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Dear Authors, Editors, Peer Reviewers and Readers of the Vojnosanitetski pregled,
I thank you for your cooperation and support in the last year
and wish you all the best in the coming 2020!

Marry Christmas and Happy New Year!

Cordially,
Prof. Silva Dobrić, PhD
Editor-in-Chief

Poštovani autori, urednici, recenzenti i čitaoci Vojnosanitetskog pregleda,
Uz zahvalnost na saradnji i podršci u protekloj godini,
želim vam sve najbolje u nastupajućoj 2020!
Srećna Nova godina i Božićni praznici!

Srdačno,
prof. dr Silva Dobrić
glavni i odgovorni urednik

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ORIGINAL ARTICLES / ORIGINALNI RADOVI

Sir William Osler (July 12, 1849–December 29, 1919) was a Canadian physician and one of the four founding professors of the famous Johns Hopkins Hospital. Osler created the first residency program for specialty training of physicians, and he was the first to bring medical students out of the lecture hall for bedside clinical training. He is considered the Father of Modern Medicine and one of "the greatest diagnosticians ever to wield a stethoscope".
This year, December 29, marks the 100th anniversary of his death.

Ser Viljem Osler (12. jul 1849–29. decembar 1919), kanadski lekar, jedan je od četvoro profesora utemeljitelja čuvene bolnice Johns Hopkins. Osler je napravio prvi program specijalističkog usavršavanja lekara i bio je prvi koji je izveo studente medicine iz predavaonice kako bi uz krevet pacijenta obavljali praktične vežbe. Smatra se ocem moderne medicine i jednim od „najvećih dijagnostičara ikada koji su rukovali stetoskopom".
Ove godine, 29. decembra, navršava se 100 godina od njegove smrti.

ORIGINAL ARTICLES

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The association between the level of physical activity with spinal posture and physical fitness parameters in early adolescence

Udruženost nivoa fizičke aktivnosti sa stavom kičmenog stuba i parametrima fizičke sposobnosti i ranoj adolescenciji

Aurelija Sidlauskiene*, Birute Strukcinskiene†, Juozas Raistenskis*, Rimantas Stukas*, Vaiva Strukcinskaite*, Raimondas Buckus*

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Abstract

Background/Aim. A low level of physical activity and sedentary lifestyle affects the body posture in adolescents. The aim of this study was to assess the core relation between the level of physical activity and spinal posture as well as physical fitness parameters in 11–14 years old teenagers. **Methods.** The cross-sectional study included 532 children, aged from 11 to 14 years. The study was carried out at three Lithuanian schools in 2011–2013. The Youth Physical Activity Questionnaire (YPAQ) was used to assess physical activity. Spinal posture was assessed according to the Hoeger visual posture assessment method. Physical capacity was evaluated using a 6-min walking test (6 MWT) and by calculating maximum oxygen consumption (VO_2max). Other physical fitness such as the general balance, flexibility, explosive leg power and abdominal muscle endurance were evaluated by applying the European Fitness Test (Eurofit). According to time spent doing moderate to vigorous intensity physical activities (MVPA), the sample was divided into 2 groups – a low activity level group and moderate to vigorous intensity physical activity level group. We compared the spinal posture evaluation results and physical fitness parameters between groups as well as correlations between the duration of MVPA, spinal posture evaluation results and physical fitness parameters. **Results.** The study showed that 22.2% of teenagers had a low physical activity level and 16% of teenagers had an incorrect posture. The teenagers of low physical activity group were less physically fit and had the poorer posture than teenagers in the MVPA group. During the 6MWT, the teenagers in the low physical activity group walked on average 63.2 m less ($p = 0.002$), and their VO_2max was 0.8 mL/kg/min lower ($p = 0.006$) than that of teenagers in the MVPA group. The teenagers in the low physical activity group also did not perform as well in the explosive leg power and abdominal muscle endurance tests compared to teenagers in the MVPA group. Correlations between the duration of MVPA and spinal posture evaluation results as well as some physical fitness parameters were very weak. **Conclusion.** The teenagers of low physical activity were less physically capable and had poorer posture than the teenagers in the MVPA group.

Key words:
adolescent; exercise; physical fitness; posture; spine.

Apstrakt

Uvod/Cilj. Nedovoljna fizička aktivnost i sedentaran stil života utiču na stav kičmenog stuba kod adolescenata. Cilj rada bio je da se proceni povezanost nivoa fizičke aktivnosti i stava kičmenog stuba, kao i parametara fizičke sposobnosti kod mladih u ranom adolescentnom dobu (11– 14 godina života). **Metode.** Studijom preseka obuhvaćena su 532 adolescenta, uzrasta 11–14 godina. Studija je sprovedena u tri litvanske škole, u periodu od 2011. do 2013. godine. Za procenu fizičke aktivnosti korišćen je Upitnik fizičke aktivnosti omladinaca – *the Youth Physical Activity Questionnaire* (YPAC). Fizička aktivnost je procenjivana korišćenjem šestominutnog testa hodanja i određivanjem maksimalne potrošnje kiseonika (VO_2max). Ostali parametri fizičke sposobnosti, kao što su opšti balans, savitljivost, eksplozivna snaga noge i izdržljivost trbušnih mišića procenjivani su primenom Evropskog testa fizičke sposobnosti – *the European Fitnes Test* (Eurofit). Prema vremenu posvećenom fizičkoj aktivnosti ispitanici su podeljeni u dve grupe – grupa sa niskim nivoom fizičke aktivnosti (I grupa) i grupa sa umerenim do visokim nivoom fizičke aktivnosti (II grupa). Upoređivani su rezultati stava kičmenog stuba i parametara fizičke sposobnosti među grupama, kao i povezanost trajanja fizičke aktivnosti umereno-visokog intenziteta, stava kičmenog stuba i parametara fizičke sposobnosti. **Rezultati.** Ukupno 22,2% adolescenata imalo je nizak nivo fizičke aktivnosti, a 16% nepravilan stav kičmenog stuba. Ispitanici I grupe bili su manje fizički sposobni i imali su lošiji stav kičmenog

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stuba od ispitanika II grupe. Za vreme šestominutnog testa hodanja, adolescenti I grupe hodali su prosečno 63,2 m manje (p = 0,002) i njihov VO_2max bio je za 0,8 mL/kg/min niži od VO_2max adolescenata II grupe (p = 0,006). Adolescenti I grupe bili su slabiji u eksplozivnoj snazi nogu i testu izdržljivosti trbušnih mišića u poređenju sa adolescentima II grupe. Nađena je slaba korelacija između trajanja fizičke aktivnosti umerenog do jakog intenziteta i stava kičmenog stuba, kao i nekih parametara fizičke sposobnosti. **Zaključak.** Adolescenti sa slabom fizičkom aktivnošću bili su manje fizički sposobni i imali lošiji stav kičmenog stuba nego adolescenti sa fizičkom aktivnošću umerenog do jakog intenziteta.

Ključne reči:
adolescenti; vežbanje; sposobnost, fizička; telo, držanje; kičma.

Introduction

Physical activity in youth is associated with many health benefits in school-aged children and young people [1, 2]. Despite the known importance and associated benefits of regular physical activity in promoting lifelong health and well-being, some studies suggest that levels of physical activity decline dramatically during adolescence [3, 4]. The World Health Organization (WHO) recommends for children and teenagers to apply at least 60 min of moderate to vigorous intensity physical activity daily [5]. However, data suggest that the majority of young people do not meet these guidelines, with approximately 80% of 13–15 year olds worldwide who are insufficiently physically active [6].

Childhood and adolescence is a period of rapid growth and development, since the dramatic physiological and psychological changes take place at these ages. Most postural problems occur in this period. The body posture depends on many factors, but it is worth emphasizing that a low level of physical activity and sedentary lifestyle also has a significant impact on the postural parameters [7, 8].

Physical fitness is considered to be a useful health marker already in childhood and adolescence [9]. Physical fitness is generally considered to be "the ability to perform daily tasks without fatigue." Muscular strength, muscular endurance, cardiovascular endurance, joint flexibility, and body composition are the health-related fitness components of fitness [10]. Physical fitness is in part genetically determined, but it can also be greatly influenced by environmental factors. Physical activity is one of the main determinants [11, 12].

However, little is known about the relationship between the physical activity level and spinal posture as well as the physical fitness parameters such as physical capacity, balance, flexibility, muscle power and endurance in the stage of early adolescence.

Methods

Study design and population

The cross sectional study was carried out at three Lithuanian schools in 2011–2013. The study was performed by the cluster sampling method. All schoolchildren in the 5th –7th grades were invited to participate. The participation rate was 84.7%. The study population consisted of 532 children, aged from 11 to 14 years (12.99 ± 0.96 years).

The subjects were examined during the first half of the day during the physical education classes and according to the research protocol. The Youth Physical Activity Questionnaire (YPAQ) was filled in at home by the respondents with the help of their parents.

The subject's selection criteria were: 11–14 year old teenagers and the written parental permission. The subject's rejection criteria were: teenagers younger than 11 and older than 14 years of age; unwillingness to participate in the study; teenagers excused from participating in physical education classes.

The study was conducted with the approval of the Lithuanian Bioethics Committee (Protocol No.1.17/3/2011). The informed consent was obtained in written form from the parents of each participating child.

Assessment of physical activity

The study used YPAQ [13]. The questionnaire listed various physical activities, and participants had to indicate the frequency and duration of the activities they undertake over the course of a week, indicating the activities they undertake on weekdays and weekends. This questionnaire was also used to evaluate the nature, frequency and duration of physical activities and passive activities in various settings, e.g., at school and during free time [14].

Every activity was assessed based on the Compendium of Energy Expenditures for Youth (2008) [15] according to the appropriate Metabolic Equivalent for Task (MET) level, and the intensity of physical activity was also assessed. Activities were categorized according to intensity into low-intensity (< 3 MET), medium-intensity (3–6 MET) and vigorous intensity (> 6 MET) groups.

Time spent doing medium to vigorous intensity physical activity (MVPA) and screen time was calculated based on the data collected from the questionnaire. The total time spent doing MVPAs was determined by summing up the duration of MVPAS over the course of one week. The MET min of physical activity were calculated by using the following formula: duration × frequency × MET intensity [15]. Based on the modified recommendations for the evaluation of physical activity [16], the participants were categorized according to their total MVPA into physical activity levels: I – low physical activity < 1,260 MET-min/week; II – moderate-vigorous physical activity > 1,260 MET-min/week.

The questionnaire was translated into the Lithuanian language. The back translation was performed, compared and discussed. The cultural adaptation was performed and the final version of questionnaire was conducted, and tested during the pilot study.

Assessment of spinal posture

Spinal posture was assessed according to the Hoeger and Hoeger [17] visual posture assessment method. The positioning of ten body segments was awarded 1, 3 or 5 points, where 1 is poor, 3 is average and 5 is good. Head, shoulder, spine, hips, knee and ankle positions were assessed in the frontal plane; neck and upper back, trunk, abdomen, lower back and legs were assessed in the sagittal plane. General posture evaluations were calculated by adding the total number of points acquired from the evaluation of body segments: 50-45 points – excellent posture, 44-40 points – good, 39-30 – average, 29-20 – poor, less than 19 – extremely poor.

Assessment of physical fitness parameters

The 6-minute walking test (6 MWT) was used to assess physical capacity. This test is reliable for evaluating the physical capacity not only for the patients with a wide range of diagnoses but also for the healthy children [18–20]. During the test, the participants were separated into groups of 8. The participants were instructed to walk for 6 min along the boundary lines of a standard volleyball court (54 m). The test results were registered as the distance travelled in 6 min, expressed in m.

Physical capacity was also assessed by a maximum oxygen consumption (VO_2max), which was calculated with the results of the 6MWT and data about the body mass index (BMI) entered into a formula developed by Vanhelst et al. [21]: VO_2max (mL/kg/min) = 26.9 + 0.014 × distance travelled during the 6MWT (in m) – 0.38 × BMI (kg/m^2).

Physical fitness was also evaluated by applying The European Fitness Test (Eurofit) [22] in the following order:

Flamingo Balance Test

The test assesses general balance. During the test the participant must stand on a balancing beam of a set height on one leg. Ability to balance was measured by recording the number of attempts (not falls) to maintain balance on the balancing beam in 1 min, with the time recorded by chronometer.

Sit-and-Reach Test

The test of flexibility during which the participant reaches their hands as far as they can while sitting on a horizontal surface with their legs straight. The result recorded is the furthest point reached by the tips of the fingers, measured in cm. For the result to be accurate, the participant must maintain this position for about 2 sec. The test is slowly carried out twice (the sec time after a brief resting period). The better result is recorded (measured in cm reached on the cm ruler on top of the measurement box).

Standing Long Jump Test

The test assesses explosive leg power in the act of jumping from a standing position and pushing off with both feet. The test is carried out twice, and the greater distance jumped is recorded in cm.

Sit-ups Test

The test evaluates abdominal muscle endurance. The participants must complete as many sit-ups as possible in 30 sec. Correct position: straight back, fingers interlocked behind the head, knees bent at a 90 degree angle, sole of the foot on the floor. The participant must lie back and touch the exercise mat with their shoulders, and then sit up into the initial position with their elbows touching their knees. The participants must do a preparatory sit-up before the test begins. The recorded result is the number of correctly completed sit-ups in 30 sec.

Statistical analysis

The statistical analysis was carried out with the IBM SPSS 20 software package.

A descriptive statistical analysis of the data was conducted, calculating the arithmetic mean of quantitative data, standard deviations (SD), minimum (min) and maximum (max) data limits and frequency distributions of qualitative data.

The one-sample Kolmogorov-Smirnov test was used to test the goodness-of-fit of the data distribution to a standard normal distribution. The parametric analysis (Student's *t*-test) was applied in order to compare the indicators from the normal distribution, and nonparametric analysis (Mann Whitney *U*-test) was applied to the nonnormal distribution and ordinal variables.

The Pearson's and Spearman's correlation coefficients (r) were used to determine the correlations (with the Pearson's coefficient for normally distributed interval variables and the Spearman's coefficient for interval variables that do not satisfy the condition of normality and ordinal variables). The correlation was very weak if $r < 0.2$; weak if r was in the interval of 0.2–0.39; moderate if r was in the interval of 0.4–0.69; strong if r was in the interval of 0.7–0.89 and very strong if r was greater than 0.9. The differences considered statistically significant if p was less than 0.05 ($p < 0.05$).

Results

The analysis of physical activity data demonstrated that the teenagers spent from 7.1 min to 408.6 min doing MVPA every day (91.4 ± 66.8 min on average). On weekends, the teenagers spent more time (16.2 min on average) doing MVPA than on school days.

The subjects were classified into low-activity group and MPVA (Table 1).

The teenagers in the low physical activity group made up 22.2% of the sample population, with the teenagers in the MPVA group taking up the remaining 77.8%.

The analysis of posture evaluation in the different physical activity groups demonstrated that the posture of teenagers in the low physical activity group was evaluated as poorer than that of teenagers in the MPVA group: the average sum of Hoeger points received by the teenagers in the low physical activity group reached 36.7 ± 7.7 points, whereas the teenagers in the MPVA group were evaluated with an average of 39.1 ± 7.2 ($p = 0.008$).

Table 1
Characteristics of adolescent groups according to different intensities of physical activity

Characteristics	Group (intensity)	
	low n = 118 (22.2%)	moderate-vigrous n = 414 (77.8%)
Age (year), mean ± SD	12.89 ± 1.09	13.02 ± 0.97
Gender, n (%)		
girls	83 (70.3)	205 (49.5)
boys	35 (29.7)	209 (50.5)

SD – standard deviation.

Table 2
Spinal posture assessment in different body segments

Body segments	Spinal posture (%)		
	poor	average	good
Frontal plane			
head	3.6	30.8	65.6
shoulders	8.7	66.1	25.2
spine	3.6	66.1	30.3
hips	2.9	45.8	51.3
knees and ankles	4.4	33.9	61.7
Sagittal plane			
neck and upper back	2.9	32.9	64.2
trunk	3.9	37.2	58.9
abdomen	2.9	38.3	58.8
lower back	7.7	51.6	40.7
legs	2.2	20.8	77.0

Table 3
Physical fitness parameters in teenagers

Physical fitness parameters	Min–max	Mean ± SD
6 MWT distance (meters)	340.0–980.6	625.2 ± 146.4
VO_2max (mL/kg/min)	21.6–34.8	28.4 ± 2.6
Balance (n/min)	1.0–40.0	13.7 ± 6.9
Explosive leg power (cm)	29.0–225.0	157.6 ± 25.7
Flexibility (cm)	2.0–43.0	22.3 ± 7.8
Abdominal muscle endurance (n^1/30 s)	12.0–90.0	26.7 ± 5.9

SD – standard deviation; 6MWT – 6 min walking test; VO2max – maximum oxygen consimption; n – number of attempls (not falls) to maintain balance; n^1 – number of correctly completed sit-ups.

The results of the spinal posture assessment in different body segments are presented in Table 2.

The analysis demonstrated that the shoulders in the frontal position and the lower back in the sagittal position received the least points: 8.7% of teenagers had poor shoulders position and 7.7% of teenagers had poor lower back position. The analysis of distribution according to the general posture evaluation showed that 53% of the participants had excellent or good posture, 31% had average posture and 16% had poor or extremely poor posture.

The analysis of physical fitness parameters revealed that the distance travelled during the 6MWT ranged from 340.0 to 980.6 meters. The teenager's VO2max ranged from 21.6 mL/kg/min to 34.8 mL/kg/min. The results of the other physical fitness parameters (balance, flexibility, explosive leg power, abdominal muscle endurance) are presented in Table 3.

A statistically significant and positive but very weak link was identified between the spinal posture results and the duration of MVPA (r = 0.186; $p < 0.001$) (Figure 1). This indicates that the posture of teenagers who were more physically active received higher evaluations.

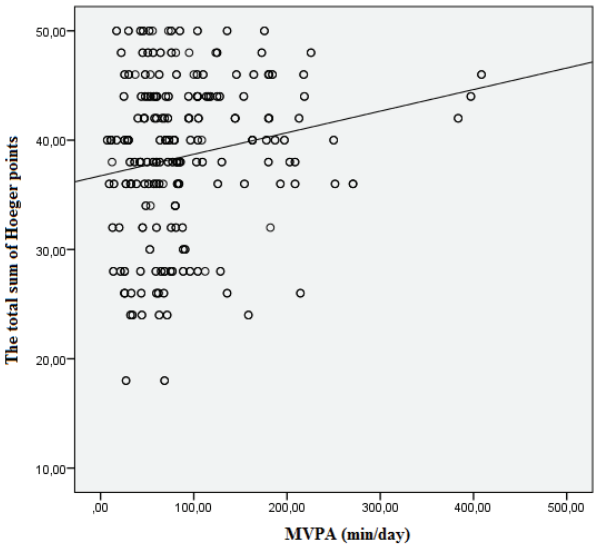

Fig. 1 – Correlation between the moderate-vigorous physical activity (MVPA) and posture of teenagers (r = 0.186; $p < 0.001$).

Table 4

Physical fitness parameters in the low group and moderate-vigorous physical activity (MVPA) group

Physical fitness parameters	Low physical activity group (n = 118) mean ± SD	MVPA group (n = 414) mean ± SD	*p*-value
6 MWT distance (meters)	571.8 ± 117.1	635.0 ± 149.2	0.002*
VO_2max (mL/kg/min)	27.7 ± 1.7	28.5 ± 2.8	0.006*
Balance (n/min)	13.4 ± 6.1	13.8 ± 7.1	0.582
Explosive leg power (cm)	153.3 ± 25.4	158.8 ± 25.7	0.05*
Flexibility (cm)	23.2 ± 7.2	22.0 ± 7.9	0.174
Abdominal muscle endurance (n_1/30s)	25.4 ± 4.7	27.0 ± 6.2	0.005*

6 MWT – 6-minute walk test; VO_2max – maximal oxygen consumption; SD – standard deviation; n – number of attempts (non falls) to maintain balance; n1 – number of correctly completed sit-ups.
***statistically significant.**

The results of the physical fitness tests indicate that, during 6MWT, the teenagers in the low physical activity group walked on average 63.2 m less (p = 0.002), and their VO_2max was 0.8 mL/kg/min lower (p = 0.006) than that of teenagers in the MVPA group.

The teenagers in the low physical activity group did not perform as well in the explosive leg power and the abdominal muscle endurance tests compared to the teenagers in the MVPA group. On average, the low physical activity teenagers jumped a distance 5.5 cm shorter (p = 0.005) and completed 1.6 fewer sit-ups (p = 0.050) than the teenagers in the MVPA group. No statistically significant differences were found in the results of the balance and flexibility tests. The results of the assessment of physical fitness parameters are presented in Table 4.

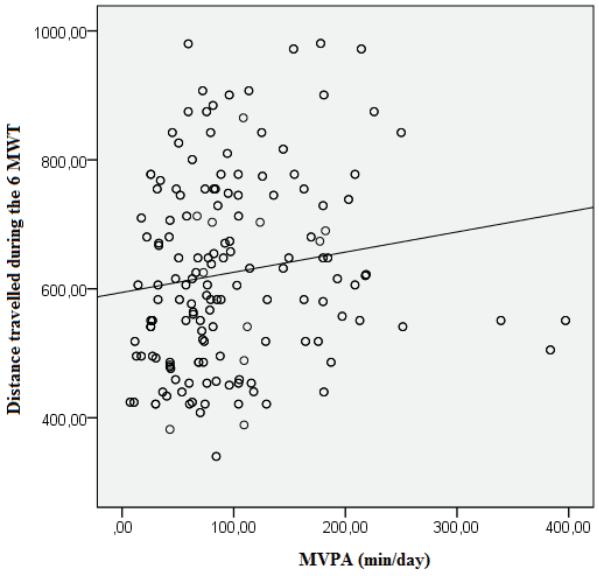

Fig. 2 – Correlation between the moderate-vigorous physical activity (MVPA) and the distance travelled during the 6-min walking test (6 MVT) of teenagers (r = 0.148; p = 0.010).

MVPA of teenagers had a statistically significant and positive but very weak link with the distance travelled during 6MWT (r = 0.148, p = 0.010) (Figure 2) and VO_2max (r = 0.155; p = 0.009) (Figure 3), which demonstrates that the teenagers who were more physically active achieved better results in the physical capacity test.

The correlation analysis of the other teenagers' physical fitness parameters and MVPA revealed a very weak positive correlation between the duration of MVPA and explosive leg power (r = 0.101; p = 0.040).

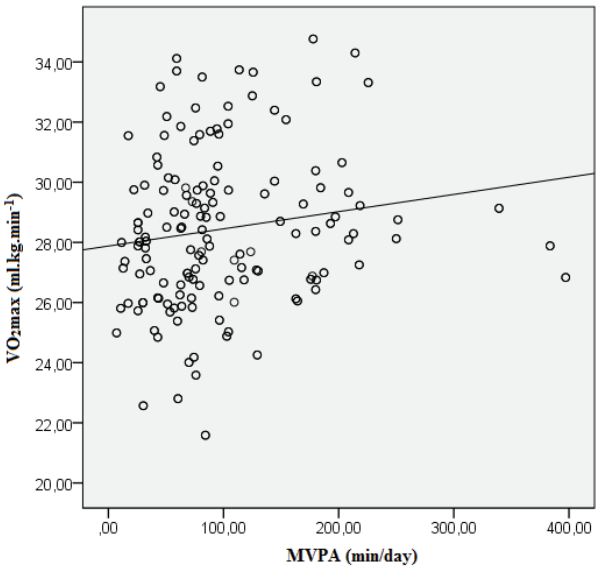

Fig. 3 – Correlation between the moderate-vigorous physical activity (MVPA) and maximum oxygen consumption (VO_2max) of teenagers (r = 0.155; p = 0.009).

Discussion

Scientific research demonstrates that the majority of adults and children across the entire world are not sufficiently physically active, and this level of physical activity keeps decreasing in all age groups [23–25]. Aside from this, physical activity naturally decreases as children grow up [26, 27]. Scientific research has established that only 1 in 5 children in the European Union is sufficiently physically active to satisfy recommendations for physical activity [28]. The results of our study also revealed that slightly more than a fifth of 11–14 year old teenagers could be classified in the low-level activity group. We also observed that there were more girls in the low-level activity group than boys.

Physical activity is a lifestyle factor that can determine an individual's physical capacity [29], which apart from being an important indicator of health in childhood and adulthood, is also a significant risk factor for cardiometabolic diseases [30]. Scientific studies have provided evidence for the link between the aerobic capacity of children as well as teenagers and medium-high intensity physical activity [31]. Our study also showed that the teenagers in the low physical activity group travelled a shorter distance during the 6MWT and had lower VO_2max than the teenagers in the CMVPA group. The other authors analyzed differences in the health-related physical capabilities among 12, 14 and 16 year old Lithuanian teenagers from 1992 to 2002, and observed that the physical capacity of children and teenagers was decreasing. A comparison of data from 1992 and 2002 revealed a strong decrease in the boy's and girl's physical capacity. The authors attribute this change to a reduced level of daily physical activity [32].

The correlation analysis between the physical activity and physical capacity indicators found a link between the duration of MVPA and the distance travelled during the 6MWT ($r = 0.148$) as well as VO_2max ($r = 0.155$), which means that the teenagers who are more physically active perform better on the physical capacity tests. An overview by Kristensen et al. [11] also indicates that studies of children's physical activity and physical capacity frequently find weak to moderate correlations between these two factors (with the r coefficient varying from 0.14 to 0.33). Thus, even though aerobic power in childhood is determined by genetic factors [33, 34], physical activity is probably an important factor that influences the children's physical capacity.

Physical activity is also important for maintaining a correct posture. Studies have shown that moderate physical activity increases abdominal strength and reduces the risk of back pain [35]. Mucha et al. [36] found that the young people aged 14–16 years characterized by increased physical activity had a more correct value of lumbar lordosis, the angle of the sacrum, the difference in the distance of the scapula's from the spine, and a greater spinal range of motion in the sagittal and frontal planes than their peers with the average and low physical activity level. Our study also revealed that the teenagers in the low physical activity group had poorer posture: the posture of teenagers in the lower physical activity group received on average 2.4 points less than the teenagers in the MVPA group.

The correlation analysis demonstrated that the duration of teenagers' MVPA had a very weak, positive correlation with the total sum of Hoeger points ($r = 0.186$), which also indicates that the more physically active teenagers had better posture. Latalski et al.[7], who assessed the posture and physical activity of 14 year old teenagers in Poland and the Czech Republic, also found a link between the low levels of physical activity and incorrect posture [7]. The study performed by Wyszyńska et al. [37] also revealed that the physical activity level determined the variability of the parameter characterizing the body posture. However, as an overview of scientific literature indicates, even though it has long been established that the physical activity influences posture, this link has not been sufficiently studied [38].

The results of our study also revealed a link between the teenager's physical activity and their physical fitness. It was observed that the teenagers of low-level physical activity did not perform as well in the explosive leg power and abdominal muscle endurance tests as teenagers in the MVPA group. A positive very weak correlation was noticed between the duration of teenagers' MVPA and explosive leg power ($r = 0.101$). Martinez-Gomez et al. [39] also determined that the high intensity physical activity had a positive effect on teenagers' muscle power and strength. Given that the physical fitness components relate in different ways to the different health outcomes, physical activity programs should be designed to improve not only the levels of cardiorespiratory fitness but also the muscular fitness and speed/agility [9]. It is also important to emphasize that adolescence is characterized by some specificities of the metabolism and the reactions of the organism. Physiologically, early adolescence is dominated by puberty and sexual development [40]. During normal puberty, height and body weight increase, bone mass and muscle mass increase, blood volume expands, and the heart, brain, lungs, liver, and kidney all increase in size [41], so puberty affects almost all bodily systems. However, very few studies have been conducted to examine the association between the teenagers' physical activity and physical fitness parameters such as balance, flexibility, muscle power and endurance.

The main limitation of this study was that the sample is not large enough for regressive analysis and model construction, which would have allowed to establish causal links between physical activity and spinal posture as well as physical fitness parameters in early adolescence. The other limitation of the study was the indirect method for predicting VO_2max, which may be less accurate than the direct methods. Thus, it would be useful to continue research on this subject.

Conclusion

In the early stages of adolescence, the spinal posture and physical fitness parameters had very weak correlations with the physical activity level. On the other hand, the teenagers of low physical activity were less physically capable than the teenagers in the moderate to vigorous physical activity group. The incorrect posture, weak leg power and weak abdominal muscle endurance were more frequent among teenagers of low physical activity. The findings underline the need for interventions to increase physical activity level and improve the spinal posture as well as physical fitness in teenagers.

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Exploration of personality in the patients with the inflammatory bowel disease

Istraživanje osobina ličnosti obolelih od hroničnih zapaljenskih bolesti creva

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Abstract

Background/Aim. Inflammatory bowel diseases (IBD), which include the ulcerative colitis (UC) and the Crohn's disease (CD), are chronic diseases, the course of which is under the influence of numerous psychosocial factors. The aim of this study was the exploration of the personality traits of patients with IBD. **Methods.** This cross-sectional study has been conducted at the University Clinical Hospital Centre Zvezdara, Belgrade, Serbia. The study involved 150 patients suffering from IBD of both genders, out of which 50.7% and 49.3% of the patients suffering from UC and CD, respectively. The main inclusion criteria were: age 18 to 65 years and confirmed the diagnosis of UC or CD in remission. The sociodemographic and disease related data were collected from the hospital medical records. The personality traits related data were collected using the self-report forms of The Revised NEO Personality Inventory (NEO PI–R) and the inventory for the Assessment of Dysregulation (DELTA 10). **Results.** At the domain-level, the significant differences between IBD sample and normative sample were found in the Neuroticism ($p < 0.01$) and the Disintegration ($p < 0.01$). At the facet-level, the IBD sample scored significantly higher than the normative sample on Anxiety ($p < 0.01$), Assertiveness ($p < 0.01$), Tender-Mindedness ($p < 0.01$) and Dutifulness ($p < 0.01$), and the significantly lower scores on Warmth ($p < 0.01$), Excitement Seeking ($p < 0.01$), Positive Emotion ($p < 0.01$), Actions ($p < 0.01$), and on the all facets of Disintegration except Depression, Somatoform Dysregulation and Social Anhedonia ($p < 0.01$). The differences between UC and CD were found only at the facet-level. The facets that adds the most predictive power to the discriminative function is the General Executive Impairment, followed by Warmth, Self-Discipline, Depression and Mania. **Conclusion.** The IBD patients showed to differ from the general population in terms of basic personality structure at the domain-level, and at the facet-level. The differences between the UC and CD patients can be found only at the facet-level. Screening of the personality traits and early detection of the IBD patients who are at a greater risk of mental disorders and bad psychosocial functioning can enable their adequate prevention and improve the course of the disease.

Key words:
inflammatory bowel diseases; colitis ulcerative; crohn disease; personality; surveys and questionnaires.

Apstrakt

Uvod/Cilj. Zapaljenske bolesti creva (ZBC) koje uključuju ulcerozni kolitis (UK) i Kronovu bolest (KB) su hronične bolesti čiji je tok pod uticajem brojnih psihosocijalnih faktora. Cilj našeg istraživanja je bio ispitivanje crta ličnosti obolelih od ZBC. **Metode.** Ova opservaciona studija preseka je sprovedena u Univerzitetskom kliničkom bolničkom centru „Zvezdara", Beograd, Srbija. U studiju je bilo uključeno 150 osoba obolelih od ZBC, oba pola, sa dijagnozama UK (50,7%) i KB (49,3%). Glavni kriterijumi za uključivanje su bili: starost od 18 do 65 godina i dijagnostikovani UK ili KB u fazi remisije. Za sve ispitanike su iz bolničke medicinske dokumentacije prikupljeni sociodemografski i podaci o bolesti. Procenjivanje crta ličnosti obavljeno je primenom upitnika za samoprocenu *the Revised NEO Personality Inventory* (NEO PI – R- Revised) i Upitnika za procenu dezintegracije (DELTA 10). **Rezultati.** Na nivou domena, značajne razlike između obolelih od ZBC i normativnog uzorka nađene su na Neuroticizmu ($p < 0,01$) i Dezintegraciji ($p < 0,01$). Na nivou faceta, u poređenju sa normativnim uzorkom, oboleli od ZBC su imali značajno više skorove na facetima Anksioznost ($p < 0,01$), Asertivnost ($p < 0,01$), Blaga narav ($p < 0,01$) i Dužnost ($p < 0,01$) i značajno niže skorove na facetima Toplina ($p < 0,01$), Potraga za uzbuđenjem ($p < 0,01$), Pozitivne emocije

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($p < 0,01$), Akcija ($p < 0,01$), kao i na svim facetima Dezintegracije osim Depresije, Somatoformne disregulacije i Socijalne anhedonije ($p < 0,01$). Razlike između UK i KB nađene su samo na nivou faceta. Faceti koji najviše doprinose prediktivnoj snazi diskriminativne funkcije su Opšta egzekutivna disfunkcija, za kojom slede Toplina, Samodisciplina, Depresija i Manija. **Zaključak.** Oboleli od ZBC se po strukturi ličnosti razlikuju od opšte populacije na nivou domena i na nivou faceta. Razlike između UK i KB su nađene samo na nivou faceta. Rutinska provera crta ličnosti i rana detekcija onih ZBC bolesnika koji su u većem riziku za razvoj mentalnih poremećaja i lošeg psihosocijalnog funkcionisanja može omogućiti njihovu adekvatnu prevenciju i poboljšanje toka bolesti.

Ključne reči:
creva, zapaljenske bolesti; kolitis, ulcerativni; kronova bolest; ličnost; ankete i upitnici.

Introduction

The chronic inflammatory bowel diseases (IBD), which include the Crohn's disease (CD) and the ulcerative colitis (UC), are chronic diseases of the gastrointestinal tract, of the unknown etiology and not enough clarified pathogenesis which usually begins in early adulthood. The course of IBD is unpredictable and characterized by episodes of relapse and remissions. Incidence and prevalence of IBD have increased in all regions of the world [1], including the Eastern Europe [2], thus becoming a significant health issue. Based on the current knowledge, IBD is the result of interaction between the genetic predisposition and the environmental factors [3, 4]. The holistic biopsychosocial model of the disease [5] observes the impact of the psychosocial factors on the occurrence and the course of the disease. According to the latest European Crohn's Colitis Organization (ECCO) guidelines, there are no reliable data on the correlation between the psychosocial factors and the IBD etiology, but they could affect the course of the disease [6]. Although the results of the recent studies are controversial, a large number of them shows that stress, anxiety and depression are risk factors for the disease relapse [7], low health-related quality of life [8] and low adherence [9].

The personality is a significant predictor of psychosocial functioning and physical health. The personality traits, being the basic units of the personality structure, can be defined as the biologically determined predispositions of an individual for relatively permanent thinking patterns, feelings and behaviour in similar life situations. They can be evaluated through the personality inventory in which they are structured into domains [10]. The Five Factor Model (FFM) of personality is nowadays a predominant paradigm in the personality psychology. The model assumes the existence of five basic personality dimensions (Neuroticism, Extraversion, Openness, Agreeableness and Conscientiousness), seen as five biological dispositions [10, 11]. Also, the major effects of individual traits may be modified through their mutual interaction [12]. However, the FFM does not include the adaptively important phenomena of behavior, which would suggest the existence of a special regulatory mechanism, outside the premises of the FFM, but which lies in the basis of integration/disintegration of the psychic processes. For the purpose of a more detailed exploration of the basic personality structure, FFM was supplemented with the Disintegration as the sixth, basic dimension of personality, which refers to the psychosis proneness [13]. Thus, within this model, the basic personality structure was defined by the Five Factor Model [11] and Disintegration [13, 14].

A number of studies within the health psychology suggested that the personality traits significantly correlate with the various health aspects and reactions to the disease including the somatic complaints, reaction to stress, anxiety, depression and coping strategies [15]. Numerous studies of the personality traits of those suffering from IBD, and their relation to the psychological factors which affect the course of the disease give controversial results [16–18]. According to the available knowledge about the relations between the personality traits and the psychosocial functioning of the patients on the one hand and the effect of the psychosocial factors on the course of IBD on the other, further research of the personality traits are necessary among those suffering from IBD.

The aim of our study was to investigate the personality traits of patients with IBD in remission.

Methods

Study design / Setting / Participants

The study was designed as an observation cross-sectional and was conducted at the Clinical Hospital Centre "Zvezdara" in Belgrade, Serbia. The sample consisted of 150 patients with IBD and it was divided into two subgroups according to the type of diagnosis: UC (N = 76) and CD (N = 74). The inclusion criteria were: age from 18 to 65 years, diagnosed with UC or CD in the remission confirmed by a gastroenterologists (based on the recommended clinical, radiological, endoscopic and histological criteria [3, 4, 19–21], the absence of other chronic diseases, the absence of current or previous psychiatric morbidity and treatment, the ability to fill in the questionnaire and accept participation in the research. The exclusion criteria were: CD or UC in relapse confirmed by a gastroenterologist [19–21], the presence of other chronic somatic illnesses, inability to fill in the questionnaire (illiteracy, blindness, significant mental handicap), and rejection of participation in the study. The patients who met the inclusion criteria were invited to participate in the study and received verbal and written information about the study. All patients are provided the written informed consent before enrolling in the study.

Data sources / Instruments

Sociodemographic data (gender, age, education level, employment status, marital status, children, place of residence) and disease-related data (type of diagnosis, age at diagnosis, duration of the disease, the total number of relapses

since the onset of disease), were obtained from the hospital medical records. The personality traits related data were collected using the self-report forms of the instruments:

The Revised NEO Personality Inventory (NEO PI - R)

The questionnaire was designed to operationalize the Five Factor Model (FFM) of personality [11, 22]. NEO PI-R measure the five broad basic dimensions (domains) each of which is represented and measured by the six lower-level traits (facets): Neuroticism (N) – anxiety, hostility, depression, self-consciousness, impulsiveness, vulnerability, Extraversion (E) – warmth, gregariousness, assertiveness, activity, excitement seeking, positive emotion, Openness (O) – fantasy, aesthetics, feelings, action, ideas, values, Agreeableness (A) – trust, straightforwardness, altruism, compliance, modesty, tender-mindedness and Conscientiousness (C) – competence, order, dutifulness, achievement striving, self-discipline, deliberation [10]. The instrument consisted of 240 five-point Likert-type items, with 48 items measuring each of five broad dimensions (8 per facet). The instrument was translated into Serbian and empirically tested on a normative sample in Serbia [23].

DELTA 10

DELTA 10 is the inventory for the assessment of general proneness to psychosis named Disintegration [13, 14]. The instrument measures ten facets: General Executive Impairment (GEI), Perceptual Distortions (PD), Paranoia (P), Depression (D), Flattened Affect (FA), Mania (M), Somatoform Dysregulation (SOD), Enhanced Awareness (EA), Social Anhedonia (SA), and Magical Thinking (MT). The instrument consists of 120 five-point Likert-type items. DELTA 10 showed to be a sound measure of Disintegration [14].

Statistical analysis

Descriptive statistical measures were calculated and presented for all relevant sociodemographic and disease-related characteristics, as well as each personality domain and facet. The data were presented as the arithmetic mean ± standard deviation for continues variables and as the absolute number and percentages for discrete variables. In all analyses, the two-tailed tests were used. The *P* values estimated by the independent sample *t*-test for equality of means and by the χ^2-test to compare proportions. A normality of distribution was tested using the Kolmogorov-Smirnov test. The α – Cronbach alpha was used to measure the reliability of the instruments. The stepwise discriminant canonical analysis was used in order to examine personality-based differences between UC and CD. In order to make a comparison between personality traits of the IBD patients and the general population a series of *t*-tests were calculated for each personality domain and facet, along with the Cohen's effect size measure. For the purpose of this analysis we used data obtained from the IBD patients within this study as well as the normative sample data [23, 14]. Following the mean differences calculation for each domain and facet, due to the multiple comparisons made, the Bonferroni correction was used. The data were analyzed using the IBM SPSS 21 statistical package.

Ethical consideration

The study was approved by the local Research Ethics Committee of the University Clinical Hospital Center Zvezdara, and by the Ethics Committee of the Faculty of Medicine, the University of Belgrade, Serbia (No. 29/II-18). The written informed consents were obtained from all participants in this study. The study was conducted in accordance with the Helsinki Declaration.

Results

The study involved 150 IBD patients of both genders (49.3% females). The sample consisted of two subgroups of participants according to the type of diagnosis: UC (50.7%) and CD (49.3%). The sociodemographic characteristics of the whole sample and two subsamples are provided in Table 1. The majority of the whole sample consisted of the secondary school graduated (52%), and the university educated individuals (36.7%), while a smaller part of the sample reported finishing only elementary school (4%), or higher school (7.3%). Sixty-seven percent of participants were employed, 28.7% were unemployed, while 4.7% of participants were retired. The majority of participants are married/cohabiting (44%), 38.7% reported as not married, while 15.3% of them were divorced/separated and 2% were widower. The majority of participants were living in urban area (96.0%). As shown in Table 1, there were no significant differences between UC and CD subsamples with respect to the sociodemographic characteristics ($p > 0.05$).

The disease-related characteristics of the whole sample and two subsamples are provided in Table 2. There were no significant differences between UC and CD subsamples with respect to the age of onset of disease, disease duration and the total number of relapses from onset of disease.

The descriptive characteristics of NEO PI-R and Disintegration domains and their respective facets for the whole sample (IBD) and subsamples (UC and CD) are summarized in Table 3. The Cronbach's alpha was used to measure reliability of the instruments.

The differences in NEO PI-R and Disintegration scores between whole sample (IBD) and the normative sample of Serbia [14, 23] are provided in Table 4. At the domain level, the statistically significant differences were observed only in Neuroticism ($p < 0.01$) and Disintegration ($p < 0.01$). The differences between the IBD sample and the normative sample in relation to other domains were not statistically significant. The patients with IBD were characterized by the higher scores on Neuroticism and lower scores on Disintegration than the normative sample. At the Neuroticism domain, the Anxiety facet made the largest contribution to the discrepancy between the IBD sample and the normative sample. Compared to the normative sample, the IBD patients scored significantly higher on the Assertiveness facet of Extraversion ($p < 0.01$), while at the same time achieved the significantly lower scores on Warmth, Excitement Seeking and Positive Emotionality facets within the Extraversion domain ($p < 0.01$).

Table 1

Sociodemographic characteristics of the inflammatory bowel disease (IBD) sample and the subsamples (ulcerative colitis – UC and Chron's disease – CD)

Variable	Whole sample (n = 150)	UC subsample (n = 76)	CD subsample (n = 74)	*p*-value
Current age (years)				
mean ± SD	37.16 ± 11.48	36.79 ± 12.84	37.54 ± 9.97	
range	19–63	19–63	20–63	
Gender, n (%)				
male	76 (50.7)	40 (52.6)	36 (48.6)	0.626
female	74 (49.3)	36 (47.4)	38 (51.4)	
Education (years), n (%)				
elementary (1–8)	6 (4.0)	4 (5.3)	2 (2.7)	
secondary (9–12)	78 (52.0)	39 (51.3)	39 (52.7)	0.862
higher school (13–16)	11 (7.3%)	6 (7.9)	5 (6.8)	
university (≥ 16)	55 (36.7)	27 (35.5)	28 (37.8)	
Employment status, n (%)				
unemployed	43 (28.7)	28 (36.8)	15 (20.3)	
employed	100 (66.7)	44 (57.9)	56 (75.7)	0.064
retiree	7 (4.6)	4 (5.3)	3 (4.0)	
Marital status, n (%)				
single (never married)	58 (38.7)	31 (40.8)	27 (36.5)	
married/cohabiting	66 (44.0)	35 (46.1)	31 (41.9)	0.398
divorced/separated	23 (15.3)	8 (10.5)	15 (20.3)	
widowed	3 (2)	2 (2.6)	1 (1.3)	
Children, n (%)				
having children	73 (48.7)	40 (52.6)	33 (44.6)	0.325
not having children	77 (51.3)	36 (47.4)	41 (55.4)	
Place of residence, n (%)				
rural area	6 (4.0)	4 (5.3)	2 (2.7)	0.424
urban area	144 (96.0)	72 (94.7)	72 (97.3)	

SD – standard deviation; *p* – values estimated by the independent sample *t*-test for equality of means and by the χ^2 test to compare proportions.

Table 2

Disease-related characteristics of the inflammatory bowel disease (IBD) sample and the subsamples (ulcerative colitis – UC and Chron's disease – CD)

Variable	Whole sample (n =150)	UC subsample (n = 76)	CD subsample (n = 74)	*p*-value
Age at the onset of disease (years)				
mean ± SD	29.25 ± 10.49	29.30 ± 11.75	29.19 ± 9.08	0.947
range	14–60	14–60	14–58	
Duration of disease (months)				
mean ± SD	96.50 ± 92.73	92.47 ± 101.14	100.64 ± 83.72	0.592
range	2–420	2–420	2–335	
Total number of relapses				
mean ± SD	3.98 ± 2.77	3.86 ± 2.78	4.11 ± 2.79	0.579
range	1–15	1–15	1–15	

SD – standard deviation; *p* – values estimated by the independent sample *t*-test.

Table 3
Descriptive characteristics of NEO PI-R + Disintegration domains and their respective facets for the whole sample (IBD) and subsamples (UC and CD)

Domains and facets	Whole sample (IBD) (n = 150)	α	UC subsample (n = 76)	α	CD subsample (n = 74)	α
	mean ± SD		mean ± SD		mean ± SD	
Neuroticism (N)	95.48 ± 21.75	0.897	96.14 ± 21.85	0.892	94.80 ± 21.77	0.904
anxiety	19.05 ± 5.57	0.764	19.34 ± 5.55	0.756	18.74 ± 5.62	0.776
hostility	15.06 ± 5.00	0.696	14.96 ± 5.43	0.740	15.16 ± 4.56	0.636
depression	15.06 ± 5.74	0.759	14.86 ± 5.52	0.707	15.27 ± 5.99	0.807
self-consciousness	16.27 ± 3.83	0.351	16.79 ± 3.86	0.368	15.73 ± 3.75	0.334
impulsiveness	17.31 ± 3.82	0.462	17.16 ± 3.90	0.421	17.46 ± 3.76	0.514
vulnerability	12.74 ± 4.78	0.760	13.04 ± 5.08	0.770	12.43 ± 4.46	0.747
Extraversion (E)	101.13 ± 17.46	0.818	101.54 ± 15.67	0.754	100.70 ± 19.22	0.864
warmth	19.19 ± 3.42	0.396	19.51 ± 3.10	0.168	18.85 ± 3.70	0.559
gregariousness	16.59 ± 5.31	0.747	16.57 ± 5.26	0.708	16.62 ± 5.39	0.788
assertiveness	15.63 ± 4.24	0.546	15.57 ± 4.16	0.488	15.69 ± 4.35	0.602
activity	18.73 ± 4.62	0.607	18.39 ± 5.10	0.646	19.07 ± 4.07	0.542
excitement seeking	14.09 ± 5.46	0.629	14.36 ± 5.18	0.539	13.82 ± 5.76	0.711
positive emotion	16.90 ± 5.16	0.693	17.14 ± 4.85	0.644	16.65 ± 5.48	0.734
Openness (O)	106.61 ± 18.48	0.852	106.39 ± 18.88	0.851	106.84 ± 18.9	0.855
fantasy	16.53 ± 5.01	0.724	16.57 ± 5.10	0.730	16.50 ± 4.95	0.724
aesthetics	18.54 ± 5.70	0.743	18.39 ± 5.53	0.693	18.69 ± 5.91	0.793
feelings	21.38 ± 4.04	0.600	21.26 ± 4.36	0.632	21.50 ± 3.71	0.558
actions	12.61 ± 3.93	0.499	12.47 ± 3.78	0.421	12.76 ± 4.11	0.570
ideas	18.42 ± 6.02	0.789	18.55 ± 6.62	0.831	18.28 ± 5.38	0.727
values	19.13 ± 3.18	0.206	19.14 ± 3.46	0.333	19.11 ± 2.89	0.042
Agreeableness (A)	119.66 ± 18.05	0.866	118.57 ± 19.86	0.882	120.78 ± 16.05	0.841
trust	19.35 ± 4.61	0.718	18.86 ± 5.17	0.763	19.86 ± 3.91	0.638
straightforwardness	21.35 ± 4.65	0.671	21.16 ± 4.45	0.594	21.55 ± 4.87	0.739
altruism	23.18 ± 4.12	0.711	23.01 ± 4.07	0.659	23.35 ± 4.19	0.765
compliance	17.06 ± 5.20	0.706	16.62 ± 4.92	0.654	17.51 ± 5.47	0.751
modesty	16.92 ± 4.24	0.586	17.21 ± 4.96	0.679	16.62 ± 3.34	0.379
tender-mindedness	21.79 ± 3.61	0.497	21.71 ± 4.00	0.571	21.88 ± 3.19	0.381
Conscientiousness (C)	129.26 ± 20.39	0.903	128.28 ± 22.18	0.913	130.27 ± 18.47	0.889
competence	21.89 ± 3.95	0.613	21.33 ± 4.15	0.625	22.47 ± 3.68	0.582
order	20.03 ± 3.58	0.329	19.97 ± 3.82	0.327	20.08 ± 3.34	0.347
dutifulness	25.55 ± 3.96	0.696	25.11 ± 4.42	0.742	26.01 ± 3.40	0.611
achievement striving	21.00 ± 4.99	0.714	21.03 ± 5.44	0.755	20.97 ± 4.51	0.655
self-discipline	20.95 ± 5.26	0.793	21.26 ± 5.28	0.786	20.62 ± 5.25	0.804
deliberation	19.84 ± 4.90	0.762	19.58 ± 4.81	0.726	20.11 ± 5.01	0.797
Disintegration	2.22 ± 0.46	0.962	2.24 ± 0.46	0.960	2.20 ± 0.46	0.966
GEI	2.22 ± 0.65	0.863	2.35 ± 0.65	0.845	2.09 ± 0.64	0.871
PD	1.72 ± 0.57	0.835	1.73 ± 0.56	0.816	1.70 ± 0.57	0.858
P	2.06 ± 0.61	0.855	2.11 ± 0.64	0.856	2.01 ± 0.57	0.857
D	2.13 ± 0.64	0.823	2.12 ± 0.64	0.814	2.15 ± 0.64	0.839
FA	2.28 ± 0.53	0.702	2.30 ± 0.58	0.731	2.25 ± 0.49	0.666
SOD	2.05 ± 0.56	0.786	2.05 ± 0.56	0.775	2.04 ± 0.56	0.801
MT	2.27 ± 0.67	0.797	2.27 ± 0.71	0.811	2.27 ± 0.63	0.783
EA	2.45 ± 0.72	0.804	2.50 ± 0.74	0.808	2.39 ± 0.69	0.802
M	2.83 ± 0.73	0.842	2.81 ± 0.69	0.806	2.86 ± 0.77	0.873
SA	2.18 ± 0.61	0.789	2.13 ± 0.61	0.778	2.22 ± 0.61	0.805

NEO PI-R – Revised NEO Personality Inventory; IBD – inflammatory bowel disease; UC – ulcerative colitis; CD – Chron's disease; GEI – General Executive Impairment; PD – Perceptual Distortions; P – Paranoia, D – Depression; FA – Flattened Affect; SOD – Somatoform Dysregulation; MT – Magical Thinking; EA – Enhanced Awareness; M – Mania; SA – Social Anhedonia; VC – ulcerative colitis; SD – standard deviation; α – Cronbach alpha.

Table 4

Differences between whole sample (IBD) and the normative sample

Domains and facets	*t*	Cohen's *d*
Neuroticism (N)	**3.527****	**0.323**
anxiety	**5.771****	**0.543**
hostility	-0.172	0.016
depression	2.343*	0.220
self-consciousness	2.151*	0.190
impulsiveness	2.791**	0.251
vulnerability	2.859**	0.264
Extraversion (E)	-1.080	0.098
warmth	**-4.794****	**0.420**
gregariousness	-0.519	0.048
assertiveness	**6.896****	0.643
activity	2.103*	0.195
excitement seeking	**-3.530****	**0.332**
positive emotion	**-3.739****	**0.354**
Openness (O)	-1.402	0.128
fantasy	-0.582	0.053
aesthetics	-2.550**	0.241
feelings	1.475	0.135
actions	**-5.099****	**0.467**
ideas	1.018	0.096
values	-0.222	0.020
Agreeableness (A)	-0.079	0.008
trust	0.206	0.019
straightforwardness	1.744	0.166
altruism	-0.601	0.057
compliance	-1.218	0.117
modesty	-3.158**	0.290
tender-mindedness	**3.293****	**0.310**
Conscientiousness (C)	1.991*	0.184
competence	0.848	0.078
order	2.346*	0.209
dutifulness	**3.389****	**0.312**
achievement striving	1.663	0.157
self-discipline	0.162	0.015
deliberation	1.224	0.113
Disintegration	**-6.276****	**0.568**
GEI	**-3.939****	**0.365**
PD	**-7.627****	**0.627**
P	**-4.297****	**0.374**
D	0.358	0.031
FA	**-6.171****	**0.522**
SOD	-3.015**	0.254
MT	**-6.779****	**0.584**
EA	**-8.162****	0.733
M	**-4.163****	**0.392**
SA	-1.696	0.151

IBD – Inflammatory bowel disease; GEI – General Executive Impairment, PD – Perceptual Distortions, P – Paranoia, D – Depression, FA – Flattened Affect, SOD – Somatoform Dysregulation, MT – Magical Thinking, EA – Enhanced Awareness, M – Mania, SA – Social Anhedonia; NEO PI-R normative sample n = 474 [68], DELTA 10 normative sample n = 1001 [72]; *t* – *t*-test, Cohen's *d* – effect size, *$p < 0.05$, **$p < 0.01$; Values of the *t*-test that exceed alpha level (0.05/46 comparisons = 0 .0011) after the Bonferroni correction are marked bold.

Among the Openness facets, the only significant difference emerged for the Action facet ($p < 0.01$). The IBD sample scored significantly higher on the Tender-Mindedness facet of Agreeableness domain ($p < 0.01$). Among the Conscientiousness facets, the IBD patients can be the most strikingly distinguished from the general population by means of their significantly higher scores of Dutifulness ($p < 0.01$). Regarding Disintegration, the significant differences between the IBD group and the normative sample were found in all facets except Depression, Somatoform Dysregulation and Social Anhedonia ($p < 0.01$). More specifically, compared to the normative sample, the patients with IBD had the significantly lower scores on General Executive Impairment, Perceptual Distortions, Paranoia, Flattened Affect, Magical Thinking, Enhanced Awareness and Mania facets of Disintegration.

Table 5
The statistical significance of canonical correlation

r	W-L	χ^2	df	*p*
0.451	0.797	33.073	5	0.001

r – canonical correlation; W-L – Wilks' Lambda; χ^2 – Chi-square test; df – degrees of freedom.

In order to examine which traits discriminated the best between two subgroups of the patients, the stepwise discriminant analysis was conducted, with the facets of NEO PI-R and DELTA10 taken as the predictors of the diagnostic group. Since the Box's M test indicated the equality of population covariance matrices [Box's M = 13.611, F (15,88054.71) = 0.875, p = 0.593], the analysis was carried out using the within-groups covariance matrices. The results indicated a significant canonical correlation (Table 5).

The variables that showed to add predictive power to the discriminant function are presented in Table 6. The facets that added the most to the discriminative power were General Executive Impairment facet, followed by Warmth, Self-Discipline, Depression, and Mania. The UC group, in contrast to the CD group was characterized by the elevated levels of General Executive Difficulties, accompanied by the higher levels of Warmth and Self-Discipline and lower levels of Depression and Mania.

Centroids for the UC and CD groups were 0.495 and -0.509, respectively. In all, results indicated that two groups could be distinguished based on their personality traits.

The proportion of successful classification of cases into the diagnostic groups using the discriminant function was overall at the satisfying level – 70.7% (Table 7).

Table 7
Classification results

Subsamples	Predicted group membership		Total
	UC	CD	
UC	55 (72.4%)	21 (27.6%)	76
CD	23 (31.1%)	51 (68.9%)	74

UC – ulcerative colitis; CD – Chron's disease.

Discussion

In this study, we investigated the personality traits of the IBD patients in remission, whereby applying the Five factor + Disintegration personality model. The study was carried out both for the entire sample of the IBD patients as well as for two subsamples separately (UC and CD). The personality traits were examined both at the domain-level and at the facet-level. Although the interest for the characteristics of persons suffering from IBD has been present since the first half of the 20th century, the findings are contradictory, which might relate to the methodological and conceptual limitations. The previous studies used various methodological approaches (mixed sample both of UC and CD, children and adults, patients in remission and in relapse), various theoretical personality models and various instruments for measuring the personality traits. Also, in previous studies, the personality traits were analyzed solely at the domain-level, whereas in some studies, only selected personality traits were observed (most often the extraversion and neuroticism). All of the aforementioned makes the comparison of results more difficult. To the best of our knowledge, the present study is the first to use the full FFM and Disintegration trait to investigate the patients with IBD. However, there are a large number of studies which confirm the connection between the personality traits and those psychosocial factors which were proven to influence the course of the chronic diseases in general, and therefore IBD as well.

The findings of our study suggest that the IBD patients differ from the normative sample in Serbia, in regard to the personality structure. The IBD patients have higher scores in the domain of Neuroticism, especially on Anxiety facet.

Table 6
Discrimination between ulcerative colitis (UC) and Chron's disease (CD): standardized canonical discriminant function coefficients and structure matrix

Parameters	Standardized canonical discriminant function coefficients	Structure matrix
Depression	-0.716	-0.058
Warmth	0.437	0.193
Self-Discipline	0.517	0.121
General Executive Impairment	1.854	0.411
Mania	-0.707	-0.068

Such findings are in compliance with those which other researchers obtained, which implies that the high scores of Neuroticism were observed in 70% of patients with UC, and in 62% of patients with CD [16]. Neuroticism, defined as the tendency to experience negative emotions (such as fear, sadness, anger, guilt) is related with vulnerability to stress and specific disposition to depression [24, 25], anxiety [26] and other mental disorders [27]. High Neuroticism is also associated with less adequate emotion regulation and ineffective coping [27, 28]. Earlier researches showed that the IBD patients were at a risk of high frequency of mental disorders [29], especially anxiety and depression [7, 29, 30]. Among the IBD patients, depression and anxiety are associated with frequent relapses, hospitalizations, operations and low quality of life [31], due to which many authors suggest the routine screening to anxiety and depression, aimed at early initiation of their treatment [32]. We can assume that among the IBD patients who have the high scores on Neuroticism, there is a high risk of developing anxiety and depression. Therefore, it might be that early detection of this risky group of patients, prior to the occurrence of symptoms of anxiety and depression, enabled adequate prevention of mental disorders and improvement of the course of the disease [33].

At the level of other NEO PI-R domains we found no significant difference between the IBD patients and the general population. We did find out that the IBD patients had the lower scores at Extraversion and Openness domains as well, but they were not statistically significant. However, these findings can be of practical significance, since some studies showed that among personality traits, neuroticism and extraversion had the highest effects on well-being [34, 35]. Although other researchers discovered a significant difference between the IBD patients and the normal group for other domains (e.g., lower extraversion and openness), we cannot compare these findings with ours since various tests for the assessment of personality were used.

At the level of facets, we found out that, in comparison to the general population, the IBD patients had the lower scores at the following facets: Warmth, Excitement Seeking, Positive Emotion and Actions, and the higher scores for Anxiety, Assertiveness, Tender-Mindedness and Dutifulness. Among the IBD patients, we also discovered the high scores for Impulsiveness and Vulnerability, but the differences in comparison to the general population were not significant. Low Warmth was connected to difficulties in establishing emotional connections and poor friendly compassion towards other people and can be a predictor of low social support and a bad doctor-patient relationship. Low Excitement Seeking is related to the decreased desire for excitement and stimulation and can be connected to the old age and behavior related to a life burdened by a chronic condition. Low Positive Emotion is in relation to a low tendency to experience positive emotions (e.g., happiness, love) and is also associated with an increased risk of developing chronic conditions [36]. Low Actions, which refers to preference of familiar patterns and routine and dislike of changes, is often seen among people who suffer from chronic diseases for a longer period of time. Also, those who scored lower on Positive Emotion and Actions had more chronic diseases [36]. High Assertiveness is connected to the strength and social domination whereas Tender-Mindedness is connected to sympathy and care for other people. High Dutifulness is in relation to scrupulosity and abiding by rules and moral obligations, which in favorable combination with other facets can result in better adherence.

In our study, the IBD patients showed the lower scores in the domain of Disintegration in comparison to the general population. There are no published studies which investigated this personality dimension among the IBD patients. The lower scores at Disintegration imply that the IBD patients are less prone to psychotic-like experiences than the general population.

We did not find a difference between the UC and CD patients at the level of observed domains. This is in compliance with the results of previous studies which shows that these two IBD diagnostic types are not different in the personality structure [16, 37]. In one of the studies [16], a higher level of Neuroticism and Extraversion was observed in the patients with UC, whereas the patients with CD exhibited a higher level of Openness to experience and Agreeableness, but these differences were not statistically significant. However, when comparing UC and CD at the facet-level, it turned out that the differences between UC and CD exist and that they can have a practical significance. The dominant difference refers to the General Executive Impairment (GEI) facet within Disintegration: the patients with UC had more obvious executive dysfunctions than the patients with CD. The contribution of other facets in deciphering UC from CD is lower. The patients with UC had higher scores at Warmth and Self-Discipline facets, which could mean that the UC patients are more sensitive to the lack of social support and have a higher tendency to low adherence than the CD patients. We also found that the patients with UC had the lower scores at Depression and Mania facets, which suggests that the UC patients are more prone to mood swings, i.e., they are more prone to the cyclothymia than the CD patients.

So far, the studies that dealt with researching the persons suffering from chronic diseases were mostly focused on the domain level. Our findings show that certain differences can be discovered only at the facet-level and that facet-level associations can go in opposite directions and obscuring the effect at the broad domain-level. This implies the significance of analysis of personality structure at the facet-level as well, which is in compliance with the recommendations of other authors [35].

The limitations of our study refer primarily to a relatively small sample and the results that we obtained should be confirmed in some studies involving larger samples. Our sample comprised of patients from one reference tertiary centre which disables the generalization of results. Also, the patients who previously received the psychiatric treatment were not included in the study as well as persons in relapse, which mitigated the difference between our sample and the general population. The limitations of our study refer to its design as well (observational study). The future prospective studies would enable the research of impact of personality traits on the course of the disease, as well as the observation of

changes in the personality traits during the course of the disease. The research on correlations between the personality traits and psychosocial factors which are proven to affect the course of IBD (anxiety, depression, coping) are also necessary.

In case of theoretical implications of our study, they primarily refer to the necessity of researching the personality traits not only at the domain-level but also at the facet-level. Given that we found the significant differences between IBD and the general population, at the level of Disintegration, the observance of this dimension is also important for future researches. A larger number of studies in this field and the comparison of their results could enable the conceptualization of specific type of personality suffering from IBD, which would be more vulnerable and prone to the worse course of the disease. In that sense, the interdisciplinary cooperation is necessary among the gastroenterologists, psychiatrists and the psychologists.

The examination of personality of the IBD patients has the important clinical implications. The routine application of personality tests among the IBD patients, especially immediately upon setting the diagnosis, would enable early detection of those patients who are at a greater risk of anxiety, depression and inadequate coping, according to their personality traits. Thus detected patients would be the focus group for various forms of psychosocial interventions, psychotherapy and possibly pharmacotherapy. Consequently, anxiety, depression and inadequate coping would be prevented, which would also prevent their negative impact on the course of IBD.

Conclusion

Based on personality traits, we found that the IBD patients differ from the general population in the domains of Neuroticism and Disintegration. At the level of other domains, we found no difference, but the difference was found at the level of facets to which they belong. Among the patients with UC and CD, we found no difference at the domain-level, but we did discover it at the facet-level, predominantly at GEI within Disintegration. The screening of the personality traits can be used to early detection of the IBD patients who are at a greater risk for mental disorders and bad psychosocial functioning. These patients would be the focus group for early psychosocial interventions, psychotherapy or pharmacotherapy which would prevent anxiety, depression and inadequate coping. In accordance with the proven relationship between personality and psychosocial functioning of the IBD patients, an implementation of these procedures can improve the course of the disease.

Conflict of interest

The authors fully declare that there is no conflict of interest.

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Production of immunoregulatory cytokines in clinically asymptomatic periapical lesions depends on the lesions size

Zavisnost produkcije imunoregulatornih citokina u klinički asimptomatskim periapeksnim lezijama od veličine lezije

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Abstract

Bacground/Aim. Development of periapical lesions (PLs) involves a complex cross-talk between pathogenic microorganisms from the root canal and host immune mechanisms. In these processes proinflammatory cytokines are involved in stimulation of inflammation and osteodestructive mechanisms, whereas anti-inflammatory cytokines, with the immunoregulatory functions, have the opposite effects. How this balance is controlled is still the subject of numerous studies. The aim of this study was to examine whether the local production of interleukin (IL)-10, IL-27 and transforming growth factor (TGF)-β in human asymptomatic PLs depends on the lesion size and how levels of these immunoregulatory cytokines correlate with the cellular composition of PLs. **Methods.** The study was conducted on 30 PLs which were collected at the Clinic for Stomatology of the Military Medical Academy, Belgrade, Serbia. The PLs were divided according to their size into small- and large-size lesions (n = 12 and n = 18, respectively). The inflammatory cells (PL-ICs) were isolated from PLs and cultivated for 24 hours in culture medium supplemented with phorbol myristate acetate and Ca^{2+} ionophore. Cytospins were processed for immunocytology by using monoclonal antibodies to cell subsets. The levels of cytokines in culture supernatants were determined by the ELISA method. Statistical analysis was performed using the Student *t*-test and the Spearman's correlation test. The values of $p < 0.05$ were considered to be significant. **Results.** The levels of IL-10 and TGF-β were significantly higher in the PL-ICs cultures of large-size lesions than in small ones ($p < 0.01$ and $p < 0.05$, respectively). In contrast, the levels of IL-27 were higher in the cultures of small-size lesions than in small ones ($p < 0.05$). Although the total number of PL-ICs and the proportion of mononuclear phagocytes were higher in the large-size PLs ($p < 0.01$ and $p < 0.05$, respectively), their main composition was not significantly different between the groups. The proportions of B cells/plasma cells ($CD19/CD38^+$ cells), $CD8^+$ T cells and $CD14^+$ cells were significantly higher in the large-size PLs ($p < 0.005$; $p < 0.05$; $p < 0.05$, respectively). In contrast, the proportion of total T cells ($CD3^+$ cells) was higher in the small-size lesions ($p < 0.05$). No statistically significant correlation was found between the levels of these cytokines and the composition/phenotype of PL-ICs. **Conclusion.** This study suggests that IL-10, IL-27 and TGF-β may play different roles in suppression of unwanted immune/inflammatory reactions in asymptomatic PLs, depending on the extension of pathological process as judged by the size of lesions.

Key words:
periapical diseases; cytokines; microbiota; immunologic factors; inflammation.

Apstrakt

Uvod/Cilj. Razvoj periapeksnih lezija (PLs) prolazi kroz složenu interakciju između patogenih mikroorganizama iz kanala zuba i imunskih mehanizama domaćina. U ovim procesima proinflamacijski citokini stimulišu zapaljenske reakcije i destrukciju kostiju dok anti-inflamacijski citokini sa imunoregulacijskim svojstvima imaju suprotan efekat. Način kako je taj balans kontrolisan je i dalje predmet brojnih studija. Cilj ovog rada bio je da se ispita da li lokalna produkcija interleukina (IL)-10, IL-27 i transformišućeg faktora rasta beta (TGF-β) u humanim asimptomatskim PLs zavisi od njihove veličine i kako nivo ovih citokina korelira sa ćelijskim sastavom PLs. **Metode.** Istraživanje je sprovedeno na

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30 PLs koje su ekstrahovane u Klinici za stomatologiju VMA. Lezije su podeljene na grupu malih lezija (n = 12) i grupu velikih lezija (n = 18). Inflamatorne ćelije izolovane iz lezija (PL-ICs) kultivisane su u toku 24 časa u medijumu za ćelijske kulture sa dodatkom forbol miristat acetata i Ca^{2+} jonofora. Citospin preparati obojeni su pomoću imunocitohemijskih metoda korišćenjem monoklonskih antitela prema ćelijskim subpopulacijama. Nivo citokina u supernatantima ćelijskih kultura određen je ELISA metodom. Za statističku obradu podataka korišćen je Studentov *t*-test i Spearman-ov test korelacije. Vrednosti razlika $p < 0,05$ smatrane su statistički značajnim. **Rezultati.** Nivoi IL-10 i TGF-β su bili statistički značajno viši u supernatantima PL-ICs velikih lezija ($p < 0,01$, odnosno $p < 0,05$), za razliku od IL-27 čiji su nivoi bili veći u kulturama malih lezija ($p < 0,05$). Iako je ukupan broj PL-ICs i procenat mononuklearnih fagocita bio viši u velikim lezijama ($p < 0,01$, odnosno $p < 0,05$), njihov osnovni ćelijski sastav nije se bitnije razlikovao između grupa. Procenat B ćelija/plazma ćelija (CD19/CD38$^+$ćelija), CD8$^+$ T ćelija i CD14$^+$ ćelija je bio veći u velikim lezijama ($p < 0,005$; $p < 0,05$; $p < 0,05$), za razliku od procenta ukupnih CD3$^+$T ćelija koji bio je veći u malim lezijama ($p < 0,05$). Nisu nađene korelacije između nivoa ispitivanih citokina i ćelijskog sastava/fenotipa PL-ICs. **Zaključak.** Ova studija ukazuje na to da IL-10, IL-27 i TGF-β najverovatnije imaju različitu ulogu u suzbijanju neželjenih imunskih/inflamacijskih reakcija u asimptomatskim PLs, zavisno od ekstezivnosti patološkog procesa, procenjivanog na osnovu veličine lezije.

Ključne reči:
periapeksne bolesti; citokini; mikroorganizmi; imunski faktori; zapaljenje.

Introduction

Periapical lesions (PLs), common chronical pathological processes of the oral cavity, are triggered by bacterial infection of pulpal and endodontic environment [1]. Their pathogenesis involves a complex host immune/inflammatory response to the bacteria and their products in order to eliminate the invading microorganisms. However, the same mechanisms may also result in the tissue injury, followed by the destruction of soft and mineralized tissues surrounding the root apex [2]. The breakdown of tissues is triggered by different host mediators, which independently or cooperatively stimulate proteolysis and bone resorption processes [1–5]. Simultaneously, the regulatory mechanisms are triggered with the aim to suppress the inflammation and tissue destruction and to restrict the PL development [3, 4].

PLs consist of granulation tissue, proliferating epithelium or cyst infiltrated by different inflammatory cells (ICs) [5]. The compostion of ICs as well as functional and phenotypic properties of both infiltrating and stromal cells depend on the activation status of PLs which is under control of a series of cytokines. Cytokines play a major role in the modulation of immune/inflammatory reactions within PLs, and are critical determinants of lesions outcome [2, 6]. In this context, it is believed that the T-helper 1 (Th1) immune response, mediated by interferon-γ (IFN-γ) is involved in the progression of PLs and bone destruction, whereas T-helper 2 (Th2) cytokines, such as interleukin 4 (IL-4), IL-5, IL-10 and IL-33, are described to be important for the humoral immune response and to limit or attenuate the tissue damage [2]. IL-17 may play a role in exacerbating inflammation[7]and osteolytic processes [8] within PLs. On the other hand, the Foxp3$^+$CD4$^+$ CD25$^+$ subset of T regulatory cells (Tregs) and Tr1 cells exert suppressive effects on inflammatory osteolysis, in which cytokines Transforming growth factor beta (TGF-β) and IL-10 seem to play a crucial role [9–11]. The functions of Th17 and Tregs cytokines are interconnected and the Th17/Tregs archetype was suggested to influence the PLs outcome [12, 13] through the balance between the production of the osteoclastogenic factor named a receptor activator of nuclear factor kappa-B ligand (RANKL) and its antagonist osteoprotegerin (OPG) [6, 14]. Our previous results showed that IL-27 may have both pro-inflammatory and immunoregulatory functions in PLs [15].

Pro-inflammatory cytokines prevail in symptomatic PLs, whereas the production of immunoregulatory cytokines characterizes predominantly asymptomatic PLs [3, 6, 16]. Since restriction of inflammation within PLs may occur at different stages of their development, the aim of this study was to examine whether the local production of three immunoregulatory cytokines (IL-10, IL-27 and TGF-β) in human asymptomatic PLs depends on the lesion size and how the levels of these cytokines correlate with the cellular composition/phenotype of such PLs. Similar data have not been published yet.

Methods

Patients

The study was conducted on human PLs (n = 30) at the Military Medical Academy (MMA), Belgrade, Serbia after an approval from the Ethics Committee of MMA and obtaining an informed consent from the patients.The average age of the patients was 31 years (range: 19–59 years). The patients had no malignant or autoimmune diseases and did not use immunosuppressive /immunomodulatory drugs. In addition, the patients had not been treated with antibiotics one month before the PLs excision. The clinical part of the study was performed at the Department for Oral Surgery, Clinic for Stomatology, MMA, at the time of teeth extraction or apicoectomy. The immunological part of the study was performed at the Institute for Medical Research, MMA. PLs were radiographically diagnosed using the standard equipment for intraoral (Carestream CS 2200 rentgenaparature) and extra-oral (ortopantomography and Dental Cone Beam Computed Tomography – CBCT) radiography of the maxillofacial region. Intraoral radiographs were obtained using the Digora imaging system (Soredex Corporation, Helsinki, Finland), size 2 photostimulable storage phosphor (PSP) plate sensors (40.0 mm × 30.0 mm) and the Scanora software. Extraoral tomographs were made by the Vatech aparature in the

OrtoDent digital diagnostic center, Belgrade, following the rules for patients positioning according to the manufacturer's instructions. The gained ortopantomography shots were calibrated by the Vatech program software for panoramic pictures (Ez dent-i), while the 3D CBCT analyses were done in the Ez 3D plus and Ez 3D-i Vatech program software. The size of radiolucent PLs on the radiographs and tomographs was analyzed by the above mentioned softwares and the smallest and largest diameters were measured. All PLs were clinically asymptomatic.

The lesions were divided according to their size into the small and large PLs. The small lesions (n = 12) were those whose greatest diameter was less than 3.5 mm. The lesions whose smallest diameter was higher than 4.5 mm were classified as the large lesions (n = 18). No further distinctions between specimens were made regarding sex, age, and etiology or tooth type. The examples of radiographs/tomographs of one small- and one large-size PLs are given on Figures 1 and 2. After extraction, PLs were immediately placed in a medium consisting of RPMI-1640 (Sigma, Munich, Germany) and antibiotics/antimycotics, and transported to the laboratory.

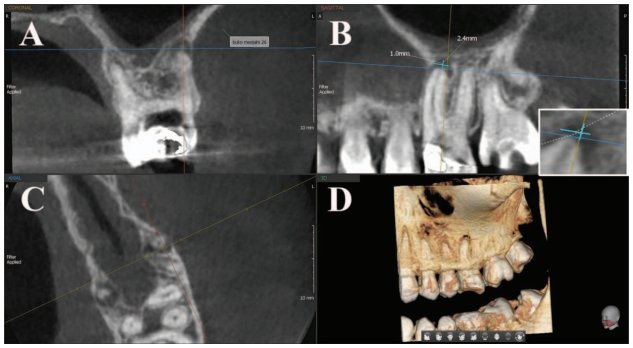

Fig. 1 – The images of one representative small-size periapical lesion (teeth 26): A) Sagittal cross-section (bucomesial and palatinal root); B) Coronary cross-section (bucomesial root); C) Axial cross-section (rout apex); D) 3D reconstruction – Dental Cone Beam Computed Tomography – CBCT. The insert represents a higher magnification of the measured lesion size.

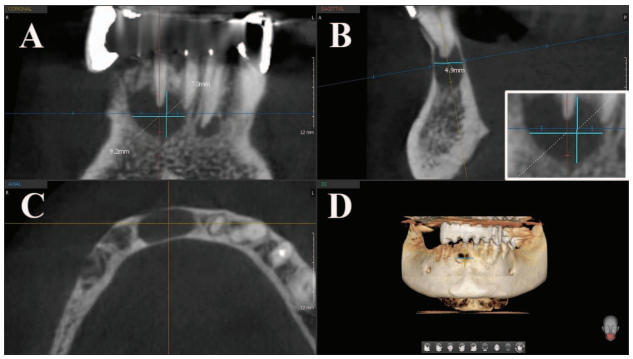

Fig. 2 – The images of one representative large-size periapical lesion (teeth 41): A) Coronary cross-section of the root; B) Sagittal cross-section of the root; C) Axial cross-section (largest bucomesial size); D) 3D reconstruction (Dental Cone Beam Computed Tomography – CBCT). The insert represents a higher magnification of the measured lesion size.

Preparation of inflammatory cells

The inflammatory cells from PLs (PL-ICs) were isolated using a procedure previously optimized by our research group [5, 17]. Briefly, periapical tissue was placed in a Petri dish containing 1 mL RPMI-1640 medium and cut into 2–3 mm diameter pieces using a scalpel. The tissue was then digested for 15 min with 0.05% collagenase type IV (Sigma) and 0.02% DNAse (Sigma) in 10 mL RPMI-1640 medium in a cell incubator at 37ºC. After that, the tissue was pressed through a stainless-steel mesh using a syringe plunger, filtered and resuspended in RPMI-1640 medium containing 1 mm EDTA. The released cells were washed twice by centrifugation in the RPMI medium containing 0.5 Mm EDTA at room temperature (400 g for 10 min), and counted. The viability of cells, determined by Trypan Blue dye, was between 90% and 95%. After that, cytospins were prepared from each sample of PL-ICs using a cytocentrifuge (MPW-350, Poland) on the poly-l-lysine-coated glass slides.

The cytospins were stained with May-Grünwald-Giemsa or used for immunocytochemistry. The cellular composition was analyzed by using a light microscope (Olympus). The identification of cells was done by the morphological criteria. A minimum of 500 cells was analyzed on each cytospin. The results are given as percentages of cell subsets.

Immunocytochemistry

For immunostaining, anti-CD3, -CD4, -CD8, -CD14, -CD19, - CD38 and -HLA DR unconjugated monoclonal antibodies (mAbs) were obtained from Serotec, Oxford, UK. Rabbit anti-mouse unconjugated- and peroxidase conjugated-Ig, as well asan alkaline phosphatase anti-alkaline phosphatase (APAAP) complex were purchased from DAKO, Copenhagen, Denmark. Cytospins were fixed with 2% pararosaniline (Sigma) for 2 min at room temperature, washed with phosphate-buffered saline (PBS) for 10 min, blocked with rabbit serum for 20 min and washed with PBS. Cytospins were incubated with the primary antibodies for 60 min at room temperature followed by washing with PBS. When the staining was further processed for the immunoperoxidase method, the slides were blocked with 0.3%H_2O_2 in PBS for 20 min and then incubated with appropriate dilution of anti-mouse peroxidase conjugated Ig. The immunoperoxidase reaction was developed with diaminobenzidine. When the immunostaining was performed with the immunoalkaline phosphatase method, the slides were incubated with anti-mouse unconjugated Ig and then with APAAP. The reaction was finished by using Fast Red, as a substrate. Both enzyme substrates were obtained from Sigma. The controls were the samples incubated with an irrelevant mAb, mouse anti-rat CD4 (OX-38), Serotec, non-reactive with human cells. To identify B cells and plasma cells, cytospins were stained with the combination of anti-CD19 mAband anti-CD38 mAb. T cells were identified based on the positivity with anti-CD 3 mAb and subsets of T cells based on the staining with anti-CD4 and anti-CD8 mAbs, respectively. CD14 was a marker of mononuclear phagocytes, whereas HLA-DR was a marker

of antigen-presenting cells. Cytospins were analyzed by light microscopy. On each cytospin at least 500 cells were counted. The percentages of positive cells were determined on the basis of total counted cells.

Cell cultures

PL-ICs were cultivated in 96-wells, with the round-bottomed plates (ICN, Costa Mesa, CA) (1×10^5 cells/well, 200 μL) in the complete culture medium consisted of RPMI-1640 medium supplemented with 10% fetal calf serum (FCS) (Sigma) and the standard culture solutions of antibiotics [17]. Phorbol myristate acetate (PMA) (20 ng/mL) (Sigma) and Ca^{2+} ionophore (A 23187, 1 M) (Sigma) were used for the cell stimulation [18]. After 24 h, the cell supernatants were collected, centrifuged and frozen at -70ºC until the levels of cytokines were determined.

Cytokine assays

The concentrations of IL-10, IL-27 and TGF-β in culture supernatants were detected by using the specific ELISA kits (R&D, Minneapolis, USA) following the instructions of the manufacturer. The levels of cytokines were determined on the basis of standard curve, constructed by known concentrations of these cytokines. The results are presented as pg/mL.

Statistical analysis

Statistical analysis was performed by using the Student *t*-test and Spearman's correlation test. The values of $p < 0.05$ were considered to be significant.

Results

The first aim of this study was to examine the composition of PL-ICs isolated from clinically asymptomatic lesions divided according to their size. As presented in Table 1, the total number of PL-ICs from the small-size lesions was lower compared to the number of PL-ICs isolated from the large size lesions ($p < 0.01$). However, their main composition was not significantly different, except that the large-size lesions contained a higher proportion of mononuclear phagocytic cells ($p < 0.05$).

The results related to the production of three immunoregulatory cytokines in the cultures of PL-ICs were presented in Figure 3. The levels of IL-10 and TGF-β were significantly higher in the PL-ICs cultures of larger-size lesions ($p < 0.01$ and $p < 0.05$, respectively). In contrast, the level of IL-27 was higher in the cultures of small-size lesions ($p < 0.05$).

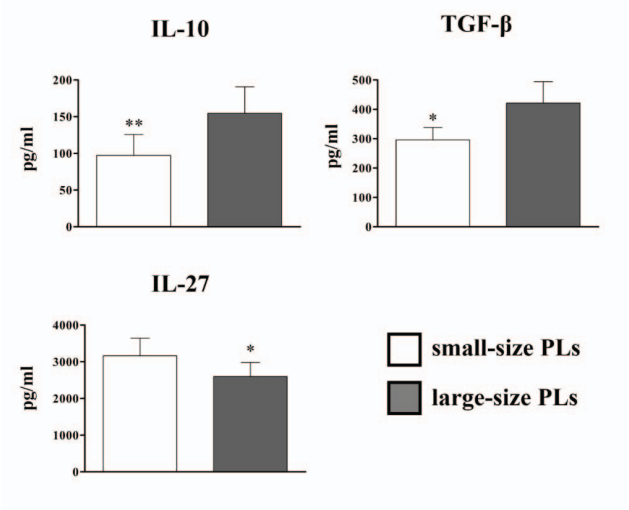

Fig. 3 – The levels of interleukin (IL)-10, IL-27 and transforming growth factor (TGF)-β in culture supernatants of asymptomatic periapical lesions (PL)-inflammatory cells (PL-ICs). Values are given as mean ± standard deviation for n = 12 (PL-ICs from the small-size lesions) or n = 18 (PL-ICs from the large-size lesions).
*** = $p < 0.05$; ** = $p < 0.01$, compared to the values of small-size (PLs).**

In order to explain whether these differences are associated with the cell subset composition in PLs, phenotypical analysis of PL-ICs was performed on the cytospin preparations by using two immunocytochemistry methods. Table 2 and Figures 4 and 5 show that the proportion of B cells/plasma cells ($CD19/CD38^+$ cells), the subset of T cells ($CD8^+$) and $CD14^+$ cells were higher in the large-size lesions ($p < 0.005$; $p < 0.05$; $p < 0.05$, respectively). In contrast, the proportion of total T cells ($CD3^+$ cells) was higher in the small-size lesions ($p < 0.05$). No significant differences were observed in the proportion of $CD4^+$ T cells and $HLA\text{-}DR^+$ cells.

When the levels of cytokines in each group of lesions were correlated with the cellular composition of these lesions, no statistically significant correlation was found (data not shown).

Table 1

Total cellularity and cellular composition of periapical lesions (PLs)

Cell type	Small-size PLs (n = 12)	Large-size PLs (n = 18)
	mean ± SD	mean ± SD
Total number of cells ($\times 10^6$)	1.1 ± 0.8	2.3 ± 1.0**
Lymphocytes (%)	50.2 ± 7.2	48.0 ± 10.2
Plasma cells (%)	14.8 ± 6.6	17.1 ± 4.9
N.granulocytes (%)	16.4 ± 6.9	12.1 ± 7.5
Macrophages (%)	12.2 ± 3.7	17.9 ± 6.8*
Mast cells (%)	4.1 ± 1.6	2.9 ± 2.2
Other cells (%)	2.3 ± 1.9	2.0 ± 1.8

SD – standard deviation; *$p < 0.05$; **$p < 0.01$, compared to the values of small-size PLs.

Table 2

Phenotypic characteristics of inflammatory cells isolated from periapical lesions (PLs)

Markers	Small-size PLs (n = 12)	Large-size PLs (n = 18)
	mean ± SD	mean ± SD
CD3	38.2 ± 6.9	32.4 ± 7.1*
CD4	19.2 ± 5.2	22.3 ± 6.5
CD8	6.8 ± 4.6	12.2 ± 5.8*
CD19/38	20.4 ± 6.9	30.8 ± 5.5***
CD14	13.8 ± 4.7	21.7 ± 8.6*
HLA-DR	15.1 ± 3.6	20.9 ± 8.8

SD – standard deviation; *$p < 0.05$; ***$p < 0.005$, compared to the values of small-size PLs.

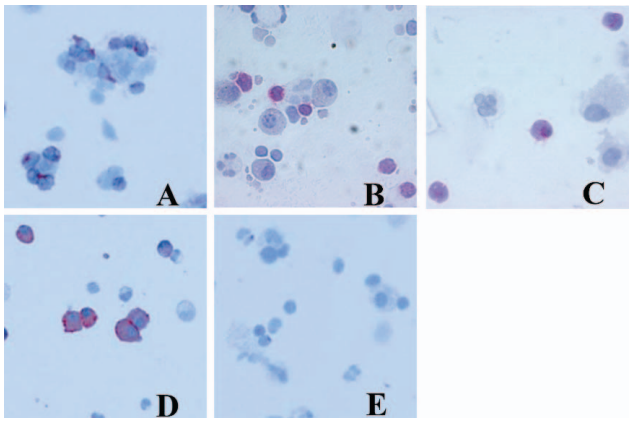

Fig. 4 – Representative images of cytospins stained with monoclonal antibodies (mAbs) to the lymphocyte cell subsets by an immunoalkaline phosphatase method: A) CD3; B) CD4; C) CD8; D) CD19/CD38; E) Negative control (Magnifications: × 600).

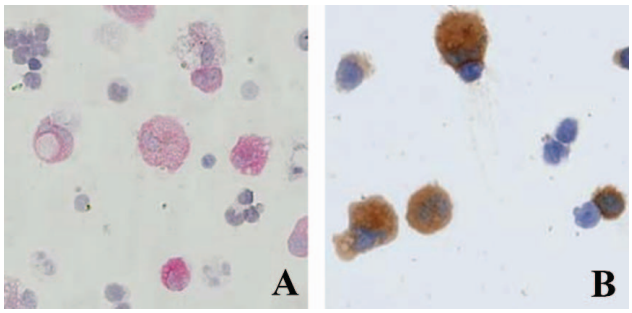

Fig. 5 – Representative images of cytospins stained with mAbs to CD14 (A) and HLA-DR (B).
A) immunoalkaline phosphatase method;
B) immunoperoxidase method [Magnifications: × 600].

Discussion

This is the first study investigating the production of immunoregulatory cytokines (IL-10, IL-27 and TGF-β) in clinically asymptomatic PLs, which were analyzed according to the size of PLs. IL-10 and TGF-β were implicated in the suppressive mechanisms mediated by different PL cells, including $CD4^+CD25^+Foxp3^+$ Tregs [19]. In contrast, IL-27 was identified as both pro-inflammatory and immunosuppressive cytokine in PLs [15]. We decided to study the clinically asymptomatic lesions based on a large number of data showing that immunosupressive mechanisms are more operative in this type of lesions [3, 4, 6, 20]. In addition, the large-size lesions are usulally an advanced stage of the PL development, characterized as large granulomas or cysts. In contrast, the small-size lesions usually represent an early stage of PL induction [6, 20]. The division into these two groups of PLs was arbitrary and based on the size of our PL collection. To avoid possible errors in the PL measurement [21] we excluded from the study those PLs whose greatest diameter was higher than 3.5 mm and PLs whose smallest diameter was less than 4.5 mm.

Both types of PLs had similar cellular content, composed predominantly of mononuclear infiltrating cells, which is a typical hallmark of asymptomatic lesions, whereas the proportion of granulocytes which are characteristic for symptomatic lesions (acute phase of PL development or PL exacerbation) was lower [3, 17, 20]. Our results showed that ICs from the large-size PLs produced higher levels of IL-10 and TGF-β than ICs from the small-size PLs. This finding correlates with a higher proportion of mononuclear phagocytic cells, which are an important source of these immunosuppressive cytokines [22]. Except macrophages, IL-10 is produced by dendritic cells, different subsets of T and B cells and various innate immune cells [23]. TGF-β is additionaly secreted by different stromal cells [24] and both cytokines are produced by Tregs [25]. In our previous paper [19], we showed positive correlations between the frequency of Tregs and the levels of IL-10 and TGF-β in culture supernatants of mononuclear cells (MNC) isolated from PLs. It is interesting that all Tregs, isolated from PLs, expressed IL-10, but only a half of them were TGF-β-positive [19]. Based on all these data, it can be supposed that the proportion of Tregs in the large-size PLs is higher than that in the small-size lesions, but this hypothesis, which has not been tested so far, needs to be proved in further experiments.

IL-10 and TGF-β have the potent anti-inflammatory properties that plays a central role in limiting the host immune response to pathogens, thereby preventing the tissue damage, including osteolysis in PLs [3, 7, 20]. Dysregulation of IL-10 is associated with the enhanced immunopathological processes in response to infection and increased risk for the development of autoimmune diseases [23]. It is interesting that the systemic administration of IL-10 for autoimmune therapy was shown to be paradoxically proinflammatory, whereas the localized IL-10 delivery proved to be therapeutic [26]. IL-10

mediates its immunosuppressive activity by the heterodimeric IL-10 receptors (IL-10R1, IL-10R2), which is expressed at varying degrees in many cell types, especially in monocytes and macrophages. The ligation of receptor is followed by the activation of Janus kinase/signal transducers and activators of transcription (JAK/STAT) signaling pathway, and subsequent transcription of various immunomodulatory genes, which, in turn, inhibit the production of pro-inflammatory mediators, stimulate the release of anti-inflammatory molecules such as interleukin-1 receptor antagonist (IL-1RA), soluble tumor necrosis factor-α (TNF-α) receptor, and interleukin IL-27 and down-regulate antigen presentation and phagocytosis [23, 26]. Additionally, through the release or via physical interactions with T cells, IL-10 can directly or indirectly enhance the Treg function [26], but it is also able to activate a certain type of immune cells, including the B cells, and to stimulate their proliferation. Such finding and association of IL-10 with the Th2 immune response are in agreement with a higher percentage of $CD19^+/CD38^+$ B cells/plasma cells and with previous findings that the advanced stage of PL development is associated with the predominance of humoral immune response [3, 20, 23].

TGF-β participates in several important pathophysiological processes and plays a bidirectional activity in the immune regulation. Its dual role in modulating the macrophage function is rapidly gaining recognition. TGF-β functions as a macrophage suppressing agent and as a monocyte activator [24]. It also exerts the inhibitory and stimulatory effects on the bone marrow cells, stimulates the chemotactic activity of osteoblasts and supports the differentiation of mesenchymal stem cells into osteoblasts and chondroblasts [24, 27]. In our previous paper, we showed that the production of TGF-β by PL-ICs is upregulated by the mesenchymal stem cells isolated from PLs [28]. TGF-β also stimulates the production of extracellular matrix and collagen type I, the molecules which are of key importance for the promotion of tissue healing [29]. It is known that healing is a characteristic of advanced stage of the PL development [20], a finding which is in correlation with our present results.

IL-27 is a relatively new cytokine member of the IL-6/IL-12 family, exerting both pro-inflammatory and anti-inflammatory properties. It consists of the Epstein-Barr virus-induced gene 3 (EBI3) and p28 subunits and acts through the IL-27 receptor complex formed by WSX-1 and gp130 subunits. DCs and activated macrophages are its main source [30]. In the PLs endothelial cells [15] as well as the mast cells [31] are also $IL\text{-}27^+$. It is well documented that IL-27 plays an important role in the initial immune response by stimulating the production of Th1 cytokines, which are important for the development of granulomatous diseases (tuberculosis, sarcoidosis and Crohn's disease) [32]. However, IL-27 has general inhibitory effects on the activity of Th1, Th2, Th17 and regulatory T cells [30]. The inhibitory effect of IL-27 on osteoclasts [33] is in accordance with this hypothesis, suggesting the role of this cytokine in the restriction of unwanted immune responses and bone destruction in PLs. In our previous experiment, we showed that the production of IL-27 by PL-MNC, especially in symptomatic PLs, was a significantly higher compared to its level in the cultures of peripheral blood MNC and correlated with the frequency of $CD14^+$ and $CD3^+$ cells. Exogenous IL-27 stimulated Th1 and downregulated the Th17 cytokine production by PL-MNC from symptomatic PLs, but the downregulated Th1 and Th2 responses in asymptomatic PLs suggested its complex biological functions in PLs [15].

We showed in this study for the first time that the small-size asymptomatic PLs produce the higher levels of IL-27 than the large-size PLs. These results suggest that the immunoregulatory mechanisms are differently regulated during the progression of PL development, having in mind the opposite production of IL-10 and TGF-β. Our results also suggest that, during initiation of the PL development, IL-27 may have a more important down-modulatory role in suppressing the production of Th1 and Th2 cytokines, than the other two cytokines may have. However, under the certain conditions, IL-27 can stimulate the Th1 response, which is mediated by the activated $CD4^+$ T-cells, recently migrated into the periapical tissue. This finding was already observed in our previous paper, in which we showed that exogenous IL-27 stimulated the production of IFN-gama by PL-MNC, especially during the exacerbation phase of the PL development [15].

Conclusion

The results of this study, performed on clinically asymptomatic PLs, showed that the small-size lesions differ from the large-size lesions in terms of IL-10, IL-27 and TGF-β production. ICs from the small-size PLs produce significantly much higher quantity of IL-27 than the large-size PLs. It was the opposite with IL-10 and TGF-β. Cumulatively, it can be hypothesized that these immunoregulatory cytokines may play different roles in the suppression of unwanted immune/inflammatory responses in asymptomatic PLs, depending on the extension of the pathological process.

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Declaration of interest

Authors declare no conflict of interest.

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Oncological outcome audit of multivisceral resections for primary colorectal cancer: a single centre experience

Pregled onkoloških rezultata multivisceralnih resekcija kod primarnog kolorektalnog karcinoma: iskustvo jednog centra

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Abstract

Background/Aim. Colorectal cancer still presents a major health problem, with around 10% of patients in whom the tumor invades surrounding structures or organs. These patients are usually challenging even for an experienced colorectal surgical team. The decision for performing multivisceral resection (MVR) is often made intraoperatively, with no sufficient data on the tumor and patient condition. The percentage of perioperative morbidity and mortality is high and oncological outcome is often unfavorable.The aim of this study was to investigate the poor oncological outcome risk factors after MVR in the patients with colorectal carcinoma. **Methods.** This was a retrospective analysis of patients operated at the Department for Colorectal Surgery of the First Surgical Clinic, Clinical Centre of Serbia, Belgrade. The *en bloc* multivisceral resection for the primary adenocarcinoma of the colon and rectum was uniformly performed. Data were collected in prospectively designed database. Follow-up period was minimum 2 years. The patients were analyzed in terms of histopathological, demographical and follow-up data. Survival and time to recurrence were evaluated using the Kaplan–Meier method and the log-rank test. **Results.** Two hundred and thirteen patients were included in the study Their mean age was 59.9 ±12.0 years. The follow-up period was 33.8 ±29 months. Histopathology confirmed the true tumor infiltration of surrounding organ/structure in 126 (59.2%) patients. The R0 resection was confirmed in 173 (81.2%) patients. Five-year overall survival was 43.4%. Five-year survival for colon patients was 45.9% and in the rectal cancer group 40.9%. In the N0 group of patients, the overall survival in 5-year period was 66.7%. The N1 and N2 status proved the adverse effect on survival (overall 5-year survival 31.3% and 15.9%, respectively). The five-year local recurrence rate in the R0 group of patients was 17.7% and the percentage of distant metastases was 66.3%. **Conclusion.** The multivisceral resections are demanding procedures requiring a highly specialized surgical team and a high volume hospital. The oncological outcome of these procedures is still unfavorable. In the cases with the node positive disease, or the R1 resection, the perspective is poor. On the other hand, in the absence of these unfavorable factors, we can expect a good oncological outcome. More meticulous preoperative staging and aggressive preoperative treatment can further improve the results.

Key words:
colorectal neoplasms; neoplasm invasiveness; neoplasm staging; surgical procedures, operative; prognosis.

Apstrakt

Uvod/Cilj. Kolorektalni karcinom još uvek predstavlja veliki zdravstveni problem sa oko 10% slučajeva kod kojih je tumor zahvatio okolne strukture/organe. Kompleksno lečenje ovih bolesnika je izazov, čak i u specijalizovanim centrima. Odluka o izvođenju multivisceralne resekcije (MVR) se često donosi intraoperativno, bez dovoljno informacija o tumorskom statusu ili samom bolesniku. Perioperativni morbiditet i mortalitet je visok, a onkološki ishod lečenja je često nepovoljan. Cilj rada je bio da se izlože faktori rizika lošeg ishoda nakon MVR kod bolesnika sa kolorektalnim karcinomom. **Metode.** Sprovedena je retrospektivna studija bolesnika operisanih na III Odeljenju, Prve hirurške klinike Kliničkog centra Srbije. *En bloc* MVR zbog primarnog adenokarcinoma kolona i rektuma je bila učinjena kod svih bolesnika. Podaci su bili prikupljani u prospektivno dizajniranoj bazi podataka. Postoperativno

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praćenje je iznosilo minimum dve godine. Analiza je obuhvatala patohistološke, demografske i podatke postoperativnog praćenja. Preživljavanje i vreme do recidiva bolesti je procenjivano na osnovu Kaplan-Meier i long-rank testa. **Rezultati.** Dvesta trinaest bolesnika je bilo uključeno u studiju. Prosek godina iznosio je 59,9 ± 12,0, a praćeni su prosečno 33,8 ± 29 meseci. Patohistološki, tumorska infiltracija okolnih organa/struktura dokazana je kod 126 (59,2%) bolesnika. R0 resekcija je potvrđena kod 173 (82,1%) bolesnika. Ukupno petogodišnje preživljavanje je iznosilo 43,4%. Kod bolesnika sa karcinomom kolona preživljavanje je iznosilo 45,9%, a kod onih sa karcinomom rektuma 40,9%. Ukupno petogodišnje preživljavanje u N0 grupi bilo je 66,7%. N1 i N2 kategorije su imale loš uticaj na petogodišnje preživljavanje (ukupno preživljavanje 31,3% i 15,9%, respektivno). Petogodišnji lokalni recidiv u R0 grupi iznosio je 17,7%, a procenat udaljenih metastaza je bio 66,3%. **Zaključak.** Multivisceralne resekcije su zahtevne procedure, zahtevaju visoko specijalizovan hirurški tim u ustanovi sa velikim brojem takvih sučajeva. Onkološki rezultat ovih operacija je još uvek nezadovoljavajući, bez velikih varijacija u odnosu na lokalizaciju bolesti (kolon/rektum). Kod slučajeva sa pristunim nodalnim metastazama, ili kod onih sa R1 resekcijom, perspektiva je veoma loša. Sa druge strane, kod onih gde ne postoje navedeni faktori rizika, možemo da očekujemo razumno dobar rezultat lečenja. Rezultate je moguće unaprediti sprovođenjem detaljnog preoperativnog određivanja stadijuma bolesti i primenom agresivne neoadjuvantne terapije.

Ključne reči:
kolorektalne neoplazme; neoplazme, invazivnost; neoplazme, određivanje stadijuma; hirurgija, operativne procedure; prognoza.

Introduction

Colorectal cancer, despite all efforts made in the early discovery, preoperative therapy, surgery and adjuvant treatment, still presents the major health problem [1, 2]. In countries with developed screening programs, early colorectal cancer becomes an important issue with almost 20% –25% of all treated patients. On the other side, surgeons still have at hands a considerable number of patients with advanced disease [3]. Among these, the patients with locally advanced tumors are of special interest to us. By using the good pretreatment staging, neo and adjuvant treatment, and most importantly, the high quality surgery, the cure of the disease is still achievable. We have around 10% of patients with tumor invading the surrounding structures or organs, i.e., T4b adenocarcinomas [3]. These patients are usually challenging to treat even for an experienced colorectal surgical unit [2, 4–7, 9]. The reasons for this are numerous: the decision for performing multivisceral resection (MVR) is made intraoperatively with no sufficient data on the tumor and patient condition, the percentage of perioperative morbidity and mortality is high and finally, the oncological outcome is often unfavourable. Many explanations are offered, but it still remains a matter of debate among experts. The *en bloc* resection is a well-established method of choice. Any fragmentation of partial resection is followed by a poor oncological outcome with unacceptable morbidity and mortality, thus, we have to avoid the emergency of unplanned MVR in order to achieve the good treatment result. Meticulous preoperative staging is of paramount importance. In addition, we have to be aware of unfavourable prognostic factors that would prevent us of performing, or lead us to employ the neoadjuvant approach prior to the MVR. Postoperatively, the group of high-risk patients is the candidate for the intensive adjuvant treatment and more aggressive follow-up regimen. Among mentioned above, the impact of lymph node metastases on the treatment outcome [6–8] was discussed controversially in a number of papers and was described to be associated with an adverse outcome.

Having at hand relatively large population of patients with locally advanced colon and rectal carcinomas in our unit where the MVR was performed, we performed a retrospective analysis of all patients operated between 1995 and 2011.

The type of operation and details of pathological report were analysed in order to establish the risk factors for a poor oncological outcome after the MVR.

Methods

The study was a retrospective analysis of prospectively collected data of consecutive cohort of patients in the period between September 1995 and December 2011. All patients were operated at the Department for Colorectal Surgery of the First Surgical Clinic, Clinical Centre of Serbia, Belgrade. The procedures were performed by the same surgical team and included the *en bloc* MVR for the primary adenocarcinoma of colon and rectum. The patients with distant metastases and those with the intraoperative tumor fragmentation were excluded. The study was approved by the Ethics Committee of the Faculty of Medicine in Belgrade. The preoperative workup included: endoscopy with biopsy, cystoscopy, ultrasound, pelvic/abdominal computed tomography/magnetic resonance imaging (CT/MRI).

The patients were operated following a standardized open approach with high ligation of the corresponding lymphovascular bundle. Additionally, if the infiltration of adjacent organs was suspected, or the mobilization, or the sharp dissection was not feasible, a primary MVR was performed. The procedures were classified according to the primary colorectal operation regardless of the extent of additional resection. The sixth edition of the Union for International Cancer Control (UICC) classification from 2002 was used to categorize colorectal adenocarcinomas. Rectal cancer was diagnosed according to the distance, measured by a rigid proctoscope (first 15 cm). In the patients with rectal cancer, a neoadjuvant treatment was not standardized according to the modern guidelines. Only the most advanced cases fit for this mode of treatment, selectively received neoadjuvant chemoradiotherapy. The majority

of patients in the neoadjuvant treatment group were planned for abdominoperineal amputation.

Patients' hystopathology and operation data were collected in prospectively designed database. The follow-up period was minimum 2 years according to the standardized protocol.

The data was evaluated by using the descriptive statistical methods. The patients with the MVR were analysed in terms of the histopathological, demographical and follow-up data. The survival and time to recurrence were evaluated by using the Kaplan–Meier method and the log-rank test. The p value < 0.05 was considered significant.

Results

Initially, 213 patients were included in the study. Their basic characteristics are shown in Table 1.

Table 1

Clinical and histopathological features of patients with multivisceral resections for primary colorectal carcinoma

Characteristics of patietns	Multivisceral reactions n (%)
Sex	
female	94 (44.1)
male	119 (55.9)
Tumor localization	
colon	107 (50.2)
rectum	106 (49.8)
TNM status	
T3	87 (40.8)
T4	126 (59.2)
N0	95 (44.6)
N1	50 (23.5)
N2	68 (31.9)
N1 + N2	118 (55.4)
R status	
R0	173 (81.2)
R1	40 (18.8)

TNM staging system of malignant neoplasms – tumor-lymph node-metastasis.

The mean age was 59.9 ± 12.0 years. The average follow-up period was 33.8 ± 29 months. A total of only 22 (20.75%) patients received the neoadjuvant treatment (the rectal cancer patients). The number of colon and rectum cancer patients was comparable, almost equal (107 vs. 106, respectively). The most frequently, MVR was performed in the patients with sigmoid cancer. MVR involved the partial, or complete removal of single additional organ in 129 (60.6%) patients. The most commonly affected organ was the urinary bladder in 47 (22.1%) patients. The partial resection of the small intestine was necessary in 44 (20.7%) patients, and the abdominal wall was resected in 62 (29.1%) patients. The data are shown in Table 2. The infiltration of removed organs/structures was comparable to the overall infiltration rate of around 59%. This percentage was roughly present when analysing percentage of resected/infiltrated organs (Table 2).

Table 2

List and number of the resected and infiltrated organs

Organs	Resected organs n (%)	Tumor infiltration present n (%)
Pelvic wall	10 (4.69)	4 (1.88)
Abdominal wall	62 (29.11)	42 (19.72)
Diaphragm	3 (1.41)	1 (0.47)
Liver	8 (3.76)	6 (2.82)
Gallbladder	5 (2.35)	2 (0.94)
Duodenum	4 (1.88)	2 (0.94)
Pancreas	8 (3.76)	6 (2.82)
Stomach	3 (1.41)	2 (0.94)
Spleen	9 (4.23)	3 (1.41)
Kidney	3 (1.41)	2 (0.94)
Appendix	15 (7.04)	8 (3.76)
Other parts of colon	1 (0.47)	1 (0.47)
Small intestine	44 (20.66)	31 (14.55)
Urinary bladder	47 (22.07)	32 (15.02)
Uterus	17 (7.98)	10 (4.69)
Adnexa	30 (14.08)	13 (6.10)
Ureter	5 (2.35)	2 (0.94)
Vagina	30 (14.08)	17 (7.98)
Prostate	18 (8.45)	10 (4.69)
Sem. vesicles	23 (10.80)	9 (4.23)
Sacrum	9 (4.23)	2 (0.94)
Other	5 (2.35)	1 (0.47)

Sixteen (7.5%) patients died in the first month after the operation. The procedures and average number of lymph nodes harvested are presented in Table 3.

Table 3

List of performed procedures and the average number of lymph nodes harvested

Type of surgical procedure	Surgical procedure n (%)	Average number (n) of lymph nodes harvested
Low anterior resection of the rectum	49 (23.01)	28.94
Resection of the rectum with partial mesorectal excision	39 (18.31)	28.44
Abdominoperineal resection of the rectum	35 (16.43)	20.34
Hartmann's procedure	27 (12.68)	26.63
Right hemicolectomy	28 (13.15)	28.64
Left hemicolectomy	11 (5.16)	30.09
Subtotal colectomy	7 (3.29)	47.00
Total pelvic exenteresis	5 (2.35)	25.40
Partial resection of the colon	5 (2.347)	17.60
Total colectomy	4 (1.88)	59.71

Table 4

Oncological outcomes, overall, according to localization (colon/rectum) and two favorable categories T3N0R0

Neoplasm	5-year OS (%)	5-year DFS(%)	5-year LR(%)	5-year DM(%)
Overall	43.40	31.01	26.75	65.29
Colon	45.97	34.40	25.90	62.67
Rectum	40.99	27.63	27.83	67.85
Colon T3N0R0	82.63	49.62	17.93	20.18
Rectum T3N0R0	87.50	71.09	6.20	22.22

OS – overall survival; DFS – disease free survival; LR – local recurrence; DM – distant metastases.

The histopathology exam confirmed the true tumor infiltration of the surrounding organ /structure in 126 (59.2%) patients. We were not able to confirm the true tumor infiltration in 87 (40.8%) cases – the R0 resection was confirmed in 173 (81.2%) patients (Table 1).

We analysed the prognostic factors overall and cancer-specific survival, time to local/distant recurrence.

Of interest to us, in terms of oncological outcome, was the group of patients with the favourable prognostic factors, i.e., with no nodal deposits and R0 resection. The results in the favourable categories, for colon and rectum carcinomas, are presented in Table 4.

The five-year overall survival was 43.4% (Figure 1), 45.9% for the colon patients and 40.9% for the patients from the rectal cancer group. The T stage proved to influence the oncological outcome of MVR and a significant difference was noted between the T3 and T4 tumors in terms of overall survival (OS) (60.03% and 32.97% at 5 years, respectively) (Figure 2). In the N0 group of patients, the situation was significantly better since OS in the five-year period was 66.7%. The N1 and N2 status proved the adverse effect on survival (overall survival 31.3% and 15.9% respectively) and no patients in this stage lived for 5 years. These results include both the R0 an R1 patients (Figure 3). The local recurrence rate in the R0 group of patients was 17.7% (Figure 4), and the percentage of distant metastases in the five-year period was 66.3% (Figure 5).

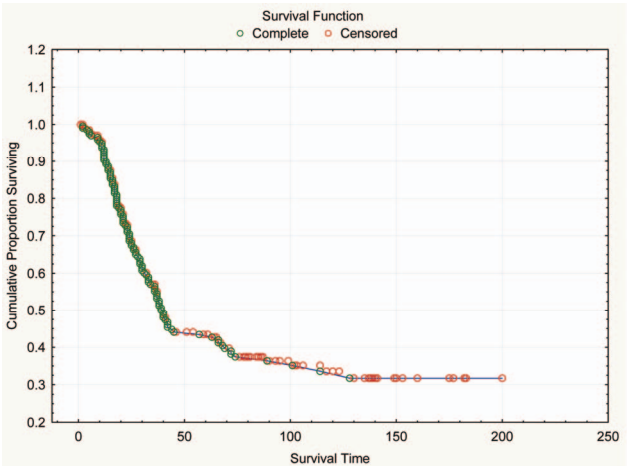

Fig. 1 – Kaplan-Meier curve – overall survival (43.4%) at 60 months.

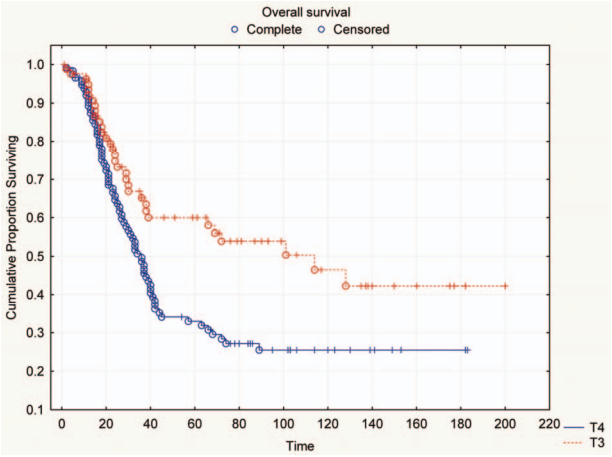

Fig. 2 – Kaplan-Meier curve – Overall survival according to the T stage.

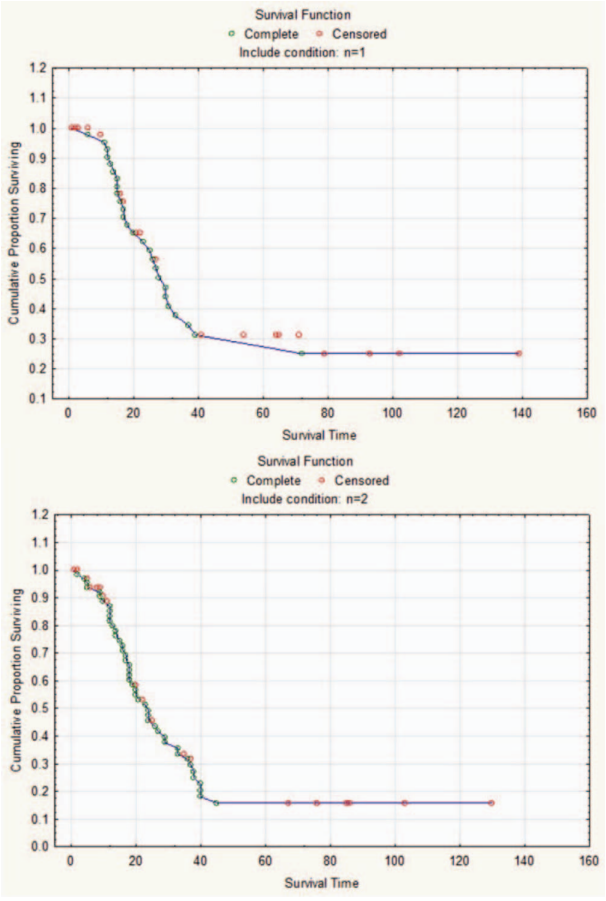

Fig. 3 – Kaplan-Meier curve – overall five-year survival in patients with the N1 and N2 status (31.3% and 15.9%, respectively).

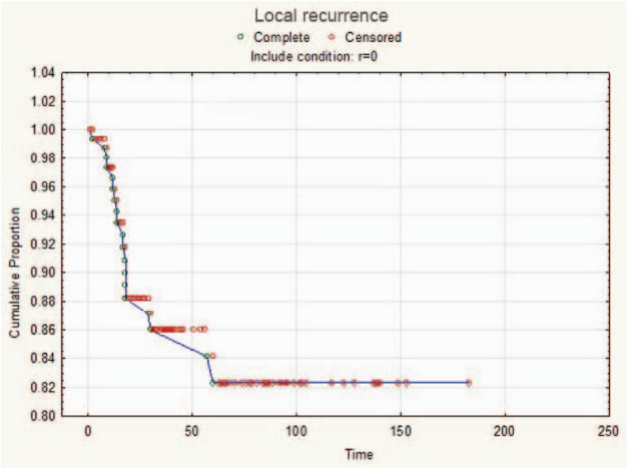

Fig. 4 – Kaplan-Meier curve – the local recurrence for the R0 resections trend. The local recurrence at 5 years – 17.7%.

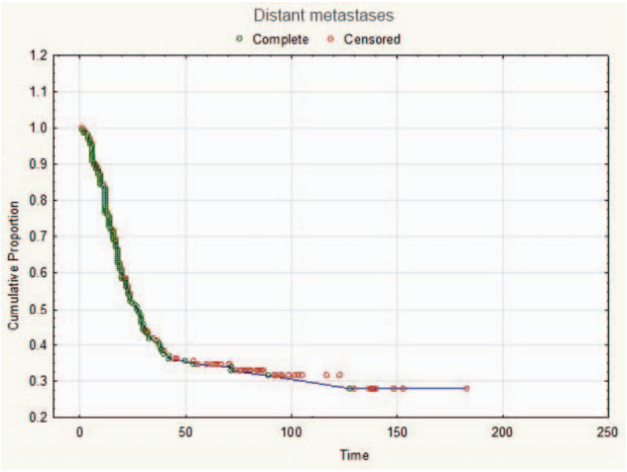

Fig. 5 – Kaplan-Meier curve – the distant metastasis trend. Distant metastases at 5 years – 66.3%.

Discussion

Multivisceral resections for primary colorectal cancer are extremely complex procedures. One, maybe the most important aspect of these procedures, is a need for the multidisciplinary approach. By doing so, we can expect a good outcome in terms of the morbidity, mortality and favourable oncological result [7].

There is a number of papers published on this subject, but a direct comparison of results is often difficult. The reason for this is a wide variation of inclusion criteria. In our series, the invasion of neighbouring organs was confirmed by a pathologist in 59.2% of the cases, which was comparable to the majority of other series (malignant invasion in 34% to 58%) [10]. An existence of peritumor fibrosis makes a distinction between the adhesions and tumor invasion impossible. In these cases any attempt of division or dissection results in the R1 or R2 resection with the poor outcome. The *en bloc* resection is uniformly advised in order to avoid the tumor cell dissemination and tumor fragmentation [5–7, 11–13]. The organs/structures most frequently resected in this series were: abdominal wall, urinary bladder and small intestine. This can be explained by the fact that the majority of colon carcinomas were located in the sigmoid colon which is comparable to similar studies [6, 10, 14, 15].

An R0 resection, according to the published data is a favourable prognostic factor [16, 17]. In our study, the R0 resection was achieved in 81.2% which corresponds to the upper range of published data (range 72%–91%) [14, 15, 18–20]. The R positive resection was, on the other side, associated with the poor prognosis [9, 19]. Similar conclusion can be reached by looking into our series data, where the patients with the R1 and R2 resection had the poor oncological outcome [9].

The main limitation of our study, besides its retrospective nature, is the group of patients with rectal cancer. In this group, there was a number of them included in the period when the neoadjuvant treatment was not standardized nor uniformly employed. Hence, the proportion of patients with the neoadjuvant therapy was fairly low, 20.75%. This can explain the relatively high percentage of local recurrence in this group (five-year local recurrence for R0 group was 19.1%), followed by the poor overall and cancer specific survival. The role of neoadjuvant chemoradiotherapy in the local control of rectal cancer is very well known. We can even expect the complete response in the patients planned for the multivisceral resection [14].

Our results confirm that the neoadjuvant therapy is the preferable way to treat the patients with the locally advanced rectal cancer.

In the group of patients with colon cancer, we achieved the results comparable to other studies [7, 10, 15, 20].

The five-year local recurrence rate of 26.8% is comparable to the mentioned studies. Adding the absence of neoadjuvant treatment in the rectal cancer group, we can say that these results are acceptable.

In our study, the lymph node involvement was significantly and independently associated with the decreased survival rate. The same conclusion was reached in the studies that emphasized this problem [6, 20].

In the context of our results, concerning both patient groups (colon and rectal cancer), we have to stress that a high quality surgery can yield an acceptable oncological outcome even in the absence of appropriate neo and/or adjuvant approach. For this reason, further education and constant improvement of surgical practise should be performed, instead of over-relying on the effect of chemo- and radiotherapy.

Conclusion

We can state that the multivisceral resections have no desired oncological outcome. The results for colon and rectal carcinomas are with no dramatic differences. It is difficult to evaluate properly the oncological outcome of rectal cancer patients, since a small proportion of them received adequate neoadjuvant therapy. We administered this mode of therapy only to the patients with "ugly" carcinomas, most frequently planned for the abdominoperineal amputation. The patients with the node positive disease, or R1 resection had the ex-

tremely poor outcome. Fortunately, a significant proportion of those without the mentioned risk factors had the acceptable outcome. Based on the results and published papers, we can conclude that the meticulous preoperative staging and preoperative therapy both for the colon and rectal patients was the way for further improvement of oncological outcome of multivisceral resections.

Acknowledgements

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Frequency and risk factors for injury of the inferior alveolar nerve during surgical extraction of the impacted lower third molars

Učestalost i faktori rizika za povredu donjeg alveolarnog nerva u toku hirurške ekstrakcije impaktiranih donjih trećih molara

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Abstract

Background/Aim. The injury of inferior alveolar nerve during a surgical extraction of impacted lower third molars, followed by sensory disturbance, is, for the patient, an extremely unpleasant complication. The aim of this study was to determine the frequency of this complication after the third molar surgery and its frequency depending on a tooth position and tooth relation to the mandibular canal. **Methods.** In this study, 800 surgical extractions of the impacted lower third molar were performed. The position of the impacted tooth was recorded according to the Winter classification, as well as the ratio of their root tips to the mandibular canal using the Tanaka et al. and Rood and Shebab classifications. **Results.** The frequency of the recorded post extraction sensory disturbance was 2.25%, most frequently when teeth were in the mesioangular position. Concerning Tanaka and al. classification, the incidence of injuries was inversely proportional to the increase of distance between roots and mandibular canal with the statistical significance in cases where mandibular canal overlaps more than a half of the root of the tooth (p = 0.001). Considering the radiological signs recommended by Rood and Shehab, a higher frequency of the inferior alveolar nerve injury was recorded when illumination in the area of the root tips was present and when the loss of linear overshadowing characterized by the "roof" and the "bottom" of the mandibular canal were observed, or diversion of the canal and root deflection, but without a statistical significance. **Conclusion.** The superposition of the mandibular canal with the lower third molar roots at the panoramic radiographies may increase a possibility of the inferior alveolar nerve injury. The angulations of the impacted lower third molar as well as the vicinity of the tips of its roots to the content of the mandibular canal, do not significantly affect the frequency of the nerve injury.

Key words:
molar, third; tooth, impacted; tooth extraction; mandibular nerve; oral surgical procedures; paresthesia.

Apstrakt

Uvod/Cilj. Povreda donjeg alveolarnog nerva prilikom hirurške ekstrakcije donjeg impaktiranog umnjaka, praćena poremećajem senzibiliteta, za pacijenta je izuzetno neprijatna komplikacija. Cilj ovog istraživanja je bio da se utvrdi učestalost ove komplikacije posle hirurškog vađenja impaktiranih donjih umnjaka, zavisno od položaja donjeg umnjaka i njegovog odnosa prema mandibularnom kanalu. **Metode.** U studiju je bilo uključeno ukupno 800 hirurški ekstrahovanih impaktiranih donjih umnjaka. Preoperativno je analizom digitalnog ortopantomografskog snimka evidentiran položaj impaktiranih umnjaka prema Winter-ovoj klasifikaciji, kao i odnos njihovih vrhova korenova prema mandibularnom kanalu primenom klasifikacije Tanaka-e i saradnika, kao i Rooda-e i Shehaba-e. **Rezultati.** Učestalost poremećaja senzibiliteta iznosila je 2,25%, najčešće kada je umnjak bio u mezioangularnom položaju. Imajući u vidu klasifikaciju Tanaka-e i saradnika, uočen je značajno veći broj povreda u slučajevima kada mandibularni kanal superponira više od polovine korena impaktiranog umnjaka (p = 0,001). Praćenjem rendgenoloških znakova preporučenih od strane Rooda i Shehaba, veća učestalost povreda donjeg alveolarnog nerva zapažena je u slučajevima kada je postojalo rasvetljenje u predelu vrhova korenova i gubitak linijskog zasenčenja „krova" i „poda" mandibularnog kanala ili kada je zapaženo skretanje kanala ili defleksija korenova, ali bez statističke značajnosti. **Zaključak.** Superpozicija mandibularnog kanala sa korenovima donjeg trećeg molara na ortopantomografskom snimku može povećati mogućnost

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povrede donjeg alveolarnog nerva. Angulacija impaktiranih donjih umnjaka, kao odnos vrhova njihovih korenova prema sadržaju mandibularnog kanala, ne utiču značajno na učestalost povrede nerva.

Ključne reči:
molar, treći; zub, impakcija; zub, ekstrakcija; n. mandibularis; hirurgija, oralna, procedure; paresteija.

Introduction

The injury of the inferior alveolar nerve during a surgical extraction of the impacted lower third molars is relatively rare complication, but for the patient an extremely unpleasant one. It can occur indirectly (infection in the post-extraction area, pressure of postoperative hematoma and/or oedema on the nerve), or directly (injuries due to intimate contact of the lower third molar roots and the nerve) [1]. This injury manifests by a permanent or transient sensitivity disturbance in the area of nerve innervation of varying intensity. Clinically, sensory deficits are classified as: paraesthesia (neuropraxia and axonotmesis) and anaesthesia (neurotmesis) [2]. Neuropraxia is the easiest degree of the sensitivity disturbance and presents the appearance of mild paraesthesia in the form of tingling, burning and numbness in the nervous distribution area [3]. It is usually due to ischemia or compression of the nerve, while its structure is still preserved [4]. Axonotmesis is a severe sensory disturbance that manifests itself as hyperalgesia (overstimulation on stimuli) or allodynia (pain caused by harmless stimuli) [5]. It occurs due to the interruption of certain axons in the structure of the nerve with consequent Wallerian degeneration, but still with the preserved myelin coating [6]. Neurotmesis is a permanent and complete absence of sensitivity caused by a complete breakdown of the morphological continuity of the nerve.

The degree of restoration of sensitivity depends on the extent of the damage. The outcome after neuropraxia is most often a complete recovery of sensitivity by the end of the fourth month at the latest [7]. Axonotmesis is usually accompanied by an incomplete recovery of sensitivity, especially if the disturbance lasts longer than six months [4], while anaesthesia in neurotmesis, which lasts longer than a month, is usually permanent [7]. A higher degree of recovery can be expected in younger people, with a good general condition, with good vascularization of the tissue, without the presence of a foreign body and with a preserved epineural coat [8].

The aim of this study was to determine the frequency of occurrence of sensory deficits in the innervation distribution of the lower alveolar nerve after surgical extraction of the impacted lower third molar, as well as the frequency of this complication, depending on a tooth position and its relation to the mandibular canal.

Methods

The research was conducted as a prospect clinical study in the period from 2009 to 2017 at the Department of Oral Surgery, Faculty of Medicine in Kosovska Mitrovica. The study included 687 people of both sexes from the northern part of Kosovo and Metohija, aged 17–60 years, who had 800 surgical extractions of the impacted lower third molars. The study excluded people with a history of some neurological disease. The preoperative plan involved an analysis of digital panoramic radiographs of each patient. The position and angulation of impacted teeth were analysed according to the Winter classification. The study included the teeth in the most common positions - mesioangular, vertical, horizontal and distoangular [9]. Also, the relationship of the root tips of the impacted teeth and the mandibular canal was analysed using the classification by Tanaka et al. [10] and Rood and Shehab [11] (Figures 1 and 2). The cases that were classified in the first three classes by Tanaka et al. [10] were further analysed by the Rood and Shehab classification. According to this classification, seven X-ray signs or indications of a close contact between the tips of the roots of the lower third molar and the contents of the mandibular canal were proposed (Figure 2).

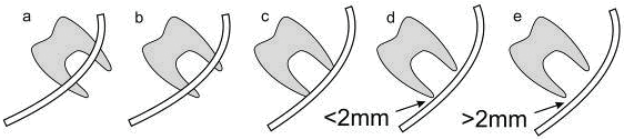

Fig. 1 – Classification by Tanaka et al. [10]: a) Class I – the canal overlaps more than a half of the root of the tooth; b) Class II – the canal overlaps less than a half of the root of the tooth; c) Class III – the root reaches to the upper limit of the mandibular canal; d) Class IV – the distance between the root and the top of the canal is less than 2 mm; e) Class V – the distance between the root and the upper edge of the canal is more than 2 mm.

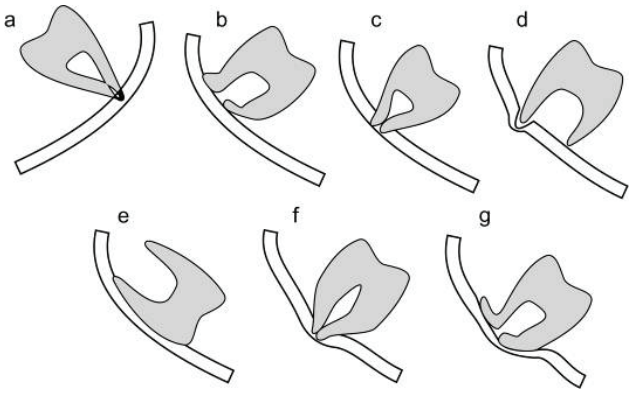

Fig. 2 – Classification by Rood and Shehab [11]: a) X-ray illumination of tooth root; b) deflection of tooth root; c) narrowing of the root; d) lightness and bifid root apex; e) interruption of the "white" line, i.e., X-rays of linear shadow, which delineates "roof" or "bottom" of the mandibular canal (interruption of white line of the canal); f) diversion of the canal; g) narrowing of the canal.

In all patients included in this study, the surgical extractions of the impacted third molars were performed in local block anaesthesia for the inferior alveolar nerve (HCR 4% + Epinephirne 1:100.000, 1.7 mL), with the use of standard triangular flap and buccal approach to the impacted lower third molar. Osteotomy and, if necessary, a separation of crown and/or roots of the impacted teeth was performed using the round and fissure carbide drills with a minimal trauma to the surrounding jawbone and mandatory cooling with saline to prevent or reduce the surgical trauma of the mandibular canal. After the extraction, antibiotic and/or analgesic therapy was prescribed when needed.

The post-operative follow-ups were performed on the first and the seventh post-operative day. To the patients with the sensitivity disturbances registered in the innervated area of the lower alveolar nerve, the complex of vitamin B (Beviplex N, Galenika A.D. Serbia) was administered in the next four weeks, in a dose of one coated tablet daily, and, in the same period of time, infrared therapy was applied locally (Infrared lamp, Medisana IRL), three times a week, lasting 15 minutes, from a distance of 40 cm. The patients with persistent paraesthesia after this period were called for screening twice a month until the total absence of symptoms. Anamnesis and three types of clinical tests were used to determine the existence of the sensory deficiency:

Pin-prick test was performed with a dental probe placed on the surface of the skin or mucous membrane, and then used for a light prick of the tissue, with the simultaneous assessment of painful perception of the patient. Each area was examined three times consecutively, bilaterally, and the patient was asked to point to any difference in sensation between the two sides. The test was performed in the skin area of the corners of the mouth and mucous membranes from the vestibular side in the area of the canines [12].

The light touch test was performed by a tactile stimulation of the patient's skin. A ball of cotton wool was used to gently touch the skin at the corners of the lips, concentrically, gradually spreading towards the periphery until the patient felt touch, which made it possible to map the area with a loss of sensation [12];

The two-point discrimination test – this test was performed using a ten-millimetre scale divider. The tips of the divider at a distance of 2 mm were placed on the skin at the corners of the mouth with continuous pressure, and the patient was asked whether he felt pressure in one or two points. Whenever the patient gave an incorrect response, the distance of the divider peaks for the next test would increase and whenever the patient gave the correct answer the distance of the divider peaks for the next test would be reduced until the smallest distance in which the patient could feel the pressure in two spots was defined. The distance obtained was compared with the reference values. With the preserved sensitivity in the corner of the lips, the pressure in two spots could be different at their distance from 2 mm to 4 mm and in the lower edge of the mandible at the distance of 8 mm to 10 mm [13]. The minimum distance of two spots where the pressure might vary was usually greater on the side of the damage [14].

The results were presented in percentages and ratios. A binomial test was used to analyse the frequency of events of interest. For the multiple variables testing, Bonferroni correction was applied. All p values less than 0.05 were considered significant. All analyses were performed in the R programming language and environment (R Core Team, 2014).

Results

Of the total number of surgically extracted the impacted lower third molars, the occurrence of sensory disturbances was recorded at 18/800 or 2.25%. In all patients, in the observed period of 6 months, full recovery of sensitivity occurred.

Of all recorded cases with the injuries of the lower alveolar nerve, the largest number occurred after the surgical extraction of the impacted lower third molars in the mesioangular position, and the least was registered at distoangular position (Table 1). The statistical analysis of the obtained results showed no statistically significant difference in the incidence of lower alveolar nerve injuries compared to the position of the impacted third molar.

By monitoring a percentage of the sensitivity outbreak in the innervated zone of the inferior alveolar nerve, depending on the relationship of the molar roots and the mandibular canal, the highest percentage of sensitivity disturbance was registered in Class I, then in Class II and the smallest in Class III, according to the Tanaka et al. classification [10] (Table 2). The statistically significant difference was observed in Class I, in the cases where mandibular canal overlapped more than a half of the root of the tooth ($p = 0.001$).

After additional analysis of all registered cases of sensitivity outbreaks in the innervated zone of the inferior alveolar nerve in different classes (Tanaka et al. [10]), to which the classification by Rood and Shehab [11] was applied, it was concluded that the nerve injuries were more frequently met when the „white" line of the root canal and canal diversion was interrupted and when the root deflection and narrowing of the canal were previously radiographically observed (Table 3), but without a statistical significance.

Table 1

Nerve injury in regard to the angulation of the impacted lower third molar (Winter [9] classification)

Position of impacted lower third molar	n	Sensitivity disturbance, n (%)		p – value
		with	without	
Mesioangular	336	11 (3.3)	325 (96.7)	0.196
Vertical	242	4 (1.7)	238 (98.3)	0.826
Horizontal	118	2 (1.7)	116 (98.3)	1.000
Distoangular	104	1 (1.0)	103 (99.0)	0.734
Total	800	18 (2.3)	782 (97.7)	

***p* – values were calculated by comparing each ratio with the ratio 18:782.**

Table 2
Nerve injury in regard to the relationship of the molar roots and the mandibular canal (Tanaka et al. [10] classification)

Class*	n	Sensitivity disturbance, n (%)		*p* – value
		with	without	
I	264	13 (4.9)	251 (95.1)	0.001†
II	157	4 (2.6)	153 (97.4)	0.783
III	188	1 (0.5)	187 (99.5)	0.138
IV	106	0	106 (100)	0.181
V	85	0	85 (100)	0.270
Total	800	18 (2.3)	782 (97.7)	

*** – Description of particular class see under Fig. 1.**
***p* – values were calculated by comparing each ratio with the ratio 18:782; † – statistically significant.**

Table 3
Nerve injury in regard to the relationship of the molar roots and the mandibular canal (Rood and Shehab [11] classification)

Parameters	n	Sensitivity disturbance, n (%)		*p* – value
		with	without	
Illumination of root	172	7 (4.1)	165 (95.9)	0.362
Deflection of root	174	3 (1.7)	171 (98.3)	0.498
Narrowing of root	0	0	0	-
Lightness and bifid root apex	12	0	12 (100)	1.000
Interruption of white line of canal	140	5 (3.6)	135 (96.4)	0.612
Diversion of canal	67	2 (3.0)	65 (97.0)	1.000
Narrowing of canal	44	1 (2.3)	43 (97.7)	1.000
Total	609	18(3.0)	591 (97.0)	

***p* – values were calculated by comparing each ratio with the ratio 18:591.**

Discussion

Surgical extraction of impacted lower third molars is a common therapeutic procedure in everyday oral surgery. Indications for it are numerous, although it might be followed by several possible complications with the injury of the inferior alveolar nerve being one of the most serious and unpleasant. According to relevant literature, the frequency of this complication ranges from 0.5% [15, 16] to 8% [17, 18]. In our study, the recorded frequency was 2.25%, which is roughly the registered value reported by Rood and Shehab [11]. Some authors state that the frequency of permanent sensory deficit is between 0.35% [19] and 1.1% [20], but there are also those ones who do not record it [16, 21], which is in correlation with the results of this study.

Some authors considered the possible risk factors that could predict a possibility of occurrence of this complication. In this respect, the incidence of injuries of the inferior alveolar nerve was monitored in relation to a number of factors, such as: gender and age of respondents, experience of a surgeon and a degree of tooth impaction. However, as the most commonly cited factors are angulation of the impacted third molar and the relationship of its roots to the mandibular canal.

According to our results, the favourable position for the injury of the lower alveolar nerve was the mesioangular position of the tooth, but it was not significant. The lowest frequency of the occurrence of this complication was recorded with lower third molars in the distoangular position. Selvi et al. [22], Hasegawa et al. [23] and Nguyen et al. [24] recorded similar results to ours. However, there are authors who reported different results. Thus, Jerjes et al. [20], showed that the most risky situation for the emergence of a nerve injury is a vertical position and the least risky is horizontal position. The results of our study, however, indicate the importance of careful preoperative planning as well as using an adequate surgical approach in order to prevent the development of this complication.

The relationship between the tips of the roots of the impacted lower third molars and the content of the mandibular canal was also analysed according to their mutual distance, on the panoramic radiographs, using the X-ray classification by Tanaka et al. [10] and the classification by Rood and Shehab [11]. The results showed that the incidence of injuries was inversely proportional to the increase of a distance between the tip of the tooth roots and the canal. Such results suggest that the presence of a superimposition of roots with a mandibular canal on standard radiographs requires the additional three-dimensional imaging techniques in order to perceive more accurately the relationship of the root tips of the impacted lower third molar to the content of the mandibular canal. Observation of the radiological signs in this study, recommended by Rood and Shehab [11], showed a higher possibility of the inferior alveolar nerve injury during the extraction if the illumination in the area of the root tips was present, or the loss of linear overshadowing characterized by the "roof", i.e., "bottom" of the mandibular canal, as well as when the canal diversion, root deflection and narrowing of the canal were present, which is in accordance with the results of Rood and Shehab [11]. Valmaseda-Castellón et al. [19] state that only the canal diversion can predict a possible injury of the nerve, while Blaeser et al. [25] point out the illumi-

nation of the roots, the loss of the "white" line and the canal diversion as the most risky x-rays indicators. However, in contrast to these studies, in our research, in spite of the greater interrelation of these radiographic signs with the frequency of the occurrence of sensory disturbances, no statistical significance was recorded with any of the mentioned parameters.

It should be noted that in this study articaine was used as local anaesthetic. Some authors state that articaine can induce paraesthesia after the inferior alveolar nerve block. The reason for that, as they state, is the higher concentration of anaesthetic (4%) compared to lidocaine (2%) and, eventually, a greater potential to cause neurotoxicity. However, it is the lingual nerve that is more often affected with this complication (about 89% of all cases) [26].

Conclusion

The incidence of the inferior alveolar nerve injury during a surgical extraction of impacted lower molars is approximately 2.25%. The superimposition of the mandibular canal with the lower third molar roots at the panoramic radiography significantly increases a possibility of nerve injury. The angulation of the impacted tooth does not significantly affect the incidence of nerve injury after the surgical extraction, nor does the relationship of the tips of tooth roots to the content of the mandibular canal.

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Sex assessment from the proximal femur in the Spanish population based on three-dimensional computed tomography metric analysis

Procena pola metričkom analizom tomografskih trodimenzionalnih snimaka proksimalnog femura u populaciji Španije

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Abstract

Background/Aim. The studies published in recent years have shown that the linear measurements on the three-dimensional computed tomography (3D-CT) clinical images of the hip bone, skull or breastbone can serve as a reliable alternative method for sex estimation. In spite of the fact that the proximal femur exhibited high dimorphism when examining the skeletal material, there is still a lack of morphometric studies dealing with the CT imaging of this anatomical region that would confirm the relevance of the previously obtained results. The aim of this study was to validate the reliability and precision of some proximal femur measurements obtained *in vivo* from the 3D-CT models and to compare the accuracies of our findings with those formerly reported by other relevant research. **Methods.** A total of 146 CT scans (73 male and 73 females) were selected to take 6 measurements using the traditional osteometric methods. The 3D reconstruction was done at 1mm and 1.25 mm thick slices with OsiriX (v.4.1). The univariate and multivariate discriminant functions (DFs) were formulated for assessing sex. **Results.** The vertical diameter of neck and the vertical diameter of head were found to contribute the most when considered independently (90.4%–91.8%). When combining these with the other dimensions, the prediction accuracy increased up to 97.3%. The accuracy of CT measurements is in accordance with those obtained in the traditional morphometric studies on the skeletonized femurs of contemporary populations. The 3D-CT approach showed remarkably higher percentage of predictive ability in comparison with the 2D technique. **Conclusion.** 3D-CT is a suitable tool for the objective quantification of osteological data. The medical scans and measurements on living individuals offer a valuable source of data from which the highly reliable skeletal standards can be developed for estimating sex, even from the fragmented remains. The method proposed here can be highly useful especially in the identification of mass disaster victims when the direct osteometry is difficult to apply and maceration of the remains is not an option.

Key words:
anthropometry; femur; multidetector computed tomography; sex.

Apstrakt

Uvod/Cilj. Studije objavljene poslednjih godina pokazale su da linearna merenja na trodimenzionalnim tomografskim snimcima (3D-CT) lobanje, karlične ili grudne kosti pacijenata mogu poslužiti kao pouzdana alternativna metoda za procenu pola. Uprkos tome što je proksimalni okrajak butne kosti pokazao visok dimorfizam prilikom analize skeletnog materijala, još uvek nema dovoljno morfometrijskih studija 3D snimaka te anatomske regije koje bi potvrdile relevantnost prethodno dobijenih rezultata. Cilj rada bio je da se ispita pouzdanost i preciznost merenja proksimalnog femura pomoću *in vivo* načinjenih 3D-CT snimaka modela. **Metode.** Ukupno 146 tomografskih snimaka pacijenata (73 muškarca i 73 žene) odabrani su za 6 merenja uz primenu tradicionalnih osteometrijskih metoda. Posredstvom softvera Oziriks (verzija 4.1) urađena je 3D rekonstrukcija na slajsovima debljine 1 mm i 1,25 mm. Preciznost CT merenja u korelaciji je sa merenjima dobijenim u tradicionalnim morfometrijskim studijama kostiju femura modernih populacija. U poređenju sa dvodimenzionalnom, znatno viši procenat preciznosti u određivanju pola postignut je korišćenjem trodimenzionalne tehnike. **Rezultati.** Ustanovljeno je da su vertikalni prečnik vrata i vertikalni prečnik glave femura, analizirani pojedinačno, dali najbolje rezultate u utvrđivanju pola (90,4%–91,8%). U kombinaciji sa ostalim dimenzijama, nji-

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hova preciznost porasla je na 97,3%. **Zaključak.** Za objektivnu kvantifikaciju osteoloških podataka CT je prikladna tehnika. Klinički snimci i *in vivo* merenja pružaju validan izvor podataka pogodan za izradu pouzdanih standarda za procenu pola, čak i iz fragmentiranih ostataka. Metod koji se ovde predlaže može biti od izuzetne koristi u identifikaciji žrtava masovnih katastrofa kada maceracija ostataka i direktna osteometrijska merenja ne mogu biti opcija.

Ključne reči:
antropometrija; femur; tomografija, kompjuterizovana, multidetektorska; pol.

Introduction

The assessment of suitability of poorly preserved or fragmented skeletal remains as the only source of data available for sex diagnosis is a task that forensic anthropologists frequently deal with. Due to its robustness and density, the femur is the anatomical area less susceptible to damage and can be better preserved than other long bones. When the shaft or distal end is missing, the proximal femoral epiphysis can be highly useful in the fragmentary forensic contexts. Being an important insertion area of muscles and upper body weight transmission, the upper extremity is undoubtedly affected in terms of size and shape, which could subsequently have effect on its dimorphic potential [1, 2].

A review of published literature showed that the proximal femur had been largely examined to estimate the efficacy in the sex assessment. For this purpose, some researchers have identified triangle on the posterior aspect [3, 4], while others have focused on different features of the proximal epiphysis [5, 6]. In the cited studies, the metric data were recorded on the modern cadaveric *femora* following the traditional morphometric techniques. They were subsequently subjected to the univariate and multivariate discriminant function (DF) analyses (DFA).

As for the single dimensions, the vertical diameter of the femoral head (VHD) and the vertical diameter of the femoral neck (VND) were those that proved to assign most correctly the males and females in a variety of population e.g., Spanish, Guatemalan and Thai [7–9]. However, all scholars agreed on the important fact that the percentages of correct classifications vary considerably within the same and among different ethnicities as a consequence of specific genetic, environmental, sociocultural and secular changes that the proximal femoral epiphysis undergoes over time [10]. So, the existent methods are constantly being re-examined and novel techniques accomplished in order to establish the more reliable standards for the estimation of sex.

With regard to the use of image-processing techniques for the prediction of sex from the proximal femur, the published literature primarily explored how the classical osteometric measurements performed when employed on digital radiographs [11–13], and secondly, they compared the level of accuracy obtained directly on the dry skeletal material with the standard digital images measuring the precision of the same dry element [14]. All these goals were carried out to validate the relevancy of some morphometric parameters in the forensic examinations as well as to provide the population-specific patterns for sexing proximal femoral epiphysis.

In recent years, computed tomography (CT) has proved to be a suitable tool for the estimation of sex (e.g., *tali* and *radii*, *os coxae* and *sternum*), providing reliable and precise results comparable to those obtained by the traditional morphometrics [15–17]. However, the number of studies that made use of the clinically relevant CT database to quantify the sex differences in the proximal femur and develop the accurate standards for that purpose is still low in the current literature [18, 19].

Therefore, the aims of this study were: to examine how accurately the proximal epiphysis of the femur predicted sex in a sample of adult living population of Spain employing the data derived from the CT scans and traditional osteometry; to explore and validate some discriminant functions obtained from the skeletal remains in the sex assessment using the medical imaging dataset to formulate new discriminant functions based on the same sample, and, to compare the classification success rates achieved in several ethnicities for the same dimensions by means of the same or different approaches.

Methods

This study was performed on a randomly selected sample consisting of a total of 146 CT clinical scans (73 male and 73 female subjects) aged between 17 and 84 years (male mean age was 62.63 ± 14.86 and female one was 56.44 ± 13.09 years) who were referred to the abdominopelvic, abdominal and thoracoabdominal CT scanning between 2009 and 2011. The material examined was conceded to the Laboratory of Physical Anthropology at the University of Granada by Castilla-La Mancha Health Care Service (SESCAM). The subjects with a history of femoral pathology, or surgery were excluded from the study. To describe the anthropometric measurement error and assess the side differences, a random sample comprising 30 specimens (approximately 20% of the cases) were measured twice by the first anthropologist on different days and it was also analysed by the second examiner. This sample confirmed the symmetry and then, for the rest of the sample, only one side was measured.

Some DFs built in our recent study [4] from a data set of 186 adults' femurs (109 female and 77 male), derived from the San José identified skeletal collection housed in the Laboratory of Anthropology at the University of Granada, Spain, were employed to validate their efficacy on the sample obtained from the medical imaging data.

In compliance with the Spanish Law (Article 16, Law 41/2002; see also [20]), the patients' data were anonymized at the source before the anthropologists received them, with only the sex and the age information retained. The CT scans we used for this study were saved in the DICOM files. The post-processing was performed using the OsiriX (v.4.1 32 bit) for

the Mac OS X (10.7.2.). The 3D reconstruction was done based on 1mm and 1.25 mm thick slices and six linear measurements were obtained in the anterior and posterior views of surface rendering images (resolution 512×512 pixels).

Following the standard anthropometric techniques and literature (see below), the observers located the referent points of the variable on the surface of 3D models by rotating the bone, so that the found starting and ending points best fitted to the described length. The distances and their respective values in centimetres were subsequently established by the same software.

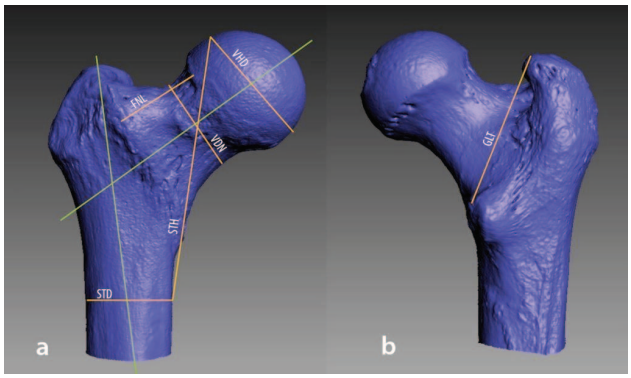

Fig. 1 – Anatomic dimensions measured on: a) anterior view and b) posterior view of a 3D-model of proximal epiphysis of the right femur.

The selected dimensions are illustrated in Figure 1 and described as follows:

- *Greater-lesser intertrochanteric distance* (GLT): the intertrochanteric distance corresponds to the distance between the apex of the greater trochanter and the apex of the lesser trochanter [21].
- *Subtrochanteric transverse diameter* (STD): measured at the upper third of the femur in the area of the maximum expansion of the bone inferior to the lesser trochanter [22].
- *Vertical diameter of the femoral neck* (VDN): diameter of the femoral neck in a cranio caudal axis [22].
- *Vertical diameter of the femoral head* (VHD): diameter of the femoral head in the cranio caudal axis [22].
- *Femoral neck length* (FNL): distance between the base of the femoral head and the intertrochanteric line [23].
- *Length STH*: the straight distance measured between the inferior point of the length STD and the superior point of the length VHD, respectfully. It is a distance devised by Kranioti et al. [14] and also used in a study on an Egyptian population [11].

The assessment of classification accuracies was performed by applying the discriminant functions (DFS) developod from the San Jose sample of dry *femora* to the medical image sample data.

Statistical analyses

The statistical analysis was performed using the software program SPSS v.24 (IBM, Somers, NY, USA). The descriptive statistics of the anatomic dimensions were obtained for each of the measurements. The normal distribution of data was evaluated by the Kolmogorov-Smirnov test. To assess the side differences, the paired *t*-test was applied and to describe the anthropometric measurement error, the technical error of measurement (TEM), the relative technical error of measurement (%TEM), and the coefficient of reliability (R) were calculated [24–26]. The comparison between the mean values of both groups was performed using the *t*-test. The stepwise DFA was performed to formulate the univariate and multivariate discriminant equations. The leave-one-out classification procedure was used to demonstrate the accuracy rate of the original sample and the one created by the cross-validation. The posterior probabilities were computed for each model. The *p*-value of less than 0.05 was considered statistically significant. We previously determined the method to be acceptable when at least 85% of individuals were correctly classified, with sex-bias lower than 5%.

Results

The results of the measurement error for each variable are presented in Table 1. The intraobserver %TEM and R values range from 0.988% to 2.396% and from 0.950 to 0.988, respectively, while interobserver %TEM and R vary between 1.160%–2.468% and 0.951–0.981, respectively. The symmetry was also confirmed by the paired *t*-test at a significant level of 0.05.

Table 1

Intra and inter-observer TEM, %TEM reliability by the anthropometric measure

Paremeter	Intra-observer			Inter-observer		
	TEM	%TEM	R	TEM	%TEM	R
STD	0.0432	1.267	0.950	0.0493	1.341	0.951
STH	0.1215	1.378	0.966	0.1162	1.329	0.970
GLT	0.0741	1.274	0.976	0.1062	1.818	0.951
VDN	0.0419	1.300	0.981	0.0587	1.829	0.971
VHD	0.0420	0.988	0.988	0.0501	1.160	0.981
FNL	0.0711	2.396	0.951	0.0733	2.468	0.951

TEM – technical error of measurement; %TEM – relative TEM; R – coefficient of reliability; STD – subtrochanteric trensverse diameter; STH – subtrochanteric length; GLT – greater-lesser intertrachanteric distance; VDN – vertical diameter of the femoral neck; VHD – vertical diameter of the femoral head; FNL – femoral neck length.

Table 2

Measures of central tendencies and comparisons of means according to sex (equal number of scans – 73)

Variable	Male		Female		*t*	df	*p*-value
	mean	SD	mean	SD			
STD	35.2397	2.26015	31.7781	2.70541	8.390	144	0.000*
STH	93.4671	4.69817	82.3493	4.80483	14.135	144	0.000*
GLT	61.1027	2.77994	54.7027	3.20208	11.895	144	0.000*
VND	35.1630	2.69817	30.1247	1.99129	12.837	144	0.000*
VHD	47.2890	2.85708	40.6397	2.23872	15.652	144	0.000*
FNL	31.4863	2.55589	26.7219	2.06197	12.396	144	0.000*

SD – standard deviation; df – degrees of freedom; * $p < 0.05$;
For the abbreviations see under Table 1.

Table 2 presents the mean and the standard deviation by the sexes for each measurement. The average for males exceeds the average for females in all cases. Furthermore, the results of Student's *t*-test reported in the same Table demonstrate highly significant differences between the sexes.

The DFs to the medical image sample data and the classification accuracies obtained range from 74.4% to 90.7%. The results are given in Table 3.

Table 3

Classification accuracies obtained after applying the DFs developed from the San José sample of dry *femora* [4] to the patients' clinical data

Measures	Male	Female	Sex bias	Total
VDN	96.8%	52.0%	44.8%	74.4%
VHD	93.3%	88.0%	5.3%	90.7%
GLT	82.7%	86.7%	-4.0%	84.7%

For the abbreviations see under Table 1.

Table 4 presents the coefficients of seventeen discriminant function equations, four univariate (1 to 4), six bivariate (5 to 10), six three-variate (11 to 16) and one using four variables which have the fewer attribution errors and better separate the two groups. The sectioning points are all zero (making the corresponding calculations). From the univariate functions, the threshold values can be calculated as the absolute value of the constant divided by the coefficient of the variable (the slope model). In this study, the threshold value for STH is 87.91 mm, for GLT 57.90 mm, for VND 32.563 mm and for VHD 43.97 mm.

These classification rules developed, can be considered an accurate and easy way to help differentiate sex. The use of the discriminant coefficients in Table 4 is as follows: multiply each measurement by the appropriate coefficient and add to the constant; a value greater or equal than the sectioning point, zero (≥ 0) is classified as a male, and a value less than zero (< 0) is classified as a female. For example, using the discriminant function 12, an adult with the following measurements:

$$DF_{12} = 0.236VND + 0.196GLT + 0.181FNL - 24.271$$
$$= 0.236*34.9 + 0.196*71.2 + 0.181*31.8 - 24.271 = 3.676 > 0,$$

is classified as male.

The Wilk's Lambda values, which measure how well each function separates cases into groups, were calculated (smaller values indicate greater discriminatory ability of the function). Table 4 also presents the accuracy percentages, cross-validated accuracy percentages and posterior probabilities for all of the DFs developed. The percentage of correct assignation ranges from 85.6% to 97.3% (85.3% to 97.3% after cross-validation).

Out of six variables analysed in the current paper, five coincided in several studies focused on sexing the proximal femur. Their classification accuracies are compared in Table 5.

Discussion

Computed tomography is increasingly proving its forensic relevancy in the osteological sex assessment. This imaging technique facilitates easy, rapid, non-invasive and direct examination of unknown deceased individuals. This way, an extensive and time-consuming maceration procedure can be avoided. The acquisition of 3D volume rendered images enables detailed inspecting and visualizing of any osseous structures and consequently accurate virtual measurements. The CT scan method can be a highly useful option in the mass fatality incidents where the state of recovered remains (fragmented, semifleshed, mummified, charred) does not allow the traditional forensic procedures to be carry out correctly (e.g., the manual data acquisition). Additionally, in the absence of suitable skeletal collections, the multislice CT (MSCT) scans can serve as a reliable alternative source of contemporary data from which specific morphometric standards for the estimation of sex can be developed, or validated.

In the present study, we aimed to explore how accurately the proximal epiphysis of femur predicts sex in a sample of adult living population of Spain by applying the traditional osteometry to the data derived from the 3D scans and to compare the accuracies of our findings with those formerly obtained in other relevant studies. To that end, several anthropometric measurement errors were calculated for the six variables selected for the study. According to Weinberg et al. [26], the REM scores revealed a very good inter- and intraobserver reproducibility. Following Ulijaszek and Kerr [24], we took into account a cut-off value of 0.95, i.e., a measurement error of up to 5%, which leads us to consider the R values greater that 0.95 to be sufficiently precise.

Table 4

Discriminant functions, accuracy, cross-validation and posterior probabilities obtained from the hospital-patients sample

Function/ variable	Coef.	Wilks´ Lambda	Accuracy (%)				Cross-validated[a] (%)	Posterior probilities
			male	female	total	bias		mean
Function 1		0.419	89.0	86.3	87.7	-2.7	87.7	87.34
STH	0.21045							
Constant	-18.50000							
Function 2		0.464	83.6	87.7	85.6	4.1	85.6	84.83
GLT	0.33351							
Constant	-19.31091							
Function 3		0.448	90.4	90.4	90.4	2.8	90.4	86.82
VND	0.45203							
Constant	-14.71960							
Function 4		0.370	90.4	93.2	91.8	2.8	91.8	91.47
VHD	0.389612							
Constant	-17.12947							
STD	0.17490							
Function 5		0.369	93.2	90.4	91.8	-2.8	91.8	90.74%
STH	0.17901							
Constant	-21.59744							
Function 6		0.353	93.2	91.8	92.5	-1.4	92.5	92.18
VND	0.15665							
VHD	0.28941							
Constant	-17.82491							
Function 7		0.317	91.8	94.5	93.2	2.7	93.2	93.13
VHD	0.25877							
STH	0.10737							
Constant	-20.81522							
Function 8		0.272	95.9	95.9	95.9	0	95.9	94.67
VHD	0.29443							
GLT	0.20170							
Constant	-24.62348							
Function 9		0.317	91.8	95.9	93.8	2.0	93.8	93.89
VHD	0.29012							
FNL	0.20679							
Constant	-18.77340							
Function 10		0.330	90.4	94.5	92.5	-4.1	92.5	92.35
GLT	0.23071							
FNL	0.28401							
Constant	-21.62475							
Function 11		0.263	94.5	97.3	95.9	-2.8	95.9	94.75
VND	0.12288							
VHD	0.22045							
GLT	0.19644							
Constant	-25.06789							
Function 12		0.270	93.2	97.3	95.2	-4.1	95.2	95.20
VND	0.23569							
GLT	0.19564							
FNL	0.18101							
Constant	-24.27112							
Function 13		0.264	93.2	95.9	94.5	-2.7	94.5	94.32
VND	0.20790							
GLT	0.18852							
STH	0.09881							
Constant	-26.37226							
Function 14		0.248	94.5	98.6	96.6	-4.1	95.2	95.54
VHD	0.23507							
GLT	0.18053							
FNL	0.15530							
Constant	-25.30742							

Table 4 (*continued*)

Function/ variable	Coef.	Wilks´ Lambda	Accuracy (%)				Cross-validated[a] (%)	Posterior probilities
			male	female	total	bias		mean
Function 15		0.249	95.9	95.9	95.9	0.0	95.9	95.60
VHD	0.21379							
GLT	0.17950							
STH	0.07898							
Constant	-26.73553							
Function 16		0.258	97.3	97.3	97.3	0.0	97.3	95.23
GLT	0.17528							
FNL	0.19992							
STH	0.11677							
Constant	-26.23283							
Function 17		0.230	95.9	98.6	97.3	2.7	95.9	96.31
VHD	0.16697							
STH	0.07223							
GLT	0.16251							
FNL	0.14270							
Constant	-25.30742							

Coefficients and constants are to construct the discriminant equations; [a] Jackknife leave-one-out method for cross validation is used.
For the abbreviations see under Table 1.

Table 5
Accuracy (%) of the five coincident variables of the proximal femur explored in several populations by means of different methods

Variable / Method	STD	STH	GLT	VND	VHD
3D-CT patient images	$Spanish_1$ 74	$Spanish_1$ 88.7	$Spanish_1$ 85.3	$Spanish_1$ 92 $Spanish_4$ [19] 90.4 Turkish [18] 88	$Spanish_1$ 92 Turkish [18] 86
CT patient scans					$Spanish_4$ [19] 93; AC [13] 76.9
Digital radiographs (living subjects)	Egyptians [11] 66.7	Egyptians [11] 79.2		Egyptians [11] 100 Iranians [12] 77	Egyptians [11] 91.2 Iranians [12] 78
Digital radiographs (dry bone)				Greeks [14] 85.7	Greeks [14] 80
Dry bone (physical measurements)	Portuguese, $Spanish_3$ [5, 7] 73–74.24		$Spanish_2$ [4] 85.5 CI [3] 84.3	$Spanish_2$ [4] 91.9 EA, AA, Guatemalans & French [6, 8, 10] 83.85–90.1	$Spanish_2$ [4] 89.8 Chileans [28] 86, Thai [9], CI [1] 91.3–92.7

$Spanish_1$ (current study); $Spanish_2$, $Spanish_3$ and $Spanish_4$ (other studies on Spanish population [4, 7, 19]); EA – European Americans; AA – African Americans; CI – Central Indians; AC – Anatolian Caucasians.
Mathematical model commonly used by the cited authors was discriminant function analysis except for Clavero et al. [19] who applied logistic regression.
For the abbreviations see under Table 1.

The ranges obtained in Table 1 confirmed a high level of repeatability for all the dimensions considered, indicating that accurate osteometric measurements can be obtained from the reconstructed 3D-CT image data and that this approach seems to be suitable and reliably for the assessment of the proximal femoral epiphysis.

Three models developed in our recent study on dry *femora* [4] were validated on the sample of 3D image data. Two out of three functions show a possible applicability in sexing skeletal remains. The poor result that VDN exhibited for the female group could have to do with the secular changes that affected female VDN, contrary to the male one which was not notably altered by this trend, as stated previously in the studies conducted on French, Caucasians and Afro-Americans, born prior to the turn of century as well as those born after 1910, respectively [6–10]. The authors of the cited

studies assert that the secular increases in the female neck morphology decreased the distance between the male and female distributions and consequently led to a decrease in the overall classification success accuracy rate. It is possible that our original skeletal sample [4], tested on the clinical data, was also affected by the trend due to the fact that 39.78% of it comprised the individuals born before 1909 (see Table 3, also Ref. 10). This analysis was carried out to ascertain whether the imaging-based models performed worse, better, or were comparable to those previously formulated from the sample comprising skeletal remains. Due to the fact that the compatibility was established for the two functions, the forensic contexts will determine which of them will be more appropriate to apply. Nevertheless, the formulae obtained from the CT scans are supposed to be used when they show better predictive ability; if the dry bone standard for the determinate variable is not available; or, in the identification of mass disaster victims when traditional forensic methods cannot be a choice. In such circumstances, when a rapid and accurate sex assessment is a crucial factor, both forensic pathologists and forensic anthropologists who work closely in the identification of human remains can use the CT scans. In case of degraded and contaminated DNA and severe soft tissue injuries, the identification tasks can be very complicated for a forensic pathologist with the CT inclusive. If the bone fragments are better preserved, the imaging technique will be in favour of the forensic anthropologist. After the scanning of recovered remains, the 3D reconstruction and the elimination of the soft tissue will be provided by the imaging software. The measured data will undergo the multivariate statistical analysis. Then, the results of discriminant functions will be compared with the corresponding sectioning points established for each function (zero, in this case). The bones are classified as male or female based on whether the discriminant scores were higher or lower than the sectioning points. Finally, the formulae previously developed for the examined anatomical region would be applied to assess the sex of the deceased person.

The multivariate DFA, to which our CT-scan data was subjected, showed that the most accurate single parameters were VND and VHD with 90.4% and 91.8% of correct classification after the cross-validation, respectively. Because of their high correlation, a model comprising both variables would not be as useful as other patterns achieved when any of these were combined with GLT and FNL. Although the latter performed more poorly as an independent model, it gave noteworthy results in the groups with GLT, VDN and VHD, which was the main reason to include it in the finally selected ones. We emphasize that GLT and STH as single prediction models were less sexually dimorphic than the others obtained here (below 88%). However, they gave more accurate functions when joined together, or combined with other variables selected for the study. When these formed a group based on two, three and four variables (see Table 4), the prediction accuracy increased up to 97.3%.

We assessed the percentages of correctly classified individuals for five dimensions that the present survey and the studies on a variety of different ethnicities had in common when different approaches for assessing the proximal femur were employed. We found that there is no significant difference for the Spanish population in the measurements taken by MSCT when compared with the measurements of defleshed bones, except for VHD, which better assigned sexes on virtual models. On the other hand, our accuracy rates are generally in consonance with those obtained from the skeletal samples of other populations. Furthermore, the CT measurements provided remarkably higher percentage of correct classification with respect to those obtained from the 2D digital radiographs both of living subjects and skeletal remains. As Rubin et al. [27] asserted, the standard radiographs are somewhat limited for a precise morphometric analysis due to the lack of 3D data on a planar X-ray which most likely introduce errors to the final geometry. Such distortion was not observed in our 3D-CT images, which was subsequently reflected on the percentage of correct classification (see Table 5 and Ref. 11 and 13).

Overall, our results suggested that 3D-CT is a suitable alternative tool for objective quantification of osteological data that can provide highly accurate models for estimating sex. The standards developed here should be considered as specific for the Spaniards. The possible applicability to the other Mediterranean populations needs to be examined on comparative samples of osteometric and CT data. Moreover, further research based on the morphometric evaluation using the CT imaging technique are needed in order to expand the number of anatomically relevant features that could enable novel and reliable modern population standards applicable for identification in forensic settings.

Conclusion

This study demonstrates that the clinical 3D-CT images-based linear measurements are reliable alternative method for the assessment of the proximal epiphysis of the femur in the modern adult population of Spain. Overall differences between the traditional bone measurements on skeletal sample- and the 3D-CT patient's images, respectively, are negligible for the Spaniards and can be alternatively used. They are generally in consonance with those previously accomplished from groups of different geographical origin. In comparison with the 2D technique, it was the 3D-CT approach that provided a remarkably higher percentage of predictive ability. The discriminant functions can be extremely useful in the assessment of fragmented femurs, especially in the mass disaster victim identification, where a direct morphometry is difficult to apply and the image processing techniques such as computed tomography is the only option remained.

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Conflict of interest

The authors declare no conflict of interest regarding this article. This paper is part of the principal author's PhD thesis within the Doctoral Programme in Biomedicine: "Human Evolution, Physical and Forensic Anthropology".

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HIF-1α and SOX2 expression in cervical squamous cell carcinoma

Ekspresija HIF-1α i SOX2 kod skvamoznih karcinoma grlića materice

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Abstract

Background/Aim. Hypoxia is one of the major changes that occurs in the tumor microenvironment. It has been observed that there are pluripotent cancer cells in the cancer cell population that affect tumor growth and their resistance to therapy. The aim of this study was to examine the expression of hypoxia-inducible factor-1 alpha (HIF-1α), endogenous marker of hypoxia, and SOX2, marker of the pluripotent stem cells existing in the normal adult tissues, in the cervical squamous cell carcinoma (SCC). **Methods.** The study was conducted in 90 women with invasive cervical SCC, divided into two groups – 60 women in the Group A, with FIGO IB1 < 20 mm tumors (no metastases in the lymph nodes), and 30 women in the group B with tumors FIGO I–II (positive lymph nodes). The basic clinical parameters were determined by standard histopathological analysis, and the expression of HIF-1α and SOX2 by immunohistochemical examination. **Results.** There was a significant difference between the groups A and B, in the expression of HIF-1α (p = 0.024), but not in the expression of SOX2 (p = 0.566). Expression of HIF-1α was significantly higher in the group with lymph node metastases and invasion of lymphovascular spaces (p <0.001) but not associated with tumor size (p = 0.291) or lymphocytic stromal response (p = 0.940). The tumor grade significantly influenced the expression of HIF-1α (p = 0.013). The expression of SOX2 did not significantly correlate with any of the established clinical tumor parameters. **Conclusion.** A significant difference in the expression of HIF-1 α between the group with and that without metastases in lymph nodes in invasive cervical SCC could distinguish HIF-1α as a parameter of poor prognosis of the disease. The prognostic significance of SOX2 as well as a significant correlation between expression of HIF-1α and SOX2 were not established.

Key words:
uterine cervical neoplasms; neoplasm staging; prognosis; hypoxia; biomarkers.

Apstrakt

Uvod/Cilj. Hipoksija je jedna od bitnih promena koja se dešava u mikrookolini tumora. Zapaženo je da u populaciji kancerskih ćelija postoje pluripotentne kancerske *stem* ćelije koje utiču na rast tumora i njihovu rezistenciju na terapiju. Cilj rada je bio da se kod bolesnica sa skvamoznim karcinomom (*squamous cell carcinoma* – SCC) grlića materice ispita ekspresija faktora 1 alfa indukovanog hipoksijom (*hypoxia-inducible factor-1 alpha* – HIF-1α), endogenog markera hipoksije i SOX2, markera pluriponentnih stem ćelija koje postoje u normalnim tkivima odraslog čoveka. **Metode.** U ispitivanje je bilo uključeno 90 žena sa invazivnim SCC grlića materice podeljenih u dve grupe – 60 žena u grupi A, sa tumorima stadijuma FIGO IB1 < 20 mm (bez metastaza u limfnim nodusima) i 30 žena u grupi B, sa tumorima FIGO I–II (sa pozitivnim limfonodalnim statusom). Osnovni kliničkopatološki parametri su bili određeni standardnom patohistološkom analizom, a ekspresija HIF-1α i SOX2 imunohistohemijskim ispitivanjem. **Rezultati.** Između grupa A i B utvrđena je značajna razlika u ekspresiji HIF-1α (p = 0,024), ali ne i u ekspresiji SOX2 (p = 0,566). Ekspresija HIF-1α bila je značajno viša u grupi sa limfonodalnim metastazama i invazijom limfovaskularnih prostora (p < 0,001), ali nije bila povezana sa veličinom tumora (p = 0,291) niti jačinom limfocitnog odgovora (p = 0,940). Gradus tumora je značajno uticao na ekspresiju HIF-1α (p = 0,013). Ekspresija SOX2 nije značajno korelisala ni sa jednim utvrđenim kliničkopatološkim parametrom tumora. **Zaključak.** Značajna razlika u ekspresiji HIF-1α između grupa sa i bez metastaza u limfnim nodusima kod invazivnog SCC grlića materice mogla bi izdvojiti HIF-1α kao parametar loše prognoze bolesti. Nije utvrđen prognostički značaj SOX2, niti značajna uzajamna korelacija ekspresije HIF-1α i SOX2.

Ključne reči:
materica, neoplazme; neoplazme, određivanje stadijuma; prognoza; hipoksija; biološki pokazatelji.

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Introduction

Cervical cancer is the third most common malignant tumor of women in the world, and Serbia is ranked fourth in Europe [1]. The most common histological type of invasive cervical cancer according to the WHO classification is squamous cell carcinoma (SCC) with a frequency of 70%–80%, while the frequency of other types is much lower [2]. The therapeutic procedure is determined according to the clinical the International Federation of Obstetrics and Gynecology (FIGO) stage of the tumor disease. There are four FIGO stages, FIGO I–IV, which are determined regardless of the histological type of tumor. Small invasive cervical cancer is a FIGO IA, and FIGO IB1 indicates tumor smaller than 20 mm. Standard treatment for the stages FIGO IA1 and FIGO IA2, FIGO IB1 and IB2, as well as FIGO IIA is surgical, while primary treatment for the stages from FIGO IIB to FIGO IV is chemoradiotherapy.

In the last decades numerous attempts have been made to determine the prognostic factors that influence the transformation of preinvasive stage to malignant one, the tumor spreading process, and its metastatic potential, by determining the tumor immunophenotype and its environment. It is considered that one of the factors of the malignant transformation of the cell are changes in its microenvironment, which include, among other things, damage of blood vessels, the formation of hypoxic focus and the maintenance of hypoxia by the activation of signal molecules, such as hypoxia-inducible factor 1-alpha (HIF-1α). HIF-1α is part of the transcriptional complex involved in the regulation of many aspects of tumor biology. Increased activation of HIF-1α or hypoxia causes the transition from oxidative to glycolytic metabolism and leads to increased expression of angiogenesis markers [vascular endothelial growth factor (VEGF)] and of numerous metabolic markers, such as: glucose transporter 1 (GLUT-1), c-met and carbonic anhydrase 9 (CA9), which in recent years have been the subject of intensive research, not only as prognostic parameters of the disease, but also for potentially targeted therapy [3].

Karsten and Goletz [4] found that one of the important reasons for the difference between cancer and normal stem cells is the effect of microenvironment, where hypoxia plays an important role. SOX2 was initially known as a protein that maintains stem cells in the mature tissue of an adult (including brain tissue), and then it has been confirmed that it is one of the leading transcriptional proteins that affect the induction of pluripotent stem cells [5]. Excessive expression and genetic amplification of SOX2 are, however, associated with the formation of SCC in various tissues [6]. The assumption is that enhanced SOX2 expression affects the proliferation of cancer stem cells and provides worse prognosis [7].

The aim of this study was to determine the expression of HIF-1α and SOX2 and to compare it between FIGO IB1 and FIGO I–II stages. In addition, we wanted to determine whether there was a relationship between the level of expression of these two markers, as well as their relationship with clinicopathological parameters: FIGO stage of tumor disease, tumor size, tumor grade, lymphovascular invasion (LVSI), and metastases in the lymph nodes.

Methods

The study included 90 patients with a histopathologically confirmed diagnosis of cervical SCC. They were surgically treated at the Gynecology Clinic of the Clinical Center of Serbia in Belgrade, Serbia. All women had radical hysterectomy with lymphadenectomy and on the basis of a definitive finding, the entire group was divided into two groups: the group A – small invasive carcinoma of the stage FIGO IB1 < 20 mm (60 patient), and the group B – all cases of cervical SCC in which metastases existed in lymph nodes (30 patients).

From the group A, with small invasive carcinoma, we excluded: patients with microinvasive carcinoma of the stage FIGO IA1 and FIGO IA2, patients with stage FIGO IB1 in which the tumor was greater than 20 mm, patients of the stage FIGO IB2 and more.

The group B included patients with positive lymph nodes, regardless of the FIGO stage, so in this group there were women in the stages of FIGO IA1, FIGO IB1 (irrespective of the size of the tumor), FIGO IB2, FIGO IIA and FIGO IIB. The number of positive lymph nodes was not important for inclusion in this group.

The common criteria for both groups were: women who previously had not any other type of oncological treatment of their illness, and those without other malignancies, including gynecological malignancies outside the cervix.

Biopsy material in paraffin blocks was processed in the laboratory of the Department of Pathology of the Clinical Center of Serbia in Belgrade. The obtained samples of cervical tumor tissue, fixed in 10% neutral buffered formalin and embedded into paraffin blocks were analyzed on standard hematoxylin-eosin colored cross-sections of 4 μm in order to establish a pathohistological diagnosis. The study included cases of squamous differentiation, whereas adenocarcinomas, adenosquamous carcinoma and other rare types of tumors were excluded. The pathological report determined the size of the tumor, degree of differentiation, nuclear grade, lymphovascular invasion, lymphocytic response, parametrial and vaginal wall involvement, number of lymph nodes taken out and number of positive lymph nodes.

From the obtained preparations, the most homogeneous field for further processing is selected using the tissue microarray (TMA) method. From the selected paraffin blocks, from the field of the most homogeneous tumor tissue, 2 tissue cylinders (per patient) were taken, using a 2 mm puncture needle, and then inserted into a new paraffin block (TMA block), where a 60-cylinder series was placed. Each TMA block was cut at a thickness of 4 μm, and after deparaffinization and heat treatment an immunohistochemical (IHC) analysis was performed.

Immunohistochemical analysis

Immunohistochemistry was performed on archived formalin-fixed, paraffin-embedded tissue, using a manual

method for HIF-1α antibody (EP1215Y, Abcam) at 1 : 100 dilution. Pretreatment antigen retrieval by microwave heating in 10 mM citrate buffer pH 6 was performed according to the manufacturer's directions and current laboratory protocol, applying avidin-biotin complex method (UltraVision™ Detection System, Termoscientific) using 3,3'-diaminobenzidine (DAB) as chromogen. Nuclear and cytoplasmatic staining were analysed. The positive control was ovarian carcinoma.

HIC stain for SOX 2 antibody (SP76, Ventana) was done automatically (Leica Bond), according to the manufacturer's directions and resulted in nuclear staining of variable intensity in the positive cases.

Expression was quantitatively analyzed by determining the intensity of coloring and the percentage of positive tumor cells. The quantitative scale for the intensity and percentage of colored tumor cells is categorized into 4 and 5 classes, respectively in the following way: coloring intensity – 0 (colorless), 1+ (poor), 2+ (medium), 3+ (strong), and percentage of positive carcinoma cells in relation to the total number of tumor cells – 0 (< 5%), 1+ (6%–25%), 2+ (26%–50%), 3+ (51%–75%), 4+ (> 75). The final IHC score is calculated as a combination of intensity and percentage scores (range 0–12). The values of < 4 indicated a weak positivity, 5–8 moderate, and 9–12 a strong positivity of expression.

Statistical analysis

Results are presented as count (%) or mean ± standard deviation depending on data type. Groups are compared using *t* test, ANOVA, χ^2 test (Pearson and Cochrane-Armitage test), Kruskal-Wallis and Mann-Whitney *U* test. To assess correlation between variables Spearman's correlation was used. All *p* values less than 0.05 were considered significant. All data was analyzed using SPSS 20.0 (IBM Corp. Released 2011. IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp.).

Results

In the group A, 60 cases of small invasive tumors were analyzed. The average age of women was 45.2 years, the oldest patient was 53 years old and the youngest one 37, with a standard deviation rate of ± 10.1 years. In the group B, the youngest woman had 28 and oldest 71 year, with an average of 51.9 ± 11.8 years.

In the group A all women were clinical FIGO IB1 stage, with the largest dimension of a tumor of up to 20 mm. In the group B, all women had metastatic disease in lymph nodes, but their FIGO stages were different: 2/30 patients were at the stage FIGO IA1, 10/30 were at the stage FIGO IB1 (all > 20 mm), 9/30 women had FIGO IB2 stages (tumor > 40 mm), 4 women had infiltration of the vagina – FIGO IIA stage, and 5 women had a parametrial infiltration – FIGO IIB stage. For both groups, the total tumor volume was calculated.

All prognostic parameters of the tumor were determined by histopathological analysis. The average tumor volume in the group A was 1,420 (625–2,405) mm³, and in the group B 19,775 (8,000–40,500) mm³. For the degree of differentiation, the Broders classification was used. The group A had the highest number of moderately differentiated carcinomas (G2) – 66.7%, and much less well differentiated (G1) 3.3% and poorly differentiated cases (G3) 31.3% which makes 63.5% of G2, 100% of G1 and 72% of G3 tumors, of the total number of cases in both groups. In the group B there were no well-differentiated cases; the G2 tumor was present in 23/30 (76.7%) cases and G3 in 7/30 (23.3%) cases, corresponding to 36.5% of G2 and 28% of G3 SCCs of a total of 90 analyzed in the groups A and B.

Lymphovascular invasion was not present in 40 cases in the group A, while 33.3% of patients in this group had an invasion of blood and/or lymph vessels.

After standard analysis and determination of clinicopathological parameters, an IHC analysis of HIF-1α and SOX2 was performed. Results for HIF-1α were classified into 3 categories, where the first category comprised cases with a negative and weak positive reaction, in the second category there were cases of moderate positivity, and in the third category there were tumors that showed strong expression. The SOX2 expression was also divided into three groups: cases with a negative reaction, cases with weak positive expression, while cases of moderate and severe expression are classified in the same group. Due to statistical processing, the number of categories was adjusted to the number of samples in both groups.

In the group A, only one (1.7%) case showed a negative reaction to HIF-1α, 20 (33.3%) cases were weak positive, 14 (23.3%) were strongly positive, and the most showed moderately positive reactions – 25/60 (41.7%) cases. In the group B, 19/30 cases had a negative or weak, 5/30 moderate and 6/30 strong reaction to HIF-1α. The obtained results showed a statistically significant difference between the group A and the group B ($p = 0.024$) in the expression of hypoxia markers. However, in the expression of SOX2, no significant difference was found between these groups ($p = 0.566$). Only 11/60 tumors in the group A and 6/30 of SCC in the group B had moderate or strong positivity, while the majority of cases were either negative or poorly positive (Table 1).

We analyzed the correlation between the expression of HIF-1α and the defined clinical-pathological parameters and found that it existed in relation to a tumor grade ($p = 0.013$), lymph node metastases and a LVSI ($p = 0.006$), but it was not expressed in relation to a tumor size or volume ($p = 0.291$) nor a strength of the inflammatory stromal response ($p = 0.940$) (Table 2).

The expression of SOX2 was analyzed in relation to clinicopathological parameters. No statistically significant association was found in any category: in relation to a tumor grade ($p = 0.331$), invasion of vascular spaces ($p = 0.645$), and a lymphocytic stromal response ($p = 0.916$) (Table 3).

There was no significant association in the expression of HIF-1α and SOX2 ($\rho = 0.132$; $p = 0.215$) (Figures 1 and 2).

Table 1

Clinicopathological characteristics and levels of expression of HIF-1α and SOX2 in patients with cervical squamous cell carcinoma

Variable	Metastases		*p* value
	no	yes	
Age (years), mean ± SD	45.2 ± 10.1	51.9 ± 11.8	0.006[a]
Tumor dimensions (mm), mean ± SD			
length (mm)	14.3 ± 4.6	29.8 ± 12.0	< 0.001[b]
width (mm)	11.9 ± 4.2	28.8 ± 12.7	< 0.001[b]
depth (mm)	8.6 ± 4.2	22.1 ± 9.7	< 0.001[b]
volume (mm^3)	1,420 (625–2,405)	19,775 (8,000–40,500)	< 0.001[b]
LVSI, n (%)			
no	40 (100)	0	
lymph or vascular	5 (74.4)	2 (28.6)	< 0.001[c]
both lymph an vascular	15 (34.9)	28 (65.1)	
Tumor grade, n (%)			
1	2 (100)	0	
2	40 (63.5)	23 (36.5)	0.821[d]
3	18 (72.0)	7 (28.0)	
Stage, n (%)			
IB1 < 20 mm	60 (92.3)	5 (7.7)	< 0.001[c]
other	0	25 (100)	
HIF-1α, n (%)			
no/weak	21 (52.5)	19 (47.5)	
moderate	25 (83.3)	5 (16.7)	0.024[c]
strong	14 (70)	6 (30)	
SOX2, n (%)			
no	33 (70.2)	14 (29.8)	
weak	16 (61.5)	10 (38.5)	0.566[d]
moderate/strong	11 (64.7)	6 (35.3)	
Lymhocytic stromal response, n (%)			
weak	19 (61.3)	12 (38.7)	
moderate	16 (59.3)	11 (40.7)	0.041[d]
strong	25 (86.2)	4 (13.8)	

LVSI – lymphovascular invasion; HIF-1α – hypoxia-inducible factor 1-alpha; SD – standard deviation.
[a]*t*-test; [b]Mann-Whitney *U* test; [c]Pearson χ^2 test; [d]χ^2 test for trend (Cochrane-Armitage test).

Table 2

Expression of HIF-1α in relation to clinicopathological characteristics in patients with cervical squamous cell carcinoma

Variable	HIF-1α			*p* value
	no/weak	moderate	strong	
Age (years), mean ± SD	49.6 ± 10.7	46.1 ± 10.8	45.2 ± 12.1	0.253[a]
Tumor dimensions, mean ± SD				
length (mm)	22.5 ± 12.4	16.8 ± 9.2	17.3 ± 7.5	0.095[b]
width (mm)	19.2 ± 13.3	16.0 ± 9.3	16.6 ± 9.9	0.752[b]
depth (mm)	14.7 ± 10.1	10.3 ± 6.1	13.3 ± 10.1	0.242[b]
volume (mm^3)	2,630 (1,125–22,775)	1,590 (750–3,780)	2,625 (960–5700)	0.291[b]
LVSI, n (%)				
no	13 (32.5)	20 (66.7)	7 (35)	
lymph or vascular	2 (5)	1 (3.3)	4 (20)	0.006[c]
both lymph and vascular	25 (62.5)	9 (30)	9 (45)	
Grade, n (%)				
1	1 (2.5)	1 (3.3)	0	
2	32 (80)	21 (70)	10 (50)	0.013[d]
3	7 (17.5)	8 (26.7)	10 (50)	
Lymhocytic response, n (%)				
weak	16 (41)	8 (27.6)	7 (36.8)	
moderate	10 (25.6)	9 (31)	8 (42.1)	0.940[d]
strong	13 (33.3)	12 (41.4)	4 (21.1)	

For the abbreviations see under Table 1.
[a]ANOVA; [b]Kruskal-Wallis test; [c]Pearson χ^2 test; [d]χ^2-test for trend (Cochrane-Armitage test).

Table 3
Correlation between the expression of SOX2 and clinicopathological parameters in patients with cervical squamous cell carcinoma

Variable	SOX2			*p* value
	no	weak	moderate/strong	
Age (years), mean ± SD	48.5 ± 12.3	44.3 ± 9.3	49.5 ± 9.7	0.229[a]
Tumor dimensions, mean ± SD				
length (mm)	19.7 ± 10.5	17.6 ± 10.9	21.8 ± 11.1	0.534[b]
width (mm)	18.3 ± 11.5	16.6 ± 13.5	17.1 ± 6.7	0.423[b]
depth (mm)	13.5 ± 9.7	11.9 ± 9.2	12.5 ± 7.4	0.910[b]
volume (mm^3)	2,250 (1,000–8,000)	1,710 (1,050–3,780)	3,000 (960–11,500)	0.726[b]
LVSI, n (%)				
no	20 (42.6)	13 (50)	7 (41.2)	
lymph or vascular	2 (4.3)	3 (11.5)	2 (11.8)	0.645[c]
both lymph and vascular	25 (53.2)	10 (38.5)	8 (47.1)	
Grade, n (%)				
1	2 (4.3)	0	0	
2	35 (74.5)	15 (57.7)	13 (76.5)	0.331[d]
3	10 (21.3)	11 (42.3)	4 (23.5)	
Lymhocytic response, n (%)				
weak	16 (35.6)	7 (28.0)	8 (47.1)	
moderate	15 (33.3)	9 (36)	3 (17.6)	0.916[d]
strong	14 (31.1)	9 (36)	6 (35.3)	

For the abbreviations see under Table 1.
[a]ANOVA; [b]Kruskal-Wallis test; [c]Pearson χ^2 test; [d]χ^2-test for trend (Cochrane-Armitage test).

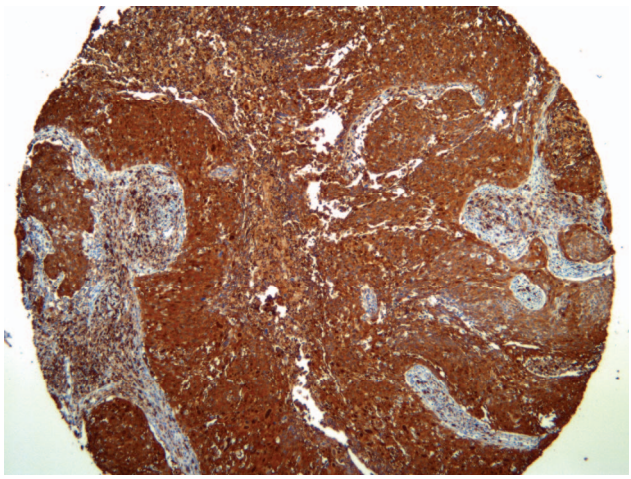

Fig. 1 – Imunohistochemical expression of hypoxia-inducible factor-1 alpha (×40).

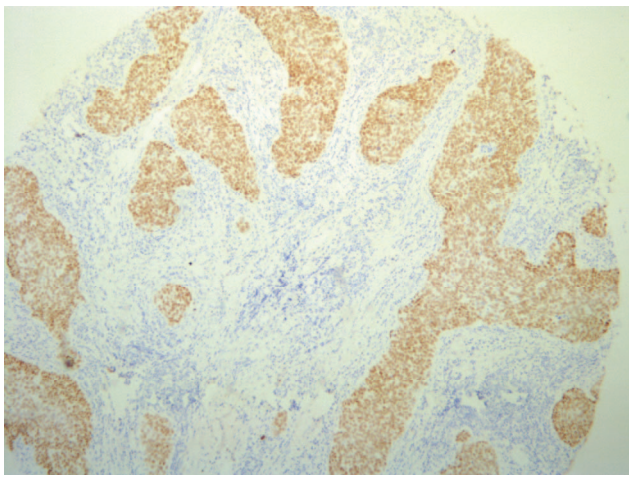

Fig. 2 – Imunohistochemical expression of SOX2 (×100).

Discussion

Screening programs in highly developed countries record mainly cases of premalignant changes in the cervix. However, for the rest of the world, invasive cervical cancer is still one of the leading causes of morbidity and mortality in the female population. In Serbia, this malignant tumor is at the 3rd place at the rate of mortality, because a large number of patients appear in advanced stages of the disease, when additional radiation or combined chemoradiotherapy is required.

One of the main reasons for the weaker success of additional therapy is hypoxia that occurs in solid tumors, and many studies are focused on enhancing oxygenation in tumors. HIF-1 as a parameter of hypoxic conditions in a changed microenvironment has been the target of numerous trials aimed at finding targeted therapy, during the last decade. It has been confirmed that HIF-1 is composed of two domains: alpha and beta, and HIF-1α consists of three different components: HIF-1α, HIF-2α and the least known HIF-3α.

Most of the studies have been carried out to estimate the expression of HIF-1α and its effects on various tumor characteristics, such as neoangiogenesis, metabolic reprogramming, genetic instability, cancer stem cells proliferation, invasiveness and metastasis, immune response of the host, survival and resistance to applied therapies. It has been shown that enhanced HIF-1 expression is a parameter of poor prognosis for many malignant tumors, both in the cases of the primary process and in metastatic disease.

Numerous, early trials were performed on tumors of the breast and ovaries, and then extended to other gynecological tumors, and tumors of other localities, irrespective of the type of differentiation. In most of those papers, the influence of HIF-1α marker is observed in correlation with the vascular endothelial growth factor marker of neoangiogenesis. The summarized results of the study by Jin et al. [8], observing the prognostic effect of HIF-1α marker expression on tumors of gynecological origin, show a significant but still insufficiently clear role of this hypoxia marker. The meta-analysis included 59 different studies with the conclusion that HIF-1α was associated with FIGO stage, tumor grade, lymph node metastases, 5-year survival and recurrence rate. The results were as follows: expression in the stage III–IV or in the presence of metastases in lymph nodes was significantly higher than in the stage I–II with or without metastases in lymph nodes, respectively ($p < 0.00001$, $p < 0.0001$, respectively); compared to tumor grade (gradus 3 vs. gradus 1: $p < 0.00001$; gradus 3 vs. gradus 2: $p = 0.002$; gradus 2 vs. gradus 1: $p < 0.00001$ p < 0.00001), 5-year survival without disease ($p = 0.001$) and 5-year survival ($p < 0.000$).

However, the results regarding the relationship of HIF-1α and cervical carcinoma are heterogeneous and partly contradictory. In the study of Huang et al. [9] it has been stated that the excessive expression of HIF-1α in invasive carcinoma is present in 94.6% of subjects, with a significant association with FIGO stage ($p = 0.024$) and tumor size ($p = 0.003$), which our research did not show.

In the work of Kim et al. [10], HIF-1α strong positivity was present in 39.7% of analyzed cervical cancers and correlated significantly with FIGO stage ($p < 0.001$) and lymph node positivity ($p < 0.001$). Our findings are similar. The increased activity of HIF-1α causes a decrease in the activity of E cadherin, an adhesive cellular molecule that suppresses the invasion and metastases by maintaining cell integrity. However, there was no significant association of expression of HIF-1α with tumor size ($p = 0.210$) and invasion of blood and lymph vessels ($p = 0.725$).

In our study, 50 cases of a total of 90, showed moderate and strong positivity for HIF-1α. According to Iwasaki et al. [11], 28 cases of 54 were positive for HIF-1α, with significant association with FIGO stage ($p = 0.0349$) and tumor type (more significant expression in adenocarcinomas compared to squamous) but no association with lymph node metastases ($p = 0.5615$), LVSI ($p = 0.2350$), or tumor size ($p = 0.5826$). Our results obviously partially coincide with the already published results of other authors, but there are some disagreements. It is interesting that each study compared to the previous one had deviations in significance, at least in some histopathological parameters.

There are several ways to explain differences in the obtained results. The antibodies used in the IHC analysis in studies are by different manufacturers and this is considered to be one of the explanations why controversial results were obtained [12]. The degree of cytoplasmic and nuclear positivity also varies, which affects the assessment of the strength of expression. Some authors include only nuclear positivity, while in other papers both nuclear and cytoplasmic staining are considered in estimating positive reaction [10]. An important reason is the interobserver difference in the interpretation of the results, considering the fact that automated digital analysis of expression is not used in most cases [13].

In recent studies that indicate an increased expression of HIF-1α as a prognostic parameter for endometrial carcinoma, there are also some other explanations for the observed differences. There is no established cut off value that defines excessive expression of HIF-1α, and no clearly defined field in which expression is determined. In most papers related to cervical carcinomas, the IHC reaction is determined only in the carcinoma islands, while in the case of endometrial carcinoma it is the case of the inverse influence of stromal and carcinogenic positivity of HIF-1α – high stromal and low carcinoma HIF-1α positivity isa parameter of poor prognosis [14].

In the paper of Seeber et al. [15], correlation between necrosis and HIF-1α expression is also observed in endometrioid carcinoma, with the emphasis only on nuclear positivity in cancer cells in three different aspects: perinecrotic positivity, diffuse positivity or mixed (combinations of the preceding two), which indicates the difference in interpretation and the discordance in determining the positivity of the hypoxia markers. We should not neglect the fact that the general condition of a patient can also affect variations in the values of HIF-1α, e.g. hyperglycemia or hemoglobinemia [16].

Interestingly, in some studies it has been shown that there is no significant difference between the increased expression of HIF-1α in high grade squamous intraepithelial lesions and in invasive carcinoma, which is considered to be evidence that this endogenous metabolic marker is involved in early stages of tumor development, associating it with highly oncogenic human papilloma virus (HPV) [17, 18]. The HPV E6 oncoprotein, the p53 tumor suppressor gene inactivator, increases the stability of HIF-1α and enhances the expression of HIF-1α -dependent VEGF in hypoxic conditions, so it is believed that HIF-1α is not only a prognostic parameter but also plays an important role in progression from preinvasive lesion to invasive disease.

Much less is known about the association of SOX2 and the tumor process. The research was directed to finding the link between SOX2 and cancer stem cells, with the conclusion that its overexpression and genetic amplification were associated with the formation of SCC in various tissues, including the lungs and esophagus [19]. In the review paper of Weina and Utikal [20], the obtained results are summarized, and through various mechanisms, by determining the genetic amplification, it is concluded that SOX2 can be a prognostic

marker, a metastatic indicator, a biomarker, or a potential targeted therapy for certain types of tumors such as pancreatic, esophageal SCC, carcinomas of lungs and oral regions, and, in particular, in tumors of neurogenic origin.

However, for cervical cancer, gene amplification is listed as unknown, and that is the mechanism through which it works. Ji and Zheng [21] in their work state that the SOX2 nuclear expression, as an IHC reaction, was significantly higher ($p = 0.05$) in SCC cells than in the normal epithelium [21], with the conclusion that the expression is much higher as the tumor was less differentiated, but for all other clinical pathological parameters there was no significant difference.

Our results are concordant with results of the research of Yang et al. [22], in which, unlike in the study by Ji and Zheng [21], there was no statistically significant difference in the degree of tumor differentiation. The digital software system analyzed the cytoplasmic positive reaction of SOX2 in the carcinoma cells, and the obtained expression did not correlate with clinicoathological parameters – FIGO stage of tumor disease ($p = 0.519$), histological tumor grade (0.594), tumor size ($p = 0.493$), vascular invasion ($p = 0.592$) and lymph node metastases ($p = 0.181$).

In their study, Kim et al. [23] showed a somewhat different result, revealing a significant difference in the tumor size category ($p = 0.015$), but not in FIGO stages ($p = 0.519$), the degree of tumor differentiation, lymphovascular invasion, or lymph node metastases ($p = 0.879$), which is in accordance with results of Yang et al. [22], as well as our results.

Interesting observations are given in the paper by Stewart and Crook [24]. They noted that most of the squamous cells in squamous intreapithelial lesions were SOX2 positive, and that most of the cancer cells in the FIGO IA1 group (early invasive tumors) were completely negative or weakly positive, and that corresponds with our results. The authors suggest that the explanation might be in the disorder of SOX2 regulation during the initial stages of the invasive processes, or that the progression of cervical SCC may include cyclical changes in the activity of SOX2.

In our study there was no link between HIF-1α and SOX2 expression in the two investigated groups, i.e. no significant coexpression of these two markers was found. There is a small number of studies determining their connection. Miyazawa et al. [25] showed a significant relationship between expression of HIF-1α and two other stem cells markers, NANOG and OCT4, that coexpress with SOX2, using prostate carcinoma samples. Mathieu et al. [26] link hypoxia or its endogenous HIF marker with increased transcription of stem cell markers: NANOG; SOX2, OCT4 (and others) within eleven cancers including cervical cancer. However, most papers are not based on the application of IHC methods, but on molecular-genetic analyzes, so perhaps the difference in the obtained results can be explained by differences in the specificity and sensitivity of the applied methods. Keith et al. [27] conclude that HIF-1 influences glucose metabolism disorder, while HIF-2 is included in OCT4-regulated stem cell pluripotency (OCT4 is coexpressed with SOX2). In our research we used HIF-1α, not HIF-2α, so maybe this is a part of the explanation why we did not get the link between the HIF-1α and SOX2 markers.

Conclusion

The importance of SOX2 as a prognostic parameter for cervical SCC, as well as the correlation of the HIF-1α and SOX2 expression, has not been established. However, the significant difference between small invasive carcinomas without lymph node metastases and invasive carcinomas with lymph nodes metastases (regardless of FIGO stage) in expression of HIF-1α confirms the role of hypoxia in the tumor microenvironment, in the process of invasion and metastasis. This is defining HIF-1α as a poor prognostic parameter. The increase in oxygenation in the tumor microenvironment and/or inhibition of this marker activity could be a basis for targeted therapy. So far, certain inhibitors of HIF-1α were used in the treatment of advanced breast carcinoma and renal cell carcinoma, so it is possible to expect their usage in advanced stages of cervical cancers, as well. However, before treatment and for determination of HIF-1α expression, it is important to work out a standardization of IHC and/or other molecular methods with a goal of results validity estimation.

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Drug utilisation trends in a physical rehabilitation hospital

Trend primene lekova u bolnici za fizikalnu medicinu i rehabilitaciju

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Abstract

Background/Aim. Drug utilisation monitoring could identify drug-related problems and hence improve the awareness of irrational drug use. The objective of this study was to analyse the drug utilisation patterns in a rehabilitation hospital over the period 2011–2016. **Methods.** The Anatomic Therapeutic Chemical classification/Defined Daily Dose (ATC/DDD) methodology was used to monitor the drug utilisation expressed as a number of DDD per 100 patient-days (HPD). The values of DDDs were obtained from the World Health Organisation (WHO) Collaborating Centre for Drug Statistics Methodology. Utilisation trends were analysed by means of the Compound Aggregate Growth Rate (CAGR), which is defined as an average annual change rate of some value during the period of interest. **Results.** The number of patient-days increased during the six years period; the CAGR being1.8% annually. At the same time, the total number of dispensed DDDs as well as the number of DDD/HPD decreased with the CAGR of -2.0% and -3.7% respectively. The average drug cost per patient-day varied from BAM 1.38 in 2013 to 0.95 in 2016; the CAGR being -1.8%. The most utilised drugs belonged to the ATC groups C, A, B, M and N and they contributed to an average of 77% of all drugs used each year. On the top of the list of most utilised drugs were: hydroxocobalamin, thioctic acid, enalapril, diclofenac, amlodipine, acetylsalicylic acid, pantoprazole, paracetamol and bromazepam. **Conclusions.** The overall drug utilisation in the hospital was modest and almost equal in 2016 compared to 2011. Besides the leading consumption of vitamin B12 and thioctic acid, this study points out some interesting prescribing patterns, such as predominant use of diclofenac over ibuprofen, and overuse of proton pump inhibitors. There is a need for educative interventions among physicians in order to improve their prescribing practice.

Key words:
hospitals; physical and rehabilitation medicine; drug therapy; drug utilisation.

Apstrakt

Uvod/Cilj. Praćenjem potrošnje lekova mogu se identifikovati farmakoterapijski problemi i unaprediti racionalna primena lekova, te je cilj ovog istraživanja bio da se utvrdi i analizira potrošnja lekova u bolnici za fizikalnu medicinu i rehabilitaciju tokom perioda 2011–2016. godine. **Metode.** Potrošnja lekova praćena je i analizirana pomoću ATC/DDD metodologije (anatomsko-terapijsko-hemijska klasifikacija/definisane dnevne doze) i izražena kao broj DDD/100 bolničkih dana. Korišćene su vrednosti DDD prema Svetskoj zdravstvenoj organizaciji. Trend potrošnje lekova analiziran je putem parametra *Compound Aggregate Growth Rate* (CAGR) koji se definiše kao prosečna godišnja stopa promene neke vrednosti u periodu posmatranja. **Rezultati.** Broj bolničkih dana rastao je tokom šestogodišnjeg perioda (CAGR 1,8% godišnje). Ukupan broj DDD lekova izdatih na odeljenja i broj DDD/100 bolničkih dana pokazali su pad (CAGR -2,0% i -3,7%, respektivno). Dnevna potrošnja lekova je finansijski varirala od 1,38 KM u 2013. do 0,95 KM u 2016. godini, sa CAGR -1,8%. Najviše su korišćeni lekovi iz ATC grupa C, A, B, M i N koji su zajedno činili u proseku 77% godišnje potrošnje lekova tokom posmatranog perioda. Najzastupljeniji lekovi po potrošnji bili su hidroksikobalamin, alfa-lipoinska kiselina, enalapril, diklofenak, amlodipin, acetisalicilna kiselina, pantoprazol, paracetamol i bromazepam. **Zaključak.** Potrošnja lekova u našoj ustanovi bila je skromna i gotovo bez promena 2016. godine u odnosu na 2011. godinu. Osim prominentne potrošnje vitamina B12 i alfa-lipoinske kiseline, usta-

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novljena su i neka odstupanja od racionalnog propisivanja lekova poput prekomerne upotrebe diklofenaka u odnosu na ibuprofen, te značajna potrošnja inhibitora protonske pumpe. Potrebne su ciljane i kontinuirane edukacije radi unapređenja prakse propisivanja lekova.

Ključne reči:
bolnice; medicina, fizikalna; lečenje lekovima; lekovi, korišćenje.

Introduction

Therapeutic practice is primarily based on evidence provided by pre-marketing clinical trials, which could be later transferred into therapeutic guidelines and clinical protocols. However, complementary post–marketing data based on drug utilisation analyses also contribute to improvement of drug therapy [1]. The World Health Organisation (WHO) defined the drug utilisation research (DUR) as "the marketing, distribution, prescription, and use of drugs in a society, with special emphasis on the resulting medical, social and economic consequences" [2].

Drug utilisation monitoring could identify drug-related problems and hence improve the awareness of irrational drug use. Data on drug utilisation in correlation with morbidity data, clinical and economic health care outcomes and quality of care could be of special value [3–5]. It could also provide a feedback to physicians and the recommendations for health professionals to improve the prescribing practice [6, 7]. Drug utilisation could have varied patterns among the different health institutions as well as among the different countries. In order to compare and discuss the differences and structure of drug utilisation, it has to be expressed in the internationally accepted units and daily doses (DDD) has to be defined [8]. The DDD per 1,000 inhabitants per day (DDD/TID) is often used to derive a rough estimate of prevalence of drug use in the population being studied. Until recently, we have published several papers related to the drug utilisation analyses [9–13].

When the hospital drug use is considered, the drug consumption figures should preferably be presented as numbers of DDD per 100 hospital bed-days (DDD/HBD) [14]. Despite advances in the DUR area, there is a paucity of information on hospital drug utilisation in our country [15]. In general, studies on the inpatients' drug use are often incomplete, mainly because it is difficult to obtain precise, accurate and structured data necessary for this type of analysis.

The aim of this study was to determine the drug utilisation trends at the biggest rehabilitation hospital in The Republic of Srpska (Bosnia & Herzegovina). Moreover, this research intends to identify the deviations of prescribing behaviour from the recommendations and guidelines as well as to propose measures for practical improvement and rationalisation of drug use.

Methods

This was a retrospective, six-year (2011-2016) drug utilisation analysis performed at the physical rehabilitation hospital (Institute of Physical Medicine and Rehabilitation "Dr Miroslav Zotović", Banja Luka). With a total capacity of 576 beds, this is the largest hospital of such kind in the country, specialised in physical medicine and rehabilitation, orthopaedic surgery and baromedicine. This study included only drugs dispensed by the hospital pharmacy department to the adult rehabilitation wards with total of 480 beds. The paediatric rehabilitation unit as well as orthopaedic surgery ward were excluded from the study due to the limitations of ATC/DDD methodology and different patterns of drug use, respectively.

The data related to the drug utilisation and hospital occupancy rate were obtained from the hospital information system, and the statistical analysis was performed by the hospital pharmacy department. All drugs were identified by the generic names and coded according to the Anatomical Therapeutic Chemical (ATC) classification. For this purpose, the data on ATC codes and the DDDs for dispensed drugs were taken from the ATC/DDD Index, 2016 [14]. DDD was established for 224 out of total of 286 different dispensed drugs. Drugs with no declared DDD (for example infusions or anaesthetics) were excluded from the analysis.

The annual number of patient-days was obtained as a sum of inpatient-days and outpatient-days for a specific year. We preferred a term "patient-day" instead of "bed-day" because both inpatient and outpatient data were included in our results. Other authors have also proposed the "patient-day" as a denominator [8]. For the analysis of the total drug costs, the average drug costs per patient-day and number of different International Nonproprietary Names (INNs) were included.

The data on drug utilisation (for drugs with the DDD) were expressed as a number of DDDs per 100 patient-days (DDD/HPD) as follows:

$$DDD/HPD = \frac{total\ number\ of\ units\ x\ unit\ strength\ x\ 100}{DDD\ x\ number\ of\ patient\ days}$$

Utilisation trends were analysed by the Compound Aggregate Growth Rate (CAGR). CAGR is defined as an average annual change rate of some value during the period of interest, according to the following formula [16]:

$$CAGR = \left(\frac{value\ in\ last\ year}{value\ in\ first\ year}\right)^{\frac{1}{number\ of\ years} - 1} - 1$$

The drug utilisation data in DDD/HPD were processed at the first and the second ATC level. The most utilised drugs, at the level of INN, were analysed in details. All DDD-designated drugs were also analysed according to their administration route.

Results

The total number of 1.104,552 patient-days in the six-year period was comprised of 1.034,577 inpatient and 69,965 outpatient days, with an average of 184,092 patient-days/year. The annual number of patient-days increased during the six-year period; the difference between 2011 and 2016 being 16,051 patient-days, with CAGR of 1.8% annually. At the same time, the total number of dispensed DDDs as well as the number of DDD/HPD decreased with CAGR of -2.0%, and -3.7%, respectively (Table1).

The total cost of dispensed drugs during the six-year period was 1.232,033 BAM. Although the annual cost of all drugs showed some fluctuations, there was no significant difference between the first and the last year. The cost of drugs with the established DDD in 2016 was lower than in all other years, with CAGR being -1.0%, while the annual cost of drugs with no DDD significantly increased over the years, and CAGR was 4.5%. The cost of drugs per DDD showed no significant difference; the CAGR being 0.3%. The average drug cost per patient-day varied from BAM 1.38 in 2013, to 0.95 in 2016; the CAGR being -1.8% (Table 2).

The drugs that belonged to the groups A, B and C were the most frequently used medicines during the whole study period. They contributed to an average of 77% (range 72%–88%) of all drugs used each year. During the first four years (2011–2014), the cardiovascular drugs (group C) were the most frequently used. However, in 2015 and 2016, the most utilised ones were the drugs acting on blood (group B) and the drugs acting on the alimentary tract and metabolism (group A), respectively. The drugs acting on the nervous system (group N) were in the fourth place representing 7.42%–9.55% of all medicines used (Table 3). According to the administration route, oral formulations were dominant (62.9%).

On the list of top 20 most utilised drugs, the group A was represented by 7 drugs: thioctic acid (the second most utilised drug), pantoprazole, ranitidine, omeprazole, bisacodyl, lactic acid-producing organisms and metformin. The use of proton pump inhibitors (PPIs), particularly pantoprazole, significantly increased over the years; from 0.07 DDD/HPD in 2011 to 3.44 DDD/HPD in 2016; the CAGR being 119.3%. The use of omeprazole was very stable over the years with an average of 2.25 DDD/HPD. At the same time, the use of ranitidine significantly decreased from 4.90 and 5.31 DDD/HPD in 2011 and 2012, respectively, to 2.18 DDD/HPD in 2016 (the CAGR being -15%; Table 4).

The most utilised drug in the group B was hydroxocobalamin (vitamin B12) with an average of 23.88 DDD/HPD, followed by acetyl salicylic acid (ASA) and clopidogrel. The use of ASA in 2016 (3.58 DDD/HPD) was significantly lower than in 2011 (8.14 DDD/HPD), or in 2012 (9.34 DDD/HPD); the CAGR being -15.2% (Table 4). Other drugs of the group B used in hospital were rivaroxaban, enoxaparin, dalteparin, but their use was negligible.

The group C was represented by enalapril, which was the third most utilised drug in the hospital (average DDD/HPD was 14.32), followed by amlodipine (in the fifth place). The use of amlodipine had a constant increase from 2011 to 2013 (6.04, 7.03 and 10.01 DDD/HPD, respectively), but it significantly decreased in the following three years (6.44, 5.02 and 3.87 DDD/HPD in 2014, 2015 and 2016, respectively; Table 4). Furosemide and hydrochlorothiazide were also present on the top 20 list.

Table1

Hospital drug utilisation parameters at the physical rehabilitation hospital (2011–2016)

Year	2011	2012	2013	2014	2015	2016	CAGR[%]
Patient-days	172,148	175,599	185,977	193,358	189,271	188,199	1.8
Total DDD	237,354	292,305	372,426	242,785	223,160	214,432	-2.0
DDD/HPD	137.88	166.46	200.25	125.56	117.90	113.94	-3.7

DDD-defined daily dose; HPD – 100 patient days; CAGR – Compound Aggregate Growth Rate.

Table 2

Drug expenditure at the physical rehabilitation hospital (2011–2016, BAM)[a]

Year	2011	2012	2013	2014	2015	2016	CAGR [%]
Total drug cost (BAM)	178.682	216.677	256.495	216.363	185.880	177.937	-0.1
Cost of drugs with established DDD (BAM)	152.899	180.116	213.360	183.905	158.619	145.752	-1.0
Cost of drugs per DDD (BAM)	0.64	0.62	0.57	0.76	0.71	0.68	0.3
Cost of drugs with no DDD (BAM)	25.783	36.561	43.135	32.457	27.261	32.184	4.5
Cost of drugs with DDD/Total drug cost (%)	85.6	83.1	83.2	85.0	85.3	81.9	-0.9
Average drug cost/patient-day (BAM)	1.04	1.23	1.38	1.12	0.98	0.95	-1.8

[a] 1 BAM= 0.5113 EUR; DDD – defined daily dose; CAGR – Compound Aggregate Growth Rate.

Table 3

Hospital drug utilisation at the first level of ATC classification (expressed as DDD/HPD; 2011–2016)

ATC	Group name	DDD/HPD						
		2011	2012	2013	2014	2015	2016	CAGR [%]
A	Alimentary tract and metabolism	28.25	34.50	41.22	33.01	30.11	29.59	0.9
B	Blood and blood forming organs	34.14	41.53	55.40	22.45	34.83	27.73	-4.1
C	Cardiovascular system	35.86	47.54	58.68	36.10	24.50	26.40	-5.9
G	Genitourinary system and sex hormones	0.13	0.05	0.14	0.09	0.00	0.00	-100.0
H	Systemic hormonal preparations, excluded sex hormones and insulin	2.93	2.96	3.55	2.73	2.40	2.41	-3.8
J	Anti-infectives for systemic use	8.09	8.45	9.93	7.69	7.09	7.91	-0.4
M	Musculoskeletal system	12.26	12.74	11.80	7.85	5.63	5.55	-14.6
N	Nervous system	13.17	14.96	15.28	10.10	8.75	9.17	-7.0
P	Anti-parasitic products, insecticides and repellents	0.01	0.00	0.00	0.00	0.00	0.00	-21.1
R	Respiratory system	3.05	3.73	4.26	5.52	4.60	5.18	11.2
Total		137.88	166.46	200.25	125.56	117.90	113.94	-3.7

ATC – Anatomical Therapeutic Chemical classification; DDD – defined daily dose; HPD – 100 patient-days; CAGR – Compound Aggregate Growth Rate.

Table 4

Top 20 drugs expressed as DDD/HPD (2011–2016).

No	ATC	INN	DDD/HPD						
			2011	2012	2013	2014	2015	2016	CAGR[%]
1	B03BA03	Hydroxocobalamin	21.42	24.91	39.32	11.31	26.42	19.93	-1.4
2	A16AX01	Thioctic acid	13.15	16.74	22.45	19.24	16.48	13.73	0.9
3	C09AA02	Enalapril	13.56	19.10	18.56	12.20	9.96	12.59	-1.5
4	M01AB05	Diclofenac	9.82	9.03	6.46	4.44	3.45	4.00	-16.4
5	C08CA01	Amlodipine	6.04	7.23	10.01	6.44	5.02	3.87	-8.5
6	B01AC06	Acetylsalicylic acid	8.14	9.34	9.28	5.17	3.62	3.58	-15.2
7	A02BC02	Pantoprazole	0.07	0.69	2.08	1.77	1.88	3.44	119.3
8	N02BE01	Paracetamol	3.48	3.66	3.74	2.78	2.75	3.36	-0.7
9	N05BA08	Bromazepam	2.97	4.52	4.19	3.09	2.37	2.61	-2.5
10	R01AA08	Naphazoline	0.25	0.41	0.86	2.79	1.92	2.28	56.1
11	B01AC04	Clopidogrel	3.28	5.55	4.92	3.33	2.63	2.19	-7.8
12	A02BA02	Ranitidine	4.90	5.31	4.68	3.05	2.30	2.18	-15.0
13	A02BC01	Omeprazole	1.37	2.14	2.36	2.74	2.89	2.05	8.4
14	A06AB02	Bisacodyl	1.48	1.53	1.46	1.66	1.54	1.92	5.3
15	J01MA02	Ciprofloxacin	1.69	1.74	1.98	1.88	1.76	1.72	0.3
16	A07FA01	Lactic acid producing organisms	0.94	1.05	1.27	1.08	1.71	1.69	12.3
17	H02AB02	Dexamethasone	2.13	2.32	2.29	1.94	1.46	1.64	-5.1
18	C03CA01	Furosemide	1.35	2.01	1.66	1.10	0.98	1.48	1.9
19	A10BA02	Metformin	1.44	1.81	2.28	1.30	1.25	1.34	-1.4
20	C03AA03	Hydrochlorothiazide	0.35	1.07	1.99	1.64	1.26	1.34	30.9

ATC – Anatomical Therapeutic Chemical classification; DDD – defined daily dose; HPD – 100 patient-days; CAGR – Compound Aggregate Growth Rate.

Diclofenac (group M) was in the fourth place of the top 20 list of the most utilised drugs in the hospital. However, its use significantly decreased over the years; from 9.82 DDD/HPD in 2011 to 4.00 DDD/HPD in 2016; the CAGR being -16.4%. Other nonsteroidal anti-inflammatory drugs (NSAIDs) used in the hospital were ibuprofen, meloxicam, ketoprofen and dexketoprofen, but their use was significantly lower than the use of diclofenac (Table 4).

The use of paracetamol (group N) was very constant over the time with an average of 3.29 DDD/HPD. On the other hand, the use of tramadol was very low (on an average 0.03 DDD/HPD) with a mild decreasing trend (CAGR -

0,1%). Bromazepam was the second most used drug in this group and its utilisation was very stable, with an average of 3.2 DDD/HPD. Other drugs from the group N were sertraline, diazepam, gabapentin, carbamazepine, paroxetine, but their use was almost negligible (Table 4).

Naphazoline was the only one drug from the group R (respiratory drugs) on the top 20 list. The utilisation of this drug was very low in years 2011–2013, ranging from 0.25–0.86 DDD/HPD, but it significantly increased during the following three years (3.09, 2.37 and 2.28 DDD/HPD in 2014, 2015 and 2016, respectively). Its CAGR was 56.1% (Table 4).

There was only one antibiotic (group J), ciprofloxacine, on the list of top 20 with an average use of 1.79 DDD/HPD. Other antibiotics, like amoxycillin, co-amoxiclav, co-trimoxazole, cefalexin, nitrofuranotin were used as well, but in much smaller quantities.

Discussion

The results of this study showed that there was a constant increase in the total drug utilisation from 2011 to 2013, followed by a significant decrease until the end of 2016. The same trend was observed in the financial values, as well, while at the same time the number of patient-days showed slight, but constant increase. This resulted in the decreased drug cost per patient-day from BAM 1.04 in 2011 to BAM 0.95 in 2016. On the other hand, the total number of utilised DDDs decreased, resulting in a bit higher average cost per DDD. This could be partly because of the increased use of new, more expensive drugs, but may also be a consequence of the increased drug prices. To a certain number of drugs, the DDD was not assigned, so they were analysed in the financial context only. Nevertheless, it can be concluded that the utilisation of these drugs follows similar trends as the drugs with the DDD. As a matter of fact, the hospital pharmacy personnel is strictly obliged to dispense the generic drugs only, which is a proven indicator of reduced drug costs, according to the WHO [17, 18].

Compared to some other studies [8], the overall drug utilisation in this study was quite low. There could be several reasons for that. First of all, the study was performed at rehabilitation hospital where the physical procedures are dominant and pharmacological treatment was used only as a complementary one. Secondly, purchasing and dispensing the drugs were based on the principles of rational prescribing, according to the hospital formularies with generic prescribing and careful management of available resources, including the transparent public drug procurement system. Furthermore, before coming to rehabilitation hospital, most of the patients were treated in some other healthcare facilities and they would usually bring their own medication upon admission to hospital.

An increased occupancy rate, which was related to the shorter hospital length of stay in recent years has led to the increased number of patients treated in hospital and consequently to the increased drug consumption. However, rehabilitation is usually a time-demanding process, with the rare acute medical conditions, and thus with a stable drug consumption. Domination of oral dosage forms is consistent with the morbidity patterns, and only a minority of patients had a serious medical reason for parenteral therapy. The parenteral drug administration was a choice when oral forms were not available, or when a rapid onset of drug action was needed. The significant differences between the DDD and the actual dose for some drugs had a strong impact on the study results.

The most prescribed drugs were from the ATC groups B, A and C. The group B was prominent mainly because of the widespread use of vitamin B12. Although vitamin B12 is known to be important for nerve function, there is no consensus about the optimal dose of vitamin B12 supplementation [19, 20]. It is obvious that prescribing of a very high dose of vitamin B12 (2.5 mg versus 20 μg according to the DDD for hypovitaminosis) in off-label indication [21] (e.g., diabetic neuropathy and radiculopathy) led vitamin B12 to the first position. This finding is also in correlation with a study of Janković et al.[8] Other authors reported the administration of ten-fold dose of vitamin B12 in comparison with the recommended dietary allowance as oral, but not as parenteral form [22]. The rationale for this off-label prescribing are the convincing results of some randomised clinical trials that showed an antinociceptive potential of vitamin B12 in the patients with the low back pain and diabetic neuropathy [23, 24].

Thioctic acid was the mosf requently used drug in the group A. A daily dose of this drug for diabetic neuropathy (600 mg) is three-fold the DDD, which might be misleading because actually 4.6 of 100 patients were receiving this drug despite of the consumption *de facto* of 13.73 DDD/HPD [25, 26]. Although it is known that aspirin decreases the incidence and the mortality of vascular disease and cancer [27], the utilisation of the low-dose aspirin decreased over recent years. It is possible that its wider prescription and use are seriously impeded by physicians' concerns of gastrointestinal bleeding [28].

The increased trend of PPIs utilisation (omeprazole and pantoprazole) was complementary to the decreased use of ranitidine. This could be due to recommendations for extensive use of these drugs for gastrointestinal protection in the patients on the long-term NSAID treatment [29]. A relevant literature survey also suggested that PPIs produce more sustained gastric acid suppression, as compared to H_2-blockers and promote the ulcer healing despite the continued NSAID use [30]. However, it was reported that the long term use of PPIs is associated with a wide range of adverse effects, including increased risk of infection, reduced intestinal absorption of iron (anaemia) and calcium (bone fractures), and more recently the kidney damage and dementia [31]. Therefore, the additional efforts are needed to reconsider the appropriate use of PPIs.

As expected, NSAID diclofenac and analgesic drug paracetamol were within the top 10 drugs at the positions 4 and 8, respectively. Diclofenac is a widely used medicine for relieving pain and inflammation, particularly in painful conditions such as arthritis [32]. Despite of some decline, the

diclofenac utilisation remained unreasonably high, compared to the relatively low use of ibuprofen. This might be the reflection of a poor prescribing practice with respect to the European Medicines Agency recommendations for the patients with cardiovascular risk. To be specific, the data from reviews suggested an increased relative risk of arterial thromboembolic events, which were sometimes greater for diclofenac than for other commonly prescribed NSAIDs and in some cases as great as, or even greater than, the one seen with certain cyclooxygenase-2 (COX-2) inhibitors [33].

Depression is one of the main comorbidities in the patients with stroke, amputations and diabetes [34] and it could be one of the reasons for a significant sertraline use, in addition to a wide accessibility of this drug in the primary healthcare [12]. A spare use of antiepileptics and antidepressants other than sertraline was obvious, despite the fact that the current treatment recommendations suggest tricyclic antidepressants, selective serotonin-norepinephrine reuptake inhibitors and anticonvulsants as adjuvant analgesics of choice for the treatment of neuropathic pain [35].

Excessive consumption of naphazoline nasal drops in recent three years could be explained by the increased number of hyperbaric oxygentherapy (HBOT) treatments in the hospital after the installation of a multiplace hyperbaric oxygen chamber in 2014. It is known that HBOT is associated with a risk of middle and inner ear barotrauma followed by otalgia, ear fullness, hearing loss, and tinnitus as the most prevalent symptoms [36, 37]. Nevertheless, it seems that the topical nasal decongestants may not be effective in preventing the middle ear barotrauma during the hyperbaric oxygen therapy [38].

Overall antibiotic utilisation was relatively stable with a small increase in 2013. The hospital has developed its own antibiotics guidelines, the Guidelines for the Prevention and Treatment of Urinary Tract Infection, as well as the Guidelines for Chronic Wound Infection. Moreover, the local Drug and Therapeutic Committee is obliged to periodically report on the utilisation of antibiotics in the hospital. Despite the hospital guidelines for the antimicrobial therapy, doctors are very prone to prescribe the broad-spectrum antibiotics for empirical treatment of bacterial infections. Hence, ciprofloxacin (both oral and parenteral) was found on the list of top 20 drugs. In general, the hospital monitoring of antimicrobial utilisation is important in order to establish the relationship between their use and the occurrence of resistance. Monitoring also reveals trends in prescribing and allows comparisons to be made among different hospitals [39].

The present study has several limitations. The main one is that the data on drug utilisation were available only for drugs dispensed from the hospital pharmacy department; hence personal patient's drugs were not included. Therefore, it remains unknown whether the total drug consumption follows the observed trends. Also, the data are collective and not analysed according to diagnoses. Consequently, additional studies are needed to determine the prescribing patterns and drug utilisation for major conditions treated in the hospital.

Conclusion

It could be concluded that the general pattern of drug utilisation is consistent with the most common conditions treated in the hospital. Additionally, this study points out a few deviations in prescribing habits and these points could be a target for the future educational activities. Our results are not surprising as there is a lot of space for harmonisation of the prescribing practice with the current recommendations and clinical guidelines.

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Significance of KIT and PDGFRA mutations in gastric gastrointestinal stromal tumor imatinib-naive surgically treated patients

Značaj mutacija KIT i PDGFRA kod bolesnika operisanih zbog gastrointestinalnog stromalnog tumora želuca bez primene imatiniba

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Abstract

Background/Aim. KIT (KIT proto-oncogene receptor tyrosine kinase) and PDGFRA (platelet-derived growth factor receptor alpha) gene mutations represent major molecular forces inside the gastrointestinal stromal tumors (GIST). Aim of this study was to evaluate these mutations in the patients who underwent surgical resection of gastric GIST, but without imatinib mesylate treatment. **Methods.** Retrospective clinical study included patients who were operated on due to gastric GIST from November 2000 till November 2016. A molecular analysis of paraffin embedded tumor tissue was performed, and the patients with the presence of KIT and PDGFRA mutations were further evaluated, with regard to the pathological tumor stage, disease recurrence and overall survival. **Results.** Out of 45 patients in total, 43 patients had KIT and PDGFRA mutations, and 2 patients were classified as the wild type GIST. After curative resection, 11 patients were classified as a low risk GIST, 8 as an intermediate risk and 26 as a high risk GIST. The KIT mutations were present in 37 patients, most commonly as deletion in exon 11. The PDGFRA mutations were present in 6 patients. The presence of KIT mutation had a strong statistical correlation with the mitotic index ($p = 0.021$). After the ten-year follow-up, all patients with the PDGFRA mutations were alive, while those with the KIT mutations had a survival rate of 71% ($p = 0.31$). **Conclusion.** The presence of KIT exon 11 deletion in the patients with primarily resected gastric GIST is associated with the higher mitotic index and worse overall survival than those present with the PDGFRA mutations. This results suggest prognostic significance towards more aggressive behaviors.

Key words:
gastrointestinal stromal tumors; genes; mutation; digestive system surgical procedures; neoplasm metastasis; prognosis; survival.

Apstrakt

Uvod/Cilj. KIT (KIT *proto-oncogene receptor tyrosine kinase*) i PDGFRA (*Platelet-derived growth factor receptor alpha*) genske mutacije predstavljaju osnovne molekularne promene u grupi gastrointestinalnih stromalnih tumora (GIST). Cilj ove studije bio je da se analiziraju KIT i PDGFRA mutacije u grupi bolesnika koji su operisani zbog primarnog GIST-a želuca, ali bez terapije imatinib mesilatom. **Metode.** Načinjena je retrospektivna klinička studija koja je uključila bolesnike operisane zbog GIST-a želuca u periodu od novembra 2000. do novembra 2016. godine. Načinjena je molekularna analiza na parafinskim kalupima tumorskog tkiva, a kod bolesnika kod kojih su identifikovane KIT i PDGFRA mutacije sprovedena je dalja analiza, sa posebnim osvrtom na patološke karakteristike tumora, recidiv oboljenja i ukupno preživljavanje, te procena uticaja analiziranih genskih mutacija na navedene promene. **Rezultati.** Od ukupno 45 bolesnika, 43 bolesnika imala su prisutne mutacije na KIT i PDGFRA genima, dok su dva bolesnika klasifikovani kao "*wild type*" GIST. Po učinjenoj kurativnoj resekciji, 11 bolesnika je klasifikovano u grupu GIST-a niskog stepena, 8 u grupu srednjeg, a 26 bolesnika u grupu visokog rizika od metastaziranja. KIT mutacije su bile prisutne kod 37 bolesnika, najčešće u formi deleciji na egzonu 11. PDGFRA mutacije bile su prisutne kod 6 bolesnika. Prisustvo KIT mutacija imalo je visoku statističku korelaciju sa mitotiskim indeksom ($p = 0,021$). Nakon desetogodišnjeg praćenja, svi bolesnici iz grupe sa PDGFRA mutacijama su bili živi, dok je stepen preživljavanja bolesnika sa KIT mutacijama iznosio 71% ($p = 0,31$). **Zaključak.** Prisustvo KIT egzon 11 delecija kod bolesnika kod kojih je sprovedena primarna hirurška intervencija zbog GIST-a želuca povezana je sa visokim mi-

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totskim indeksom i lošijem ukupnom preživljavanju, ali bez statistički značajne razlike u odnosu na bolesnike kod kojih je bila prisutna PDGFRA mutacija. Ovi rezultati ukazuju na prognostički značaj u pravcu agresivnog ponašanja tumora.

Ključne reči:
gastrointestinalni stromalni tumori; geni; mutacija; hirurgija digestivnog sistema, procedure; neoplazme, metastaze; prognoza; preživljavanje.

Introduction

Gastrointestinal stromal tumor (GIST) represents a most common mesenchymal neoplasm of the digestive system [1]. These tumors can be located in every part of alimentary tract, but most frequently GISTs are encountered in stomach [2].

Gastrointestinal bleeding is leading symptom, followed with dyspepsia, abdominal pain and discomfort, but its presentation can range from palpable masses in the abdomen to completely asymptomatic ones [3]. Unlike the carcinomas, GISTs lack dominant infiltrative type of growth, and rarely can involve the lymph nodes. However, once these tumors have reached the malignant potential, very aggressive hematogenous spread may occur, predominantly affecting the liver. This malignant potential in GISTs is outlined by three major factors, tumor site (location), size, and mitotic rate, and the risk systems based on these factors: were proven and implemented in rutine clinical practice [4]. Genetic alterations in GIST are relatively well-known, mostly through the assessments of tyrosine kinases inhibitor KIT and the platelet-derived growth factor receptor alpha (PDGFRA) gene mutations. Both genes play crucial role in GIST pathogenesis, as their activation has significant influence on the cell proliferation, apoptosis and other important cell functions [5].

Molecular changes in KIT and PDGFRA genes represent lead driving power in pathogenesis of gastrointestinal stromal tumors. These genes are the members of tyrosine kinase receptors, type III [6, 7]. With knowledge that kinase receptors play significant role in the treatment of GIST patients, proper understanding the mutations in KIT and PDGFRA may have a beneficial role in further understanding of tumor biology and subsequently may help in making proper decisions in each individual GIST patient. Various types of PDGFRA and KIT mutations in GISTs are frequently described in the relevant literature so far. Concerning the KIT gene, the mutation in exon 11 is most commonly described, followed with the mutations in exon 9, seldom in exons 13 and 17 [8]. Deletion mutation in exon 11 (codons 557 and 558) was linked to more aggressive clinical course of the disease [9]. On the other hand, the PDGFRA mutations, most frequently encountered in exons 12, 16 and 18, are less frequent than the KIT mutations, but their presences is associated with favorable pathologic and clinical tumor behavior [10, 11].

Clinical studies on KIT and PDGFRA in gastric GIST so far have been able to prove the significance of these mutations on tumor behavior, mostly reflected in disease relapse, metastatic potential and overall survival, although the studies that brought up this issue tended to have conflicting findings [12]. This might be due to the fact that various mutations can be encountered, for example in KIT exon 11, differently affecting tumor's biology [13, 14].

The aim of this study was to evaluate the significance of KIT and PDGFRA mutations as prognostic factors in correlation to metastatic potential and overall survival in the patients who underwent primary surgical treatment of gastric GIST.

Methods

This is the retrospective clinical study conducted at the Department for Esophagogastric Surgery, First Surgical University Hospital, Clinical Center of Serbia, Faculty of Medicine, University of Belgrade. The molecular analysis was performed in a genetic laboratory, at the Institute for Neurology, Clinical Center of Serbia, Faculty of Medicine, Belgrade. It included the patients who were submitted to primary curative surgical treatment of gastric GIST without prior imatinib mesylate treatment, from November 2000 till November 2016. The Hospital Board and the Ethics Committee approved the study.

A database was created by the assessment of medical records and it included the following: demographic and clinical data, location and tumor size, type of surgical intervention, pathological and immunohistochemical examination. The follow-up records were collected prospectively and assessed the data regarding the tumor recurrence, or metastasis onset. The preoperative diagnostics was performed in all cases and it consisted of barium swallow radiography, esophagogastroduodenoscopy, endoscopic ultrasonography, computed tomography (CT) scan of thorax and abdomen.

The pathological reports were retrospecitvelly reviewed with respect to the resection margins, tumor size (the largest diameter was taken into acount), cellularity, cell type and mitotic index per 50 HPF (*High Power Field*). The immunohistochemical report included immunophenotype determination (positivity for CD117, CD34, α-SMA, desmin, S-100). The risk assessment and postoperative stratification in three categories was performed based on the criteria recommended by the National Institutes of Health (NIH), depending on the tumor site, size and mitotic index respectively.

The follow-up protocol included the clinical examinations and CT scans. The patients with the high and intermediate risk GIST were examined every 4 months during the first 3 years, followed by every 6 months during the next 2 years and once a year thereafter. The patients with a low risk GIST were examined yearly during 5 years.

Molecular analysis

The molecular genetic analysis was performed in the KIT and PDGFA genes, derived from the formalin fixed paraffin-embedded tumor samples. After the identification of tumor tissue, the sample sized 1 × 1 cm was chosen for the DNA extraction. After the paraffin removal, DNA was extracted by using the phenol-chloroform method. Isolated DNA was subsequently used for the mutations analysis in selected exons KIT and PDGFRA genes with the direct sequencing method according to Sanger. The PCR amplifica-

tion of desired regions was performed with specific primers (Verity Thermal Cycler, Life Technology, USA). This was followed by the PCR purification, and sequencing by using the BigDye Terminator v 3.1 Cycle Sequencing kit (Life Technology, USA). The sample analysis was performed by the capillary electrophoresis method using the ABI 3500 Genetic Analyzer, and for the software data processing, the Sequencher program version 4.10.1. was applied.

The presence of mutation in KIT and PDGFRA was the main inclusion criteria. Out of overall patient sample (n = 100), 43 patients had mutations in KIT and PDGFRA and 2 patients were classified as the wild type and were the subject of further analysis. Remaining 55 patients, in whom the curative surgical treatment of gastric GIST was conducted, did not possessed a tissue sample of sufficient quality for the genetic analysis. Significantly, none of the patients received KIT, so all analyses were performed in imatinib mesylate-naive tumor sample.

Statistical analysis

The data are expresed as means with percentages and standard deviations. The statistical correlations between different pathological parameters were tested by using the χ^2 test and the Fischer's extact test. The survielance curve was created using the Kaplan-Meier method, and a statistical significance was tested with the log-rank test. The level of statistical significance was set at 0.05. All analyses were performed by using the statistical software SPSS version 15.0.

Results

Overall, the study included 45 patients, of whom 21 men, and 24 women, who met the inclusion criteria. The mean age of study population was 59.1 years (range from 23 to 84 years). Bleeding was the predominant symptom in the disease presentation in 22 patients, followed by the abdominal pain in 10 patients and the abdominal discomfort in 6 patients, while dysphagia was present in 2 patients and 1 patient had the abdominal palpable mass. There were 4 asymptomatic patients.

Primary curative surgical treatment was conducted in all patients. Table 1 presents the range of surgical interventions which were performed. There was no postoperative morbidity nor mortality in the study.

After the pathological examination, 26 patients were classified as high risk GIST, 8 and 11 as intermediate and low risk, respectively, while none of the patients fulfilled the criteria for very low risk. The detailed clinical, pathological and imunohistochemistery data are given in Table 2.

The mean length of follow-up was 61.77 months (ranging from 9 to 142 months).

Table 2

Clinical and pathological characteristics of gastrointestinal stromal tumor (GIST) patients

Parameters	Values
Sex, n (%)	
male	21 (49)
female	24 (51)
Age (years), mean (range)	59 (23–84)
Symptoms, n (%)	
melena	20 (44)
haematemesis	2 (4)
abdominal pain	10 (22)
abdominal discomfort	6 (13)
dysphagia	2 (4)
palpabil mass	1 (2)
asymptomatic	4 (8)
Tumor longest diameter (mm), Mean (range)	77.84 (22–210)
Tumor dimension grups, n (%)	
≤ 2 cm	0
> 2 ≤ 5 cm	13 (29)
> 5 ≤ 10 cm	23 (51)
> 10 cm	9 (20)
Mitosis, n (%)	
≤ 5/50 HPF	24 (53)
> 5/50 HPF	21 (47)
Necrosis, n (%)	
absent	31 (69)
focal	12 (27)
spread	2 (4)
Anaplasia, n (%)	
low	43 (96)
high	2 (4)
Cellulary type, n (%)	
spindele	36 (80)
epithelioid	3 (7)
mixed	6 (13)
CD 117, n (%)	
-	6 (13)
+	39 (87)
CD 34, n (%)	
-	6 (13)
+	39 (87)
SMA, n (%)	
-	31 (69)
+	14 (31)
Resection margins, n (%)	
R0	45 (100)
R1	0
NIH risk, n (%)	
very low	0
low	11 (24)
intermediate	8 (18)
high	26 (58)

Table 1

Surgical management of the gastrointestinal stromal tumors and risk classification

Type of surgery	Low risk (n = 11)	Intermediate risk (n = 8)	High risk (n = 26)	Total (n = 45)
Wedge resection, n (%)	9 (20)	8 (18)	10 (22)	27 (60)
Subtotal gastrectomy, n (%)	1 (2)	0 (0)	4 (9)	5 (11)
Total gastrectomy, n (%)	1 (2)	0 (0)	10 (22)	11 (24)
Distal esophagectomy and total gastrectomy, n (%)	0 (0)	0 (0)	2 (5)	2 (5)

Table 3

Follow-up data of gastrointestinal stromal tumor (GIST) patients

Occurence of metastases	Total of number of patients (45) n (%)
Metastasis or local recurrence	
intraoperative metastasis	2 (4)
metastasis during follow-up	9 (20)
local recurrence during follow-up	3 (7)
no metastasis / recurrences	31 (69)
Mean time to metastasis/recurrence in months	24.83
Current status	
ANED	31 (69)
AWD	5 (11)
DOD	7 (16)
DUC	2 (4)

ANED – alive no evidence of disease; AWD – alive with disease; DOD – died of disease; DUC – died of unrelated causes.

Table 3 presents a proportion of patients with liver metastasis on admission, who were submitted to surgery due to bleeding from the primary tumor site, the patients who developed liver metastasis and a local recurrence during the follow-up period, and the patients who are disease free.

The median interval from the surgery to occurrence of metastasis, or a local recurrence was 24.83 months (2–92 months). Two patients, who had liver metastases diagnosed intraoperatively, were submitted to metastasectomy, R0 resection was reached, and the postoperative course went uneventfully.

Molecular analysis

The molecular analysis showed that the genetic mutations in KIT and PDGFRA were present in 43 patients, while 2 cases were classified as the wild type GIST. A simplified presentation of genetic mutations in KIT and PDGFRA is shown in Table 4.

Table 4

Distribution of KIT and PDGFRA mutations

Type of mutation	Total of number of patients (45) n (%)
KIT	37 (82)
Exon 9	
insertion	1 (2)
deletion	2 (4)
point mutation	2 (4)
Exon 11	
insertion	3 (7)
deletion	18 (45)
insertion-deletion	3 (7)
point mutation	8 (18)
PDGFRA	6 (13)
Exon 12	
deletion	1 (2)
Exon 16	
point mutation	1 (2)
Exon 18	
point mutation	4 (9)
Wild type KIT/PDGFRA	2 (5)

PDGFRA – platelet-derived growth factor receptor alpha.

The KIT mutations were present in 37 patients. Out of those, 18 patients had deletion on exon 11, 3 patients had insertion, 3 had the combination of insertion and deletion, while the point mutation was present in 8 of them. The exon 9 mutations were present in 5 patients, the deletion and point mutation in 2, and insertion in 1 patient. The PDGFRA mutations were present in 6 patients. Out of those, 4 patients had presence of point mutations on exon 18, while 1 had deletion on exon 12 and 1 the point mutation on exon 16.

The correlation between the pathological parameters and types of genetic mutation was shown in Table 5. A statistically significant correlation was found between the mitotic rate and KIT mutations ($p = 0.021$). There was no statistical correlation between other pathological parameters and analyzed genetic mutations.

The Kaplan-Meier curve, as well as 1, 5 and 10 years of survival rates with regard to the type of mutation are presented in Figure 1. Overall survival rate for the patients with the proven KIT gene mutation was 71% (91%, 78% and 71% during 1, 5 and 10 years, respectively). In the patients without these mutations overall survival rate was 85% (94%, 85% and 85% during 1, 5 and 10 years respectively). The patients with the proven PDGFRA mutations had the overall survival rate 100%. Both patients with the wild type mutations were also alive and disease free through the follow-up period. There were no statistically significant differences between the overall survival and the type of mutations ($p = 0.310$).

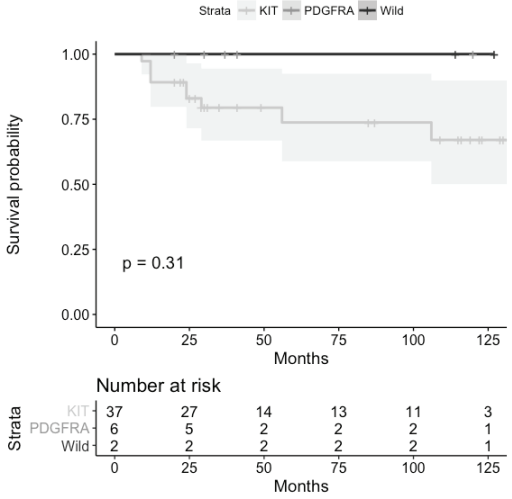

Fig. 1 – Kaplan –Meier curve showing overall survival rates with regard to different genetic mutation type. PDGFRA – platelet-derived growth factor alpha.

Table 5

Correlation between the pathological and molecular parameters

	KIT exon 9 mutations, n (%)			KIT exon 11 mutations, n (%)					PDGFRA mutations, n (%)			
	Ins	Del	PM	Ins	Del	Ins-del	PM	*pp*	Exon 12 Del	Exon 16 PM	Exon18 PM	*p*
Cell type												
spindle	1 (100.0)	0 (0.0)	2 (100.0)	3 (100.0)	15 (83.3)	2 (66.7)	7 (87.5)		1 (100.0)	0 (0.0)	3 (75.0)	
epitheloid	0 (0.0)	1 (50.0)	0 (0.0)	0 (0.0)	1 (5.6)	0 (0.0)	1 (12.5)	0.550	0 (0.0)	0 (0.0)	1 (25.0)	0.400
mixed	0 (0.0)	1 (50.0)	0 (0.0)	0 (0.0)	2 (11.1)	1 (33.3)	0 (0.0)		0 (0.0)	1 (100.0)	0 (0.0)	
Mitotic count												
≤ 5/50 HPF	0 (0.0)	0 (0.0)	2(100.0)	2 (66.7)	6 (33.3)	1 (33.3)	7 (87.5)	0.021	1 (100.0)	1 (100.0)	2 (50.0)	1.000
> 5/50 HPF	1 (100.0)	2(100.0)	0 (0.0)	1 (33.3)	12 (66.7)	2 (66.7)	1 (12.5)		0 (0.0)	0 (0.0)	2 (50.0)	
Tumor necrosis												
absent	1 (100.0)	1 (50.0)	2 (100.0)	1 (33.3)	11 (61.1)	1 (33.3)	7 (87.5)		1 (100.0)	1 (100.0)	3 (75.0)	
focal	0 (0.0)	1 (50.0)	0 (0.0)	2 (66.7)	5 (27.8)	2 (66.7)	1 (12.5)	0.613	0 (0.0)	0 (0.0)	1 (25.0)	1.000
spread	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	2 (11.1)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	0 (0.0)	
Anaplasia												
low	1 (100.0)	2 (100.0)	2 (100.0)	3 (100.0)	17 (94.4)	3 (100.0)	8 (100)	1.000	1 (100.0)	1 (100.0)	4 (100.0)	1.000
high	0 (0.0)	0 (0.0)	0 (0.0)	0 (0.0)	1 (5.6)	0 (0.0)	0 (0.0)		0 (0.0)	0 (0.0)	0 (0.0)	
NIH score												
very low	0 (0.0)	0 (0.0)	0 (0.0)	1 (33.3)	4 (22.2)	0 (0.0)	4 (50.0)		0 (0.0)	1 (100.0)	1 (25.0)	
low	0 (0.0)	0 (0.0)	1 (50.0)	1 (33.3)	2 (11.1)	1 (33.3)	2 (25.0)	0.145	1 (100.0)	0 (0.0)	1 (25.0)	1.000
intermediate	0 (0.0)	1 (50.0)	1 (50.0)	1 (33.3)	1 (5.6)	1 (33.3)	1 (12.5)		0 (0.0)	0 (0.0)	0 (0.0)	
high	1 (100.0)	1 (50.0)	0 (0.0)	0 (0.0)	11 (61.1)	1 (33.3)	1 (12.5)		0 (0.0)	0 (0.0)	2 (50.0)	

***Ins – insertion; Del – deletion; PM – point mutation; NIH – National Institutes of Health.**
For other abbreviations see under Table 4.

Discussion

The results of this study implicated that the KIT gene mutations are the most common type of mutation in gastric GIST. Although the clinical relevance of these mutations in our patient population was not clearly shown, in the discussion bellow we will try to make a thorough analysis and draw out relevant conclusion.

In the overall patient sample KIT and PDGFRA mutations were present in 45% of patients, predominantly KIT mutations in exon 11, while PDGFRA mutations were observed in 6 patients. These frequencies are lower than those noted in the literature reports so far, where the occurrence of KIT mutations was reported to be around 80%, and PDGFRA approximately about 10%. This can be explained by the retrospective nature of the study and the possibility that some of tumor samples were not preserved sufficiently to contain DNA suitable for further analysis. Yet, the overall distribution of molecular changes in this study population, reflected in the KIT versus PDGFRA mutations ratio as well as frequencies of mutation types, is in accordance to the literature results [15].

The KIT mutation was the predominant type of mutation in our study and the leading type of mutation was deletion in exon 11. This is in concordance with the report of Joensuu et al. [16], who based their study on an analysis of 11 literature reports concerning the KIT and PDGFRA mutations in GIST. The authors confirmed that the KIT mutations in exon 11 were the most common type of mutation, and presented in 293 out of 301 analyzed patients. Deletion was most common type of mutation, presented in 43% of patients. Concerning the relation of this specific mutation and other pathological parameters of GIST, our study showed that the presence of this mutation had a strong statistical correlation with the higher mitotic index. However, there was no correlation with other pathological parameters. The results of published studies shows that KIT mutations in exon 11 can be linked with more aggressive tumor behavior [17, 18]. Capelli et al. [19] showed that the patients with gastric GIST who had the KIT mutations, were presented with worse pathological parameters, such as tumor necrosis, spindle cellularity, tumor size and mitotic index. Similar results were noted by Schaefer et al. [20].

The PDGFRA mutations were by far less present in our study than the KIT mutations, which is concordance with other literature reports being around 6%. The most common type of PDGFRA mutation noted in our study was the point mutation on exon 18. There was no statistical correlation between these molecular changes and the analyzed pathological parameters. Lasota and Miettinen [14] found that the majority of GIST patients with the presence of PDGFRA mutation had predominantly the epitheloid type of tumor, with the low mitotic index and benign clinical course.

In our study, the overall survival rate among the patients who had the KIT mutations during the 10-year follow-up was 71%, while in those GIST patients with the PDGFRA mutations there was no mortality in the follow-up period. Although there is a tendency towards better overall survival in the group of patients with the PGFRA mutations, a statistical significance was not reached probably due to a small sample size inside this group. Our study involved the patients who did not receive any prior imatinib mesylate therapy. Rossi et al. [21] published their results concerning imatinib-naive GIST patients with regard to a tumor mutational status and confirmed that the KIT mutations were the independent prognostic factors of poor overall survival. The authors also identified several groups according to the mutation status and they were those with an increased risk of tumor recurrence and metastasis. Those with the greatest malignant potential had mutations in KIT exon 9 and 11, and PDGFRA in exon 18 apart from D842V.

Limitations of this study include a relatively small sample of patients and the retrospective method.

Conclusion

A risk stratification for gastric GIST is well- established and based on clinically proven classifications based on the tumor site, size and mitotic rate. The recognition and assessment of KIT and PDGFRA mutations in these tumors may play a significant role as an additional factor in the risk stratification, disease prognosis, but may be important to identify the patients who are at the highest risk for disease recurrence.

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GENERAL REVIEW

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Brain arteriovenous malformations

Arteriovenske malformacije mozga

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Key words:
brain; arteriovenous malformations; signs and symptoms; diagnosis; treatment outcome.

Ključne reči:
mozak; arteriovenske malformacije; znaci i simptomi; dijagnoza; lečenje, ishod.

Definition

Brain arteriovenous malformation (bAVM) is a pathological arteriovenous communication [1]. Morphological changes in this disease are localized on the level of blood vessel and possible changes in the surrounding parenchyma are only the consequence of changes of angioarchitecture and do not have neoplastic characteristics. Because of that, this disease terminologically has the name malformation and that is why the term angiom is incorrect since in that case it would correspond to neoplastic change [2].

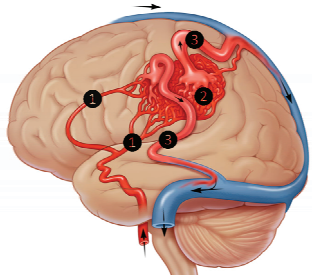

Fig. 1 – Macromorphology of brain arteriovenous malformations (bAVM): 1) arterial feeders; 2) nidus; 3) drainage veins (arrow - direction of blood flow) 3.

Pathology

Macromorphology

Macroscopically arteriovenous malformation includes three segments: artery or arterial feeders, nidus and leading back vein or veins (Figure 1). Central part of bAVM is nidus which represents a network of vascular channels where arterioles and veins are connected directly without formed capillary network. Such nidus is on hemodynamic proximal end connected with artery and on distal end with drainage vein structures. Nidus can be of different forms and scope of its size ranges within an interval from microscopic size to the size of several centimetres [3, 4].

Micromorphology

Microscopically bAVM is composed of clusterized and abnormally muscularized arterial feeders which may have duplications or destructions of elastic laminae, veins of different total diameter and wall thickness, blood vessels of indeterminate characteristics which can be formed only of fibrous tissue or may have characteristics of both arteries and veins and glial tissue which is located between blood vessels [5]. Such complex of blood vessels at bAVM is at hemodynamically proximal and distal end connected with normal blood vessels. Changes on walls of blood vessels are mostly localized in tunica media where fluctuations are detected in its thickness, its complete disappearance or division to two layers which are divided by elastic lamina [5–7].

Etiology

During angiogenesis primitive artery forms lateral sprouts which separate from the artery and then join together into a blood vessel which shall become a vein. It is postu-

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lated that defect in complete separation of the sprout from artery leads to formation of pathological communications of artery and vein which later grows into bAVM (Figure 2). Besides the stated it is probably the issue of multifactorial etiology where described embrional disorder is additionally burdened by the fact that some of genes responsible for angiogenesis are changed by separate nuclear polymorphism which represents the "second strike" on the normal angiogenesis[8].

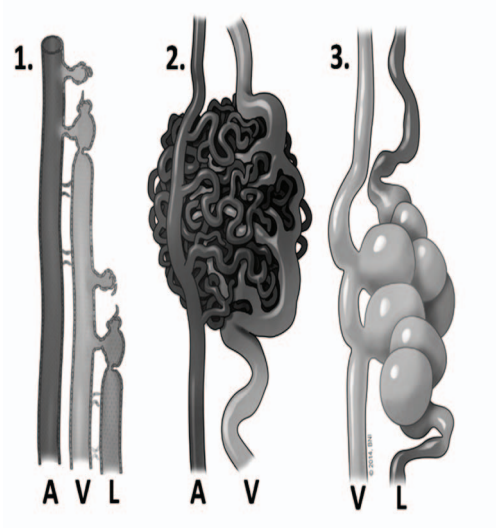

Fig. 2 – Hierarchical model of development of brain arteriovenous malformations: experimental data indicate that arteries (A) and veins (V) by infusion are formed from a common precursor (1). Similarly, lymphatics (L) are derived from venous precursors. Differentiation disorder leads to arteriovenous (2) and venolymphatic (3) malformation[8].

Epidemiology

Basically, bAVM is a rare disease. Prevalence of bAVM in general population is difficult to determine exactly in a specific time point since there are no large post mortem studies which would also provide information on some people who have asymptomatic changes which could pass unnoticed during their lifetime. Therefore, the number of 0.94 newly discovered patients at every 100,000 inhabitants during one year should be considered as a reliable data in the context of patient number and organization of health care [5].

bAVM occurs more commonly in patients with Rendu-Osler-Weber syndrome where its prevalence is estimated between 4% and 13% [9–11] and in rare Wyburn-Masin syndrome.

Division

bAVMs can be divided into three groups based on angioarchitecture, symptoms, prognosis and treatment [4].

Subpial bAVMs are the most common ones. They are localized under soft meninges, and size of nidus can range from several milimeters to several centimeters. They affect cortex and /or white matter. The form of nidus can be different, including regularly globular, ovoid, triangular, plate or completely irregular one. Arterial feeders originate from internal carotid artery branches (most commonly medial cerebral artery), vertebrobasilar branches but also branches of meningeal arteries originating from external carotid artery.

Surface pial bAVM is the most common in paediatric population and is composed of almost direct shunt. Flow is extremely strong with consequent venous dilatation. These are extremely rare lesions with great haemorrhagic potential.

Dural bAVMs are often incorrectly called dural AV fistulas. These are lesions which also include nidus localized in dural sinus. They represent about 10–15% of AVMs. Lesions of this type are most often acquired, and etiological factors are of wide spectrum including dural phlebitis, trauma, infection of paraendocranial structures (e.g. mastoiditis). Primary cause of dural bAVMs is veinous hypertension. Nidus is most often small (few millimeters) and main changes which can be detected by imaging methods are located on arterial feeders and veinous drainers [4].

Presentation

Regarding the symptoms, arteriovenous malformationscan roughly be divided into hemorrhagic and non-hemorrhagic ones. There is also a group of those accidentally detected.

Hemorrhage

Intracranial hemorrhage is the most common presentation of bAVM seen in 30% to 82% of cases. This is also the most severe complication bAVMs. Blood can be found in subarachnoid space (30%), parenchyma (23%), intraventricular (16%) or in multiple mentioned locations (31%) (Fgure 3) [5, 12]. The risk of bleeding in previously nonruptured bAVM is 2–4% per year, but in complex lesions the risks are higher [4, 13, 14].

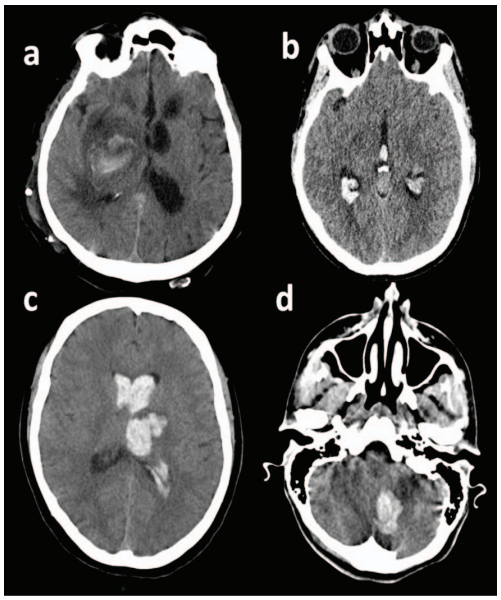

Fig. 3 – Hemorrhagic presentation of brain arteriovenous malformations (bAVM): a) intracerebral hematoma; b) isolated intraventricular hematoma resulting from rupture of intraependymal bAVM; c) combined intracerebral and intraventricular hematoma; d) intracerebellar hematoma.

Factors facilitating hemorrhagic risk

Aneurysms

Aneurysms combined with bAVM appear in large series in 7–23% of cases. The reason of possible sub-detection can lay in the fact that not all patients are submitted to digital subtraction angiography (DSA), method regarded as the golden standard [15].

By definition, aneurysms are located on arteries while vein dilatations would be terminologically correctly to call varices or vein dilatations. However, in medical terminology, all dilatations of blood vessels in correlation with bAVM are called aneurysms [16].

Generelly, aneurysms are divided into prenidal, nidal and postnidal ones. Prenidal aneurysms are further divided into: aneurysms independent of flow (aneurysms of arterial branches which bAVM does not belong to), distant flow dependent aneurysms (arterial aneurysms which belong to branches which vascularize bAVM but are at least one segment distant from nidus), close flow dependent aneurysms (aneurysms of branches that vascularize bAVM in close vicinity of nydus) (Figure 4) [15]. Vein aneurysms are the most common source of bleeding [17, 18].

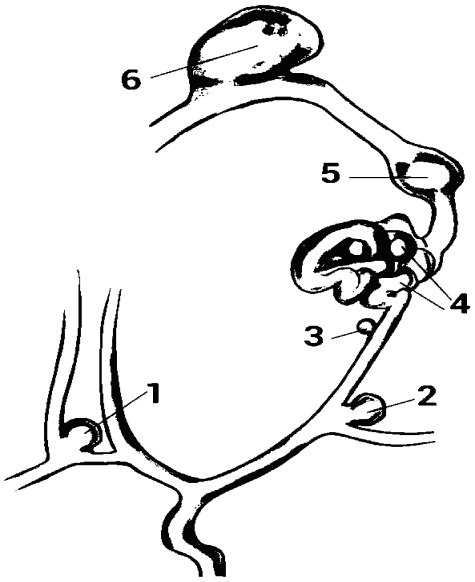

Fig. 4 – Schematic representation of aneurysms associated with brain arteriovenous malformations (bAVM). Based on their relationship with the bAVM nidus, they are divided into prenidal (arterial aneurysms), intranidal and post-nidal (venous "aneurysms"). Prenidal are divided into those that are independent of the flow (1), which are dependent on the flow (2) and are closely dependent on the flow (3). Intranidal and postnidal venous "aneurysms": saccular extensions of the nidus or the first venous drainage tract (4), varicose dilatation during the venous drainage (5) and varices (6) [15].

There is also a possibility of existence of pseudoaneurysms in nydus itself [5, 19].

It is highly probable that aneurysms represent a weak point in angioarchitecture of bAVM [5, 12] and great number of studies analyzing the risk of hemorrhage in bAVMs have come to the conclusion that existence of aneurysms increases the risk of hemorrhage [5].

Nidus size

Many studies have tried to analyze nidus size as a risk factor of hemorrhage, but this issue has remained the matter of controversy until today. Significant number of studies show that hemorrhagic complications are more common with small niduses than with bigger ones [20]. In this context two facts are to be mentioned for better understanding of the problem. Firstly, (against the theory that smaller bleed more often) bigger bAVMs more often give non-hemorrhagic symptoms and are being detected this way, so the share of hemorrhagic presentations with big bAVMs is significantly smaller than with small bAVMs which rarely give non-hemorrhagic manifestations and are more commonly detected due to bleeding. Secondly, (in favour of the theory that smaller ones bleed more often) blood pressure in small bAVMs is significantly higher than in big ones [2, 21].

Deep venous drainage

Anatomy and subsequently hemodynamics are different in surface and deep venous drainage. In the external system, anastomosis of veins are better, thus the possibility of compensation of increased inflow of blood volume and higher pressure is increased. Unlike this well developed vessel network, internal veins without anastomoses direct blood only towards Galen's vein and straight sinus. Such system has reduced capability of adaptation to hemodynamic factors which contribute to development of bleeding and because of that bAVMs with deep venous drainage are more prone to bleeding than those with surface drainage. Researches support the thesis that the risk of bleeding in cases of deep venous drainage is higher [22, 23].

Venous stenosis

Reduction in diameter of draining veins results in increase of pressure in proximal vascular bed of bAVM causing higher risk of rupture. Increased risk of bleeding in cases of stenosis of drainage veins is confirmed in several studies [5, 23].

Non-hemorrhagic manifestations

Epilepsy

This is the second most common symptom of bAVM. Most often it is the consequence of cortically located bAVMs in temporal zones in middle aged patients [24]. These seazures may occur within nonhemorrhagic manifestations, in case of draining vein thrombophlebitis, but also due to hemorrhage and edema of surrounding brain tissue [4]. According to frequency, it is an initial symptom in 16–53% of cases. They mostly present as partial attacks, while attacks of grand mal type occur in 27–35% of cases[5].

Headache

Headache is an initial symptom in 7–48% of cases[25]. These headaches are not specific and distinction between typical migrainous attacks and headaches of bAVM origin cannot be made.

Focal neurological deficit

Effects that can cause focal neurological deficit are blood stealing phenomenon, venous hypertension and mass effect.

Regarding the stealing phenomenon, bAVM are lesions which recruit significantly greater quantities of blood per volume unit compared to brain parenchyma [5]. Upon breaking compensatory mechanisms of intracranial circulation of surrounding zone of brain parenchyma due to blood deficit they become the source of focal neurological deficit. Characteristics of this deficit are in direct relation with localization of bAVM and function of surrounding brain parenchyma.

Mass effect is one of controversial issues related to bAVM. There are statements that these lesions do not produce mass, but targeted research proved its existence [4, 26]. This data is significant in explaining both the symptom of focal neurological deficit and for radiological differential diagnosis of intracranial lesions.

Accidental findings

There is a possibility of accidental detection of bAVM in patients reffered to imaging for other reasons. In such cases, bAVM can be asymptomatic or the symptoms were not recognized. Such lesions account for about 25% [4].

Imaging methods

Aims of imaging methods are [4]: diagnosis of bAVM in different clinical settings; pretherapeutic evaluation of bAVM facilitating easier decision making on the therapy type; treatment of bAVM by interventional radiology as an independent method or within multidisciplinary approach; post-therapeutic evaluation (Figure 5).

Computed tomography (CT)

Nonenhanced CT examination

CT is most often the first imaging method of evaluation of bAVM especially in cases of hemorrhagic presentation. This is mostly consequent to the organization of radiological service and usage of CT as the first diagnostic tool in brain emergencies. On CT scans, bAVM nidus can be slightly to moderately hyperdense. Calcifications may be observed as the consequence of previous minor bleedings [5]. Surrounding zone of hypodensity may be present, corresponding to gliosis or, in case of breakthrough of compensatory mechanisms, edema.

In hemorrhagic presentation a characteristic image of subarachnoid, intracerebral and intraventricular hemmorhage is observed in different combinations, typically in the region around bAVM. When bAVM is suspected on nonenhanced CT, CT angiography (CTA) is performed.

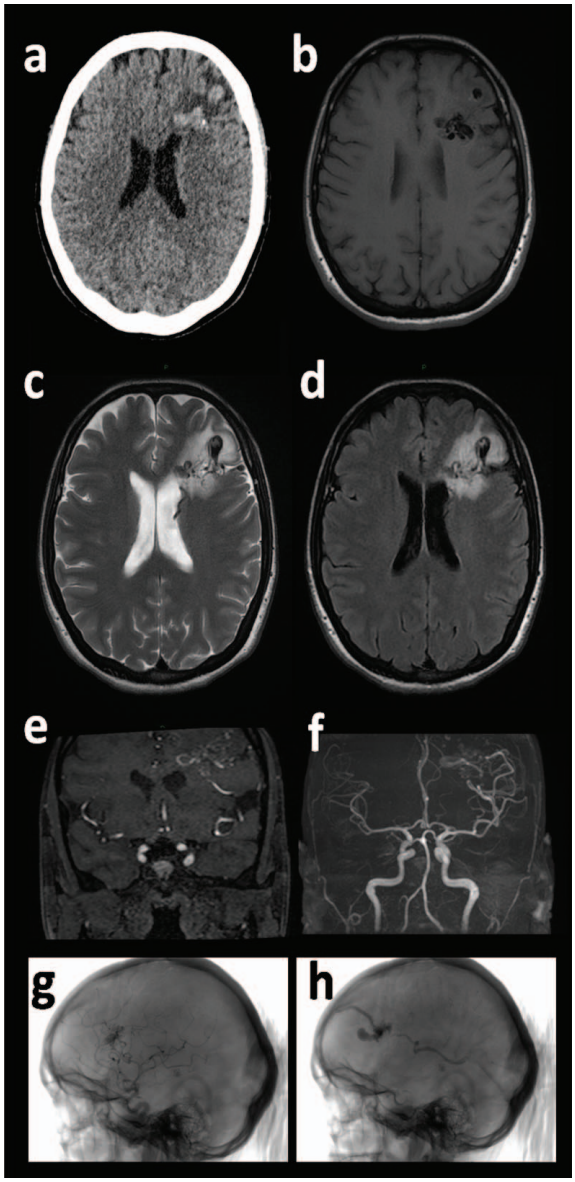

Fig. 5 – Brain arteriovenous malformations (bAVM) frontal left without signs of bleeding – display in different image diagnostic modalities.
(a) Non-contrast computed tomography (CT) examination: punctiform calcification, a hypodense structure corresponding to the blood vessels of bAVM; the surrounding hypodense corresponds to the gliosis zone (differential diagnosis with edema – no expansive effect expected in case of vasogenic edema); (b) T1-weighted (T1W) hypertension at the site of the blood vessels; (c) T2-weighted (T2W) hypertension at the site of blood vessels ("flow void"); The surrounding hyper-sensitivity corresponds to glosses; (d) Fluid-attenuated inversion recovery (FLAIR): better hyper-sensitivity demarcation; (e) Time of flight magnetic resonance angiography (TOF MRA) multiplanar structure: display of vascular structures with the flow velocity that the TOF method can detect; (f) TOF MRA projection of maximum intensity. Invasive angiography without subtraction; (g) Arterial and (h) Venous examination phase.

Computed tomography angiography (CTA)

CTA is a method able to analyze morphology of vascular network of bAVM. The principle of arterial visualization by CT is the same for all body regions, and always include administration of contrast agent [27, 28]. Contrast fills the lumen of vascular structures and allows visualization of feeding and draining vessels, as well as nidus itself, presented as conglomerate of tubular structures of contrast agent density.

As with all imaging methods there is a limit in diameter of a blood vessel which CTA can present. Therefore, small vascular structures cannot be visualized. This poses a problem in the treatment planning, since after embolization and change of hemodynamic properties, diameter of previously undetected feeders can increase and have impact on further change in morphology and dynamics of bAVM [5].

Another significant limitation of CTA, and all multiphase CT examinations in general, is high irradiation dose [29, 30].

Magnetic resonance imaging (MRI)

Basic MRI

Basic sequences (T1W, T2W, FLAIR, GRE) provide information similar to nonenhanced CT, about localization and characteristics of surrounding parenchyma and compartments. On T2W sequence blood vessels are presented as hypointense due to flow void phenomenon. The role of MRI in urgent protocol can be in revealing minimal acute subarachnoid hemorrhagic collections which are presented as hyperintenseon FLAIR sequence [4]. It is important to emphasize that, in non urgent protocols, hemosiderin deposits on GRE that are presented as hypointense are the sign of earlier hemorrhage. In this context differential diagnosis is important, however, comparison of former CT scan, which is in most cases available, makes the distinction possible.

Tissue specificity and better presentation of brain parenchyma are important advantages in analysis of surrounding brain tissue and especially in evaluation of edema which may originate as the consequence of hemorrhage or venous hypertension.

MR angiography

Time of flight and phase contrast MR angiography

Time of flight (TOF) and phase contrast MR angiography are methods which display lumen of bAVM blood vessels but they face numerous limitations. In many cases they are not capable of precise detection of anatomic change, measuring of nidus size is prone to errors, intranidal aneurysms are often invisible, and presentation of small drainage veins and blood vessels of small caliber in general is not uniformly good [5]. As with other static methods, great disadvantage is incapability of lesion blood flow dynamics analysis.

4D MR angiography

4D MR angiography (4DMRA) is noninvasive imaging method with the goal to, besides morphology change, analyze dynamics of its blood flow by adding the fourth dimension (time) to the three dimensionality of the method of MR diagnostics by successive quick high number of acquisitions. Until appearance of 4DMRA the only method by which this dynamics could be evaluated was invasive angiography which, despite of its quality, has three negative characteristics which absolutely cannot be overcome: invasive nature, application of iodine agent and radiation risk. 4DMRA originated in the attempt to find the method similar to digital subtraction angiography but without above mentioned disadvantages. Preservation of temporal and spatial resolution is satisfactory and 4DMRA represents a method which shows great efficiency in diagnostics and anatomic analysis of bAVM with good interobserver and intermethod agreement.

The method provides characterization of not only important anatomic features such as size, localization and vascular feeding and draining components but also the analysis of blood flow [30]. Also it is possible to obtain velocity-dependant mapping of brain blood flow with the possibility of marking blood vessels with different colors in correlation with the blood flow velocity [31]. However, in comparison with digital subtraction angiography (DSA), it has a sensitivity of 73.7%, specificity of 100%, positive predictive value of 100% and negative predictive value of 78.3%, due to which DSA is still the golden standard (Figure 6) [32].

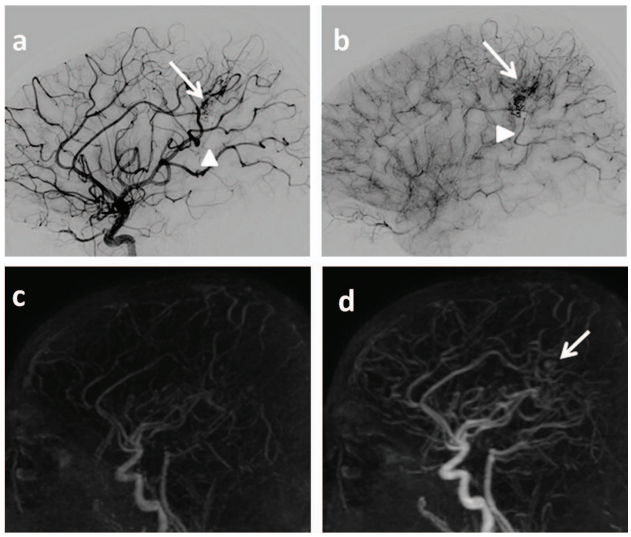

Fig. 6 – Invasive digital subtraction angiography (DSA) and 4D magnetic resonance angiography (4D MRA) studies of the same patient. Arterial (a) and venous (b) phase of DSA examination. The arrow marks the nidus, and the arrow pointers the venous drainage. Arterial (c) and venous (d) phase 4D MRA examinations. Arrow indicates nidus. Significantly better display on the DSA method than the 4D MRA. However, the 4D MRA provides an overview of hemodynamics that, prior to its development, could be achieved only by using invasive angiographic techniques.

Functional MRI

Functional MRI is a method for determining activity of brain parenchyma during performance of certain activities. In

such way, zones of brain parenchyma responsible for execution of certain activities are determined for every single examinee. This method is also aplicable in analysis of brain parenchyma related to bAVM and determination of its eloquence. The eloquence of surrounding parenchyma is one of the risk factors in further therapy which shall be mentioned further in the text within the section on Spetzler-Martin classification.

Digital subtraction angiography

Digital subtraction angiography is invasive method which comprises catheterization of certain blood vessel, transcateter application of iodine contrast agent and subsequent acquisition of x-ray images. During image processing all structures except administred contrast agent are subtracted and deleted. In this method endocranial vascular structures are underlined and their presentation is obtained without contamination of images with surrounding structures. Despite development of non invasive imaging methods (CT, MRI) the quality of data about anatomy and hemodynamics of bAVM obtained by DSA is still superb.

Based on all mentioned, nowadays diagnosis of most bAVMs is based on non invasive imaging methods, while treatment planning is based on information obtained by DSA [5].

DSA is the invasive method bearing risk of ionizing radiation and iodine contrast. Also there are risks for medical staff due to the nature of the method, with posibility of contamination by patient's blood and above mentioned ionizing radiation. Due to all mentioned, the rigorous protocol of the method must be fully respected.

Routinely, catheterization of external and internal carotid artery, as well as vertebral arteries is performed. Acquisition of images is performed in anteroposterior, profile and oblique position. Number of acquisitions in a second (f/s) should be adjusted to higher value (desired 7 f/s) due to improvement of possibilities for analysis of dynamics of vascular flow. However, one should bear in mind that greater number of acquisitions per second means greater exposure of a patient to ionizing radiation so that these values in circumstances when it is not absolutely necessary should be reduced to the level of 3 f/s.

The method also has certain disadvantages. Detection of aneurysms or intervascular communications within nidus is not perfect, neither is the possibility of analysis of nidus division into compartments in correlation with their vein drainage. The last mentioned occurs most commonly due to application of contrast agent into big blood vessels thus imbibing the whole nidus with contrast and preventing analysis of its separate parts [5, 33].

Overcoming of these deficiencies is tried by using superselective DSA during which catheterization of separate arteries nidus feeders is carried out. This allows for better analysis of nidus compartments as well as better analysis of vascular structures of nidus regarding aneurysms and intervascular communications in order to reduce superposition with surrounding vascular structures. Moreover, superselective DSA is the introduction into embolization of bAVM.

Radiological report

Diagnosis of bAVM is mostly determined on the basis of noninvasive radiological methods. Report of radiological CT or MR examination should include the following key elements [4]: 1) size – nidus dimensions (depth, width, height) and, if possible, its volume in mm^3 (attention should be paid to the distinction between nidus and vein component of BAM so that the last one is not included in calculation of nidus size; 2) localization – lobe and gyrus, depth (cortically and subcortically); supraor infratentorial; 3) venous drainage – one or more veins; deep or surface (cortical); 4) afferent arterial system: anterior, middle or posterior cerebral artery – branches of external carotid artery; 5) classification according to Spetzler-Martin – allows certain quantification of the degree of therapeutic risk; 6) signs of lesion complexity – recent or old hemmorhage; mass effect; perifocal edema; intranidal or distant thrombophlebitis; venous "aneurysms", possible signs of venous thrombosis; arterial aneurysms.

Classification

Classification systems are made to stratify and classify bAVM into groups according to the desired factor. Factors by which classifications are determined are surgical risk (Spetzler-Martin) and individual risk from bleeding (Nataf).

Classification according to Spetzler-Martin

Numeric value is scored for each category. Size: small (less than 3 cm) – 1 point; medium (between 3 cm and 6 cm) – 2 points; big (bigger than 6 cm) – 3 points; Eloquency of surrounding brain parenchyma: non-eloquent zone – 0 points; eloquent zone – 1 point; venous drainage: surface only – 0 points; deep – 1 point.

Score obtained by this classification is within interval from 1 to 5 where higher values correspond to higher surgical risk [20].

Classification according to Nataf

Based on the study of 250 patients treated by radiotherapy this classification divides patients into five groups according to risk from bleeding which is determined by hemodynamic characteristics of bAVM: Grade 1 – without risk factor, divided in two subgrades (1a – with venous engagement, and 1b – without venous engagement); Grade 2 – venous stenosis or venous reflux; Grade 3 – only deep venous drainage, and Grade 4 – intra or juxta-nidal aneurysms.

Higher grade corresponds to higher risk from bleeding and distribution of bleeding according to grades is as follows: 13% for grade 1a, 38% for grade 1b, 48% for grade 2 and 90% for grades 3 and 4 [34].

Therapy

Therapy for bAVM is complex, and absence of a single therapeutic method which would offer the cure makes the

situation even more demanding. Therapeutic approaches are endovascular embolization, radiotherapy and surgery. Decision on the treatment modality depends on a large number of factors, the analysis of which should be performed by an experienced multidisciplinary team composed of diagnostic neuroradiologist, interventional neuroradiologist, radiation oncologist and neurosurgeon.

Surgical therapy

In urgent protocol with hemorrhagic presentation a surgical treatment may be indicated with the aim of removing life-thretening hematoma. In case of surface localization and small dimensions of bAVM it can be removed during this urgent procedure.

In elective surgery the aim of operation is complete recovery from AVM. The standard microscopic technique is used, and strategy is that the treatment of arteries is performed first, followed by nidus and drainage veins treatment [5]. The success of the procedure is evaluated by postoperative imaging where complete absence of AVM is expected. In case of residual lesion, the decision on further therapy is brought multidisciplinary.

The outcome of surgical treatment on the basis of 25 series with 2,452 patients is as follows: mortality ranges from 0% to 15%, while postoperative morbidity from about 1% to 18% [35].

Radiotherapy

Radiotherapy is a noninvasive radiation method which cause proliferation of blood vessels endothelium with the aim of their obliteration by applying high energy beams on bAVM. This effect is not instant – it takes about six months for the first results to be detected, while the whole process lasts two to three years on average [36]. It is proved that equal safety is achieved by using gamma knife, cyclotron or linear accelerator as the source of radiation [5].

Degree of obliteration is mostly connected with the nidus size, and with changes less than 15 mm it is 77% ,with changes between 15 and 25 mm it is 62%, while for bigger than 25 mm it is 44% [5].

In radiological follow-up it is important to determine and analyze possible residue of the lesion and also to determine the existence of early and late radiation complications, such as disturbance of blood-brain barrier, edema and necrosis [37].

Endovascular embolization

Endovascular treatment of aneurysms is a part of therapeutic arsenal in multidisciplinary approach to the treatment of bAVM. Basically, it is a method where transcatheteric application of embolizing agent is delivered to bAVM with the aim of its occlusion.

Basic rules of endovascular treatment are as follows [5]: 1) decision on therapy should always be brought by multidisciplinary team; 2) objective of treatment is to be determined: complete or partial occlusion or prevention of bleeding or improvement of clinical symptoms; 3) procedure must be explained to the patient and/or family or custodians in detail; 4) the procedure can be carried out only by experienced neurological team (neuroradiologist, neuroanesthesiologist and instrument staff); 5) a patient shall be monitored in the intensive care unit during 24 hours after the treatment; 6) today most of interventions are performed under general anesthesia.

Intervention technique

The basic concept of embolization is nidus and drainage veins occlusion. Arteries are allowed to be occluded on the level of distal arterioles. The principle of intranidal embolization assumes placement of microcatheter top into arterioles as close as possible to drainage veins in practically occlusive position. Catheterization has to be as distal as possible. Then, after control angiogram, where expected route of embolization agent is evaluated, the embolization itself is carried out with above mentioned objective of nidus and drainage veins occlusion (Figure 7) [5].

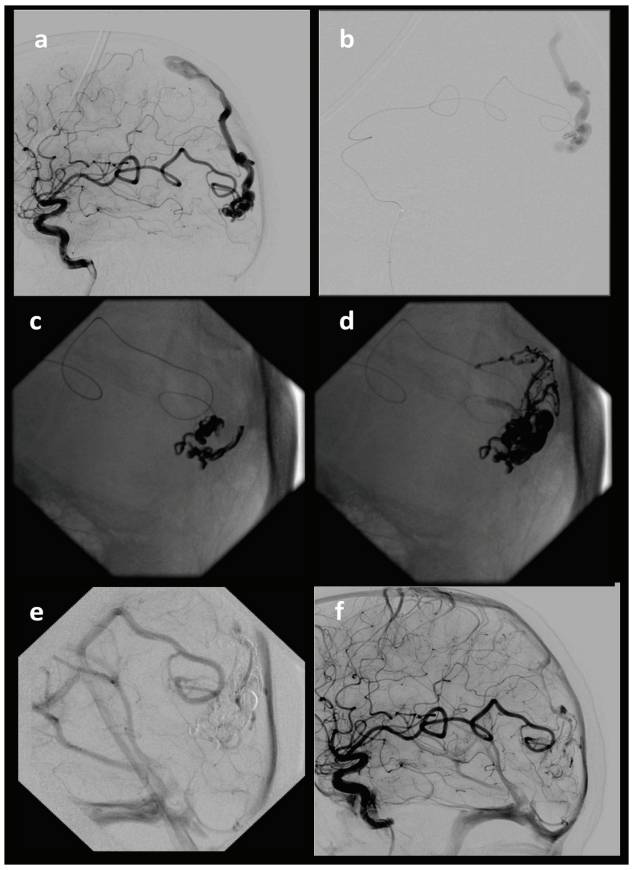

Fig. 7 – Endovascular embolization of brain arteriovenous malformations (bAVM). (a) Digital subtraction angiography (DSA) catchment of the internal carotid artery; brain arterial malformations (bAVM) presentation occipital with demarcated arterial artery, nidus and drainage vein. (b) Superselective catheterization of the arteries of the feeder; the tip of the microcatheter in the embolization position. Phases of the embolisation application: before the first (c) and the last (d) control; (e) Targeted DSA region of BAM; displaying complete occlusion of nidus; (f) Control DSA of the carotid artery after the procedure.

Material

Rough division of materials is on catheters needed for catheterization of blood vessels and reaching desired location in bAVM and embolization material itself which is applied transcatheterically.

Catheters

Catheters are further divided into leading catheters, common catheters and microcatheters.

The function of common catheters, prior to intervention, is to make images of endocranial vasculature by catheterization of extracranial segments of carotid and vertebral arteries and then in some cases they serve as support for bringing the leading catheter. Common catheters are mostly of a diameter of 5F and according to the type of their distal end they are most often vertebral or SIM catheters.

Leading catheters are most often of a diameter of 6F and their function is to provide stability for microcatheter apparatus which is placed through them. Top of the leading catheter should be brought as close as possible to endocranium and with that goal their design is a compromise of hardness and elasticity – hardness because microcatheter needs strong support first of all on the long road from the location of puncture in femoral region to endocranium and then in endocranium where it acts without external support but its point of support for further progress it finds in the hardness of the leading catheter which is placed by its top just under endocranium. Elasticity of the leading catheter is needed to overcome demanding and often winding anatomy of aorta and aorta's arch where it is often required to pass through curves of vascular structures whose angulation is in interval from blunt to extremely sharp angles. During embolization itself another role of the leading catheter is application of contrast agent with the aim of making control angiograms from which information about course of intervention in its different phases are obtained.

Microcatheters are the smallest of sets of catheters and their function is to reach nidus itself with the support of leading catheter in order to prevent embolization. Their diameter at distal end is most often 1.5 to 1.7 F and in the market microcatheters with top of diameter up to 1.3 F can be found. These are soft catheters, of extremely small diameter which are capable to catheterize even distal parts of curved intracranial vascular network by experienced hand using leading wires. Besides the fact that catheters are soft and atraumatic, their wall is equipped most often by nitinol strengthening which gives them certain rigidity thus preserving lumen width of microcatheter which is of key significance for application of embolization agent. Internal wall of microcatheter is such as to support the application of contrast agent and the whole design of some types is such that it helps blood flow in vascular bed. Top of a catheter can be fixed firmly to the rest of the catheter and according to type it can also have appropriate top which can be left glued to embolization material in order to enable pulling out of the rest of catheter after intervention [38].

Embolization material

Dominant embolization agent in practice today is Onyx. Generically, it is a mixture of ethylene-vinyl alcohol polymer (EVOH) and dimethyl sulfoxide (DMSO). EVOH dilutes in DMSO in different concentration from 6%, 6.5% and 8% thus obtaining substances of different viscosity which have different roles in embolization of bAVM. Radiological opacification required for performing the procedure under control of x-rays is achieved by application of tantalum powder. Just because of that later CT images are significantly contaminated by artifacts so that noninvasive imaging diagnostic follow up of patients treated by Onyx is directed to MRI.

Prior to usage of Onyx it is required to mix it at least 20 minutes to prevent tantalum powder settling thus resulting in weak opacification of agent. Catheters through which Onyx can be applied must be compatible with dimethyl sulfoxide (DMSO).

Advantage of Onyx in relation to other embolization agents and above all in relation to cyanoacrylic glues is that the risk from catheter gluing is reduced. This allows for greater quantity of embolization agent to be applied without replacement of the catheter. Also, Onyx behaves as liquid column without formation of drops independent from the main one [5].

Complications

Complications are divided to technical complications related to the procedure itself and clinical complications.

Technical complications

The most important technical complication is gluing the top of catheter to embolization agent and impossibility of its pulling out after intervention. Such scenario occurs in 4% of cases [39] and can result in post embolization acute hemorrhage described below [5]. There are two way of extraction: catheter traction by application of continually strengthening force until catheter is unglued or sudden strong traction of catheter. These methods are clearly related to different risks. With the aim of overcoming this issue catheters which are capable of separation of proximal end have been developed which remains confined to embolization agent but the rest of catheter can be pulled out.

Moreover, rupture of microcatheter by wire lead is possible, which must be recognized since through perforation made proximally from the top of catheter, embolization agent may be applied unwantedly thus causing ischemic complications.

Also, plugging of catheter lumen by embolization agent is possible when the whole catheter must be taken out and embolization is continued by application of a new catheter [5].

Clinical complications

Major risk of embolization of bAVM is post embolization acute bleeding. This can be the complication with mild but also devastating consequences and cause of its occurrence is embolization of drainage vein, postponed venous thrombosis, breakthrough of the level of normal perfusion

pressure, rupture of intranidal aneurysm or breaking of the wall during manipulation or extraction of catheter [5].

In the study carried out on 564 patients who were treated in 1,569 procedures, acute bleeding after embolization occurred in 1% of embolizations or 3% of patients [40].

Some of angiographic characteristics can predict development of bleeding: occlusion or slow contrast flow in drainage vein, contrast stagnation in nidus, almost complete occlusion of small bAVM with persisting small nidus and occlusion of big direct fistula in nidus. With the aim of preventing bleeding, the treatment of bAVM should be divided into several interventions except for grade 1 where whole lesion can be embolized in one act. If the vein has to be preserved then application of embolization agent should be stopped for several seconds when it reaches the vein and then continue with nidus filling. Also in the first act, weak points of the lesion such as intranidal aneurysms should be attacked [5].

Another complication is ischemia of brain parenchyma caused by embolization of arteries proximally from nidus. It occurs rarely and clinical consequences are milder [5].

Results of endovascular embolization

The results of embolization of AVM by usage of embolization agent Onyx were evaluated in the study carried out from 2005 to 2007 which included 117 patients. Complete occlusion was achieved in 23.5% of cases, occlusion 75–99% of the lesion in 33.9% of cases, occlusion 50–75% of the lesion in 27. 8% of cases, and occlusion less than half of the lesion in 14.8% of cases. Total periprocedural mortality was 4.3%. Additional treatment was carried out in 82.3% of cases of incomplete embolization and mostly by usage of radio-surgical methods [41].

Conclusion

Brain arteriovenous malformation is a rare disease of endocranialvascular system with complex morphology and hemodynamics. The most difficult complication is intracranial bleeding which is a major cause of mortality and morbidity as the consequence of bAVM. Noninvasive imaging diagnostics (CT and MRI) offers excellent possibilities of presentation of complications and acceptable possibilities in diagnostics of vascular morphology of bAVM. Golden standard in diagnostics of vascular anatomy of bAVM is invasive angiography (DSA). Therapy of a lesion is multidisciplinary and is carried out by endovascular embolization, radiotherapy and surgical intervention.

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The potential implications of exercise-induced epigenetic modifications

Potencijalne implikacije epigenetskih modifikacija uzrokovanih vežbanjem

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Ključne reči:
epigenomika; mišić; vežbanje; geni, regulacija ekspresije; zdravlje.

Introduction

Genetics provides a versatile approach and highlights the mechanisms responsible for the successful sports phenotype. Despite the stability of the genome, the environment has a potential to act as a trigger for chemical changes that activate, or silence genes and so affect the phenotype [1]. These changes could be reflected in the health beneficial epigenetic modifications that may leave a significant and permanent mark on the epigenetic profile of the individual. That means the epigenome in the adaptive response of the environmental sensitivity can adjust the metabolism and homeostasis. In contrast to some other environmental influences, exercise generates positive epigenetic changes that may be a contributing factor to improving health and better quality of life. Identification of the genetic background and the genetic determinants of variability in response to exercise is always a complex matter and sometimes exceeds the limits of known candidates genes and their gene expression. However, the individual molecular pathways information in the field of sports performance is still of paramount importance and it is one of the surest indicators of the direction and the framework needs to go. Sports scientists sometimes refer to the genetic basis of physical performance as a „biological counterpart to the holy grail“, arguing that a genetic composition is responsible for a large number of individual variations in the physical performance [2]. But, it is quite clear that this molecular information acts dynamically in relation to the environment and these epigenetic shifts in response to the exercise are worthwhile because they can be used in some trials to improve health. So, the main goal is to translate the obtained changes in the desired metabolic response and to put that initial molecular signature to practical use.

Epigenetic mechanisms: interface between gene expression and environmental cues

In recent times, environmental factors are increasingly marked as important in determining the final phenotype. In this context, regular physical activity is recognized as available and convenient component that has epigenetic capacity with many positive implications on health. The unique plasticity of skeletal muscle and the specificity of its response to homeostatic perturbation enable the integration of a set of changes within the physiological stimuli in the phenotypic response. Improving the sports performance through training is achieved as a result of the transition of gene expression to generate changes in the composition and function of skeletal muscle as well as in other tissues. These epigenetic changes are not determined by the genetic code and occur in the DNA or chromatin's structure and may affect the transcription of certain genes regardless of their primary sequence. The enhanced levels of gene transcripts can, in this manner, affect the synthesis and degradation of protein components by directly altering their normal function through changing the availability of substrate, or through an indirect mechanism

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that conduct the altered expression of growth factors, receptors and to the altered activity at gene promoters resulting in the long-term functional and structural remodeling [3, 4]. The most common epigenetic changes induced by exercise are the histone modifications, like methylation and acetylation, DNA methylation and expression of different types of microRNAs (miRNAs) [5].

What type of epigenetic mechanisms will prevail in the metabolic processes of muscle cells depends on the type, intensity, duration and frequency of exercise stimulus. The most common changes occur within the mitochondrial biogenesis and bioenergetics through different metabolic pathways of muscle fibers. As a consistent feature in many studies, the acute or long-term exercise impacts DNA methylation in a gene-specific mode. It has been reported that exercise increases the expression of many messenger RNA (mRNA) and the protein levels of genes that regulate mitochondrial function, including peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (PGC-1α), mitochondrial transcription factor A (TFAM), peroxisome proliferator-activated receptor δ (PPAR-δ), pyruvate dehydrogenase kinase isoenzyme 4 (PDK4), etc. [6, 7]. Using the human isolated contracting muscle and cultured myotubes, Barres et al. [8] demonstrated that the acute exercise changes the promoter methylation of responsive genes, indicating DNA hypomethylation as an early event in the contraction-induced gene expression. However, it was shown that the acute exercise had a dose-dependent influence on DNA methylation and required a certain intensity of exercise that initiated DNA methylation of responsible genes. Interestingly, a high-intensity exercise notably reduced promoter methylation of the following factors: PGC-1a, TFAM, myocyte-specific enhancer factor 2A (MEF2A), and PDK4 immediately after exercise, whereas PPAR-δ methylation was decreased 3 hours after exercise, so that the mechanism responsible for this exercise-induced demethylation was explained either by hydroxylation of the methyl group (5-hydroxyl methyl), which is an intermediate for demethylation, or by a loss of methyl groups [9, 10].

Another epigenetic event that regulates gene expression is the histone post-translational modifications (PTMs). The histone modifications include a number of various posttranslational modifications to the lysine rich tail regions of histones, in particular H3 and H4 [11, 12]. The modifications like phosphorylation, ubiquitination, methylation and acetylation, and their effects on transcription are different. It is known that subfamily of histone deacetylases (HDACs) has an essential role in skeletal muscle physiology and regulates genes that comprise PGC-α, carnitinepalmitoyl transferase 1 (CPT-1), medium chain acyl-CoA dehydrogenase (MCAD), hexokinase II (HKII), glycogen phosphorylase, and ATP synthase β [13].

It is still not entirely clear about ubiquitination as a potential modification that may be part of the exercise adaptation. Potthoff et al. [14] studied this issue using an animal model and found that ubiquitin-mediated proteosomal degradation of HDACs in the adaptive response to exercise, pointing out that this proteosomal degradation could take part in the adaptive response to the repeated exercise bouts.

MiRNAs are a group of short (20–24 nucleotide) endogenous posttranscriptional regulators that are capable of blocking the translation of protein-coding genes [15, 16]. They become more relevant in the regulation of cell- and tissue-specific gene expression including a role as potential biomarkers for the physiological and pathological conditions. Packed in the exosome vesicles, miRNAs are released to the circulation by nearly all cell types, including the skeletal muscles. The relevant literature data show that the most-studied miRNAs are miR-133a/b, miR-206, and miR-1, which are induced during differentiation of myoblasts into myotubes and are collectively referred to as the "myomirs" [17]. More recent studies of Nielsen et al. [18] determined that the endurance exercise and resistance training induce changes in the ci-miRNA human plasma signature. The studies showed that these changes were dynamic during the short period in the acute exercises and during the long periods of strenuous exercise. Another study of Davidsen et al. [19] reports that resistance exercise training leading to hypertrophy of human skeletal muscle is associated with selected changes in miRNA abundance. Their results indicate that miRNAs can play a major role in the phenotypic changes and noticeable intergroup diversity in a response to resistance training.

In addition, there are posttranscriptional changes in the metabolism of carbohydrates and fatty acids that occur immediately after a single bout of exercise as mitochondrial biogenesis which subsequently increase the requirements of oxygen utilization resulting in a drop in intracellular oxygen. Under these conditions of hypoxia, hypoxia-inducible factor 1 (HIF-1) a member of the HIF family of transcriptional activators which are essential for maintaining O2 homeostasis, switchs on the transcription of genes encoding glucose transporters and glycolytic enzymes, acting together with PGC-1α and initiate the mechanism of gene expression that facilitates increased oxygen supply. This complex triggers the transcription of numerous hypoxia-responsive genes of metabolic processes that would be favorable in conditions of reduced oxygen [20]. HIF1α regulates the gene expression through hypoxia response elements (HRE) present in the promoter regions of target genes. This binding can be affected through the DNA methylation and histone modification, which may maintain a favorable chromatin conformation around the HRE sites. In the presence of oxygen, HIF1α is regulated through hydroxylation, ubiquitination, and degradation by prolyl hydroxylase enzymes (PHD) [21]. In the absence of oxygen, this is inhibited which allows for the HIF1α stabilization and activation. For these reasons, HIF can be considered not only an important oxygen sensor, but also an essential regulator of adaptation induced by exercise [22].

Epigenetic stability

Discussing all these exercise-induced epigenetic modifications, the logical question is how much these changes are stable, and what the factors that determine framework of epigenetic stability are. Many have attempted to investigate the stability and inter-individual variation in DNA methylation comparing changes in DNA methylation profiles during a

short-time to longer periods and concluded that some methylation marks showed considerable variation over time, while others are highly stable [23]. In general, these processes are partly reversible, so that, for example, the histone modifications are in a continual state of change, whereas DNA methylation is considered more stable and long-term. However, the variations of methylation levels have a diverse range and are greatly affected by the gene structures and its genomic location. The epigenetic stability is defined as the persistence of modifications in the gene expression and/or epigenetic marks that influence the gene expression and such stability can exist at different temporal scales [24, 25]. It remains unclear whether the adaptive value of stable and unstable, or transient epigenetic changes may cause the long-term changes in phenotype. On the other hand, it is clear that the nature of the environmental impact that generate the epigenetic change is the most critical factor for the epigenetic stability. In support, recent advances in molecular biology has reported that epigenetic alterations induced by the environmental stressors, can create a persistent memory of the received signal called epigenetic memory. Interestingly, it is proposed that each of that stressors can promote specific alterations to the normal form of DNA methylation- epigenetic footprint, and further cause changes to the gene expression [25]. Sharples et al. [26], in attempt to explain the molecular and epigenetic mechanisms of skeletal muscle memory in humans, introduced the term „epi-memory", studying the human skeletal muscle cells isolated from the different population by generation. They showed that muscle cells had a morphological memory and can retain molecular information of the acute early lifespan in different signaling proteins and that cells possess the ability of retaining elevated methylation for at least thirty cellular divisions. They further compared this type of muscle memory with the motor learning in which learning the motor skills incorporates specific templates of movement through repetition. This implies that their understanding, confirmation and refinement of epigenetic modifications can help in future with targeted therapies, for example, in repairing muscle growth and reducing the loss of muscle mass in the aging process.

The role of epigenetic changes in response to exercise and metabolic disorders

Although the research on molecular genetics of physical exercise and health-related outcomes is still in its infancy, we need to look at the bigger picture, to link all the known and valuable facts as well as to reinforce them in healthcare practice. Exercise is one of those external factors that can modify the expression of genes and that a cascade of epigenetic changes in different tissues can preserve and improve health. So, these epigenetic mechanisms can be used for the purpose of targeted benefits of exercise and can be incorporated in the exercise prescription.

There is no doubt that the physical activity and exercise play a pivotal role in the prevention and treatment of many metabolic disorders. Large part of individual differences in the weight loss response is attributable to genetic and epigenetic factors. Recent studies about the regulation of the epigenome in human adipose tissue show a general increase in the adipose tissue of DNA methylation in response to six months of moderate exercise consisting of spinning and aerobics. Two genes, HDAC4, a histone deacetylase and NCOR2, a nuclear co-repressor, displayed the increased levels of DNA methylation and synchronous decrease in the mRNA expression in the adipose tissue in response to the exercise intervention as well as to increased lipogenesis [27]. Also, this study establish the connection between the differential DNA methylation and mRNA expression in response to exercise, thereby they confirmed the relationship between methylation and altered metabolism through the gene expression. These results may be of clinical significance and the HDAC inhibitors perhaps can be applied in the treatment of obesity and T2D [28]. Similarly, Wang et al. [29] examined DNA methylation of peripheral blood leukocytes between obese adolescent and lean controls and identified two CpG sites in the UBASH3A gene and TRIM3 gene with roles in the immune function that were differentially methylated and that methylation changes may be associated with the pathogenesis of obesity.

Existing data strongly indicate that there is a link between the obesity, energy metabolism and epigenetic modifications and support the fact that the exercises induce the expression of a number of genes that regulate glucose uptake in the skeletal muscle, including GLUT isoform 4 (GLUT4), whose increased expression is further regulated by the transcription factor MEF2 (myocyte enhancer factor 2) and with coactivator protein PPARGC1A [30]. In addition, an increase in the PGC1 expression generated by exercise is an important element for improving the insulin sensitivity in the skeletal muscle not only by increasing the glucose transporter expression (GLUT4) but also by increasing the mitochondria density and it is considered that exercises attenuate the epigenetic modifications at PGC1 and can lead to inhibition, or delay of type 2 diabetes onset [31].

Attempting to identify the epigenetic patterns which may predispose to type 2 diabetes (T2D), Nitert et al. [32] demonstrated that exercises lasting for 6 months and consisting of endurance exercise of moderate intensity, in the people with type 2 diabetes (T2D), were associated with the epigenetic changes, citing the example of decreased DNA methylation of two key transcription factors involved in the glucose uptake in the muscle and respiratory metabolism (RUNX1 and MEF2A). They further reported on differential DNA methylation of mitogen-activated protein kinase (MAPK), insulin and calcium signaling genes concluding as possible that the exercise-induced epigenetic modifications reduce the future risk of T2D among the men with the positive family history (FH+).

Other impacts of exercise-induced epigenetic modifications

The impact of exercise-induced epigenetic modifications appears to have multiple influences within all cells in organism. Accordingly, one of the exercise intensity benefits for the positive epigenetic changes in terms of mitochondrial

biogenesis was shown by Edgett et al. [33], who concluded that the intensity-dependent increases in PGC-1α mRNA following submaximal exercise are mainly due to the increases in muscle induction. Furthermore, the blunted response of PGC-1α mRNA expression following the supramaximal exercise may imply that signaling mediated activation of PGC-1a may also be blunted. According to the extensive interventional studies of Voisin et al. [34], the genes whose methylation levels change significantly after exercise in humans includ the genes involved in particular cellular metabolic states (including PGC-1a, GLUD1, PDK-4, PPAR-d, TFAM, ADIPOR1, ADIPOR2 and BDKRB2), muscle growth (MEF2A), hematopoiesis is (RUNX1) and inflammation (ASC).

Various studies have implied that epigenetic mechanisms also play a role in the definition of the onset of age-associated diseases and lifespan potential. Lopez-Otin et al. [35] postulated some hallmarks of aging like genomic instability, telomere attrition, epigenetic alterations, etc., and suggested that exercise can influence, at least partly, most of these hallmarks. The relationship between the epigenetics regulation and aging is complex and controversial, depending on the process hypo- or hypermethylation, on the type of cells, enzymes, but it seems that exercise can promote the protective effects and help to attenuate that age-deregulations [36, 37]. Genomic imprinting is a unique epigenetic phenomenon that summarizes connection of inheritance with the environment and signifies the "genotype-independent parent-of-origin" gene expression. The effect of parental origin refers to the genomic imprint, and methylation is considered the main mechanism by which the expression is modified. Such an expression of different alleles (mother or father) may take place in all cells and tissues, and it is believed that about 1% of the human genome is imprinted. These genes are of major importance in the medical context, regardless of their low percentage. In order to determine the impact of imprinted genes in human skeletal muscle, Brown [38] identified these genes and changes in DNA methylation associated with exercise. An important conclusion of this recent bioinformatics meta-analysis is that the modification of DNA methylation induced by exercise can slow down the aging process, but also to mitigate the occurrence of certain health disorders.

It is a well-established fact that the exercises, due to the increased metabolic demand, are associated with the increased formation of reactive oxygen species (ROS), but regular exercise reduces the prevalence of a wide range of ROS-associated diseases. Furthermore, the effects of exercise attend to be beneficial for the brain function and include the processes of neurogenesis via neurotrophic factors, increased capillarization, decreased oxidative damage and increased proteolytic degradation. It is known that the oxidative modification of DNA could lead to the increased apoptosis and that impaired function could be the major factors related to the brain aging and neurodegenerative diseases [39]. Moreover, the exercise-induced changes increase the resistance against oxidative stress, facilitates recovery from oxidative stress, and attenuates age-associated decline in cognition. In addition, some recent studies suggest a notable role of exercise on brain plasticity and cognitive health through the epigenetic modifications mostly by the action of brain-derived neutrophic factor (BDNF) highly expressed in hippocampus [40].

There is no strong evidence to provide a direct connection between the epigenetic modulation and changes in cardiovascular system induced by exercise, but recent data show that moderate exercise mitigate the age-dependent decrease in apoptosis associated protein (ASC) methylation, indicating suppression of redundancy pro-inflammatory cytokines through just reduction of ASC expression [41]. These epigenetic modifications just ensure proper function at the cellular level, due to the balance between the inflammatory response and anti-inflammatory genes, so any disruption of these epigenetic mechanisms could lead to the development of atherosclerosis and stenosis [42]. Keeping in mind the fact that physical activity can prevent many pathological epigenetic events, for example, through the increased expression of endothelial growth factor like (VEGF), as well as through the reduction of the many risk factors such as oxidative stress which are held responsible for cardiovascular disorders, many authors point out the role of exercise as a strong regulator of positive epigenetic modification [43–45]. Many of these key regulators of epigenetic mechanisms are associated with the modifications of DNA and histones in endothelial cells, suggesting a direct protective role of physical exercise on endothelial function. It is believed that the role of free radicals in the modulation of extracellular matrix which is regulated by epigenetic mechanisms is very important and that they participate in the development of many pathophysiological processes. In this regard, the exercises improve the antioxidant capacity and maintains cellular oxidative balance, molecular structure and architecture of the extracellular matrix through the mediating signaling cascades. Precisely in this way, the epigenetic modulation induced by exercises is a significant factor in the modification of the functional genome and heart and vascular beds [46]. Baccarelli et al. [47] in an experimental work with animals and humans argued that DNA methylation appears as a primary regulator of inflammation and atherosclerotic changes in peripheral blood leukocytes, and it is connected to several cardiovascular-related biomarkers that include homoczsteine and C-reactive rpotein. In addition, referring to the epigenetics and the cardiovascular relation, miRNAs contribute to the process of myocardium remodeling through the different signaling pathways in condition of hypertrophy and neo-angiogenesis – "the athlete's heart" , and thus protect the heart from fibrosis and pathological hypertrophy [48]. However, although a lot of factors are known and confirmed, further detailed investigations are required to explore other positive effects of epigenetic modulation induced by exercising and to incorporate them into the improved prevention, risk assessment, risk stratification and treatment of cardiovascular disorders.

Finally, the most recent tightly controlled and extensive human study showed that 3 months of endurance training in the healthy human volunteers caused the substantial DNA methylation changes at about 5,000 sites across the genome and powerful gene expression [49]. This study indicates that the

numerous changes in methylation were not a random and coincidental effect but more a well-controlled adaptive process generated as a response to endurance exercises. Thus, the increased methylation seemed to be related to remodeling of the tissue and metabolism, while decreased methylation was related to inflammation, and this can explain the benefits of exercise. DNA methylation was predominantly changed in the enhancer regions (short regions of DNA which activate gene transcription from a distance) with structural improvement for binding of myogenic regulatory factors (MRFs), myocyte enhancer factors (MEFs) and ETS proteins, so it can be assumed that the training-induced integrated epigenetic adjustment contributes to the heterogeneity in individual responses.

All of these data in the literature point out the existence of particular regions in the genome that are sensitive to the epigenetic modifications in response to exercise and there are differences depending on the type, duration and intensity of exercise. Future studies should investigate the stability of those exercise-induced DNA methylation changes and the possible effects of epigenetic alterations in different periods of training, as well as the exercise program that includes different types of speed and effort.

Conclusions and future perspectives

By understanding the epigenetic changes which are important for responses of various phenotypes, it is logical to expect that these valuable facts as part of important biological adaptation can be used to improve the health of individuals. Epigenetics provide a scientific basis for how the training intervention and other external factors can reshape the individual and provide the insight into the way the changes in gene expression through a complex network of coordinated pathways may affect the phenotype. Key epigenetic elements are responsible for regulating adiposity, numerous molecular pathways related to the inflammatory processes, energy expenditure, and glucose homoeostasis, so that the molecular events within their physiological processes are very powerful tool. It is conceivable that these observations and health benefits about the epigenetic modification within the different cells and tissues in response to exercise as readily available and efficient form of behavior intervention, could be combined for the valuable clinical information and used in practice for health improvement in the future.

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CASE REPORTS

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A fatal case of fulminant myocarditis caused by influenza A virus

Fatalni ishod fulminantnog miokarditisa izazvanog virusom influence A

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Abstract

Introduction. Myocarditis is defined as an inflammation of a heart muscle, which can be caused by a number of agents, among which viruses are the most common. Fulminant myocarditis is a rapidly progressive, life-threatening myocarditis, followed by the development of cardiogenic shock. Among viruses, there are a number of common ones, but according to our knowledge, there are only a few cases of fulminant myocarditis caused by influenza A virus described in the literature. **Case report.** We presented a 44-year-old man who was admitted to the Cardiology Intensive Care Unit because of the clinical as well as electrocardiographic signs pointing to the ST-segment elevated myocardial infarction with peracute development of heart failure and cardiogenic shock and subsequently lethal outcome, despite applied circulatory support. The urgent coronarography showed no signs of coronary artery disease, while the autopsy revealed myocarditis and the real-time polymerase chain reaction of nasopharyngeal swab revealed influenza A(H3) virus. **Conclusion.** Fulminant myocarditis is a life-threatening cardiac disease which should be treated in Intensive Care Units with both medicament and mechanical circulatory support and antiviral therapy and which, despite the applied therapy, has a high mortality rate. Influenza A virus is a rare cause of fulminant myocarditis which should be taken into consideration.

Key words:
diagnosis; influenza a virus; influenza, human; myocarditis; shock, cardiogenic; treatment outcome.

Apstrakt

Uvod. Miokarditis je zapaljenje srčanog mišića izazvano velikim brojem različitih agenasa, među kojima su najčešći virusi. Fulminantni miokarditis je rapidno progresivni, životnougrožavajući miokarditis, koji je praćen razvojem kardiogenog šoka. Različiti virusi su česti uzročnici međutim, u litaruri je, prema našem znanju, opisano samo nekoliko slučajeva fulminantnog miokarditisa izazvanog influenza A virusom. **Prikaz bolesnika.** Prikazali smo bolesnika starog 44 godine, primljenog u Jedinicu intenzivne kardiološke nege zbog kliničkih simptoma i elektrokardiografskih znakova koji su pobudili sumnju na akutni infarkt miokarda sa ST elevacijom. Zbog perakutnog razvoja akutne srčane slabosti do nivoa kardiogenog šoka i pored primenjene cirkulatorne potpore došlo je do fatalnog ishoda. Kod bolesnika je urgentnom koronarografijom isključeno postojanje koronarne bolesti, autopsijom dokazano prisustvo miokarditisa, a „*real-time*" metodom lančane reakcije polimeraze prisustvo virusa influenca A (H3) u nazofaringealnom brisu. **Zaključak.** Fulminantni miokarditis je teško kardiološko oboljenje koje se leči u jedinicama intenzivne nege medikamentnom i mehaničkom cirkulatornom potporom, uz hitno započinjanje antivirusne terapije koje, i pored primenjene terapije ima visoku stopu mortaliteta. Virus influenca A je redak uzročnik fulminantnog miokarditisa koji treba razmotriti pri postavljanju dijagnoze.

Ključne reči:
dijagnoza; grip a virus; grip; miokarditis; šok, kardiogeni; lečenje, ishod.

Introduction

Myocarditis is defined as an inflammation of a heart muscle, which can be caused by various of external antigens such as viruses, bacteria, parasites, toxins and medications as well as by internal triggers, such as autoimmune activation vs. own antigens [1, 2].

Viruses are the most common cause of myocarditis. Among viruses, the most common are: enteroviruses including coxsackie virus, adenovirus, parvovirus, hepatitis C virus, human immunodeficiency virus, while others, such as influenza A virus are rare causes of myocarditis, especially in a fulminant form. To our knowledge, there are only few

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cases of fulminant myocarditis caused by influenza A virus described in the literature [3–6].

Case report

A 44-year-old male patient, previously healthy driver instructor, was admitted to the Cardiology Intensive Care Unit because of short loss of consciousness, nausea and vomiting one hour prior to admission. A history of flu-like symptoms four days prior to admission and profuse sweating night before admission, without measured fever was provided. No history of typical angina-like chest pain was reported.

At admission, the patient was conscious, adequately communicative, oriented, normotensive (120/80 mmHg), tachycardic (heart rate of 120 beats per minute), afebrile (35.9ºC), dyspnoic and tachypnoic (respiratory rate of 30 per minute), with signs of a heart failure, Killip class II/III.

An arterial blood gas analyses obtained at admission showed pH 7.38 [normal range (nr) 7.35–7.45], pCO_2 42 mmHg (nr 35–45 mmHg), pO_2 65 mmHg (nr 90–100 mmHg), SaO2 92%, (nr > 95%), lactate 1.2 mmol/L (nr < 1.0 mmol/L), BE- 0.2 mmol/L (nr -2 to +2 mmol/L).

Electrocardiogram (ECG) at admission showed sinus tachycardia (120 beats per minute) with ST elevation of +2 mm in DII, DIII, aVF, V2-V6 leads (Figure 1). Bedside echocardiography was performed in the Intensive Care Unit, and showed normal dimensions of left ventricle (end-diastolic dimension of 4.5 cm, end-systolic dimension of 3.6 cm), with the presence of diffuse left ventricular hypokinesia, reduced ejection fraction (EF) of 45% (normal EF 50–70%) and a first grade diastolic dysfunction. Right ventricle dimension was normal, without direct or indirect signs of pulmonary hypertension.

Based on the echocardiography, one of the proposed differential diagnoses, acute pulmonary thromboembolism, was excluded. The possible other three differential diagnoses were the acute myocardial infarction, early phase of acute lung injury – pneumonia and myocarditis as well.

Immediately after admission, in order to exclude the acute myocardial infarction, transradial coronary angiography was performed, with only 30 cc of contrast media and with no complications and hemodynamic compromise during the procedure. It showed normal finding (Figure 2).

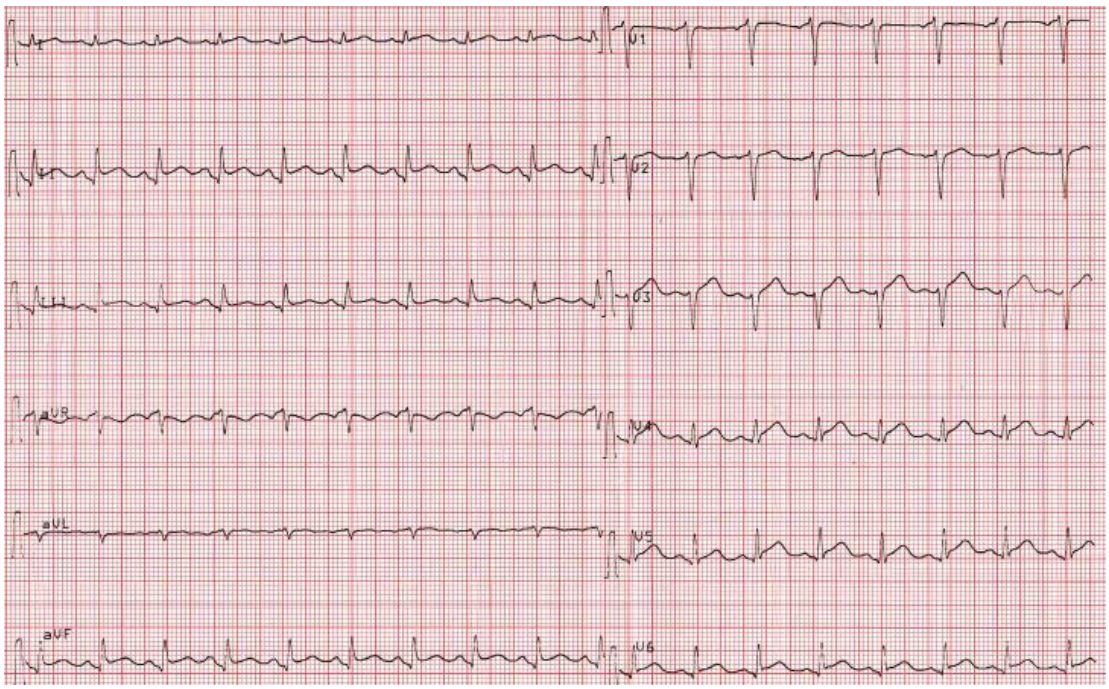
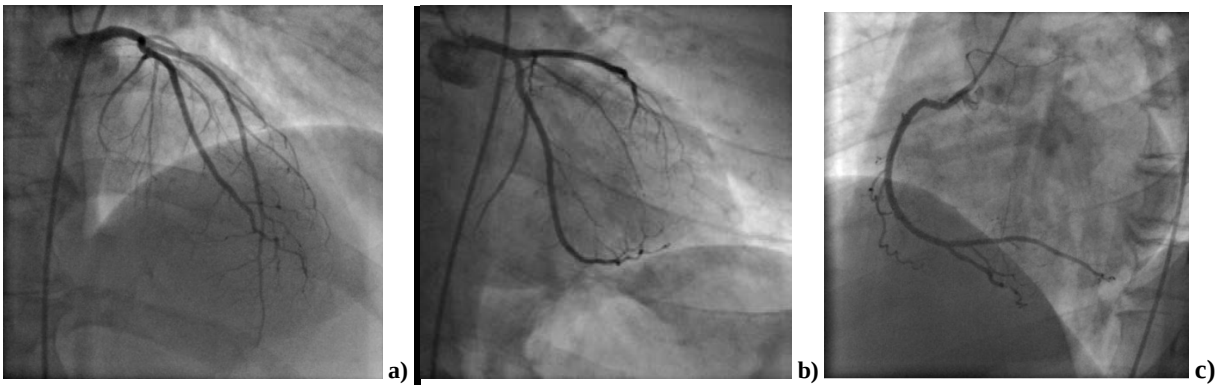


Fig. 1 – Normal coronary angiogram electrocardiogram at admission showed sinus tachicardia (120 beat/min) and ST elevation of +2 mm in diaphragmatic and anteroseptal (V2–V6) leads.



a) b) c)

Fig. 2 – a) and b) left anterior and circumflex coronary arteries; c) right coronary artery.

During the first two hours after admission to the Cardiology Intensive Care Unit, the patient was conscious, breathing spontaneously using oxygen supplementation, normotensive (systolic pressure 110–130 mmHg), tachycardic (100–120 beats per minute), and afebrile. Due to the symptoms and signs of acute heart failure, the furosemid and nitroglycerin intravenous infusion (5 μg/min) was administered with continuous invasive arterial monitoring via the left radial artery.

The laboratory tests showed the elevated levels of Troponin I, N-terminal pro-brain natriuretic peptide (NT-pro NDP) and C-reactive protein (Table 1). There were no other specific abnormalities in laboratory findings (Tables 1 and 2). The chest X-ray showed the emphasized right pulmonary hilum and a stricter bronchovascular drawing (Figure 3).

Table 1

Laboratory findings at addmission

Parameter	Value	Reference range
Troponin I (μg/L)	0.16	< 0.01
Creatine kinase – CK (U/L)	97	0–200
CK myocardial band (U/L)	25	0–25
C-reactive protein (mg/L)	7.7	0–5
Fibrinogen (g/L)	3.3	2.2–4.9
D-dimer (mg/mL)	< 500	< 500
NT-proBNP (pg/mL)	1552	125
Urea (mmol/L)	4.2	2.5–7.5
Creatinine (μmol/L)	95	65–120
Lactat dehydrogenase (U/L)	336	230–460
Aspartate aminotransferase (U/L)	38	0–40
Alanine aminotransferase (U/L)	33	0–40

NT-proBNP – N-terminal pro-brain natriuretic peptide.

Table 2

Complete blood count

Parameter	Value	Reference range
Leukocytes ($\times 10^9$/L)	6.60	4.0–10.0
Erythrocytes ($\times 10^{12}$/L)	5.23	3.9–6.0
Hemoglobin (g/L)	153	120–170
Hematocrit (g/L)	0.416	0.350–0.540
Thrombocytes ($\times 10^9$/L)	235	150–400
Lymphocytes ($\times 10^9$/L)	1.1	1.18–3.74
Monocytes ($\times 10^9$/L)	0.2	0.24–0.86
Neutrophils, ($\times 10^9$/L)	5.24	1.78–6.13
Eosinophils, ($\times 10^9$/L)	0.06	0.04–0–36

During that time, echocardiography in the Cardiology Intensive Care Unit as well as coronary angiography were performed.

During the third hour of hospitalization, there was a rapid progression of antegrade heart failure with the rapid evolution to cardiogenic shock. The patient became hypotensive (70/40 mmHg) and extremely dyspnoic, with low urine output (less then 0.5 mL/kg/hour). The fluid challenge of 250 mL of 0.9% sodium chloride was performed without adequate response, so inotropic and vasopressor stimulation was applied. Due to the rapid respiratory failure, the endotracheal intubation was performed immediately afterwards, and the patient was artificially ventilated. Bicarbonates were applied according to the blood gas analyses.

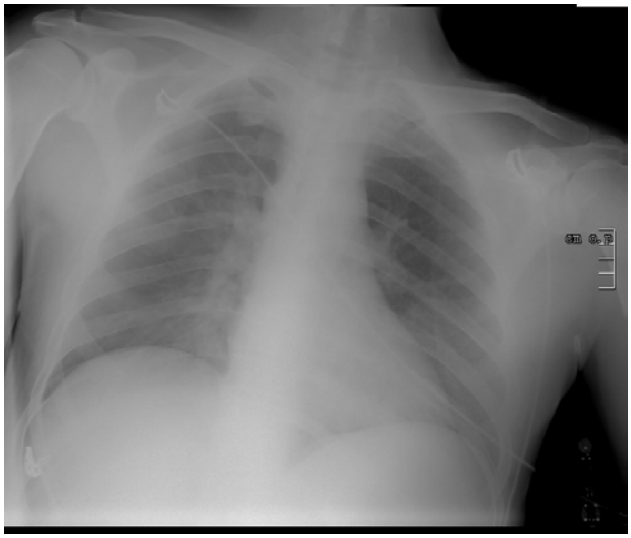


Fig. 3 – Chest radiogram showed emphasized right pulmonary hilum and a stricter bronhovascular drawing.

Despite the artificial ventilation and high inotropic (dobutamine of 10 mg/kg/min) and vasopressor (norepinephrine of 1.8 μg/kg/min) support, there was no improvement of hemodynamic status of the patient. Furthermore, the patient was markedly hypotensive with the mean arterial pressure not exceeding 50 mmHg. The deterioration of arterial blood gas analysis was noticed as well, with the high lactate levels (lactate of 9.1 mmol/L).

To evaluate the cause of rapid and refractory cardiogenic shock, bedside was echocardiography repeated and noticed the decrease in the global contractility (EF estimated of 20%), and no mechanical complications were found. At the same time, the lung ultrasound revealed pulmonary edema. A diagnosis of acute respiratory distress syndrome (ARDS) with PaO2/FiO2 ratio = 60, was proposed, too.

According to the clinical presentation, laboratory and echocardiography findings there was a high suspicion to myocarditis and viral pneumonia.

After the performed examinations, a possible correlation of the patient's condition with influenza epidemic was suggested. Due to the epidemic of H1N1 viral infection in previous months with observed poor outcomes, empiric oseltamivir phosphate (Tamiflu®) was administered. An implantation of intra-aortic balloon pump, as the only mechanical circulatory support at that time, was considered, but at the beginning of the fourth hour of hospitalization, a cardiac arrest with pulseless electrical activity occurred. Cardiopulmonary resuscitation was performed with no results and the patient died at the end of the fourth hour from the presentation to the Cardiology Intensive Care Unit.

The real-time polymerase chain reaction (PCR) of the nasopharyngeal swab was influenza A (H1N1) negative, but influenza A (H3) positive (Table 3).

Table 3

Real-time polymerase chain reaction of the nasopharyngeal swab

Virus	Result
Influenza A (H1N1)	Negative
Influenza A (H1)	Negative
Influenza A (H3)	Positive
Influenza A (H5)	Negative
Influenza A (H7)	Negative
Influenza B	Negative

On autopsy, the macroscopic histopathological analysis showed the globally slightly dilated heart, weighted 410 g. The coronary arteries were slightly atherosclerotic without significant stenosis. Myocardium was pale, grayish, softened and thin, with focal small dotty hemorrhages. There were no signs of previous or new ischemic lesions.

The microscopic histopathological analysis revealed the myocardium infiltrated with a dense inflammatory lymphocyte infiltrate, rare neutrophile and rare eosinophile granulocytes and histiocytes with the focal areas of necrotic cardiomyocytes and extensive interstitial edema (Figures 4–6).

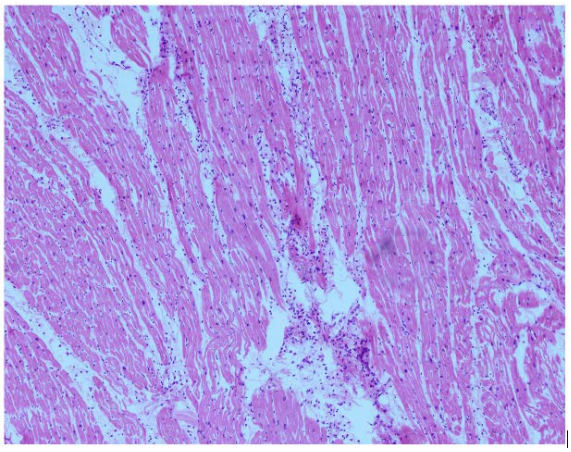


Fig. 4 – Dense lymphocyte infiltrate and interstitial edema [haematoxylin/eosin (HE), ×10].

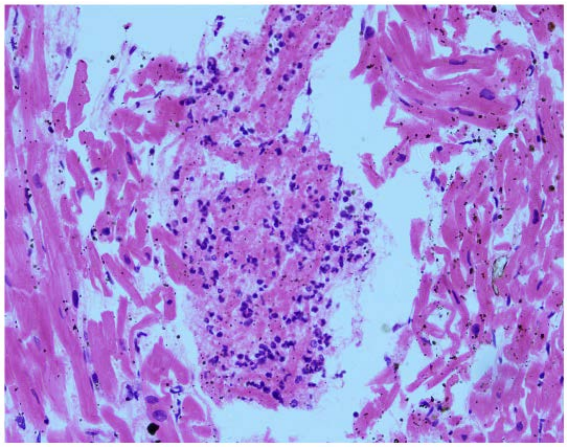


Fig. 5 – Area of necrotic cardiomyocytes with lymphocyte infiltrate and rare neutrophil and eosinophil granulocytes (HE, ×40).

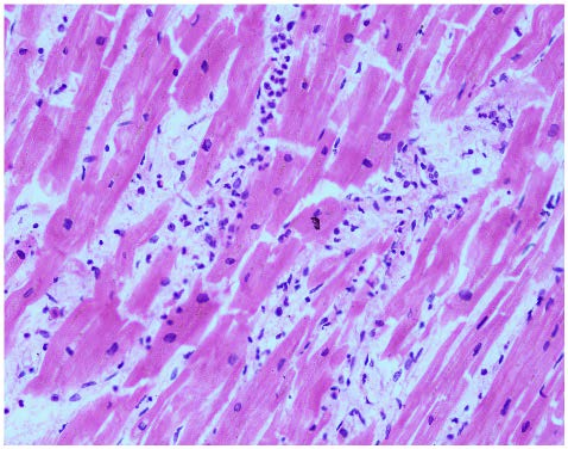


Fig. 6 – Broken cardiomyocyte fibres with interstitial edema and focal dense inflammatory infiltrate (HE, ×40).

The pulmonary histopathological findings revealed the thickened alveolar walls, interstitial edema and alveolar lumen filled with transudate – the picture of cardiogenic pulmonary edema (Figures 7–8).

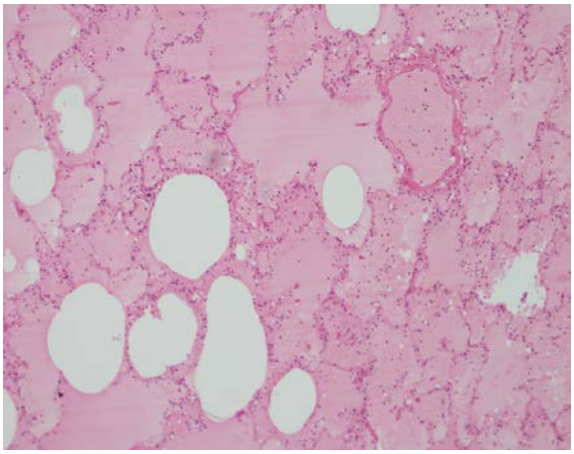


Fig. 7 – Thickened alveolar walls with interstitial edema and transudate in alveolar lumen (HE, ×10).

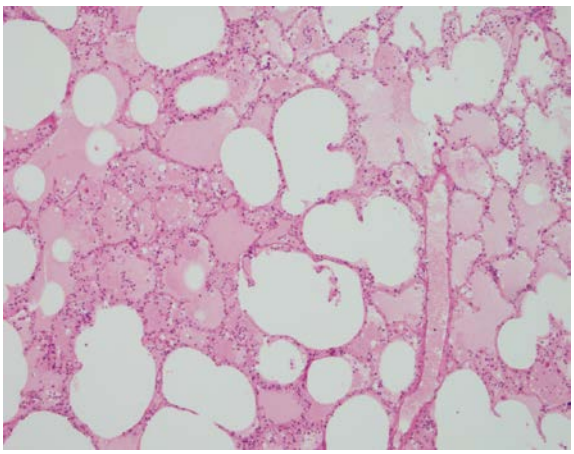


Fig. 8 – Thickened alveolar walls with interstitial edema and transudate in alveolar lumen (HE, ×10).

Discussion

Fulminant myocarditis is a rapidly progressive, life-threatening myocarditis, followed by the development of cardiogenic shock [7, 8].

Precise incidence of myocarditis is difficult to evaluate and according to some data it is about 8 to 10 per 100,000 population. A pathologic series of Fabre and Sheppard [8] showed that the inflammatory disorders, including lymphocytic myocarditis and cardiac sarcoidosis, accounted for 8.6% of all sudden adult cardiac deaths.

Viruses are the most common cause of myocarditis. Among viruses, the most common are enteroviruses including the coxsackie virus, adenovirus, parvovirus, hepatitis C virus, human immunodeficiency virus, while other viruses, such as influenza A virus, rarely cause myocarditis. Recently, by using the modern molecular methods, a simultaneous presence of more viral antigens is discovered. These viruses possibly strengthen the virulence of one another, but a genetic defect in a human immune system which leads to incapability to destroy multiple viral forms is possible, as well [9, 10].

It is shown that during influenza epidemics, 5% to 10% of infected patients may have cardiac symptoms [2]. The patients with fulminant myocarditis often have flu-like symptoms for 2–4 weeks prior the cardiac presentation. The physical examination at the time of presentation to the Intensive Care Unit shows the New York Heart Association (NYHA) class III or IV heart failure symptoms, hypotension, tachycardia, livid cold extremities, hemodynamic instability and respiratory failure [2].

In our case report, flu like symptoms (cough and sweating) were present four days prior to admission. However, body temperature was normal. To make a diagnosis even more difficult to establish, a short loss of consciousness was the main reason for the doctor's attention. In the physical finding, the heart failure symptoms and signs of heart failure Killip class II/III were present.

At the time of admission, the proposed differential diagnosis were the acute pulmonary thromboembolism, acute myocardial infarction with the heart failure, acute lung injury and fulminant myocarditis.

The initial laboratory tests usually show elevated inflammatory markers (erytrocyte sedimentation rate and C-reactive protein), however they do not confirm the diagnosis and are often increased in other circumstances such as acute pericarditis [11].

Despite that cardiac troponins are more sensitive of myocyte injury than creatine kinase enzyme, they are not specific for myocarditis, and when normal, do not exclude myocarditis. Brain natriuretic peptides and circulating cytokines are nonspecific for myocarditis, as well [12, 13].

The current European Society of Cardiology (ESC) guidelines strongly recommend the assessment of troponins, erythrocyte sedimentation rate, C-reactive protein levels in all patients while a routine viral serology testing is not recommended [14].

Except slightly elevation of troponin I (0.16 mg/L), NT-pro BNP (1,550 pg/mL) and C-reactive protein (7.7 mg/L), there were no other specific abnormalities in our patient laboratory at admission. This is also a confirmation of severe and rapidly progressive form of fulminant myocarditis.

The electrocardiographic abnormalities range from the T wave inversion to the ST segment elevation, as well as disorders in the intraventricular conduction (wide QRS complex), and both supraventricular and ventricular arrhythmias. However, the ECG signs are neither specific nor sensitive for myocarditis [15]. In combination with the elevated cardiac enzymes and echocardiography finding, the suspicion of acute myocardial infarction as differential diagnosis could be established as well.

Echocardiography is the first choice imaging technique in the evaluation of cardiac function. According to the study of Felker et al. [16], among the patients with fulminant myocarditis, echocardiogram usually shows low ejection fraction, normal end-diastolic diameter of left ventricle and increased left ventricle wall thickness due to inflammatory response resulting in interstitial oedema. In our report, bedside echocardiography, a slightly decreased global contractility (EF of 45%) was showed at first, along with a rapid progression of myocardial function and three hours after admission, EF was estimated to 20%.

Coronary angiography is recommended for all adult patients in order to exclude thrombosis of epicardial coronary artery and it is extremely important among the patients with the known risk factors for coronary disease.

Cardiac magnetic resonance imaging (MRI) is considered to be the most sensitive noninvasive imaging method for the diagnostics of myocarditis, showing a subepicardial late enhancement pattern, thereby visualizing myocarditis-related necrosis [17]. The cardiac MRI findings consistent with myocarditis should be based on the Lake-Louise criteria [18].

However, most of the patients with fulminant myocarditis are hemodynamically unstable and therefore MRI is not possible. In our report, the clinical deterioration was so rapid, that performing MRI could not be done.

Furthermore, cardiac MRI cannot replace the endomyocardial biopsy (EMB).

The EMB and histopathological analysis has a critical role in the evaluation of the patients with an unexplainable acute heart failure and it has the American College of Cardiology/the American Heart Association (ACC/AHA) class I indication in the evaluation of the hemodynamically unstable patients with the cardiac insufficiency lasting less than two weeks, with the optimal sensitivity as soon as the symptoms started [2].

Intensive myocardial inflammation with cardiomyocite necrosis is pathological, but it is a nonspecific sign of fulminant myocarditis. Histological findings can be classified according to the Dallas criteria as the active myocarditis, borderline myocarditis and negative. Despite being a gold standard for an unequivocal diagnosis of myocarditis, there

are a number of reasons for the insensitivity of Dallas criteria [17, 19].

The myocardial samples, each 1–2 mm in size, taken from the EMB should be analysed using histology, immunohistochemistry and viral PCR. A high quality EMB confirms the diagnosis of myocarditis, identifies the underlying etiology and the type of inflammation (e.g., giant cell, eosinophilic myocarditis, sarcoidosis), and therefore can guide different treatments and predict prognosis [14].

According to the current position statement of the ESC, the diagnostic criteria for clinically suspected myocarditis include: ≥ 1 clinical presentations (acute chest pain, new onset (days up to three months), or worsening of dyspnea; subacute/chronic (> 3 months) worsening of dyspnea; palpitation, syncope, aborted sudden cardiac death; unexplained cardiogenic shock) and ≥ 1 diagnostic criteria from different categories (ECG/Holter/stress test features, myocardiocytolysis markers, functional and structural abnormalities on cardiac imaging [echo/angio/cardiovascular magnetic resonance (CMR)], tissue characterization by CMR) in the absence of angiographically identified coronary stenosis ≥ 50%, preexisting cardiovascular disease or extracardiac causes that could explain the syndrome [14].

There is no specific therapy of fulminant myocarditis. Since the patients are usually hemodynamically unstable, supportive therapy is used as the first choice of therapy. In our report, endotracheal intubation, inotropic and vasopressor support were the first line of therapy. However, the result of supportive measures was very poor. There was also a lack of time to implant the intra-aortic ballon pump (IABP) as the second line therapy, mainly due to a highly progressive cardiogenic shock with the subsequent cardiac arrest.

Despite the intensive treatment, the mortality associated with fulminant myocarditis is high, from 39% for the patients with fulminant myocarditis associated with the H1N1 reported by Ukimura et al. [20] to the mortality rate of 83% reported by Saji et al. [21] in the patients with fulminant myocarditis who failed to respond to an initial supportive medical treatment without a mechanical circulatory support.

If the patient does not respond to the aggressive supportive therapy during the first few hours or days, an implantation of extracorporeal membrane oxygenation, because of its simplicity, should be considered to be the first choice, or the ventricular assist devices as an alternative. Many cases of spontaneous recovery without the need for the heart transplantation after the use of extracorporal membrane oxygenation are reported [22].

Conclusion

Fulminant myocarditis is a life-threatening cardiac disease which should be treated in intensive care units with both medicament and mechanical circulatory support and antiviral therapy and which, despite a therapy applied, has a high mortality rate. Influenza A virus is a rare cause of fulminant myocarditis, which should be taken into consideration, especially in times of epidemic.

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CASE REPORT

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Plasmablastic lymphoma as a rare cause of subocclusive events – case report and review of the literature

Plazmablastni limfom kao redak uzrok subokluzivnih smetnji

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Abstract

Introduction. The most common causes of subocclusive disorders are the adhesion, Crohn's disease and small bowel neoplasms. Plasmablastic lymphoma (PBL) is an aggressive distinct subtype of diffuse large B-cell non-Hodgkin lymphoma initially reported in the oral cavity of the HIV infected individuals. **Case report.** We presented a male patient with PBL of the small intestine as a rare cause of intestinal subocclusion, without HIV infection and negative serology for hepatitis C, hepatitis B, and Epstein-Barr infection. A 73-year-old male was admitted to our Center due to the one-year history of abdominal pain, weigh loss, non-bloody diarrhea, night sweating and pruritus. The patient underwent the ileocolonoscopic examination with the accompanying biopsy specimens. The results, based on the histopathological and immunohistochemical pattern, confirmed a diagnosis of PBL. Following the chemotherapy treatment, our patient underwent the resection of ileum. The postoperative histopathological report confirmed PBL as the final diagnosis. The patient was treated for the following 6 months with the chemotherapy according to the cyclophosphamide, doxorubicin, vincristine, and prednisolone (CHOP) protocol. Fatal outcome was due to acute myocardial infarct. **Conclusion.** PBL of the small intestine is a rare and unusual cause of subocclusive events. In our patient, an accurate histopathological verification of the detected changes in the ileum was of crucial importance for further treatment.

Key words:
crohn disease; diagnosis, differential; immunohistochemistry; intestinal neoplasms; intestinal obstruction; lymphoma, large b-cell, diffuse; plasmablastic lymphoma; treatment outcome.

Apstrakt

Uvod. Najčešći uzroci subokluzivnih poremećaja u adheziji, Kronova bolest i neoplazme tankog creva. Plazmablastni limfom (PBL) je tip agresivnog difuznog krupnoćelijskog B nehočkinskog limfoma, koji je prvi put opisan u usnoj duplji kod HIV pozitivnih bolesnika. **Prikaz bolesnika.** U radu je prikazan bolesnik sa PBL tankog creva kao retkim uzrokom subokluzije, bez HIV infekcije i sa negativnom serologijom za hepatitis B, hepatitis C i infekciju Epštajn-Barovim virusom. Muškarac, star 73 godine, primljen je u naš Centar zbog jednogodišnje istorije bolova u trbuhu, gubitku telesne mase, dijareje (bez krvi), noćnog znojenja i svraba. Bolesniku je urađena kolonoskopija sa terminalnom ileoskopijom, pri kojoj je uzeta biopsija sluznice terminalnog ileuma. Rezultati patohistološkog i imunohistohemijskog ispitivanja su potvrdili dijagnozu PBL. Posle hemioterapije, bolesniku je urađena resekcija ileuma. Rezultat postoperativne patohistološke analize je potvrdio dijagnozu PBL. Bolesnik je lečen po protokolu CHOP (ciklofofamid, doksorubicin, vinkristin i prednizolon) tokom šest meseci. Fatalan ishod je nastupio zbog infarkta miokarda. **Zaključak.** Mada redak i neuobičajen, uzrok subokluzivnih tegoba može biti PBL ileuma. Kod našeg bolesnika krucijalni značaj za dalje lečenje je imala tačna patohistološka verifikacija promena aktiviranih u ileumu.

Ključne reči:
kronova bolest; dijagnoza, diferencijalna; imunohistohemija; creva, neoplazme; creva, opstrukcija; limfomi, b-krupnoćelijski, difuzni; limfom, plazmablastni; lečenje, ishod.

Introduction

Small bowel obstruction is a major cause of morbidity in hospitals around the world. The etiology of small bowel obstruction includes the adhesions (74%), Crohn's disease (7%), neoplasia (5%), hernia (2%), radiation (1%), and miscellaneous (11%)[1]. Plasmablastic lymphoma (PBL), a rare subtype of diffuse large B cell lymphoma, usually occurs in the patients with HIV infection and is primarily found in the oral cavity[1–4]. There are also reported cases of PBL in the immunocompetent individuals involving the cervical lymph nodes, stomach, lungs, cavity, small and large bowel, and liver[5–15]. This rare lymphoproliferative disorder is characterized by its plasmablastic morphology and immunohistochemical panel.

In this case report, we described a patient with symptoms of subocclusion and suspected Crohn's disease of terminal ileum on the endoscopy examination. Since the histopathological findings of the terminal ileum biopsy did not confirm Crohn's disease, an immunohistochemical examination was required. Upon the immunohistochemical analysis, PBL was diagnosed, isolated locally on the terminal ileum. Our patient had the negative serology tests for HIV, Epstein-Barr virus, hepatitis B and hepatitis C, and did not receive immunosuppressive therapy or suffered from any chronic disease.

The Ethics Committee approval for this report was obtained by the Ethics Committee of our Center.

Case report

A 73-year-old male was admitted to our Center with the one-year history of abdominal pain, weight loss, reported non-bloody diarrhea (six times a day), night sweating, and pruritus. His medical history revealed the laparoscopic cholecystectomy in 2012, sinus arrhythmia, hypertension, and benign prostatic hyperplasia. There was no family history of malignancies. The patient was an ex-smoker and had no recent consumption of beverages or drugs.

Six months prior to the admission to our Center, the patient was examined in a regional hospital. However, the results of the previous examinations were unmarkable, except the finding on the radiographic examination of the small intestine which raised suspicion of Crohn's disease of the terminal ileum.

Upon the admission in our center, the general physical examination revealed an abdominal tenderness in the lower abdomen, with no palpable lymphadenopathy or hepatosplenomegaly. The patient had an arrhythmic heartbeat with a heart rate of 55–90 beats/min, sinus arrhythmia in electrocardiogram, an atrioventricular block type I and a suspected sick sinus syndrome.

The laboratory data included hemoglobin concentration [113 g/L (normal range: 138–172 g/L)], and normal values for the white blood cell and platelet counts, β2 microglobulin, lactate dehydrogenase, total proteins and albumins. The C-reactive protein (CRP) values (28.2 mg/L) and the erythrocyte sedimentation rate (ESR) (32 mm/h) were elevated (normal range – CRP: less than 5 mg/L; ESR under 20 mm/h). A serum protein electrophoresis did not show the presence of monoclonal protein. Both the serum carcinoembryonic antigen and CA 19-9 were within the normal range. The serological tests for HIV, hepatitis B, hepatitis C, and Epstein-Barr virus were negative. The purified protein derivative (PPD) skin test was also negative. The analyses of stool samples were negative for *Campylobacter jejuni*, *Yersinia enterocolitica*, and *Clostridium difficile*.

The conventional chest X-ray, abdominal ultrasonography and computed tomography (CT) scan showed no abnormalities. Due to the clinical presentation and radiographic signs of subocclusion, the patient was examined by a surgeon who recommended further investigation and conservative treatment. An esophagogastroduodenoscopy revealed a small hiatal hernia and no macroscopic abnormalities of the esophagus, stomach and duodenum. The colonoscopy examination and an accompanying biopsy revealed a normal colon. Endoscopy of terminal ileum showed diffuse erythema and vascular congestion of the mucosal architecture in the terminal ileum with ulcerations as the "skip lesions" between macroscopically normal presented areas of mucosa of terminal ileum.

Relative improvement of clinical symptoms including reduction of abdominal pain and decreasing number of liquid stools was achieved using mesalamine and methylprednisolone. The laboratory data, however, revealed an elevated ESR (66 mm/h) and the fibrinogen values [7.5 g/L (normal range 2–4 g/L)]. Although the histopathology report did not confirm Crohn's disease, the findings of multiple endoscopic biopsies revealed the diffuse infiltration of lymphoid cells. Re-biopsies of terminal ileum and immunohistochemical examination of the terminal ileum were therefore required. The endoscopic features of the terminal ileum during the repeated endoscopy revealed inflamed mucosa with the irregular nodular and polypoid pattern, spontaneous bleeding, and multiple large ulcers (Figure 1).

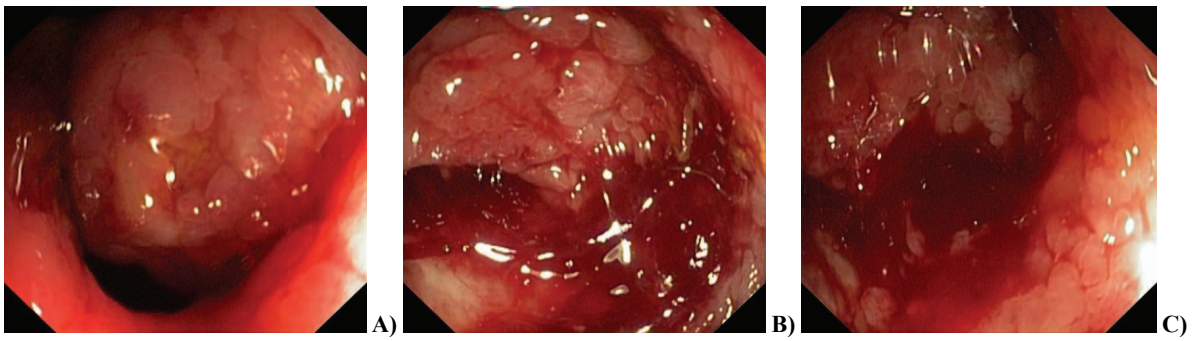

Fig. 1 – The endoscopic features of the terminal ileum during ileocolonoscopy: A) ulcer; B) the polypoid altered mucosa and spontaneous bleeding; C) the nodular alterated mucosa.

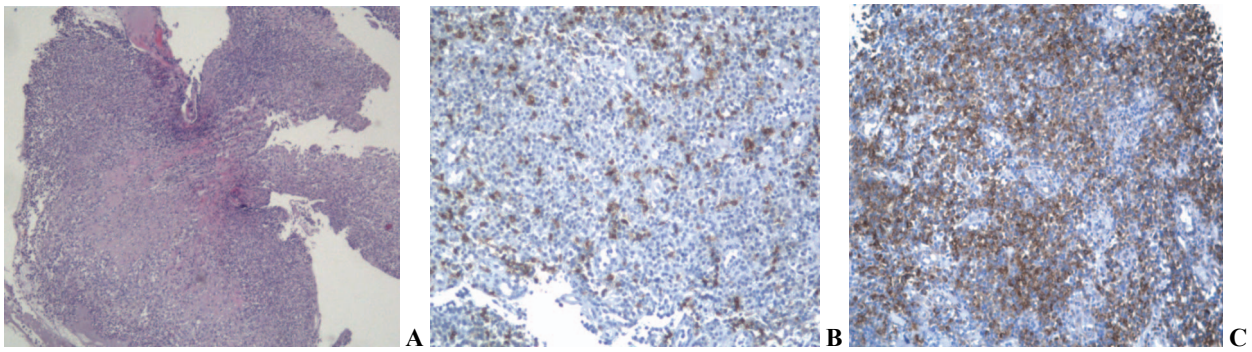

Fig. 2 – The histopathological examination of tumor tissue in the terminal ileum: A) Hematoxylin and eosin staining – diffuse neoplastic infiltration of ileum with the atypical large lymphoid cells; B) The immunohistochemical (IHC) analysis of CD20 – the lymphoid tumor cells are negative, while normal B lymphocytes are positive for the CD20 expression; C) The IHC analysis for CD38 – the lymphoid tumor cells were positive for the CD38 expression.

The histopathological examination revealed the abundancy of atypical large lymphoid cells. The immunohistochemical analyses of the biopsied tissue were positive for MUM1, CD38, and CD138, and negative for Pax5, CD20, CD3, bcl2, bcl6, CD56, and CD10; Ki67 was approximately 30% (Figure 2). The results based on the histopathological and immunohistochemical patterns confirmed a diagnosis of PBL. The patient was sent for further hematological investigation and treatment. After completing the clinical staging procedures, the patient received cyclophosphamide, doxorubicin, vincristine, and prednisolone (CHOP) chemotherapy. Due to the persistence of abdominal pain, the patient was transferred to the surgery department, where an ileum resection (50 cm in length) was performed. The postoperative histopathology report confirmed PBL as the final diagnosis.

After the postoperative recovery, the patient was receiving chemotherapy according to the CHOP protocol for the following 6 months, when the fatal outcome occurred due to acute myocardial infarction and consequently cardiac insufficiency.

Discussion

Our case report on localized PBL of the ileum presents a rare cause of subocclusive events. The patient was initially admitted to our hospital for further investigation to exclude previously suspected Crohn's disease. Due to the patient's persistent abdominal pain and radiographic signs of subocclusion, a surgical treatment was discussed without previous ileocolonoscopy.

Crohn's disease is the second most common etiological factor of intestinal obstruction that can require a surgical treatment [1]. According to the available literature, approximately 80% of patients with Crohn's disease will undergo an operation during their lifetime [2]. For the patients with Crohn's disease of the small intestine, an intestinal obstruction is the primary surgical indication [3]. During an ileocolonoscopy, the endoscopic features of Crohn's disease of the terminal ileum were described in our patient. The patient was therefore treated with mesalamine and corticosteroids. An explanation for the relative clinical improvement is that prednisolone is also included in the CHOP therapy. The final PBL diagnosis was based on the histopathology and immunohistochemical analysis with the additional confirmation by the postoperative findings. The case report of our patient once again proves the importance of histopathology and immunohistochemistry in order to establish the final diagnosis.

Although PBL was initially described in the patients with acquired immunodeficiency syndrome (AIDS) predominantly in the oral cavity, the clinical spectrum of this malignancy has since been expanded [4].

In the largest cohort study so far, conducted on 135 patients with PBL, the most of them were immunocompromised – either HIV-positive, transplanted or previously treated for systemic diseases and carcinoma [16].

However, there have been a number of patient series and reports including the HIV-negative cases and extraoral localizations**.** Over one-third of all PBL cases were first noted at extraoral locations, predominantly within the gastrointestinal tract. According to the relevant literature, the HIV-negative patients can have PBL in the stomach, small bowel, and colon [6, 9, 10, 13, 14]. PBL of the small intestine is extremely rare. Some Korean authors described the PBL cases of the small intestine associated with other locations, such as the oral cavity, jejunum and thorax [9]. According to a study conducted by some Chinese authors, the patients with localized PBL of the small intestine were immunocompromised from hepatitis B infections and had a recent radiotherapy for maxillary sinus cancer [11]. Our case report is specific because the illness, causing the small bowel obstruction, was localized to 50 cm of ileum in the immunocompetent patient with no previous medical history of radiotherapy or use of immunosuppressant drugs.

A group of Chinese authors analyzed 114 HIV negative patients with PBL from 52 published papers and concluded that PBL was localized in the gastrointestinal tract in only 15.79% of cases [17].

In a study conducted by the US authors, of 61 patients with PBL, the gastrointestinal tract was affected in 12 patients, of whom only 3 patients were immunocompetent [18].

Although the studies of other authors suggest a very low diagnostic yield terminal ileum intubation during colonoscopy, our case report points to the importance of insisting on the exploration of the small intestine in a patient with subocclusive symptoms in order to establish the final diagnosis [19, 20]. Also, in our patient, the crucial importance for further treatment was an accurate histopathological verification of the detected changes in the ileum.

Conclusion

The differential diagnosis of subocclusive events can also include PBL of the small intestine, as a rare and unusual site of the disease, in the HIV-negative patients without a previous medical history of immunosuppression.

Conflicts of interest

The authors state that they have no conflict of interest.

Acknowledgments

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CASE REPORT

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The arteriovenous hemangioma of the right ventricle – case report and literature review

Arteriovenski hemangiom desne komore

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Abstract

Introduction. Cardiac hemangiomas of the right ventricle are very rare and mostly asymptomatic benign tumors. The surgical excision is the first line treatment. **Case report.** We report a case of 69-year-old woman with an asymptomatic arteriovenous hemangioma of the right ventricle. The complete surgical excision was performed with the use of cardiopulmonary bypass and the patient was discharged on the postoperative day 6 after the uneventral postoperative course. There was no relapse during the six-month follow-up. Literature review revealed totally 35 cases of this tumors including our case **Conclusion.** Described procedure can be performed safely with the excellent long-term results.

Key words:
hemangioma; heart ventricles; cardiac surgical procedures; diagnosis; treatment outcome.

Apstrakt

Uvod. Hemangiomi desne komore srca su izuzetno retki i uglavnom asimptomatski benigni tumori. Hirurško lečenje je terapija izbora. **Prikaz bolesnika.** Kod bolesnice stare 69 godina dijagnostikovan je asimptomatski benigni tumor srca tipa arteriovenskog hemangioma desne komore. Totalna hirurška ekscizija učinjena je uz pomoć vantelesnog krvotoka. Postoperativni tok je bio uredan i bolesnica je otpuštena kući šest dana posle zahvata. Na kontrolnom pregledu posle šest meseci nije bilo znakova recidiva. Pregledom literature pronađeno je ukupno 35 slučajeva ovog tumora uključujući i prikazanu bolesnicu. **Zaključak.** Opisana procedura se može bezbedno koristiti kao terapija izbora sa odličnim dugoročnim rezultatima.

Ključne reči:
hemangiom; srce; komora; hirurgija, kardijalna, procedure; dijagnoza; lečenje, ishod.

Introduction

Hemangiomas of the heart are exceptionally rare benign tumors constituting 1%–2% of all cardiac tumors which may occur in all cardiac layers: pericardium, myocardium or endocardium. Their location in the right ventricle is highly uncommon and usually without any symptoms. The cardiac hemangiomas are clinically classified into three subcategories: capillary, cavernous and arteriovenous type [1]. This report accounts for a case of arteriovenous cardiac hemangioma, an extremely rare subtype of this tumor.

Case report

We report a 69-year-old woman without any reported symptoms who was accidentally diagnosed with the tumor of the right ventricle during a routine echocardiography. She is a nonsmoker with previous history of hypertension and under control by therapy. Transthoracic echocardiography showed a mass in the right ventricle with no tricuspid regurgitation, normal right ventricle diameter and normal left ventricular function. The cardiac magnetic resonance imaging (MRI) showed an intermediate-density mass 20 × 25 cm fixed with

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a small pedicle to the anterior wall of the right ventricle. The coronary angiography did not show signs of coronary disease (Figure 1a and b). The laboratory test results were all within normal ranges, as well as serum tumor markers.

Under general anesthesia, the median sternotomy was performed. After the institution of bicaval cardiopulmonary bypass, the heart was arrested with warm blood cardioplegia in normothermic conditions. The tumor was resected completely with a clear margin through right atriotomy, there was no involvement of the tricuspid valve (Figure 1c). There was no need for the ventricle wall reconstruction.

The histopathology exam revealed the mixture of arterial and venous vessels confirming the diagnosis of arteriovenous hemangioma (Figure 2a and c). The endothelial markers CD 31 were positive on immunohistochemical staining (Figure 2 b).

The patient was discharged on the postoperative day 6 with the uneventful postoperative course. After six-month follow-up, the patient was alive and well, with no relapse of the hemangioma showing at the control echocardiography.

Discussion

The cardiac hemangiomas are extremely rare benign tumors of the heart. They can occur in all three layers of the myocardium and can be present anywhere in the heart cavities or pericardium. They consist of small arterial or venous vessels and cavernous vascular channels, leading to the division on three subtypes: capillary, cavernous and arteriovenous. They are very uncommon in the right ventricle, especially the arteriovenous type presented in our case. The disease can appear in the patients of all ages, and the clinical presentation depends on the localization and the size of the tumor – it can vary from the asymptomatic to the signs of right ventricle congestion, but they are usually asymptomatic [2]. There are some cases described to result in a sudden cardiac death, rhythm disturbances in hemangioma localized in the vicinity of the atrioventricular (AV) node and tamponade caused by a ruptured hemangioma in the pericardium. The tumors localized in the valvular apparatus can cause the orifice obstruction and distal embolization [3].

There was an indication for a surgical procedure because of the malignant localization of the tumor (it was even supposed to be benign), as well as for the definite histopathology exam confirmation of the tumor type. Especially because the right-sided heart tumor mass is always suspicious for malignancy because the high frequency of cardiac metastases originate from the primary malignant tumor (bronchogenic carcinoma, breast, hepatic and renal carcinoma) via venous dissemination [4].

The diagnosis was made on the basis of echocardiography followed by a confirmation of the contrast-enhanced computed tomography (CT) or cardiac MRI. Coronary angiography is useful in determining the relationship with coronary arteries if necessary, or in excluding the concomitant coronary artery disease if suspected [5].

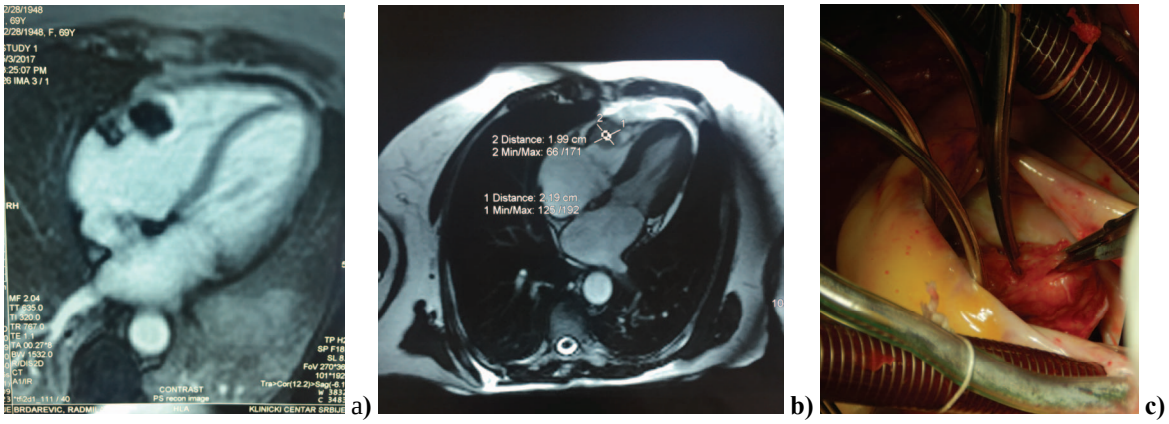

Fig. 1 – Magnetic resonance imaging (a, b) and intraoperative image (c) showing the hemangioma in the right ventricle.

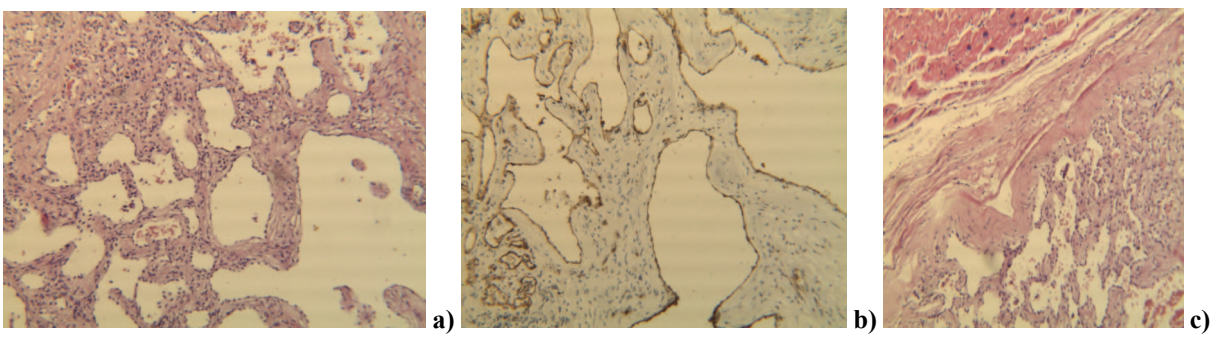

Fig. 2 – Microscopic view of the arteriovenous hemangioma of the right ventricle (a, c) and the CD 31 positive marker staining (b).

MRI was performed in order to visualize the tissue structure and the possible invasiveness of the tumor mass. The MRI and echocardiography together give more information on the tumor origin in the right heart that was histopathologically confirmed after the surgical tumor removal than the echocardiography alone, especially for the right heart localization due to a difficulty in obtaining this view on the standard echocardiography [6]. The contrast enhancement feature of the MRI is highly predictive for malignancy, as well as the ability of the MRI to show the exact localization and tissue invasiveness, in addition to pleural effusion presentation, which are all predictive for cardiac malignancy. The T1 and T2 sequence contrast density alongside the presence of the contrast enhancement can differentiate between the different types of tumor masses. The cardiac lypomas are hyperintense while fibromas and myxomas are hypointense. The cardiac hemangiomas are homogeneous, intermediate-to-high signal on T1-weighted, and diffusely hyperintense on the T2-weighted images [7].

The treatment of choice is a surgical resection with a clear margin, with the ventricle wall reconstruction if needed [8].

The first hemangioma of the right ventricle was described by Hochberg in 1950, and up to date, 35 cases have been found in the relevant literature, including the one presented here. The median sternotomy was used predominantly, and the thoracotomy was used only in two cases. The total resection with a clear margin was performed in 31 patients, while 4 patients had the biopsy done without resection. Over 90% of surgeries were performed with the use of cardiopulmonary bypass (CPB) mainly, with the aortic cross clamping and the use of cardioplegic arrest of the heart. The follow-up data was available for 80% of patients (from 6–24 months), and there were no relapses or fatal outcomes. The main localization was the anterior wall (63%) of the right ventricle, while the right ventricle outflow tract (35%) had the most dramatic clinical presentation. Three tumors had the apical ventricular localization and these hemangiomas may require the right ventricle (RV) wall reconstruction similar to the aneurysmectomy. Jiang et al. [9] reported this procedure with a satisfactory postoperative cardiac function.

Conclusion

The cardiac hemangiomas of the right ventricle are very rare and mostly asymptomatic benign tumors. The surgical excision is the first line treatment. The procedure can be safely done with the use of CPB while the complete excision is mainly achieved with a low rate of recurrence and excellent long-term survival.

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E R R A T U M

In the article by ***Vladimir Miloradović, Dušan Nikolić, Miodrag Srećković, Ivana Djokić Nikolić***. Extremly tortuous coronary arteries – When optical coherence tomography and fractional flow reserve did not help us much / Ekstremno tortuozne koronarne arterije – kada optička koherentna tomografija I frakciona rezerva protok ne pomažu mnogo.
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two authors (Vladimir Miloradović and Miodrag Srećković) have additional affiliation.

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Vladimir Miloradović*†, Dušan Nikolić*, Miodrag Srećković*†, Ivana Djokić Nikolić*.

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Primeri referenci:

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Tabele

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

Ilustracije

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

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

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