetrična konfiguracija Willis-ovog prstena, od toga tip A bio je zastuljen kod 27(48,2%), a tip C kod 14 (25%) umrlih osoba. Zastupljenost asimetrične konfiguracije ustanovljena je kod 15 (26,8%) umrlih, od toga tip B bio je zastupljen kod 9 (16%), a tip D kod 6 (10,8%) umrlih osoba. Grupa sa simetričnim Wills-ovim prstenom (73,2%) nije bila sa homogenim nalazom koji bi se uklopio u šematski prikaz simetričnih Willis-ovih prstenova tipa A i tipa C. U tu grupu, takozvane uslovno simetrične konfiguracije, bilo je svrstano 17 (30,4%) nalaza. Tip B (16%) u svim slučajevima bio je sa jednostrano smanjenim prečnikom A1 i hiperlazijom AcoA. **Zaključak.** Prisustvo 26,8% asimetričnih konfiguracija Willis-ovog prstena, što je više od jedne četvrtine, ukazuje da asimetrične konfiguracije ne predstavljaju patološku formu povezivanja krvnih sudova na bazi mozga, već jedan vid njene adaptacije. Formiranjem osnovnih tipova konfiguracije Willis-ovog prstena, možemo uočiti sklonost odgovarajućeg tipa ka hemodinamskim poremećajima i češćem formiranju patoloških promena na mestima smanjene rezistencije.

Ključne reči:
vilisov arterijski prsten; cerebrovaskularna cirkulacija; neouroanatomija.

Introduction

The forming of the blood vessel network configuration at the base of the brain and interconnecting of blood vessels during the embryogenesis is directly related to the phylogenetic development of the brain and brain structures. A blood vessel configuration at the brain base, in the form of a ring or a hexagon, stands in direct relation to the perfusion needs of certain parts of the brain during its primary differentiation [1–4].

The aim of this paper was to determine the incidence of certain blood vessel configurations at the base of the brain and understanding their symmetry or asymmetry. The potential perfusion potential of the brain could be assumed from the corresponding vessel configuration types present at its base [5, 6].

Methods

Analysis of blood vessels of the brain base was performed on the autopsied subjects randomly selected.

During autopsy, the brain was extracted from the cranial fossa using a precise technique, together with blood vessels of the skull base which were resected at the entrance of the cranial cavity (Figure 1).

All the blood vessels of the brain base were separated from it by accurate dissection and arranged on a homogeneous flat surface so as to form the typical configuration of the circle of Willis. These preparations were photographed with a digital camera (Canon PowerShot A1200 12.1 mega pixels) and analyzed on a computer (the use of Adobe Photoshop CS2).

The elements of observation were parts of the blood vessels comprising the circle of Willis. The object of observation was the anterior segment of the circle of Willis, consisting of part of the *a. carotis interna* (ICA)/(C1-ICA), above the *a. communicans posterior* (PcoA) to its bifurcation, part of *a. cerebri anterior* (ACA)/(A1-ACA) from the *a. carotis interna* bifurcation to the junction with *a. communicans anterior* (AcoA) and *a. communicans anterior* (AcoA), as well as the posterior segment consisting of *a. communicans posterior* (PcoA) and part of *a. cerebri posterior* (PCA)/(P1-PCA) from the *a. basilaris bifurcation* to the junction with *a. communicans posterior* (PcoA).

Each preparation was observed separately and its symmetry was determined by comparing the thickness of the same blood vessels on the opposite sides of the circle of Willis. Comparisons were made regarding the thickness of A1-ACA, the thickness of PCoA and P1-PCA. The narrowing of the outer diameter of the vessel, as compared to the contralateral one, by one third or more was classified as hypoplasia. No absolute values of the thickness of blood vessels were measured, but only the differences in the thickness of the observed blood vessels of the opposite sides of the circle of Willis, in order to determine its symmetry.

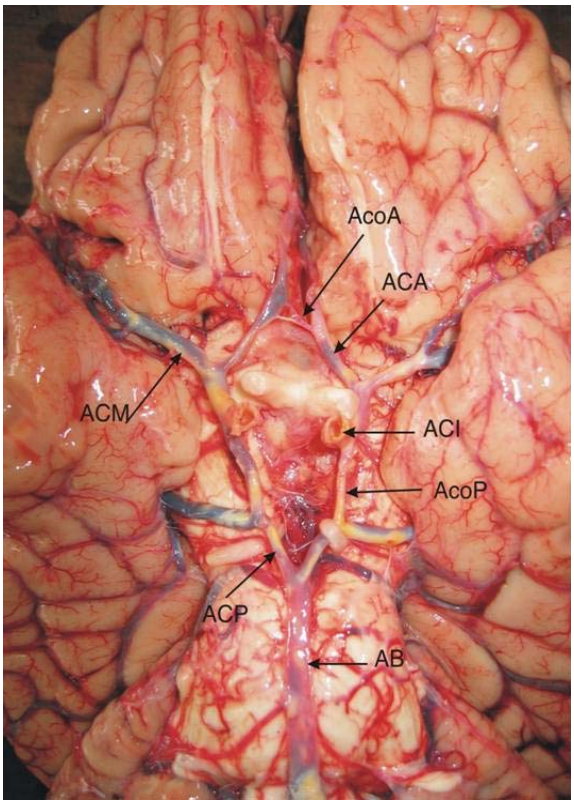

Fig. 1 – Brain preparation after the extraction from the scull AB – *a. basilaris;* ACP – *a. cerebri posterior*; AcoP – *a. communicans posterior*; ACI – *a. carotis interna*; ACA – *a. cerebri anterior*; AcoA – *a. communicans anterior*; ACM – *a. cerebri media.*

For the purpose of grouping the findings, the four basic configuration types of the circle of Willis were identified based on its symmetry or asymmetry (Figures 2 and 3).

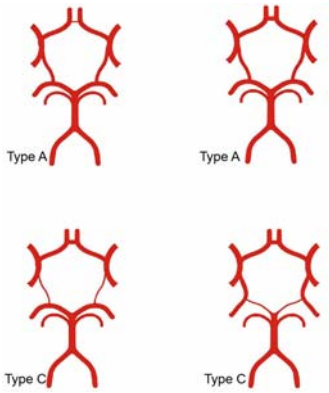

Fig. 2 – Symmetric types of the circle of Willis.

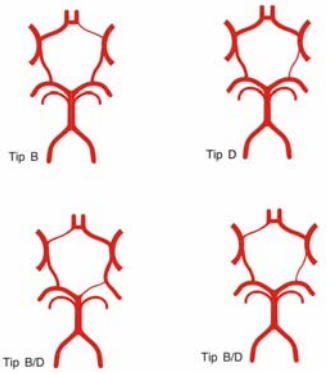

Fig. 3 – Asymmetric types of the circle of Willis.

The first configuration type (type A), is a symmetric circle of Willis. No significant differences in the thickness of the contralateral blood vessels were detected, except for possible variations of the AcoA.

The second configuration type (type B), represents an asymmetric type of the circle of Willis presenting with a narrower A1-ACA diameter unilaterally.

The third configuration type (type C), is a symmetric circle of Willis presenting with varying degrees of bilateral changes present on PCoA according to the type of hypoplasia, or the presence of a bilateral fetal-type PCoA.

The fourth configuration type (type D), is an asymmetric Willis circle because of the observed presence of unilateral hypoplastic PCoA or unilateral fetal-type PCoA. Combinations are possible with changes to the posterior and the anterior segment with the formation of asymmetric (Subtype B/D).

Types A and C belong in the group of symmetric circle of Willis, whereas types B and D are classified as asymmetric circle of Willis.

Results

In the group of 56 autopsied bodies, 36 were male and 20 female. Most of the subjects (48.2%) were above 60 years of age. The average age with regard to gender was of no significant difference. In 32 subjects a violent death occurred, whereas in 24 subjects death was the result of illness.

The highest incidence was the circle of Willis type A configuration, which was present in 27 (48.2%) subjects (Figure 4), followed by type C identified in 14 (25%) subjects (Figure 5). The asymmetric type B configuration was found in 9 (16%) subjects (Figure 6) (Table 1); the asymmetric type D

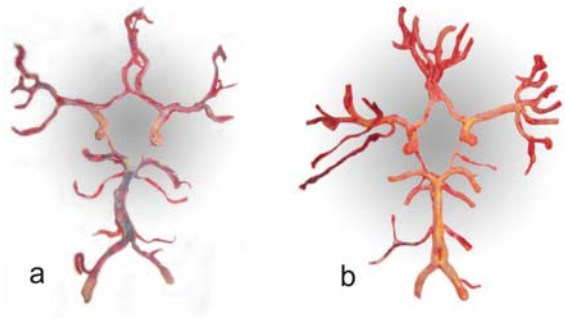

Fig. 4 – Type A of the circle of Willis.

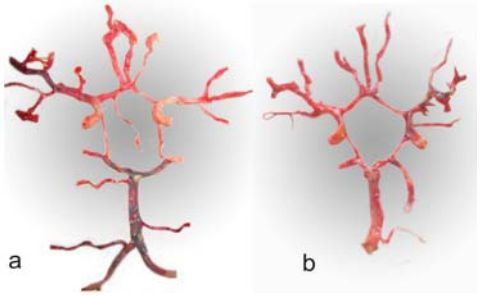

Fig. 5 – Type C of the circle of Willis.

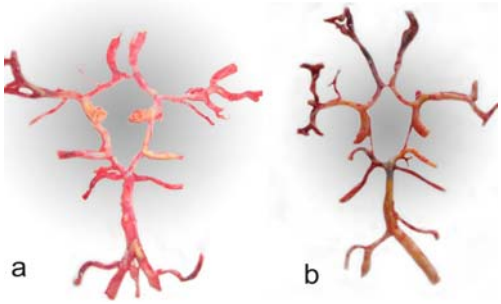

Fig. 6 – Type B of the circle of Willis.

Table 1

Type B circle of Willis changes

Change on A1	Associated changes	n	Subjects, n (%)
Hypoplasia of A1 *sin.*	Dilated ACoA	3	4
	Dilated AcoA with *a. mediana corpori callosi*	1	
Hypoplasia of A1 *dex.*	Dilated ACoA	3	5
	Dilated AcoA with *a. mediana corpori callosi*	1	
	Fenestrated ACoA	1	
Total, n (%)			9 (16)

ACoA – *a. communicans anterior.*

configuration was present in 6 (10.8%) subjects, of which 3 (5.4%) cases had mixed asymmetric subtype – B/D configuration (5.4%) (Figure 7) (Table 2). The symmetric Willis configuration was recorded in 41 (73.2%) subjects and 15 (26.8%) subjects with asymmetric Willis configuration.

Table 2

Type D – circle of Willis changes

Changes on PCoA	Associated changes	n	Subjects, n (%)
Fetal PcoA *dex.*	Hypoplasia of A1 *dex.*	2	3
Fetal PcoA *sin.*		1	
	Hypolasia of A1 *dex.*	1	
Hypolasia of PcoA *dex.*	Hypolasia *a. mediani corporis callosi*	1	3
Hypolasia of PcoA *sin.*	Hypolasia of A1 *dex.*	1	
Total n (%)			6 (10.8)

PCoA – *a. communicans posterior*; A1 – a part of *arteria cerebri inferior*.

Table 3

Symmetric circle of Willis changes

Circle of Wills	Changes of the circle of Wills	n	Subjects, n (%)
Symmetric			24 (42.8)
	Hypoplasia of PcoA bill.	9	
	Hypoplasia of PcoA bill. – *a. mediana corporis callosi*	1	
Conditionally symetric	Hypoplasia of PcoA bill – hypoplasia of ACoA	3	17 (30.4)
	Fetal PcoA bill. – *a. mediana corporis callosi*	2	
	Lnger A1 *sin.* – *a. medina corporis callosi*	1	
	Longer A1 *sin.*	1	
Total, n (%)			41 (73.2)

PCoA – *a. communicans posterior*; A1 – a part of *arteria cerebri inferior*.

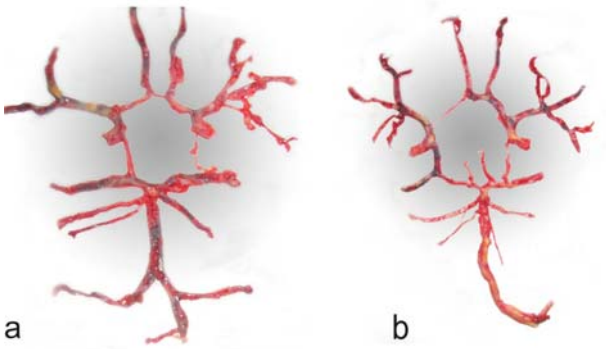

Fig. 7 – Type D and Type B/D of the circle of Willis.

The symmetric Willis group (73.2%) did not have a homogeneous finding that would fit into the schematic presentation of the symmetric Willis of type A and type C. There was a considerable deviation from the schematic presentation but this did not change the basic configuration display, 17 (30.4%) findings were classified in this group of the so-called conditionally symmetric configurations (Table 3).

The asymmetric type B configuration (16%) had in all cases a reduced A1 diameter and hyperplastic AcoA. Of this total, hypoplastic A1 *dex.* was found in 5 cases, whereas hypoplastic A1 *sin.* was detected in 4 subjects. The analysis of the asymmetric D type showed asymmetry in two cases because of the presence of unilateral fetal PCoA (one left and right), and in 4 cases because of the presence of a hypoplastic PcoA (two left and right).

Discussion

The embryological development of the cerebral blood vessels and their task to follow the development and growth of the brain parenchyma directly condition various modalities of differentiation and development of the circle of Willis [2, 7]. This leads us to conclude that we cannot talk about the normal definition of the circle of Willis, but rather about certain types of its configuration [8–11].

The presence of 26.8% of asymmetric configurations of the circle of Willis, which makes up more than one fourth of the total number of cases observed, indicates that the asymmetric configurations do not represent a pathological form of blood vessel configurations at the base of the brain, but one aspect of its adaptation (Table 4) [1, 12, 13].

Table 4

Incidence of the basic types of Willis' configuration [12–14]

Willis' configuration	Subjects, n (%)
Type A	27 (48.2)
Type B	9 (16)
Type C	14 (25)
Type D	6 (10.8)
Total, n (%)	56 (100)

In all asymmetric type B (16%) cases, hyperplasia of the AcoA was present as the adaptive process to ensure adequate perfusion because of the hypoplasia of one A1 ACA [14–16]. All this can lead to hemodynamic load and segmental dilatation of AcoA with subsequent formation of aneurysmal changes [2, 17, 18]. In addition, on the side of the Willis with the hypoplastic A1-ACA, there is a direct perfusion rush from ICA to *a. cerebri media* (ACM), ipsilaterally, which may lead to increased hemodynamic rush in the middle cerebral artery (MCA)

bifurcation area. All this suggests that type B has decreased hemodynamic reserve so, in the event of increased perfusion needs, some parts may be subjected to greater stress [19].

The asymmetric type D is without significant hemodynamic load since the asymmetric posterior segment does not result in significant hemodynamic load. However, due to the presence of asymmetry of the anterior segment as well, the asymmetric B/D type (5.4%) becomes hemodynamically loaded.

The symmetric type A was recorded in 41 (73.2%) subjects. Within this group, 17 (30,4%) subjects were found to have some kind of deviation presenting as changes to the AcoA or PCoA, or different lengths of A1-ACA, PCoA or P1-PCA. Nevertheless, these changes did not lead to deviation from the basic type of symmetric type A configuration.

The forming of the basic types of configurations of the circle of Willis is associated with a tendency toward certain types of hemodynamic disorders and more frequent pathological changes in places of reduced resistance.

Places of reduced resistance are characteristic for specific types of the circle of Willis, carrying a greater tendency towards the formation of aneurysmal changes. Symmetry or asymmetry of certain types of the circle of Willis reflect their hemodynamic characteristics in the sense of higher or lower hemodynamic reserve, which is directly related to the functionality of the collaterals and certain segments of the circle of Willis [20, 21].

Conclusion

The presence of asymmetric Willis configuration in 26.8% of the cases, which makes up more than one fourth, indicates that the asymmetric configurations do not represent a pathological form of connecting the blood vessels at the base of the brain, but rather one aspect of its adaptation. The forming of the basic types of configurations of the circle Willis is associated with a tendency toward certain types of hemodynamic disorders and more frequent pathological changes in places of reduced resistance.

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Administration of iron in renal anemia

Primena gvožđa u lečenju anemije bubrežnog porekla

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Introduction

According to the report of the World Health Organization (WHO) of 2005, the average prevalence of anemia in the world is 24.8%. Anemia is caused by iron (Fe) deficiency in 50%, and reduced iron in storage depots precedes the clinical manifestations of anemia. In the poorer countries of Asia and Africa, iron deficiency appears in more than 65% of preschool children, while in the U.S. (27.3%) and Europe (21.7%) was significantly less frequent, but it is still surprisingly high. Hypochromic anemia may occur in 1–8% of pregnant women and 10–12.7% males older than 65 years [1].

According to the WHO criteria, anemia is a decrease in hemoglobin (Hgb) below the agreed values depending on age, gender and specific residential altitude, and thus values less than 13 g/dL for males and 12 g/dL for females are considered to be diagnostic values [2]. In addition to the concentration of hemoglobin, the correlation between blood volume or the number of erythrocytes and body weight, the number of erythrocytes (E) and hematocrit (Hct) can be used to assess the severity of anemia (Table 1) [3, 4].

Table 1

Diagnostic criteria for anemia

Diagnostic criteria	Male	Female
BVW (mL/kg)	60–90	60–90
ErV (mL/kg)	25–35	20–30
HgB (g/dL)	13.4–17.1	11.9–15.1
sEPO (mg/mL/pmol/L)	0.1/5	0.1/5
Hct (%)	40.7–50.3	36.1–44.3
Er (n/mm^3)	4.3–5.7 × 10^8	3.9–5.1 × 10^8
MCV (fL)	82–98	82–98
MCH (pg)	27–33	27–33
MCHC (g/dL)	32–36	32–36
sFe (µg/dL) (µmol/L)	65–177 (11.6–31.7)	50–170 (9.0–30.4)
TSAT (%)	20–50	15–50
sTf (ng/mL)	≥ 25	≥ 11
TIBC (µg/dL)	250–350	45–80
sF (µg/L)	22–270	18–150
HRC (%)	< 2.5	< 2.5
CHv (pg)	< 29	< 29
ZPP (µg/L)	150–360	150–360
STIR (mg/L)	2.2–5.0	2.2–5.0

BVW – blood volume weight; ErV – erythrocyte volume; HgB – hemoglobin; sEPO – serum erythropoietin; Hct – hematocrit; Er – the number of erythrocytes; MCV – mean corpuscular volume; MCH – mean corpuscular hemoglobin; TSAT – transferrin saturation; sTf –serum transferrin (siderophilin); TIBI – total iron-binding capacity; HRC – hypochromic (HgB < 26 pg) erythrocytes; CHr – reticulocyte hemoglobin; ZPP – zinc protoporphyrin; STIR – soluble transferrin receptor [3, 4].

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Etiology

The causes of anemia can be divided into three main groups: decreased erythrocytes production – disruption in the stem cells or unipotent cells, impaired synthesis of hemoglobin and anemia of unknown cause or multietiologic origin; increased decomposition of erythrocytes – corpuscular and extracorpuscular hemolysis; blood loss anemia – acute and chronic bleeding [5].

Anemia in patients with chronic renal insufficiency (CRI) is of multietiologic nature. It is recorded sporadically in patients with milder forms of CRI (glomerular filtration rate – GFR ≥ 60 mL / min), and in more than two-thirds of predialysis patients (GFR ≤ 15 mL / min) [6]. By activating different mechanisms initiated by ischemia, anemia contributes to the progression of chronic renal disease, and the development and/or deterioration of many cardiovascular disorders (left ventricular hypertrophy, ischemic heart disease, heart failure, arrhythmias, etc.), reducing the volume of physical activity, weakening of mental functions, etc. Relevant clinical studies have confirmed improved cardiovascular performance after the correction of anemia in predialysis patients (Trial to Reduce Cardiovascular Endpoints with Aranesp® Therapy – TREAT) and dialysis patients [7–9].

Iron metabolism

Total iron content in an adult healthy subject is 3–4 g or 40–50 mg/kg/body mass (BM) of which 80% is functionally engaged in hemoglobin (65%), myoglobin (10%) and various enzymes (5% approx).

Women of childbearing age and pregnant women need 2.8–3.0 mg, and men need 0.8–1.0 mg of elemental iron *per* day.

Plant foods (90%) and foods of animal origin provide the intake of 18–20 mg of iron daily. Despite meager absorption (≈ 10%), 1–2 mg is absorbed daily in the duodenum and the same content is eliminated *via* feces for external balance. "Organic" iron or heme iron (Fe^{+2}) produced by heme-oxygenase is absorbed ten times faster than "inorganic" iron from foods of plant origin (Fe^{+3}), which must be previously reduced (ferri-reductase) in divalent ions of iron (Fe^{+2}) [5, 10].

Absorption is promoted by low pH and organic acids in intestinal chyme. Iron from plant foods (Fe^{+3}) and iron chelate (phytates, oxalates, carbonates, tannates, and phosphates) are less suitable for absorption. Hypo/non-acid chyme, milk and dairy products, intestinal mucosal damage, drugs (antacids, proton pump inhibitors, H_2-blockers), and competitive salt ions (Mg^{2+}, Cd^{2+}, Co^{2+}) reduce iron absorption even further [10].

By specific divalent metal transporter (DMT1) Fe^{+2} is transported from the lumen formation through the apical polarity of enterocytes to the 'intermediate depot' in the cytosol in the form of ferritin. The transport of Fe^{+2} from the cell through the basolateral membrane is controlled by hepcidin (an acute-phase reactant to inflammation) originating from the liver. Binding to ferrous iron transmembrane transporter – ferroportin, hepcidin causes its internalisation and lysosomal degradation, and Fe^{+2} remains 'temporarily trapped' inside the ferritin depot. In the absence of the inhibitory action of hepcidin, after binding to feroportin Fe^{+2} must oxidize to Fe^{+3} affected by an oxidative enzyme hephaestin (in the membrane) or serum ceruloplasmin. Then, two moles of Fe^{+3} bind to one mole of transport protein (apotransferrin), becoming the serum transferrin (siderophilin) [11].

Thus iron is delivered to the cells of particular organs through transferrin receptors (TfR) whose synthesis is not affected by proinflammatory cytokines, and their plasma concentrations may indicate the available iron. Iron in the cell is functionally allocated and included in the synthesis of heme and other proteins and enzymes [11, 12].

Causes of iron deficiency in the general population are numerous. The most common cause of iron deficiency is in plant-dominant diet (starch, pasta, rice) along with reduced consumption of meat. In addition, iron deficiency appears even with a balanced diet for the increase in iron requirement (pregnancy, lactation, growth, etc.). Intestinal absorption disorders (gastritis, bowel resection, inflammatory bowel diseases, antacids, H_2 blockers, etc.). Increased intestinal (gastritis, peptic ulcer disease, hernia, diverticulitis, hemorrhoids, parasitic infections, inflammatory bowel disease, tumors, etc.) and genitourinary (meno-metrorrhagia, calculi, tumors, chronic urinary tract infections) blood loss may cause reduction of body iron stores [5, 13].

Anemia in patients with CRI is erythropoietin-dependent and ferrous-deficient, and it is proportional to the seriousness of the renal disease. Specific causes of iron deficiency in patients with CRI are associated with restricted protein intake (meat), chronic microinflammation (hepcidin, transferrin), loss of appetite and digestive erosion, the effects of drugs (phosphate binders and drug-drug interactions) and poor patient cooperation due to digestive disturbances. In addition, anemia is the result of temporary and, if on hemodialysis (HD), permanent blood losses – on the average 3–9 mL (2–4 mg of iron) *per* dialysis session [4, 13].

Uremic toxins (parathyroid hormon – PTH etc.), impaired oxidative balance, aluminum concentration (in water used to make-up dialysate or drugs), insufficient intake and/or reduced resorption (diet / medication / microinflammation), mechanical trauma (blood pump), hemolysis (uremic toxins /oxidative stress, shortened E life-span to 70–80 days) all contribute in different ways to anemia [14].

Two extensive prospective epidemiologic studies (Predialysis Survey of Anaemia Management – PRESAM) presented that iron deficiency was found in 31–38% of CRI patients with different severity of illness, and in more than 60% of dialysis patients (Dialysis Outcomes and Practice Patterns Study – DOPPS) [15].

Iron deficiency in the body can be absolute (unavailable serum-iron and iron in deposits/stores) and relative-functional (unavailable serum-iron, although present in cellular iron storage depots). Although serum ferritin level is most reliable to determine iron stores, and transferrin saturation is used to estimate functional iron, in certain clinical

conditions they must be supplemented by other indicators that are not functionally dependent on inflammatory cytokines (Table 2) [2, 3].

Table 2

Laboratory parameters for detecting iron deficiency

Iron deficiency	Laboratory values
Absolute deficit	
sF	
non CKD/HD (μg/L)	< 15
CKD/HD (μg/L)	< 100/200
TSAT (%)	< 20
Functional (relative) deficit	
sF (μg/L)	≥ 100
TSAT (%)	< 20
HRC (%)/(pg/cell)/(g/dL)	≥ 6/< 26/< 28
CHv (pg/cell)	< 29
ZPP (μg/L)	> 360
STFR (mg/L)	> 5.0
Inadeguate response to ESAS (HgB)	
epoetines (iv/kg/week)	300–500
darbepoetin (mg/week)	100–150

sF – serum ferritin; CKD/HD – chronic kidney disease/hemodialysis; TSAT – transferrin saturation; HRC – hypochronic erythrocytes; CHv – reticulocyte hemoglobin content; ZPP – zinc protoporphyrin; STFR – soluble transferrin receptor; ESAS – erythropoiesis stimulating agents [2, 3].

Treatment

Basic principles for anemia management in chronic renal disease include the following set of measures and procedures [16, 17]:

- use of erythropoiesis-stimulating agents (ESAs): epoetin α: 50 IU / kg/i.v., 1–3 times weekly; epoetin β: 20 IU / kg /i.v./s.c., 1–3 times weekly; epoetin δ 50 IU / kg/i.v./s.c., 1–3 times weekly; darbepoetin α: 0.45 (0.75) μg/kg/sc, 1–2 times monthly; continuous erythropoietin receptor activator (CERA): 0.6 μg/kg/sc; 1–2 times monthly.
- iron supplementation (after assessing iron status/stores): p.o./i.v. supplementation.
- transfusion of erythrocytes: emergency treatment – acute bleeding; resistance to ESAs; symptomatic anemia – comorbidities.
- vitamin supplementation: C-vitamin 500 mg p.o. or i.v. at the end of dialysis; B-complex vitamins p.o./i.v. supplementation; vitamin E p.o. 1,200 mg – before dialysis; folate: 1–3 × 5 mg p.o. supplementation.
- adequate nutrition according to established standards.
- androgens can have beneficial effects – not necessarily administered.
- antioxidant glutathione may reduce resistance to ESAs – not necessarily administered.
- L-carnitine can have beneficial effects – not necessarily administered.
- optimization of dialysis: hemodialysis Kt / V ≥ 1.2; peritoneal dialysis Kt / V ≥ 1.8–2.0/weekly.
- other: dialysis modality switches – peritoneal dialysis (PD) to hemodialysis (HD), hemodiafiltration (HDF), extended daily/overnight dialysis, appropriate PD modality; ultrapure dialysate: bacteria ≤ 0.1 Colony-forming unit/mL (CFU/mL), endotoxin ≤ 00:03 enolotoxin units/mL (EU/mL).

Kidney transplantation is most notably physiological method for the treatment of renal anemia.

Recommendations for initiation of therapy and further monitoring of renal anemia by administration of ESAs and iron supplements in patients undergoing HD/PD and in predialysis period in patients with CRI are shown in Table 3 [18–23].

Iron supplementation

Iron supplementation is required in more than half of patients with advanced renal failure, particularly in those who receive ESAs, although iron supplementation is also needed in patients still without erythropoiesis-stimulating medications [20, 21].

Iron supplementation should be started after the assessment of iron availability and stores. According to the recommendations of the European Best Practice Guidelines

Table 3

General recommendation for renal anemia menagement

When to start treatment	Recommended target values	Performance indicators monitoring*
Hgb < 90 g/L	Hgb 11–12 g/L	Hgb: measure 2–4 times *per* month until steady forget value is reached, once a month later
Signs and symptoms of heart failure:	sF HD 200–500 μg/L	
EF < 40%, IHD, arrhythmias, etc.	CKD/PD 100–500 μg/L	Anticipated increase in Hgb *per* month 0.7–2.0 g/dL
GFR < 50 mL/min/1.73 m^2	TSAT 30–40%	ESAS: titrate the dose over 15 days to optimal level, than every 1–3 months
Previous corection of iron deficiency	HRC < 6%	TSAT: check once a month, than every 3 months
Target value of Hgb ≥ 11 g/dL	CHr > 29 pg	sF: check once a month, then every 3 months
Exculude other causes of anemia if GFR ≥ 50 mL/min/1.73 m^2		*more frequent testing is needed in case of bleeding, surgical interventions and *iv* iron administration

HgB – hemoglobin; EF – ejection fraction; IHD – ischemic heart disease; GFR – glomerular filtration rate; SF – serum ferritin; CKD/PD – chronic kidney disease/peritoneal dialysis; HD – hemodialysis; TSAT – transferrin saturation; HRC – hypochronic erythrocytes; CHr – reticulocyte hemoglobin; ESAS – erythropoiesis stimulating agents.

(EBPG) 2004, National Kidney Foundation / Kidney Disease Outcome Quality Initiative-NKF-KDOQI 2006/2007, European Renal Best Practice (ERBP) 2008, oral iron therapy is indicated for patients with CRI who do not undergo hemodialysis, peritoneal dialysis patients and those patients who obtained kidney transplants, especially if they do not take ESAs. The use of oral iron may continue with the beginning of administration of ESAs, but parenteral use is more effective and more tolerable for the patients [21, 22].

The synthesis of one gram of hemoglobin was assumed to require 20 mg Fe for women and 25 mg for men, and on the basis of BM and the difference between expected and actual values of hemoglobin iron deficiency can be calculated and supplemented, and iron stores can be replenished [Target-Hgb (g/dL)] × [TM (kg) × 0.24] + 1,000 mg (for men)/600 mg (for women) [23, 24].

Peroral iron supplementation

Although most commonly used supplements are organic complexes of either divalent or trivalent iron bound to different protein or sucrose carriers, in many countries simple iron salts either organic or inorganic are still in use. Heme iron is 20 times better absorbed than iron from ferrous fumarate, almost without side effects [22].

The Serbian Prescribed Drug Register [23] determines ferrous fumarate, ferric hydroxide-polymaltose complex and iron protein succinylate may be present in the national market (Table 4).

Common side effects are nausea, anorexia, flatulence, vomiting, abdominal pain, diarrhea/constipation, etc. Toxic effects include proinflammatory, proatherogenic and prooxidant effects connected with serious (20–100 mg/kg) or fatal consequences (200–250 mg/kg or sFe > 5 mg/L) due to excessive intake of iron supplements.

Coadministration may affect drug-drug interactions and reduce their effectiveness, e.g.: penicillamine, bisphosphonate, ciprofloxacin, ofloxacin, norfloxacin, levodopa, levothyroxine, mycophenolate, methyldopa, calcium, magnesium, etc.

Peroral iron supplementation is contraindicated in patients with sensitization, hemochromatosis, hemosiderosis, concurrent use of parenteral Fe, active peptic ulcer and intestinal diseases, etc. To develop greater tolerance and avoid interactions with other drugs or food, a single daily dose is recommended, heme-iron polypeptide products are particularly effective and tolerable [24, 25].

Parenteral iron supplementation

Since target hemoglobin is slowly achieved and because of numerous side effects and interactions with other medications that have to be used regularly, patients are reluctant to take the prescribed oral amount and do not follow basic instructions for administration, and thus parenteral iron supplementation has become widely recommended for the treatment of anemia [20, 22].

On the basis of kinetic and thermodynamic parameters of organic complexes of iron, parenteral supplements are divided into four groups (types I-IV) (Table 5) [11].

Table 4

Most widely used oral iron drugs

Iron complex	Trade name of the drug, manufacturer and dosage form*
Ferrous fumarate: [S.Th.D.: 2 × 1]	Heferol® Alkaloid: (caps. 350 mg/115 mg Fe)
Iron hydroxide polymaltose: [S.Th.D.: 2–3 ×1]*	Referum® Slaviamed: (tbl. 100 mg Fe; syrup 50 mg/5mL)
Iron protein succinylate: [S.Th.D.: 2 × 1]*	Legofer® Alcaloid: (sol. 40 mg/15 mL)
Ferrous sulphate: [S.Th.D.: 1–2 × 1]	Ferro gradumet® Abbot: (ferro sulphate s.r.tbl. 325 mg); Ferrograd C® Abbott: (s.r.tbl. ferro sulphate 325/105 mg + 500 mg vit.C); FGF® Abbott: (s.r.tbl. ferro sulphate 250 mg + 300 mg folic acid)
Ferrous gluconate: [S.Th.D.: 2–3 × 1–2]	Ferrous gluconate® Kent Pharmaceuticals: (tbl. 300 mg)
Heme iron polypeptide: [S.Th.D.: 2–3 × 1–2]	Proferrin ES® Colorado Biolabs: (tbl. 20 mg)

***Drugs from the National Drug Register, 2012 [23]; S.Th.D – single therapeutic dosage; Caps – capsulas; Tbl. – tablets; s.r.tbl. – slow release tablets; Sol – solution.**

Common characteristics of oral iron supplements are: maximum daily dose of 300 mg elemental iron; hemoglobin values are corrected within 2–3 weeks, normalization is achieved within 2–3 months, and iron stores are usually replenished within 6 months.

After *iv* application iron complexes are taken up by phagocytes in reticuloendothelial system in the liver, spleen, and bone marrow. Iron is released there from its

carrier and deposited in the form of cytosolic ferritin and, when needed, it is released and transported to the cell by transferrin.

Absolute indication for the use of parenteral iron is functional iron deficiency which can be one of the possible causes of treatment failures, manifested as the lack of increase in hemoglobin level despite progressively increasing amounts of ESAs.

Initial correction of hemoglobin can be achieved in 1–2 weeks with application of parenteral iron supplements, and its normalization is reachable within 3–4 weeks. General tolerance is greater, and the replenishment of iron stores is faster (6–8 weeks). These supplements are also efficient for other indications: pregnant women after the first 3 months of pregnancy, women in labour, anemic patients with malignancies, anemia in patients with heart failure, etc. Therefore, it is an acceptable iron-replenishment method in patients with hypochromic anemia, especially those who also take ESAs.

Contraindications to its use include: previous diagnosis of sensitization, asthma, allergies, atopic dermatitis, hemosiderosis, liver cirrhosis and severe hepatitis.

Serious adverse effects (SAE) include the development of an anaphylactic [after primary allergic sensitization and antigen (Ag) exposure] or anaphylactoid (after the first contact with Ag without prior sensitization) systemic reactions with potentially fatal outcome, but there are significant differences between particular products.

Milder adverse effects (AE) are common for all available remedies, but their frequency within a particular group of remedies is significantly different: dizziness, numbness, metallic taste, burning, heat, joint pains, abdominal pain, skin rash, swelling in the hands and feet, pyrexia, transient increase/drop in blood pressure, etc. [18, 24, 25].

Taking into account efficiency and reliability above all, current clinical guidelines for the treatment of anemia in dialysis patients recommend ferric gluconate and, particularly, iron sucrose since there have been no registered fatal outcomes until now, and because of rare AE and SAE if compared to other forms of parenteral iron [12, 26].

According to European Renal Best Practice / European Best Practice Guidelines (ERBP/EBPG) recommendations – optimal *iv* dose of iron supplementation in the first 6 months of therapy with ESAs, with iron status and expected increase in hemoglobin regularly checked, can be achieved following the manufacturer's instructions. Thus serum ferritin levels should be maintained between 200 and 500 μg/L and transferrin saturation should be maintained at 30–45% [27].

Ferric gluconate of 62.5–125 mg in 100 mL 0.9% NaCl can be infused over 30 min. or *iv* bolus of 5 mL 0.9% NaCl over 5 min. at the end of 6–8 dialysis sessions, or 2–4 doses

Table 5

Most widely used parenteral iron drugs

Type	Drug	Basic information on supplements			Comments
		Incidence of SAE / 10^6 application, n (%)	Incidence of fatal outcome / 10^6 dosage 100 mg, n (%)	Incidence of AE / 10^6 dosage 100 mg, n (%)	
I	Iron dextran (Dexferrum®) Iron hydroxi dedextran (CosmoFer®)	11.3–57.9 (0.5–1%)	1.4	29.2 (5.4–9.7%)	Mandatory testing before application
	Ferric carboxymaltose (Ferinject®) 2 mL = 100 mg 5 mL = 250 mg 10 mL = 500 mg	3.3		0.9–3.3%	Mandatory testing before application
II	Iron hydroxide sucrose (Venofer®) (Ferrovin®) 2.5 mL = 50 mg 5 mL = 100 mg 10 mL = 200 mg	0.6 (0.0021%)	0	4.2	Possible application in the event of intolerance to drugs of type I/III/IV No need for test dosage before application;
III	Sodium ferric gluconate (Ferrlecit®) 5 mL = 62.5 mg	0.9	0.6	10.5	No need for test dosage before application
IV	Iron sorbitol (Jectofer®) 2 mL = 100 mg (*i.m.* only)				Possible SAE and serious systemic and cardiac disorders; Withdrawn from the European market; Mandatory testing before application

Note: SAE – severe adverse effects; AE – adverse effects; i.m. - intramuscular injection.

in patients treated with peritoneal dialysis, and non-dialysis CRI patients.

Iron sucrose can be applied either as iv bolus dose of 100–200 mg in 5 mL 0.9% NaCl over 2–3 min. at the end of 8–10 dialysis sessions or 1–2 infusions of 500 mg in 250 mL 0.9% NaCl over 15 min. especially in non-hemodialysis patients [29].

Length of iron supplementation is adjustable depending on target outcomes including the assessment of iron stores, current hemoglobin levels and stability of erythropoietin. If during the treatment hemoglobin values exceed 12.0 g/dL, ESAs dose should be reduced according to current recommendations, and *iv* iron therapy should be suspended until the next scheduled assessment of the iron status. Peroral supplements may be prescribed due to patient's intolerance or poor cooperation and such patients can be treated with intermittent infusions of parenteral iron, usually at 1–3 month intervals according to clinical response [29, 30].

Conclusion

Hypochromic anemia is a rare type of iron deficiency which represents important health problem in the world. Insufficient intake of food rich in iron which is suitable for absorption or increased need for iron are the most common causes for sideropenic anemia in general population. In patients with chronic kidney disease, iron deficiency and insufficient erythropoietin synthesis are the most prominent factors for anemia. Restriction on dietary protein (meat), chronic proinflammatory state, reduced absorption of iron (effect of uremic environment and the concomitant use of drugs that hinder iron absorption), permanent dialysis (blood) losses and hemolysis are the basic reasons for absolute or relative iron deficit. The application of erythropoiesis-stimulating agents and iron compensation supplements is the main approach of treatment of iron deficiency in patients with chronic kidney disease. Before starting the treatment it is essential to determine the concentration of serum ferritin and the level of transferrin saturation. This also has to be done periodically during the process and in line with expected response. Peroral iron supplements do not absorb well and have too many side effects, predominantly in digestive tract. That is why the patients loose motivation for this kind of treatment although it is strongly recommended in patients on peritoneal dialysis and those who underwent kidney transplantation. Pareneteral iron supplements are better tolerated, the correction of hemoglobin is faster and thus erythropoiesis-stimulating agents consumption is lower. But there is also a possibility of serious adverse events and potentially life-threatening complications caused by some pharmacological forms. Nevertheless, ferric gluconate and iron sucrose complex are much better tolerated with fewer side effects and less incidence of serious adverse events. That is why these medicines are recommended as a standard in all guidelines for renal anemia treatment.

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CASE REPORTS

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Application of concentrated growth factors in reconstruction of bone defects after removal of large jaw cysts – The two cases report

Upotreba koncentrovanih faktora rasta u rekonstrukciji koštanih defekata nakon uklanjanja velikih viličnih cista

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Abstract

Introduction. Coagulation and blood clot formation in bone defects is sometimes followed by retraction of a blood clot and serum extrusion, thus producing peripheral serum-filled spaces between bony wall and coagulum. This can result in a higher incidence of postoperative complications. Stabilization of blood coagulum, which enables successful primary healing, may be accomplished by autotransplantation, allotransplantation, xenotransplantation, or application of autologous platelet concentrate and concentrated growth factors (CGF). **Case report.** Two patients with large cystic lesions in the upper and lower jaw were presented. In both patients postoperative bony defects were filled with autologous fibrin rich blocks containing CGF. Postoperative course passed uneventfully. **Conclusion.** Application of fibrin rich blocks containing CGF is one of the possible methods for reconstruction of bone defects. CGF can be applied alone or mixed with a bone graft. The method is relatively simple, without risk of transmissible and allergic diseases and economically feasible.

Key words:
jaw cysts; oral surgical procedures; platelet-derived growth factor; transplantation, autologous.

Apstrakt

Uvod. Koagulacija i stvaranje krvnog koaguluma u koštanim defektima posle uklanjanja cističnog sakusa i zatvaranja operativnog polja nekad može biti praćena istiskivanjem seruma, čime se stvaraju periferni, serumom ispunjeni prostori između zidova kosti i površine koaguluma. Ovo može izazvati češću pojavu postoperativnih komplikacija. Radi stabilizacije krvnog koaguluma i očuvanja primarnog zarastanja primenjuje se autotransplantacija, alotransplantacija, ksenotransplantacija, kao i autologni koncentrati trombocita i koncetrovani faktori rasta (KFR). **Prikaz bolesnika.** Prikazana su dva bolesnika sa velikim cističnim lezijama u gornjoj i donjoj vilici. Kod oba bolesnika koštani postoperativni defekt bili su ispunjeni autolognim fibrinskim blokovima bogatim KFR. Postoperativni period protekao je bez komplikacija. **Zaključak.** Upotreba fibrinskih blokova bogatih KFR jedna je od mogućnosti rekonstrukcije koštanih defekata. KFR mogu se koristiti samostalno ili u kombinaciji se nekim od veštačkih zamenika kosti. Metoda je relativno jednostavna, bez opasnosti izazivanja transmisionih i alergijskih bolesti, a ekonomski je isplativa.

Ključne reči:
vilice, ciste; hirurgija, oralna, procedure; faktori rasta, trombocitni; transplantacija, autologna.

Introduction

Bone regeneration processes are highly dependent on the range and extent of the defect, provided that coagulum formation process is not impaired [1]. The average healing time of small cystic defects is usually up to one year, while healing time extends with the size of a defect, ranging from two to five years for medium-size and large cysts [2]. After removal of cystic sac and closing the wound primarily, a bone defect is filled entirely. However, the initial blood clot formation is followed by clot retraction and serum extrusion, thus producing peripheral serum-filled spaces between bony wall and coagulum surface [1, 2]. This significantly interferes with protrusion of vascular epithelium and the healing process. On the other hand, the space formed by the removal of dental cysts usually provides favorable conditions for microbial growth and a risk of infection. Therefore, stabilization of blood coagulum and preservation of primary healing has be-

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en accomplished by several methods, such as autotransplantation, allotransplantation, xenotransplantation, or application of autologous platelet concentrate (APC) and concentrated growth factor (CGF) procedures [3].

Growth factors are proteins, which regulate complex processes during wound healing. Growth factors are mainly located in blood plasma and platelets and play an important role in cell migration, cell proliferation and angiogenesis during regeneration [4]. Most important and representative growth factors are: platelet derived growth factor (PDGF), transforming growth factor (TGF), vascular endothelial growth factor (VEGF), epidermal growth factor (EGF) and insulin like growth factor 1 (IGF 1) [5, 6]. First generation of platelet concentrates, platelet-rich plasma (PRP) [7] was introduced in 1998 and the second, platelet rich fibrin (PRF) [8], in 2000. PRF is a fibrin-rich gel produced with fresh venous blood taken from a patient's vein [8–10]. CGF were first developed by Sacco. CGF show a higher tensile strength, more growth factors, higher viscosity and higher adhesive strength than PRF [11]. The use of autologous fibrin does not cause any side effect and it is a safe and simple procedure for a specialist, and inexpensive and efficacious for the patients [11, 12].

The aim of this report was to describe two patients with large cystic lesions in the upper and lower jaw, respectively, whose bone defects after cystectomy were filled with by autologous fibrin rich blocks containing CGF.

Case report

Case 1

Male patient, aged 54, was presented to the Department of Oral Surgery, Clinic for Dentistry of Vojvodina, with pain and swelling in the anterior lower jaw region. Clinical examination revealed swelling in the lower vestibule, painful and fluctuant to palpation. Mild luxation of the central and lateral incisors was observed (Figure 1). Subsequent to the clinical examination, orthopantomography (OPT) and cone-beam computed tomography (CBCT) scans were taken, showing a 5 cm clearly demarcated oval radiolucency, localized in the anterior region of the lower jaw (Figure 2), and indicating massive bone destruction on the vestibular side of the mandible, with the preserved lingual bony structures.

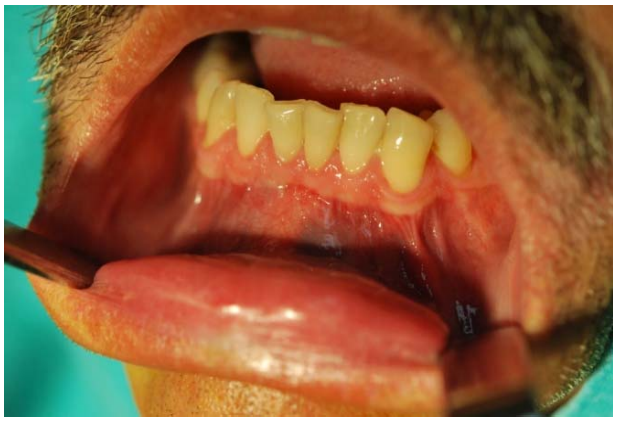

Fig. 1 – Intraoral finding (mild luxation of the central and lateral incisors).

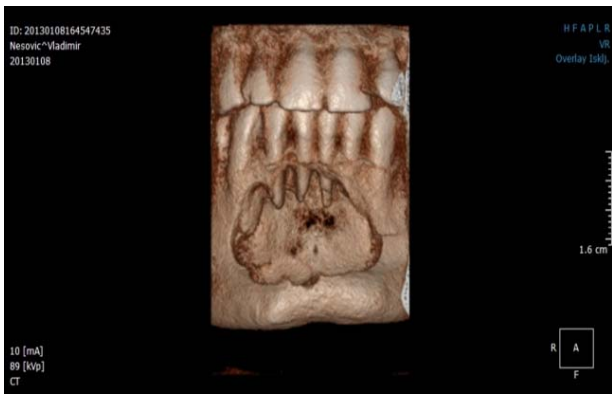

Fig. 2 – Cone-beam computed tomography scan with 3D projection of the mandible.

The planned surgical treatment included immobilization of the affected teeth by orthodontic splint, complete removal of pathological process, resection of the affected teeth and filling the defect with fibrin rich blocs containing CGF.

Fibrin blocks with CGF were made immediately before the surgical procedure from patient's blood sample (total volume 60 mL), which was distributed into six sterile 10 ccm tubes without chemical additives. The tubes were immediately centrifuged for 14 min at 2,500 rpm (Figure 3). After centrifugation, the tubes were left for 20 min until further processing. The upper layer contained the separated serum, fibrin-rich block with CGF was in the middle of the tube, whereas blood corpuscles were precipitated in the bottom layer. Isolated CGF were obtained by pouring off the serum and careful harvesting the middle and lower coagulated layers. In a sterile Petridish, the fibrin block was separated from blood corpuscles with scissors to obtain the pure CGF block, which was ready to be used [12].

Fig. 3 – Tubes after cenrifugation (fibrin blocks with concentrated growth factors).

Subsequent to preparing the CGF block, surgical procedure was performed under local anesthesia. After creating a sulcular flap between both lower second premolars, alternating blunt and sharp dissection was applied to separate and remove the cystic lesion from bone. Following cystectomy, root resection of the affected teeth was done. The resulting bone defect was restored by placing six fibrin-rich blocks with CGF, which completely filled the bone cavity (Figure 4). Finally, the flap was sutured in place with silk sutures.

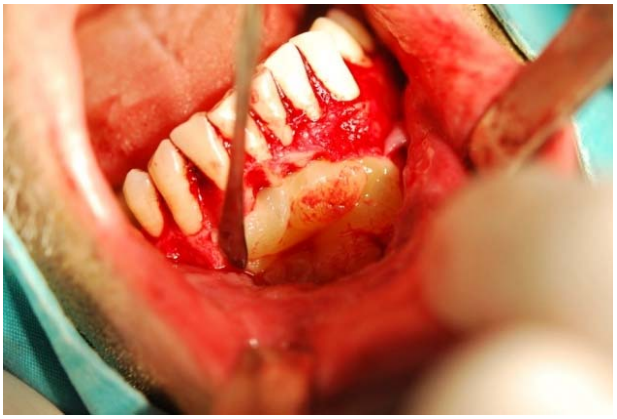

Fig. 4 – Bone defect filled with concentrated growth factors.

After surgery, the patient was instructed about appropriate hygienic-dietary regimen. A combination of clindamycin and metronidazole was prescribed. Postoperative course passed without complications and sutures were removed 7 days later. During the following six months, a uniform and steady filling of the defect by newly formed bone was recorded.

Case 2

A female patient, aged 42, was referred to the Department of Oral Surgery, Clinic of Dentistry of Vojvodina by her polyvalent dentist, for surgical treatment before prosthodontic rehabilitation. Although the patient did not have subjective problems, OPT scan revealed large round radiolucency 4 cm in diameter, extending distally towards the right maxillary sinus and cranially towards hard palate. Clinical examination revealed mild swelling in the anterior upper labial sulcus, insensitive to palpation. The mucosa above the swelling was regular in color and moisture. The teeth 11 to 13 were non-vital. A 3D CBCT scan revealed massive destruction of the upper jaw in the aforementioned region and interrupted communication towards the right maxillary sinus and nasal cavity (Figure 5).

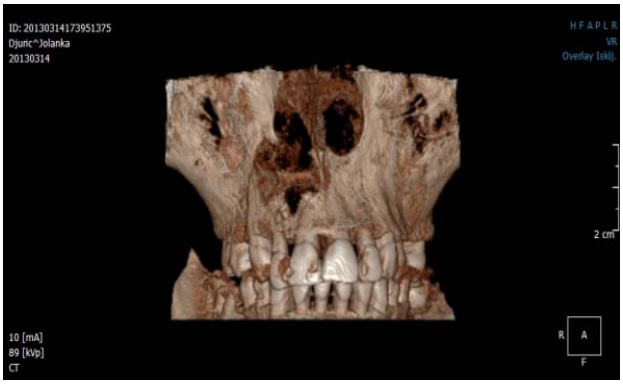

Fig. 5 – Cone-beam computed tomography scan and 3D projection of the maxilla.

After discussing the options of surgical treatment with the patient, who worried a lot about the pathological process and treatment outcome, we decided to extract the affected teeth, completely remove the pathological process applying closed surgery and restore the defect with fibrin-rich blocs with CGF. Fibrin-rich blocks with CGFs were made immediately before surgery as previously described, using five 10 ccm tubes. A full-thickness sulcular mucoperiosteal flap was made from teeth 23–17 on contralateral side in order to ensure better visibility of the operative field. After lifting the mucoperiosteal flap, osteotomy was performed on the frontal side of the maxilla above the pathological change. The pathological change was entirely removed with caution, applying blunt and sharp dissection, completely preserving sinus and nasal mucosa. The resulting bone defect was entirely filled out with CGF blocks and the flap sutured back in place (Figure 6).

The patient was prescribed a combination of clindamycin and metronidazole as the previous patient, and instructed about appropriate hygienic-dietary regimen. Sutures were removed 7 days later. During the following six months, a complete recovery and almost full reconstruction of bone defect was recorded, and the patient underwent prosthetic restoration (Figure 7).

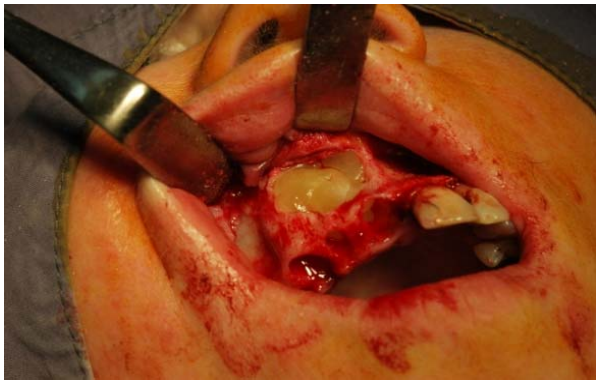

Fig. 6 – Bone defect of the maxilla filled with concentrated growth factors.

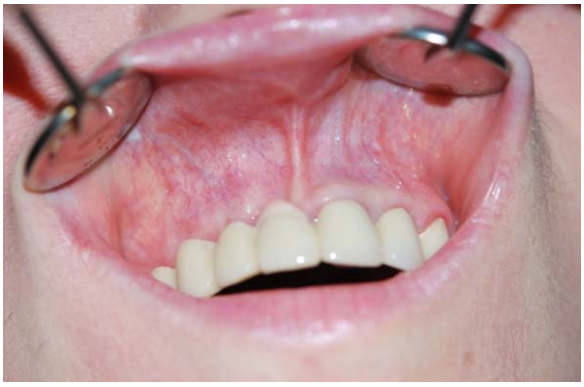

Fig. 7 – The patient after prosthetic rehabilitation.

Discussion

Reconstruction of bony defects after removing large cystic lesions in the upper and lower jaw may, at times, be associated with problems. Contraction of the coagulum, serum extrusion, and formation of dead spaces, as well as a possibility of secondary infection, significantly interfere with reparatory and regenerative processes in the jaws. A number of

authors have addressed this issue, and a range of scientific papers and reports resulted from numerous clinical studies. Modern surgical protocols imply complete removal of cystic lesion, filling the resulting bony defect and primary wound closure. The principal dilemma of the surgeon is a way of bone defect reconstruction. According to the available literature, large bony defects are commonly filled and reconstructed with autotransplants obtained from the iliac ridge, ribs or donor sites in the oral cavity. Application of autotransplants enables primary wound healing, preservation of bone contours and its fast regeneration. However, a drawback of this approach is the need for additional surgical procedure, highly specialized personnel, general anesthesia and very high expenses [2].

Application of growth factors in guided bone regeneration procedure has been well-known for long time. This procedure is of particular relevance to implantology, especially regarding diverse augmentation procedures, unfavorable anatomic conditions (horizontal and vertical augmentation, sinus lift etc.) [5]. CGF can be applied alone or mixed with bone autotransplants or other bone graft substitutes. The aforementioned indications represent small bony defects that can be easily reconstructed. The application of CGF alone in the reconstruction of large cystic defects has not yet been reported. The presented cases are a pioneering attempt of reconstructing and restoring bone defects of the upper and lower jaws thereby avoiding application of synthetic bone substitutes, secondary surgical procedures and chemical additives. The only method that is somewhat comparable with the presented cases is a lateral sinus lift procedure with filling dead spaces between the sinus mucosa and bony palate with pure CGF blocks. The published papers addressing this topic indicate that new bone tissue of satisfactory quality (density) and quantity is formed within 3–6 months, and is associated with minimum postoperative complications. Moreover, the procedure is economically acceptable to the patient [11, 12].

Conclusion

Application of fibrin-rich block with concentrated growth factors is one of the most up-to-date methods for reconstruction of bone defects in the dentoalveolar region. Concentrated growth factors are applicable alone or mixed with a bone graft. The two cases presented in this paper, demonstrate their efficiency in significant shortening of bone-healing time, particularly in massive bone defects, reducing the incidence of postoperative relapse, alleviating the postoperative course and enabling better restitution of surrounding soft tissue structures. Apart from the aforementioned cases, concentrated growth factors are applicable in implantology and periodontology, with the aim of preventing disturbance of the bone and soft tissue architecture. The method is relatively simple, without risk of transmissible and allergic diseases, and economically feasible.

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CASE REPORT

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Treatment of a large radicular cyst – enucleation or decompression?

Lečenje velike radikularne ciste – enukleacija ili dekompresija?

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Abstract

Introduction. Radicular cysts treatment involves surgical approach, more or less aggressive. However, treatment of large cystic lesions, including radicular cysts, causes some of dilemmas concerning the choice of the surgical method, especially the degree of radicalism. **Case report.** We presented a 65-year-old male patient with large radicular cyst in the mandible. A large elliptical multilocular radiolucency, located in the left side of the mandible, being in close vicinity to the mandibular canal, was registered at the orthopantomographic radiography. There was a risk of pathological fracture of the mandible. However, the cyst was completely removed by enucleation without intraoperative and postoperative complications. **Conclusion.** The presented case support the opinion that careful enucleation of large mandibular cysts may be done without complications, such as damages of surrounding anatomical structures or mandibular fracture. The authors indicate reasons for strong support of the undertaken surgical approach of treating large radicular cysts in the mandible.

Key words:
mandible; odontogenic cysts; oral surgical procedures.

Apstrakt

Uvod. Lečenje radikularnih cista podrazumeva hirurški pristup, manje ili više agresivan. Ipak, lečenje velikih cističnih lezija, uključujući i radikularne ciste, stvara nedoumice u izboru hirurške metode, posebno u stepenu radikalizma. **Prikaz slučaja.** Prikazali smo pacijenta, starog 65 godina sa velikom radikularnom cistom donje vilice. Na ortopantomogramu moglo se uočiti veliko elipsasto multilokularno rasvetljenje na levoj strani tela mandibule, u neposrednoj blizini mandibularnog kanala, Postojao je rizik od nastanka patološke frakture vilice. Ipak, cista je u potpunosti uklonjena enukleacijom bez intraoperativnih i postoperativnih komplikacija. **Zaključak.** Prikazani slučaj podržava stav da pažljivo izvršena enukleacija može da se uradi bez nastanka komplikacija, kao što su oštećenje okolnih anatomskih struktura ili prelom vilice. Autori ukazuju na razloge za snažnu podršku preduzetom hirurškom pristupu u lečenju velikih radikularnih cista u donjoj vilici.

Ključne reči:
mandibula; ciste, odontogene; hirurgija, oralna, procedure.

Introduction

The most common cysts of adult jaws are inflammatory (radicular) cysts [1], which occur after tooth pulp necrosis or insufficient root canal treatment. In children, however, non-inflammatory (developmental) cystic lesions are much more frequent than inflammatory ones [2].

Radicular cysts treatment involves more or less aggressive surgical approach. Although enucleation of jaw cysts, the so-called "cystectomy", and primary closure of the defect still represents the "state of the art procedure" [3], enabling spontaneous bone healing [4, 5], more conservative approach, comprising just decompression of the cystic cavity as the primary procedure, has become popular recently, especially in case of large jaw cysts (> 3cm^2) [6, 7].

The aim of this report was to present an adult patient with a large radicular cyst in the mandible, unusually multilocular, being in the close vicinity of the mandibular canal and with a serious risk of pathological jaw fracture, which is completely removed by enucleation without intraoperative and postoperative complications.

Case report

A 65-year-old male patient was admitted to the Department of Oral Surgery, Military Medical Academy in Belgra-

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de, because of expansion of the mandibular buccal cortex on the left side, as well as a large peri-mandibular soft tissue swelling. Intraoral examination revealed gangrenous roots of the left first and second mandibular premolars. On the panoramic radiography, a large elliptical multilocular radiolucency, located in the left side of the mandibular body, could be noticed (Figure 1).

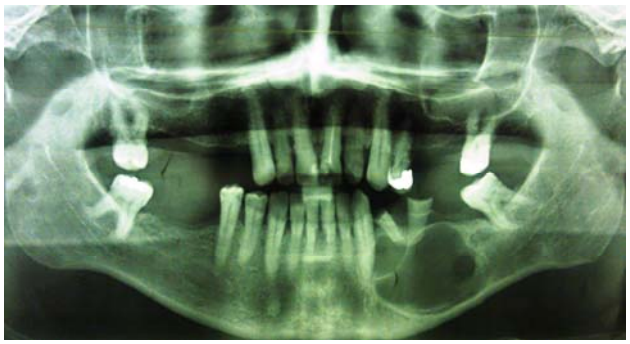

Fig. 1 – Orthopantomographic radiography at the time of examination showing large elliptical multilocular radiolucency located in the left side of the mandibular body.

Due to the existing infection at the time of examination, we made intraoral incision for drainage and commenced with antibiotic therapy (amoxicillin/clavulanate and metronidazole, orally). Seven days later, the symptoms of infection subsided and the patient underwent an incisional biopsy of the lesion under local anesthesia (4% Articain hydrochloride™, 3M ESPE). As the left mandibular canine was nonvital, endodontic treatment was performed. A few days later, a pathological finding confirmed the diagnosis of radicular cyst.

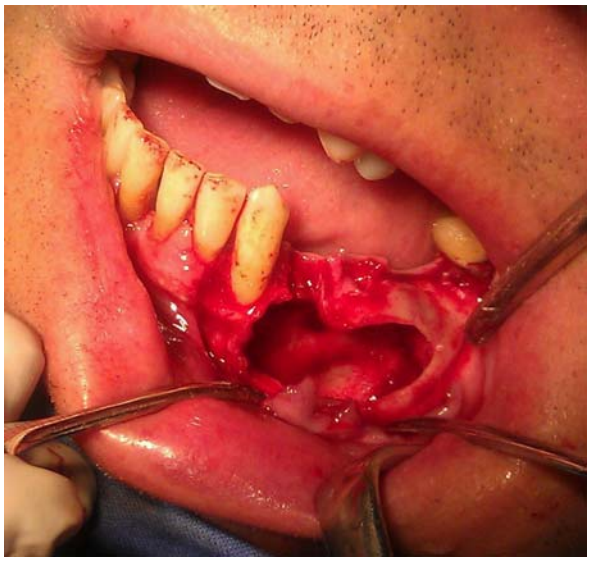

Fig. 2 – Bone defect after complete enucleation of the cystic lesion.

According to the histopathological finding, the patient was advised for surgical treatment (enucleation) of the lesion under local anesthesia. Although radiography pointed out a possible risk of injuring the mandibular canal contents, we decided to enucleate the cyst surgically. The patient was informed about the risks and adopted the surgical plan.

First, we removed gangrenous roots of the left lower premolar teeth. Surgical procedure started with incision along the superior border of the left mandible body, between the left mandibular canine and the second mandibular molar, then around the gingival margin of the left second mandibular incisor, mandibular canine and second mandibular molar, with two relaxing incisions down to the fornix. After uplifting the mucoperiosteal flap, a cortical perforation of the buccal cortex of the mandible body could be seen, as well as the membrane of the radicular cyst. After careful separation of the cystic membrane from surrounding anatomical structures and the mental nerve, lateral transposition of mental nerve was done. Then, the cyst was completely enucleated and the defect rinsed with saline (Figure 2). The filling completely blocked the defect with bone blocks (Osteovit®, B. Braun Melsungen AG, Germany) (Figure 3), and the wound was primarily closed with 4–0 silk sutures.

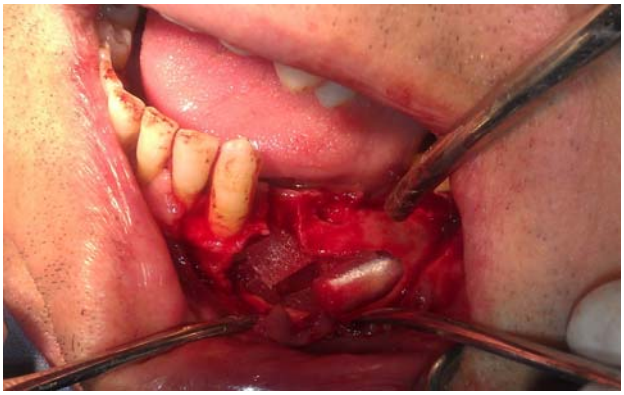

Fig. 3 – Intraoral reconstruction of the mandibular defect with heterogeneous bone blocks.

Postoperatively, the patient received antibiotics intramuscularly (ceftriaxon 2 g once daily) and orally (metronidazole 0.4g × 3 daily) for seven days, when sutures were removed. Paresthesia in the innervation area of the inferior alveolar nerve and mental nerve was present for 15 days after surgery, after which period a normal sensation returned. Other postoperative problems were not noticed. The patient was followed-up regularly, and after a 1-year period, the control panoramic radiography showed almost normal mandibular bone structure at the site of the previous defect (Figure 4).

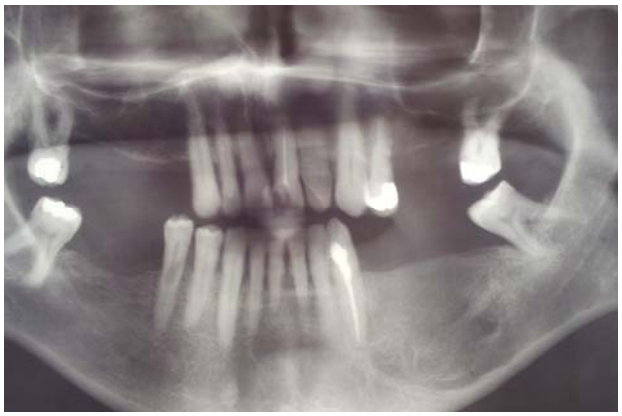

Fig. 4 – Orthopantomographic radiography 1-year after the surgery showing a successful bone healing on the left side of the mandible.

Discussion

Treatment of the large cysts lesions, including radicular cysts, has some of dilemmas concerning the choice of the surgical method, especially the degree of radicalism [8]. Recently, some authors, recommend conservative, less invasive surgical approach even in the treatment of large cysts, especially a method of decompression [6, 7]. The crucial point of this approach is a decrease of the intracystic pressure and decrease the level of inflammatory mediators in the wall of cystic lesion [6]. Decompression includes creation of a window osteotomy; insertion of a stent, its fixation to the bone and/or adjacent soft tissue, and permanent irrigation [6]. Some reported that advantages of this method could be low morbidity and low incidence of complications, protection of adjacent anatomical structures (contents the mandibular canal), keeping mandibular continuity if there is a risk of traumatic fracture of the mandible, and in children or elderly patients [6].

When selecting a method of surgery, one should always assess benefits and risks for any particular patient. However, it seems that the method of decompression has also some disadvantages. A major disadvantage is the fact that pathological tissue of the lesion stays in bone for a long postoperative period, especially if there is a need for subsequent enucleation. Minor disadvantages include dislocation or loss of the stent, the need for permanent irrigation, occlusion of the stent by detritus, discomfort for patients.

Thus, it seems that complete enucleation (cystectomy) of radicular cysts is still "state of the art procedure" [3]. Additionally, we filled the defect with bone substitute, although it is not warrant, as several studies have reported safe and regular bone healing after enucleation and simple closure of jaw cysts without using bone grafts even in cases of large defects [4, 5]. It seemed to us that the use of bone substitute will additionally decrease the possibility of local complications due to shrinkage of blood clot postoperatively.

Although positive clinical results with the use of decompression are recently reported, we believe that it cannot routinely be a definitive method of treatment, especially in cases of large radicular cysts. In the presented case, operated on by cystectomy, damages of vital neighboring anatomical elements, and pathological fracture of mandible, were not noticed. Also, we did not see any symptom of infection postoperatively. Accordingly, the presented case supports the opinion that careful enucleation of large mandibular cysts may be done without complications. Moreover, this approach significantly lowers postoperative discomfort for the patient, including needs for frequent and long-standing postoperative follow-ups, and enables early functional rehabilitation of the patient. In other words, enucleation (immediate and complete removal of pathological tissue) as a surgical approach should be the preferred method of treating large radicular cysts. The only exception could be in younger patients, with the aim of protecting unerupted permanent teeth.

Conclusion

The presented case supports the opinion that careful enucleation of large mandibular cysts may be done without complications, such as damages of surrounding anatomical structures or mandibular fracture. The authors indicate reasons for strong support of the undertaken surgical approach of treating large radicular cysts in the mandible.

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CASE REPORT

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Radiofrequency ablation of anteroseptal accessory pathway – A challenge to the electrophysiologist

Radiofrekventna ablacija anteroseptalnog aksesornog puta – izazov za elektrofiziologa

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Abstract

Introduction. Anteroseptal accessory pathways (APs) are located in the apex of the triangle of Koch's connecting the atrial and ventricular septum in the region of the His bundle. Ablation of anteroseptal pathway locations remains a challenge to the electrophysiologist due to a very high risk of transiet or permanent atrioventricular (AV) block. **Case report.** A male, 18-year-old, patient was hospitalized due to radiofrequency (RF) ablation of APs. He was an active football player with frequent palpitations during efforts accompanied by dyspnea and lightheadedness, but without syncope. Electrocardiography on admission showed intermittent preexcitations. Intracardiac mapping showed the earliest ventricular activation that preceded surface electrocardiographic delta wave in anteroseptal region very close to the AV node and His bundle. Using a long vascular sheath for stabilization of the catheter tip, RF energy was delivered at the target site starting at very low energy levels and because of the absence of either PR prolongation, as well as accelerated junctional rhythm during the first 15 sec, the power was gradually increased to 40W, so after application RF energy preexcitation was not registered. **Conclusion.** Despite this proximity to the His bundle and very high risk of transiet or permanent AV block anteroseptal APs can still be ablated successfully.

Key words:
heart conduction system; arrhythmias, cardiac; catheter ablation; treatment outcome.

Apstrakt

Uvod. Anteroseptalni aksesorni put nalazi se u vrhu Koch-ovog trougla i spaja pretkomorski i komorski septum u predelu Hisovog snopa. Ablacija u ovoj regiji predstavlja izazov za elektrofiziologa zbog visokog rizika od privremenog ili stalnog atrioventrikularnog (AV) bloka. **Prikaz bolesnika.** Bolesnik star 18 godina, hospitalizovan je zbog radiofrekventne (RF) ablacije aksesornog puta. Aktivno se bavio fudbalom. Žalio se na povremene palpitacije u toku napora, praćene osećajem kratkoga daha i omaglicama, ali bez sinkope. U elektrokardiogramu na prijemu registrovana je povremena preekscitacija. Intrakardijalni maping pokazao je najraniju komorsku aktivaciju u anteroseptalnoj regiji, neposredno uz AV čvor, odnosno Hisov snop. Upotrebom dugog vaskularnog uvodnika isporučena je RF energija niske snage i zbog odsustva produženja PR intervala odnosno nodalnog ritma tokom 15 sekundi, snaga je postepeno pojačana na 40 W, nakon čega nije registrovana preekscitacija. **Zaključak.** Uprkos blizine Hisovog snopa i visokog rizika privremenog ili trajnog AV bloka, ablacija anteroseptalnog aksesornog puta može biti uspešno urađena, ali zahteva veliku opreznost u toku procedure.

Ključne reči:
srce, provodni sistem; aritmija; ablacija preko katetera; lečenje, ishod.

Introduction

Anteroseptal accessory pathways (APs) are thin fibers composed of typical myocardial cells that allow electrical communication between atrium and ventricle. Symptoms may range from none to occasional or severe palpitations accompanied by dyspnea, chest discomfort, lightheadedness and even syncope or cardiac arrest due to rapidly conducted atrial fibrillation.

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Anteroseptal APs comprise 6% to 7% of all APs and about 80% of these APs exhibit anterograde conduction while 20% are only retrograde conducting ("concealed") [1].

Ablation of anteroseptal pathway locations remains a challenge because of the proximity to the normal cardiac conduction system [atrioventricular (AV) node and His bundle]. Inadvertent injury to these structures resulting in the need for permanent pacing.

Herein we reported a young male in whom successful radiofrequencz (RF) ablation of APs was performed.

Case report

A male, 18-year-old, patient was hospitalized due to frequent palpitations during efforts accompanied by dyspnea and lightheadedness, but without syncope. He used to be active football player. Electrocardiography (ECG) on admission showed intermittent preexcitation referring to anteroseptal accessory pathways (Figure 1).

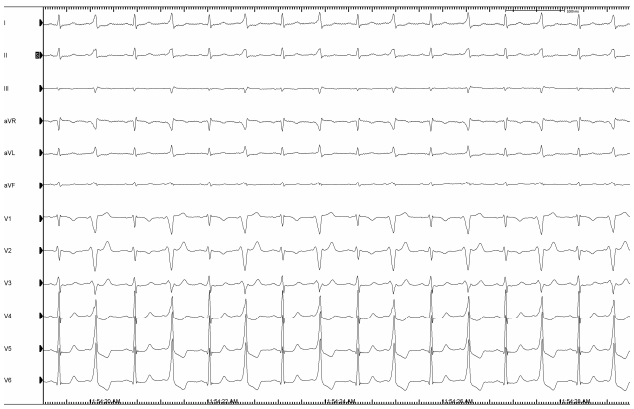

Fig. 1 – Electrocardiography (ECG) on admission showed intermittent positive delta waves in the inferior leads (DII, DIII, and aVF) and the precordial leads (V1 through V6) as well as negative delta waves in aVR.

According to basal ECG, intracardiac mapping expectedly showed the earliest ventricular activation that preceded surface ECG delta wave in anteroseptal region very close to the AV node and His bundle (preceded by 42 msec). It was also recorded incorporating atrial-AP-ventricular components as well as sharp QS deflection on the unipolar electrogram of the ablation electrode. Intracardiac recording in the periods without manifest preexcitation presented a sharp potential between atrial and ventricular electrograms referring to His deflection (Figure 2). Fluoroscopically, it was expected very close to the His bundle recording site (Figure 3).

Using a long vascular sheath for stabilization of the catheter tip, RF energy was delivered at the target site starting at very low energy levels (10 W) and because of the absence of either PR prolongation, as well as accelerated junctional rhythm during the first 15 sec, the power was gradually increased to 30 W, with the target temperature of 40° for 60 sec. During RF delivery preexcitation can be lost, but unfortunately after it was again returned. Another aplication with same characteristics was repeated, but without prolonged effects. We decided to increase the power to 40 W, so after RF energy application for 60 sec preexcitation was not registered (Figure 4). During RF delivery, the impedance was continuously monitored, and it was stable.

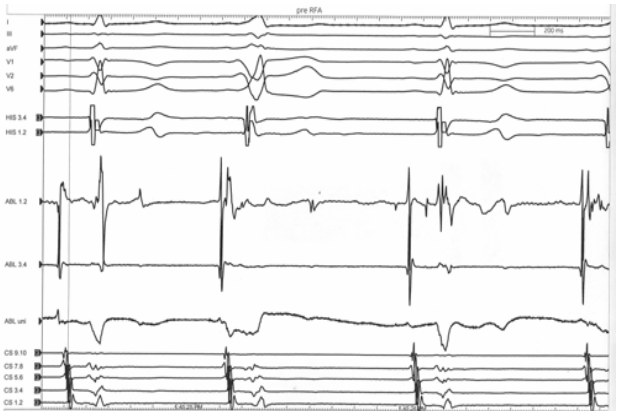

Fig. 2 – The earliest ventricular activation was close to the atrioventricular node and His bundle with continuous recording of atrial-accessory pathway-ventricular components, as well as sharp QS deflection on the unipolar electrogram during preexcitation, and in the periods without preexcitation was presented the sharp potential between atrial and ventricular electrograms referring to His deflection.

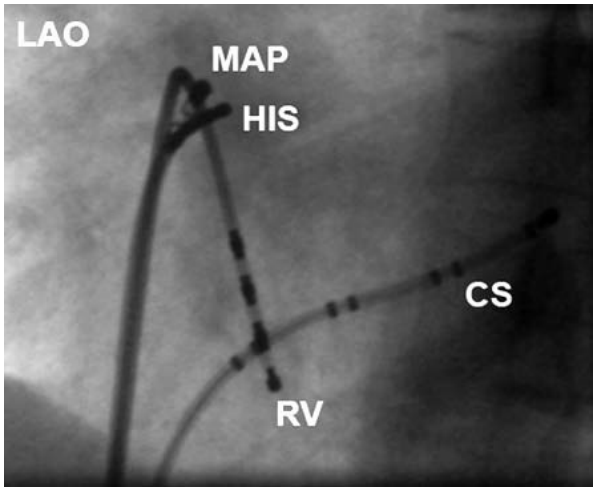

Fig. 3 – Position for ablation was expected very close to the His bundle recording site in the left anterior oblique views.

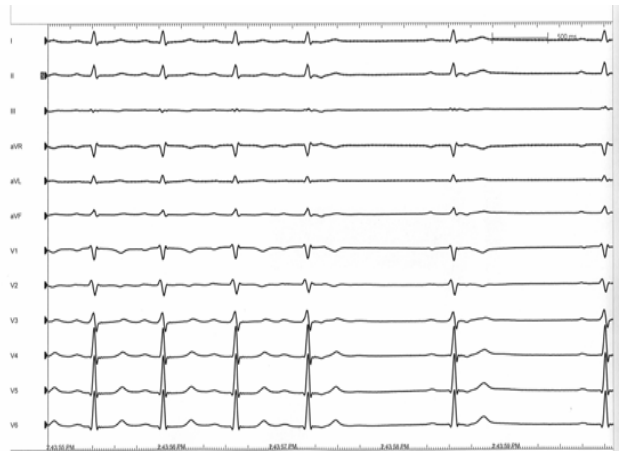

Fig. 4 – Electrocardiogram showing no signs of preexcitation after adenosine application.

Discussion

Anteroseptal APs are located in the apex of the triangle of Koch connecting the atrial and ventricular septum in the region of the His bundle.

Ablation of anteroseptal AP remains a challenge to the electrophysiologist due to a very high risk of transient or permanent AV block [1]. Studies assessing RF ablation of anteroseptal APs in children and adults report primary success rates > 90%, recurrence rates of 12–25% and risk for inadvertent AV block of 2–10% [2, 3]. It is recommended delivering as less as possible applications of RF energy to minimize the risk of damage as much as possible [1]. However, this is associated with a greater incidence of recurrences of arrhythmia [4, 5]. Accordingly, after successful ablation, patients were observed for a 30-min waiting period. The possibility of recurrence was assessed using pacing maneuvers, as well as orciprenaline or adenosine.

The target site of RF energy aplication in the presented patient was the leading one for the recommended electrophysiological characteristics: earliest ventricular activation that precede the surface ECG delta wave was 42 millisec, sharp QS deflection on the unipolar electrogram of the ablation electrode and incorporating atrial-AP-ventricular components during preexcitation [1].

During mapping this area in the presented patient, catheter was gently moved due to possible mechanical block of pathway conduction, significant for the anteroseptal APs, which was not registered [6]. The long vascular sheath helped in stabilizing the catheter tip to the target position, preventing movement of the catheter and potential very serious complications. The internal jugular vein approach may allow reliable catheter stability, especially if the attention payed to ablate from the ventricular aspect of the tricuspid annulus. It would be our next option if either PR prolongation as well as accelerated junctional rhythm occurred [1]. Although it is recommended that the APs can be safely ablated if the His deflection is less than 0.2 mV in amplitude, in the presented patient it was significantly higher referring to high risk of transiet or permanent AV block [1]. According to this, at first low power of RF energy was delivered with gradually increased level, so damage of normal cardiac conduction system was not recorded, as well as change of impedance, because the drop in impedance is a better indicator of tissue temperature than is the tip electrode temperature [1]. It is recommended that unsuccessful energy applications should be stopped after no more than 15 sec, because of possible AV node or His bundle damage.

Anteroseptal APs could be ablated over the left ventricular outflow tract and the non-coronary sinus (NCC) of the aortic valve [7, 8]. This is required in rare cases, if the catheter ablation from the right atrium fails. The left side of the anteroseptal region is a membranous structure and defined by the aortic annulus and NCC is directly related to the septum. Location and dynamic motion of the NCC leads to difficulties of access as well as keeping a stable position of the catheter, so in one case, transesophageal echocardiogram was used for assistance [9]. However, intracardiac echocardiography could be a better and more advantageous tool for precise imaging of anatomical structures and guide to successfully manage the position of the catheter tip at NCC of the aortic valve [10].

Catheter ablation based on electroanatomical mapping and contact force technology appears to be an effective treatment modality for patients with pathways close to the AV junction. Namely, the use of three-dimensional mapping systems may be helpful to denote the target sites for ablation, as well as due to the increased precision of point applications. The contact between the tip electrode of the ablation catheter and the myocardial tissue affects both the accuracy of maps and the efficacy of energy delivery [11].

Cryothermal ablation may be an alternative to RF ablation to reduces the risk of permanent block in septal arrhythmia substrates [12–16]. Due to its safety profile, cryoablation is used increasingly in pediatric patients. The advantages of cryothermal energy is reversibility of lesions during cryomapping and increased catheter stability [12–16]. The target was usually identified using a steerable quadripolar electrophysiology catheter, marked as a point on the three-dimensional mapping system. The mapping catheter was then exchanged for a cryoablation catheter with limited maneuverability, especially in younger children. Cryomapping usually was performed at -30°C at the previously marked location. If AP block was achieved, ablation was continued for 240–360 ms at -70°C to -80°C to achieve the freeze effect [12–16].

Conclusion

Our experience in the ablation of anteroseptal accessory pathways shows that despite the proximity to the His bundle and very high risk of transiet or permanent atrioventricular block, these accessory pathways still can be ablated successfully, but it requires great carefulness.

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CASE REPORT

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An experience with colistin applied in treatment of imunocompromised patients with peritonitis on peritoneal dialysis

Iskustvo sa primenom kolistina u lečenju peritonitisa kod imunokompromitovanih bolesnika na peritoneumskoj dijalizi

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Abstract

Introduction. Immunocompromised patients, such as those with multiple myeloma on peritoneal dialysis, are particularly susceptible to the occurrence of peritonitis. **Case report.** We presented a 56-year-old female patient with a 10-year history of multiple myeloma. The patient was on peritoneal dialysis since 2010. During 2012 the patient had the first episode of peritonitis that was successfully managed, but in 2013 the second episode of peritonitis occured. Analysis of dialysate culture and exit site swab revealed the presence of multiresistant *Acinetobacter* spp., which was susceptible only to colistin. Prompt colistin therapy was administered at the doses of 100,000 units/day during six days, which resulted in complete recovery of the patient, as well as improvement of local abdominal findings. Gram-negative bacteria (genus *Acinetobacter*) are common causative agents in hospital-acquired infections. Studies confirmed susceptibility of *Acinetobacter* to colistin, which was also the case with the presented patient. Intravenous administration of colistin resulted in a complete remission of this severe, life-threatening peritonitis. **Conclusion**. Patients with multiple myeloma and renal failure are highly prone to severe life-threatening infections.

Key words:
multiple myeloma; peritoneal dialysis; peritonitis; colistin.

Apstrakt

Uvod. Imunokompromitovani bolesnici, kao što su bolesnici sa multiplim mijelomom na peritoneumskoj dijalizi, posebno su skoloni nastanku peritonitisa. **Prikaz bolesnika**. Prikazali smo bolesnicu, staru 56 godina, koja je bolovala od multiplog mijeloma 10 godina. Od 2010. god. lečila se metodom peritoneumske dijalize. Prvu epizodu peritonitisa koja je uspešno sanirana, imala je tokom 2012. U 2013. ponovo je došlo do razvoja peritonitisa. Analizom kulture dijalizata i brisom izlaznog mesta peritoneumskog katetera izolovan je multirezistentni *Acinetrobacter* spp, osetljiv samo na kolistin. Primenom kolistina u dozi od 100 000 jedinica/dan tokom šest dana došlo je do potpunog poboljšanja opšteg stanja bolesnice, kao i lokalnog nalaza na trbuhu. Gram-negativna bakterija (*Acinetobacter* spp) čest je uzročnik hospitalnih infekcija. Istraživanja potvrđuju osetljivost *Acinetobacter* spp. na kolistin, što je slučaj kod prikazane bolesnice. Intravenska primena kolistina rezultovala je potpunim oporavkom od teškog, po život opasnog, peritonitisa. **Zaključak.** Bolesnici sa multiplim mijelomom i bubrežnom insuficijencijom imaju povećanu sklonost ka nastanku teških, po život opasnih, infekcija.

Ključne reči:
multipli mijelom; dijaliza, peritoneumska; peritonitis; kolistin.

Introduction

Multiple myeloma is a progressive malignant disease of plasma cells featuring the production of pathological paraproteins. Multiple myeloma cells come from pathologically altered plasma cells, which produce abnormal amounts of paraproteins. Accumulated paraproteins interfere with the production of normal antibodies in the bone marrow, and activation of natural killer cells stimulates increased osteoclast recruitment and activity finally resulting in bone defects. The process of remodelling and destruction of bone tissue leads to the release of calcium into blood, which can cause severe

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kidney disorders [1]. High amounts of paraproteins are associated with a significant increase in total blood protein levels resulting in renal function disorders in some 50% of patients. Light chains and amyloid frequently cause chronic renal failure rather than the acute one, thus indicating administration of renal replacement therapy, i.e. hemodialysis or peritoneal dialysis. Renal failure secondary to myeloma carries poor prognosis to patients. Such patients are highly prone to severe, mostly lethal infectious complications induced by Gram-negative bacteria [2, 3].

Case report

A 56-year-old female patient had a 10-year history of multiple myeloma. In 2005, end-stage chronic renal failure was confirmed, so the patient was subjected to the chronic hemodialysis (HD) program. In 2010, because of the exhaustion of vascular access, the patient underwent laparoscopic placement of a peritoneal dialysis catheter (PD) catheter [4] and was transferred to continuous ambulatory peritoneal dialysis (PD-CAPD) therapy. After the initiation of PD, the patient exhibited moderate umbilical hernia. During 2012, the patient had the first peritonitis episode with severe clinical presentation, which was successfully managed. The patient was in complete remission until February 2013, when headaches, swelling and redness in the right eye and spinal column occurred. Relevant diagnostic procedures (endocranial magnetic resonance imaging, sternal puncture, serum immunoelectrophoresis test) revealed the relapse of underlying disease, manifested as the extranodal retrobulbar mass in the right eye accompanied by skull bone infiltration (Figures 1a, b). Corticosteroid therapy was administered according to the

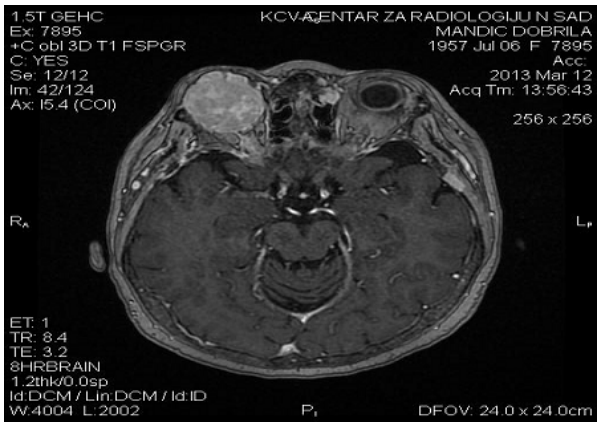

Fig. 1a – Magnetic resonance of the head – retrobulbar localization of right eye multiple myeloma.

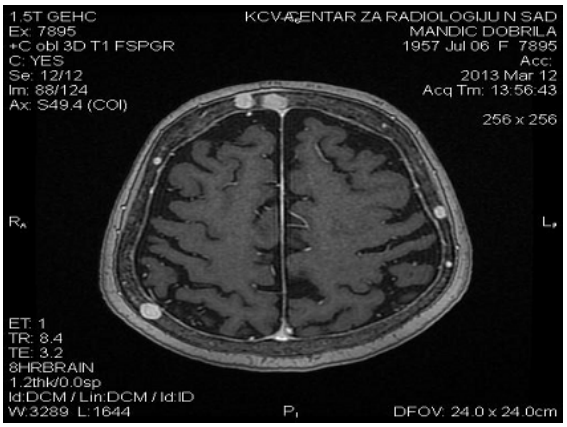

Fig. 1b – Magnetic resonance of the head – multiple myeloma infiltrates in the skull bones.

relevant protocol (40 mg/day *iv*, 1–4; 9–12; 17–20 days). After completing the therapy, abdominal pain, dialysate turbidity and peritonitis were observed and designated as a second peritonitis episode. Laboratory analysis revealed elevated levels of procalcitonin (PCT) in the serum (18.39 ng/mL) and in dialysate (4.65 ng/mL), serum C-reactive protein (CRP) (186.3 mg/L) and leukocyte count in dialysate (4850 × 10–6/L). Bacteriological examination of dialysate and the catheter exit point was in progress. Empiric antimicrobial therapy was initiated – at first III and IV generation cephalosporins, subsequently after 48 hours vancomycin and aminoglycosides and antianaerobic antibiotics, but no clinical improvement was observed. During further course of the disease, the patient developed phlegmon of the anterior abdominal wall in the region of the umbilical hernia and her overall condition worsened. The patient was examined several times by the abdominal surgeon, and computed tomography of the abdomen was performed confirming the existence of phlegmon without signs of incarcerated hernia indicating continuation of conservative therapy. After 72 hours, multiresistant *Acinetobacter* spp., which was susceptible only to colistin, was isolated from dialysate culture and exit site swab. Considering highly severe clinical status of the patient, peritoneal catheter was removed and the patient transferred to HD by placing a double-lumen catheter into the right jugular vein. Prompt colistin intravenous therapy was administered at the doses of 100,000 units/day i.v. during six days which resulted in rapid improvement of general condition of the patient as well as of local abdominal finding (Figure 2a, b) and laboratory results (PCT 0.91 ng/mL, CRP 19 mg/L). Further therapy of relapsed underlying disease was planned and continued by the hematologist.

Discussion

Peritonitis is the most common, most severe and most dangerous complication in PD patients. Most frequent causative agents are Gram-positive bacteria (*Staphylococcus spp*, *Staphylococcus aureus*, *Enterococcus*). Peritoneal dialysis-associated peritonitis is mostly characterized by milder clinical manifestations as compared to the postoperative peritonitis and responds well to outpatient treatment. Peritonitis caused by Gram-negative bacteria is rare, and may result from touch contamination of dialysis system, exit site or tunnel infection, possible inflammatory process in the abdomen, constipation, etc. Most common pathogens include *Escherichia coli*, *Klebsiella, Proteus*, more rarely *Campylobacter*, *Citrobacter*, *Acinetobacter* etc. Gram-negative peritonitis is particularly troublesome and severe resulting mostly in technique failure, catheter removal or even lethal out-

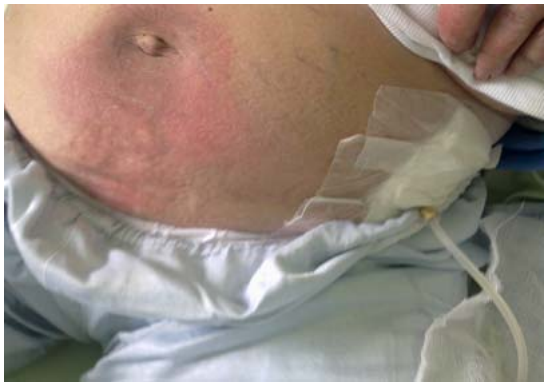

Fig. 2a – Phlegmon of the front abdominal wall before the therapy.

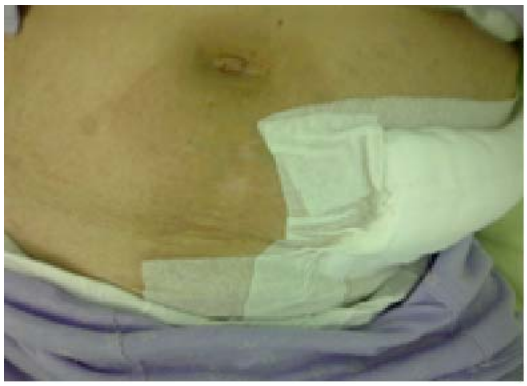

Fig. 2b – The front abdominal wall after the therapy.

come [5, 6]. Immunocompromised patients are particularly susceptible. Multiple myeloma is a disease characterised by a high incidence of severe complications, especially when renal function is disturbed and patients are subjected to any of dialysis methods (HD or PD) [7, 8]. Survival rates in such patients after 1- and 3-year therapy range from 50% and 25%, respectively [9]. Peritoneal dialysis is not the preferred program in patients with multiple myeloma and end-stage chronic renal failure, and only few cases were reported from dialysis centres in North America and Europe [2–4]. Between 1998 and 2013, three patients with multiple myeloma underwent PD at our Center [10]. Extramedular localizations of multiple myeloma are uncommon and rare cases have been described in the lungs, the larynx, skull bones and the bladder. We presented a rare case of retrobulbar localization in the right eye and skull bone infiltration. Gram-negative bacteria are common causative agents in hospital-acquired infections [11]. Bacterial species of the genus *Acinetobacter*, which may be seen as a part of normal bioflora of the human skin, mucosa and secretions is of particular importance. It is responsible for severe nosocomial infections, particularly in immunocompromised patients. The organism is especially difficult to treat because of its ability to survive and persist in hospital environment, as well as its resistance to the broad variety of antibiotics [12–14]. The presented patient was immunocompromised due to the underlying disease, particularly after receiving corticosteroid therapy and subsequent peritonitis episodes. Initial empiric antimicrobial treatment of peritonitis (cephalosporins, vancomycin, aminoglycosides) revealed no clinical improvement, since the isolated *Acinetobacter* organism was resistant to all tested antibiotics, except colistin [8, 15]. This caused serious problems in clinical practise and further peritonitis complication manifested as phlegmon in the front abdominal wall. The increased incidence of multiresistant strains of *Acinetobacter* has been reported in Serbia as well as in Italy, Greece, Slovakia, China and USA [16, 17]. Considering the multiple resistance to the range of antimicrobials, carbapenems are the drug of choice in the therapy of such infections; however, the increasing resistance to this class of antibiotics is evident. Studies conducted in Bulgaria and Korea confirmed susceptibility of *Acinetobacter* to colistin, which was also the case in the presented patient [17]. The drug is highly toxic and thus rarely administered at clinics. However, in the presented patient, intravenous administration of colistin at the doses adjusted to the rate of renal failure resulted in complete remission of this severe, life-threatening peritonitis without any adverse effects. Such severe episodes of Gram-negative peritonitis in PD patients often lead to removal of PD catheter and transfer to HD therapy, which was the case in the presented patient [18]. After complete recovery from inflammation, the treatment of the relapsed underlying disease was planned [19] taking into consideration that such cases of multiple myeloma accompanied with renal failure and extramedular localization have very poor prognosis and severe disease course.

Conclusion

Patients with multiple myeloma and renal failure are highly prone to severe life-threatening infections. Immunocompromised patients on peritoneal dialysis often develop peritonitis associated with multiresistant bacteria (*Acinetobacter* spp.), which is a highly severe complication of peritoneal dialysis, and only the prompt diagnosis and immediate administration of an appropriate therapy are the prerequisites for a positive outcome of such conditions.

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CASE REPORT

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Management of myelofibrosis during pregnancy: A case report

Lečenje mijelofibroze tokom trudnoće

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Abstract

Introduction. Primary myelofibrosis (PMF) is a clonal myeloproliferative neoplasm that occurs most commonly in the decade six of life and it is very rare in the young persons. **Case report.** We reported a 28-year-old female patient with primary myelofibrosis who had a normal pregnancy and delivery in the week 40 of pregnancy without any complications. Two years before the diagnosis of PMF she had normal pregnancy. The patient was treated with interferon-alpha and low dose aspirin during the whole pregnancy and with low-molecular-weight heparin a week before delivery and 6 weeks after. The patient had no complications during pregnancy. She delivered in term with healthy, normal baby weight. **Conclusion.** Decision about treatment strategy of pregnancy associated hematologic malignancies should be made for each patient individually.

Key words:
myeloproliferative disorders; primary myelofibrosis; pregnancy; interferon-alpha; treatment outcome.

Apstrakt

Uvod. Primarna mijelofibroza je klonsko mijeloproliferativno oboljenje koje se najčešće javlja u šestoj deceniji života, a vrlo retko kod mladih osoba. **Prikaz bolesnika.** Prikazana je 28-godišnja bolesnica sa primarnom mijelofibrozom, koja je imala normalnu trudnoću i porođaj u četrdesetoj nedelji trudnoće bez komplikacija. Tokom cele trudnoće bolesnica je lečena interferonom alfa i niskim dozama aspirina, a nedelju dana pre i 6 nedelja nakon porođaja primenjen je i niskomolekularni heparin u profilaktičkim dozama. Bolesnica nije imala komplikacije tokom trudnoće i rodila je zdravu bebu normalne težine. **Zaključak.** Odluka o strategiji lečenja trudnica sa prisutnim hematološkim malignitetima treba da bude doneta posebno za svaku bolesnicu.

Ključne reči:
mijeloproliferativni poremećaji; primarna mijelofibroza; trudnoća; interferon-alfa; lečenje, ishod.

Introduction

Primary myelofibrosis (PMF) is a clonal myeloproliferative neoplasm chacterized by a proliferation of megakariocytes and granulocytes in the bone marrow, associated with reactive deposition of fibrous connective tissue and with extramedullary hematopoises [1, 2]. It occurs most commonly in the decade six of life and it is very rare in the young ones [1, 2]. Pregnancy is a high-risk event in women with thrombocytosis, especially in patients with essential thrombocythemia and PMF. The risk of spontaneous abortion is 2.5-fold higher than in the control population, while the incidence of maternal complications is lower, 3% for major thromboembolic and 2% for major bleeding event [3]. We reported a 28-year-old female patient who had a normal pregnancy and delivery, treated with interferon-alpha, low-dose aspirin and low-molecular-weight heparin (LMWH).

Case report

A 28-year-old woman was sent to the hematologist in November 2011, due to asymptomatic thrombocytosis (platelet count 1,040 × 10^9/L). Her previous medical history was unremarkable, excluding conization of uterine cervix because of cervical intraepithelial neoplasia diagnosed in 2007. Two years before admission she had a normal pregnancy and delivery. Physical examination did not show peripheral lymphadenopathy, hepatosplenomegaly or signs of skin and mu-

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cosal bleeding. Laboratory findings (sedimentation rate, biochemistry and hemostatic findings) were normal. Except for elevated platelet count, the rest of the full blood count was within normal limits. Chest radiology was normal, too. Ultrasonography of the upper abdomen showed slightly enlarged spleen (130 × 60 mm in diameter). The bone marrow trephine biopsy showed 60% bone marrow cellularity with moderate proliferation of megakariocytes, which were mostly enlarged, hyperlobulated, polymorphic, forming clusters of variable size. Reticular fibrosis was moderate, gradus I. Kariotype was normal, 46-XX. Janus kinase 2 (JAK2) (V617F) mutation was not identified, as well as bcr-abl rearrangement. Pattern of *in vitro* growth of hematopoietic progenitors from bone marrow and peripheral blood did not speak in favor of the myeloproliferativne disease, so the revision of pathological findings of the marow trephine biopsy was done in the University of Cardif, Wales. These findings confirmed the diagnosis of prefibrotic phase of primary myelofibrosis (MF-1) with no evidence of CD34/CD117 blasts. Considering low International Prognostic Scoring System and Dynamic International Prognostic Scoring System (both 0) we decided to follow the patient with low-dose aspirin as the only treatment.

Soon after the diagnosis of PMF, the patient became pregnant. Interferon alpha therapy (3 MIU, 3 times a week) was given immediately, together with low-dose aspirin. After three weeks of therapy platelet count was reduced to normal value and sustained within normal limits during the whole pregnancy. Two weeks before delivery low-dose aspirin was stopped and LMWH was given before delivery and 6 weeks after. Fetal growth and placental circulation were monitored frequently and were normal all the time of pregnancy. The delivery was spontaneous, without complications and with normal baby weight. Stem cells from umbilical cord are saved for eventual stem cell transplantation. After six weeks, interferon therapy was stopped. One year after delivery, the platelet count of the presented patient was about 750×10^9/L. The treated with low-dose aspirin only was continued. In view of planning further therapy and possible need for allogeneic stem cell transplantation, HLA typing for her and her closest relatives (brother and sister) was done. Unfortunately, compatible donor was not found.

Discussion

PMF is at least prevalent of all myeloproliferative neoplasms in women of child bearing age – the prevalence is 0.023–0.06/100.000 in this age-group [4]. There are only a few literature data about pregnancy, complications in pregnancy, recommended treatment and delivery in these patients. Taylor et al. [5] and Gotić et al. [6] described one patient each with previous thrombosis and fetal loss with one successful pregnancy and child birth, while Tulpule et al. [7] in 2008 described two patients with 4 pregnancies. The first patient had no previous history of thrombosis and was treated with low-dose aspirin only and despite the complication (disseminated TBC infection) had the full-term normal delivery. The second woman had a history of previous thrombosis, so she was treated with LMWH and low-dose aspirin during the whole pregnancy. Despite the therapy, she had two fetal losses and one full-term normal delivery. Later on, it was shown that pregnancy in patients with chronic myeloproliferative disease has many risks [8–11], particularly increased risk for thrombosis. Harrison [12] shows that such risk is similar to risk in patients with thrombophilia and antiphospholipid syndrome. The most frequent complication in pregnant women with Philadelphia negative myeloproliferative neoplasms is abortion, while other maternal complications are relatively low with 3% for major thromboembolic and 2% for major bleeding events [3]. The presence of JAK2 mutation seemed to be an independent predictor of pregnancy complication [3]. This study also improved benefit from an intensive therapy including interferon-alpha with (out) LMWH throughout pregnancy and at least for six weeks after delivery. Fetal safety of interferon-alpha was also confirmed in the study of Yazdani Brojeni et al. [10]. Their results suggest that interferon-alpha have a protective effect against pregnancy loss and does not significantly increase the risk of major malformations, miscarriage, stillbirth and preterm delivery above general population rates [10].

Having in mind all these studies we decided to treat the presented patient with interferon-alpha and low dose-aspirin during the whole pregnancy and with LMWH two weeks before and six weeks after the delivery. We suggest such therapeutic approach as the best for patients with PMF, although the nature of the disease itself (low DIPSS score and negative JAK2 mutation) maybe contributed to good outcome of pregnancy.

The question of further treatment of our young PMF patient still remains. HLA typing of her sister and brother have been done, but no HLA-matched sibling donor was found. The only curative treatment of PMF is allogeneic hematopoietic stem cell transplantation [13]. According to French authors factors affecting favorable engraftment are splenectomy before HSCT, HLA-matched sibling donor, peripheral blood use as a source of stem cells and the absence of pre-transplant thrombocytopenia [13]. Tefferi [2] modified risk stratification of patients with PMF for further management that could be useful for patients like the presented. However, having in mind that the presented patient is still very young (at this moment 30 years) and in excellent condition with low International Prognostic Scoring System, the risk of allogeneic hematopoietic stem-cell transplantation in the light of the lack of family matched donor remain significant, particularly in innovative drug era. New drugs such as JAK2 inhibitors, mTOR (target of rapamycin) inhibitors, histone deacetylase inhibitors and pomalidomide show encouraging results in treatment of patients with PMF [14, 15]. Interferon-alpha also showed some promising results in reducing the fibrosis in Philadelphia-negative chronic myeloproliferative neoplasms [16], and it can possibly be used in combination with new drugs.

Conclusion

This case is the first reported pregnancy in primary myelofibrosis patient without the previous history of abortions, with no complications during pregnancy and normal, in term delivery with healthy, normal weight baby.

Decision about treatment strategy of pregnancy associated hematologic malignancies should be made for each patient individually.

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I N M E M O R I A M

prof. dr
MILAN M. POPOVIĆ
pukovnik u penziji
(1938–2015)

Dana 16. februara 2015. godine preminuo je u Beogradu naš poznati internista, reumatolog i imunolog – pukovnik u penziji prof. dr sci. med. Milan M. Popović.

Rođen 1938. u Bačkoj Palanci, diplomirao je na Medicinskom fakultetu Univerziteta u Beogradu 1962. godine. Magisterijum je odbranio kao trupni lekar 1967, izučavajući prevenciju reumatske groznice kod vojnika. U Vojnomedicinskoj akademiji (VMA) u Beogradu završio je specijalizaciju interne medicine 1971, a supspecijalizaciju iz reumatologije i imunologije 1973. godine.

Doktorat medicinskih nauka pripremljen u Hamersmit bolnici u Londonu, odbranio je 1976. u VMA. Profesor Interne medicine u VMA postao je 1985. godine. Bio je dugogodišnji načelnik Klinike za reumatologiju i imunologiju, načelnik grupe internih klinika i šef Katedre za Internu medicinu u VMA kao i glavni terapeut Vojske Jugoslavije. Sve ove dužnosti obavljao je savesno, odgovorno i temeljno.

Prof. dr Milan M. Popović dao je veliki doprinos razvoju kliničke reumatologije i imunologije, posebno u oblasti sistemskih bolesti vezivnog tkiva, koje spadaju u red najtežih bolesti u medicini. Prvi je u našoj zemlji (sa još dva saradnika) opisao kombinovanu sistemsku bolest vezivnog tkiva (KSBVT) 1978. godine, dokazujući da se kod jednog bolesnika mogu razviti dve i više ovih bolesti.

Posebno veliki doprinos prof. dr Milan M. Popović dao je razvoju naučnomedicinske misli u našoj zemlji kao mentor u 23 doktorata i 23 magisterijuma. Rukovodeći realizacijom ovih projekata unapredio je naša saznanja u internoj medicini.

Autor je monografija: „Terapija reumatskih bolesti“ (1999) i „Vodič za terapiju reumatskih bolesti“. Bio je koautor u knjizi „Ratna interna medicina“, a prvi autor u monografiji „Reumatske i srodne bolesti – dijagnoza i terapija“. Objavio je 423 stručna i naučna rada, od toga 103 u inostranim stručnim i naučnim časopisima.

Bio je član Srpskog lekarskog društva (u više njegovih sekcija), Srpskog imunološkog društva, Udruženja reumatologa Velike Britanije i Evropske akademije za alergologiju i imunologiju. Odlikovan je brojnim visokim odlikovanjima.

Celokupni njegov rad bio je zasnovan na principima humanosti, čime je nastavio da neguje plemenite tradicije Vojnomedicinske akademije. Zahvaljujući svestranom medicinskom obrazovanju, znanju i iksustvu, video je više i dalje od drugih. Razmišljao je i radio ispred svog vremena i bio jedan od najpoznatijih vojnih lekara našeg doba. Otvorio je vrata novim saznanjima u kliničkoj imunologiji koja su kasnijim istraživanjima postala stvarnost. Iza sebe je ostavio brojne generacije mladih lekara koji uspešno nastavljaju da idu njegovim putem.

Svojim životnim delom pomerio je granice ljudskog postojanja.

Prof. dr sci. med.
Dragan V. Mitrović,
pukovnik u penziji

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Results should be presented in logical sequence in the text, tables and illustrations. Emphasize or summarize only important observations.

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DiMaio VJ. Forensic Pathology. 2nd ed. Boca Raton: CRC Press; 2001.

Blinder MA. Anemia and Transfusion Therapy. In: Ahya NS, Flood K, Paranjothi S, editors. The Washington Manual of Medical Therapeutics, 30th edition. Boston: Lippincot, Williams and Wilkins; 2001. p. 413-28.

Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: *Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG,* editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. p. 182-91.

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Primeri referenci:

Đurović BM. Endothelial trauma in the surgery of cataract. Vojnosanit Pregl 2004; 61(5): 491–7. (Serbian)

Balint B. From the haemotherapy to the haemomodulation. Beograd: Zavod za udžbenike i nastavna sredstva; 2001. (Serbian)

Mladenović T, Kandolf L, Mijušković ŽP. Lasers in dermatology. In: *Karadaglić Đ*, editor. Dermatology. Beograd: Vojnoizdavački zavod & Verzal Press; 2000. p. 1437–49. (Serbian)

Christensen S, Oppacher F. An analysis of Koza's computational effort statistic for genetic programming. In: *Foster JA, Lutton E, Miller J, Ryan C, Tettamanzi AG,* editors. Genetic programming. EuroGP 2002: Proceedings of the 5th European Conference on Genetic Programming; 2002 Apr 3-5; Kinsdale, Ireland. Berlin: Springer; 2002. p. 182-91.

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