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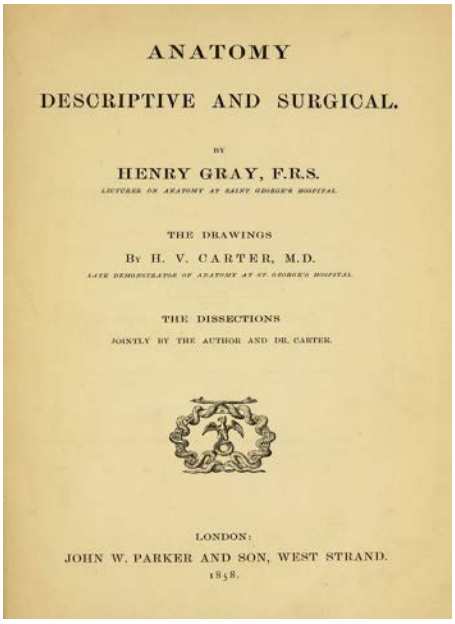

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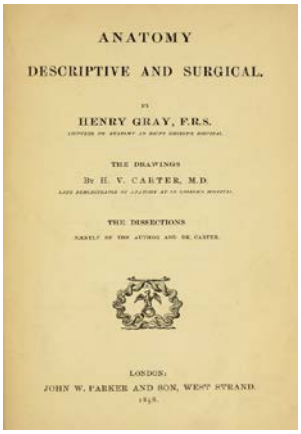

Henry Gray (1827–1861), British anatomist and surgeon, is the author of the famous textbook on anatomy. In 1852, Gray published its first edition, entitled "Anatomy. Descriptive and Surgical". The textbook had 750 pages and 363 drawings made by Henry Carter. It is still published regularly under the name "Grey's Anatomy", and is highly valued in the medical profession.
June 13 this year marks the 160th anniversary of Henry Gray's death.

Henri Grej (1827–1861), britanski anatom i hirurg, autor je čuvenog udžbenika iz anatomije. Grej je 1852. godine objavio prvo izdanje ovog udžbenika pod naslovom „Anatomy. Descriptive and Surgical". Udžbenik je imao 750 stranica i 363 crteža koje je izradio Henri Karter. Ovaj udžbenik i dalje redovno izlazi pod nazivom „Grey's Anatomy" i veoma je cenjen u medicinskim krugovima.
Ove godine, 13. juna, navršava se 160 godina od smrti Henrija Greja.

ORIGINAL ARTICLES

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Role and importance of elastography in the diagnosis of differentiated thyroid carcinomas regarding the clinical, echosonographic, biohumoral and cytological examination and correlation of these results with definitive histopathological findings – A retrospective study

Uloga i značaj elastografije u dijagnostici diferentovanih karcinoma štitaste žlezde u odnosu na klinički, ehosonografski, biohumoralni i citološki pregled i korelacija nalaza sa definitivnim patohistološkim nalazima – Retrospektivna studija

Saša Kiković[*], Dejan M. Marinković[*], Petar Ristić[*], Jelena Karajović[*], Snežana Kuzmić-Janković[*], Zorana Djuran[*], Milica Čizmić[*†], Božidar Kovačević[‡], Nemanja Nenezić[§], Zoran Hajduković[*†]

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Abstract

Background/Aim. Thyroid nodules represent a common problem in medicine. Ultrasound examination of the thyroid glands is a common method for the initial diagnosis of thyroid neoplasia enabling the selection of nodules that should undergo fine needle aspiration biopsy (FNAB). It is a noninvasive method that has a great potential in discovering suspicious lesions, enabling timely actions for further diagnostics and potential treatment. However, it is still not clear enough how the quantitative data collected from Ultrasound elastography correlate with those obtained by other diagnostic tools used for detecting thyroid nodules. The aim of this study was to estimate the importance of ultrasound elastography in diagnostics of differentiated thyroid carcinomas but also to estimate the importance of other diagnostic procedures as well. **Methods.** The research was performed on patients initially referred to the Clinic of Endocrinology in Military Medical Academy, Belgrade to evaluate the status of thyroid nodules. The data from ultrasound elastography was then correlated with those from other diagnostic procedures including clinical examination, echosonography, cytological and histopathological analyses. **Results.** Statistically significant difference between the group of patients with follicular adenoma and the group of patients with differentiated thyroid carcinoma was detected for consistency, nodules immovability, thyroglobulin (Tg) levels, presence of calcifications in the nodules and the elastographic score. Fixed nodules and those with firmer consistency were significantly more common in the group of patients with malignant lesions, in which Tg levels were higher. The elastography score 3–4 showed a high predictive value for the detection of thyroid carcinoma, unlike the elasticity score. **Conclusion**. Ultrasound elastography represents a new non-invasive method that has a very significant, high predictive value for the detection of thyroid carcinoma, especially in correlation with other diagnostic procedures.

Key words:
thyroid diseases; ultrasonography; biopsy, fine-needle; diagnosis; differential.

Apstrakt

Uvod/Cilj. Tiroidni nodusi predstavljaju veoma čest medicinski problem. Ultrazvučni pregled štitaste žlezde je najčešće primenjivana metoda za početnu procenu i izbor nodusa za iglenu biopsiju (*fine needle aspiration biopsy* – FNAB). Između mnogih metoda, ultrazvučna elastografija kao neinvazivna metoda ima veliki značaj u otkrivanja sumnjivih promena i određivanju prirode tumora. Pregled je potpuno bezbolan i jednostavan, a podrazumeva merenje

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otpora mekih tkiva, kvalitativno i kvantitativno. Međutim, još uvek nije u potpunosti jasno u kojoj meri kvantitativni podaci dobijeni ultrazvučnom elastografijom korelišu sa podacima dobijenim drugim dijagnostičkim postupcima za detekciju toroidnih nodusa. Cilj studije bio je da se proceni značaj ultrazvučne elastografije u dijagnostici diferentovanih karcinoma štitaste žlezde, ali i vrednost drugih dijagnostičkih procedura. **Metode.** Istraživanje je obavljeno na bolesnicima koji su inicijalno upućeni u Kliniku za endokrinologiju Vojnomedicinske akademije u Beogradu u cilju evaluacije nodoznih struma. Korelisani su nalazi dobijeni elastografijom sa rezultatima kliničkog pregleda, biohumoralne, morfološke (ehosonografske), citološke obrade i definitivnog patohistološkog nalaza. **Rezultati.** Od svih praćenih parametara, statistički značajna razlika između dve grupe bolesnika sa folikularnim adenomom i diferentovanim tiroidnim karcinomom je detektovana za konzistenciju, nepokretnost nodusa, vrednost tiroglobulina (Tg), prisustvo solidnih nodusa, kalcifikata i elastografski skor. Nepokretni nodusi i oni tvrđe konzistencije su bili statistički značajno češće prisutni u grupi bolesnika sa malignim lezijama, gde je bila viša vrednost T). Elastografski skor 3–4 je pokazo visoku prediktivnu vrednost za detekciju tiroidnih karcinoma, za razliku od skora elasticiteta. **Zaključak.** Ultrazvučna elastografija predstavlja novu neinvazivnu metodu koja ima veoma značajnu, visoku prediktivnu vrednost za detekciju tiroidnih karcinoma, naročito u korelaciji sa drugim dijagnostičkim procedurama.

Ključne reči:
tireoidna žlezda, bolesti; ultrasonografija; biopsija tankom iglom; dijagnoza, diferencijalna.

Introduction

Primary thyroid tumors originate from thyroid follicular epithelia or parafollicular cells of the thyroid gland, and rarely present lymphoproliferative diseases. They are clinically most often represented as localized enlargements of the thyroid gland (nodules).

The prevalence of palpatory detected nodules is about 3% (6.4% of women and 1.5% of men) [1]. In the last 20 years, an increased use of echosonography of the neck has reduced the prevalence of nodules in the general population for about 20–70%[1, 2]. Autopsy studies state that the changes in the thyroid gland due to nonthyroidal disease are found in approximately 50% of the bodies examined after death [2, 3]. Nodules of the thyroid gland are four times more common in women, in the areas with iodine deficiency, in people exposed to radiation, and in people older than 60 years [3]. The incidence of thyroid nodules is about 100 cases *per* 100,000 people *per* year [3, 4]. About 5% of these thyroid nodules are carcinoma [5, 6]. Thyroid carcinoma is not a frequent neoplasm but is the most common malignant endocrine tumor [7]. The highest incidence was recorded in Iceland and Hawaii, especially in ethnic groups of Chinese and Filipinos [8].

Diagnosis of differentiated malignant tumors is established by the standard histopathological (HP) and immunohistochemical analyses in surgical specimens of thyroid tissue after thyroidectomy performed. The diagnostic methods include: clinical examination, biohumoral treatment, ultrasound examination, scintigraphy and cytological diagnosis (FNAB – fine needle aspiration biopsy).

To detect functional nodules that do not show malignant characteristics, the serum level of thyrotropin (TSH) is determined. Functional nodules are proven by scintigraphy. In most cases, they do not have malignant features, so cytological punctures are not generally indicated at that time. Determination of the tumor marker thyroglobulin (Tg) is not routinely applied and is not recommended in the preoperative diagnostics [9, 10]. Determination of serum calcitonin levels should be performed in a patient with a family history of medullary carcinoma of the thyroid gland and multiple endocrine neoplasias [11].

Ultrasound examination of the thyroid gland includes morphological evaluation, tissue mobility analysis, and elastography analyses. The morphological analysis determines the presence, size, and the localization of focal changes, edge shapes, echogenicity, homogeneity, the presence of micro and macrocalcification, the presence of cystic component, hypoechogenic halo, and the pathologically altered lymph nodes of the neck [12]. Vascularization analyses include assessment of blood circulation to the surrounding tissue (graded 0–4) and the type of vascularization (intranodal and perinodal) [13]. It is not possible to diagnose the malignant potential with ultrasound only. Suspicious characteristics of the nodules include their hypoechogenicity, the microcalcifications, their regularly shaped edges, increased vascularization, the shape of the nodule (the anteroposterior diameter of the nodule is larger than the transversal) and the growth rate of the nodule during the sequential ultrasound examinations [14, 15].

Ultrasonic elastography is a non-invasive method in which the resistance of soft tissues to pressure is measured [16,17]. The examination is completely painless and simple. The goal of the measurement is to detect suspicious changes and to assess the malignant potential of the nodules. Resistance to the pressure of the nodules and surrounding tissue is shown in different colors, which we designate as qualitative testing of tissue resistance. The measurements are based on the fact that all tumors, especially malignant, have firmer structures, due to pronounced cell proliferation, increased tissue density, and increased vascularisation. At the pressure of the ultrasonic probe, the colors of normal tissue change from red to yellow, to green, while in tumor altered tissues it has blue or gray-black shades. The resistance can be measured quantitatively as well, both within the change itself and in the surrounding tissues, which is expressed by elastography scores and estimated by the resistance index: strain ratio (SR) [18–21]. Quantitative measurements can show up to twenty times higher resistance of the tumor tissues compared to normal ones.

In addition to the above diagnostic tests, the use of other morphological procedures [computed tomography (CT), magnetic resonance imaging (MRI), positron emission tomography (PET)] is not performed routinely. In all patients with palpatory detected thyroid glands, it is necessary to perform ultrasound diagnosis [22]. On the other hand, a cytological examination is the most accurate and cost-effective method for the evaluation of thyroid nodules. Thyroid biopsy is usually performed under the supervision of ultrasound [23–27]. Cytological examination of all nodules ≥ 10 mm is recommended as well as smaller nodules if several susceptible features are present [28, 29]. Nowadays with the advancement of ultrasound technology, it is technically possible to make a cytological examination of nodules of size below 5 mm [30]. The cytological interpretation of the findings is most commonly made by the Bethesda classification adopted by the National Cancer Institute (NCI). Cytological findings are classified into 6 diagnostic categories: I – nondiagnostic or unsatisfying; II – benign cytopathological finding; III – follicular lesion/atypia of undetermined significance; IV – follicual neoplasm or suspicious follicular neoplasm; V – suspicious on malignancy; VI – malignancy [31, 32]. Due to the insufficient sensitivity of cytological diagnosis in the distinction of follicular carcinoma from follicular adenoma, an operative procedure is required for the HP verification [17]. Cytologically confirmed benign nodules require only further monitoring. The cytological puncture under control of ultrasound should be done or repeated when an increase of the nodule is registered with palpation or ultrasound [33].

Malignant features are recorded in about 5% of solitary nodules, in the polynodal thyroid gland, or in small not palpable nodules that are randomly detected by ultrasound [34]. The risk of malignancy in patients with multiple nodules is similar to the risk in patients with solitary nodules [35]. As the size of the nodule increases, there is no increase in the risk of malignancy. In patients with multiple nodules, it is necessary to make aspirational biopsy of the suspicious nodules, or the dominant nodule in patients with multiple nodules without suspected features. Nodules suspicious to malignancy are subjected to surgical removal [36].

Most of these methods have a known predictive value during the diagnostic process of nodal changes in the thyroid gland, but for elastography, different and partially contradictory results have been shown [37]. Therefore, this study aimed to examine the significance of ultrasound elastography in the diagnosis of differentiated thyroid gland tumors.

Methods

The research was a retrospective, clinical, noninterventional cross-sectional study aimed to analyze the significance of the diagnostic test – ultrasound elastography. The study covered 58 patients (41 females and 17 males, ages 24 to 78), who were examined in the period between 2015 to 2018 at the Clinic for Endocrinology of the Military Medical Academy in Belgrade, Serbia, and who were subjected to the surgical treatment after the diagnostic procedure performed. Based on a HP analyses, patients were divided into two groups. The follicular adenoma (FA) group included patients who had been diagnosed with a benign focal lesions/follicular adenoma determined by HP and the differentiated thyroid carcinoma (DTC) group containing patients with the existence of malignant lesions (papillary and follicular – DTC) determined by HP.

The survey was conducted based on the following criteria: age > 18 years, a HP analysis verified as FA or DTC. Excluding criteria for the examined population were: patients with thyroid gland tumors that did not belong to FA or DTC, pregnant women and nursing women, patients with previous thyroid gland surgery and other thyroid diseases followed by its dysfunction (thyrotoxicosis, manifest hypothyroidism, acute and subacute thyroiditis); patients with cognitive dysfunction who could not give relevant anamnestic data, patients with other malignancies in the last 5 years (except for skin cancer), patients with multiple endocrine neoplasias, patients with severe liver and kidney failure, patients with radioactive iodine in therapeutic purposes.

The results of the clinical examination were analyzed in terms of the inspection findings or palpatory findings and laboratory analyses. The analysis of the inspection finding included the existence or absence of visible nodule changes in the normal position of the head and in the extension of the neck. The palpatory finding included palpability of the nodules (palpable or non-alopecia), nodules consistency (hard or elastic) and mobility of the nodules (movable or immobile).

Laboratory analyses were carried out at the Institute of Biochemistry of the Military Medical Academy in Belgrade, and included determining the following parameters: free thyroxine (fT4) and TSH were determined from the serum by the chemiluminescence method, on the unicell DxI 800 (Beckman Coulter) using the reference value 7.0–19.0 pmol/L, and 0.340–5.600 mIU/L, respectively; thyroglobulin was determined by chemiluminescence method, on the Elecsys 2010 (Roche) with the reference value 3.50–77.0 mg/L; calcitonin was determined from the serum on the Imulyte 2000 (Siments) using the reference values 7.0–18.0 nmoL/L; antithyroglobulin antibodies (TGAb) were measured from the serum by chemiluminescence method (the reference values were 0.00–4.00 IU/mL); anti-thyroid peroxidase antibodies (anti TPOAb) were determined from the serum using reference values < 9.0 IU/mL.

After a detailed clinical examination and laboratory analyses, all patients were examined by echosonography. Echocardial examination of the thyroid glands was carried out on the ultrasonic device of the brand Toshiba Aplio linear probe 10 MHz. In all patients, standard ultrasound examination in B-mode and ultrasound measurement were performed. Analyzed ultrasound characteristics of the thyroid gland nodule included the following: localization of the nodules, the regularity of the edges, the presence of hypoechogenic halo; homogeneity; echogenicity; presence of calcificate; extrathyroidal extension; marginal calcification; dimensions; analysis of blood flow. We used the Fukunara scoring: the nodules that were the most elastic were assigned to score 1 (mostly benign nodules), and the nodules that are least elastic were assigned to score 4 (malignant nodes) [15]. The re-

sistance was measured both quantitatively, within the same shift as in the surrounding tissues, and is expressed as the resistance index (SR – strain ratio) that represents the software calculated, quantitative measure of elasticity. In this study, all values of SR ≥ 2.5 were considered to be a predictor of malignancy of the nodules.

The finding of a cytological analysis of material obtained during FNAB was marked as a suspect malignant lesion, probably a benign lesion and a non-exclusive finding.

The sample size was calculated based on sensitivity of elastography in the diagnosis of DTC. According to the data from Tanaka et al. [36] the sensitivity of elastography in the diagnosis of differentiated thyroid carcinoma is 89.1%. The study sample was calculated taking $\alpha = 0.05$, the strength of the study $1\beta = 80\%$ and for the detection of a difference of 10% from the assumed sensitivity. By applying the formula, the established criteria for sample size included at least 49 patients. In the analysis of the obtained results, the methods of descriptive and analytical statistics were used. Data for all categories were represented as absolute and relative values. The distribution of data was tested and the data were presented according to the central tendency (arithmetic mean, median) and measurements of variability (standard deviation, range).

The difference between the two groups was analyzed by the Student's *t*-test, Man-Whitney test, chi-square (χ^2) test, and Fischer test, depending on the type and distribution of data. The relationship between the two variables was analyzed using the Person's or Spearman's coefficient of correlation, depending on the type and distribution of data. Diagnostic accuracy of elastography was expressed through the sensitivity, specificity, positive and negative predictive value of this method concerning FNAB. Receiver operating characteristic curve (ROC) was used to calculate the cut off values for continuous variables. Variables with the greatest diagnostic potential were incorporated into the mathematical model, and its diagnostic potential was determined. The significance threshold (α) for all statistical calculations was 0.05. SPSS software package (version 23.0, SRSS Inc. Chicago, IL) was used for statistical data processing of the results obtained.

Results

The study included 33 patients with FA and 25 patients with DTC. The age and gender of patients with malignant (DTC) and benign (FA) lesions were not statistically significantly different between the two groups (Table 1). The fol-

Table 1
Demographic characteristics of patients with follicular adenoma (FA) and patients with differentiated thyroid carcinoma (DTC)

Characteristics	FA (n = 33)	DTC (n = 25)	*p*
Age (year), mean ± SD	47.7 ± 12.9	51.0 ± 14.4	0.383 (*t*-test)
Gender, n (%)			
male	8 (24.2)	9 (36)	0.330 (χ^2-test)
female	25 (75.8)	16 (64)	

Table 2
Clinical and laboratory characteristics of the disease in patients with follicular adenoma (FA) and patients with differentiated thyroid carcinoma (DTC)

Parameters	FA (n = 33)	DTC (n = 25)	*p*
Inspection finding, n (%)			
no visible finding	5 (15.2)	7 (28)	0.412
node visible with neck extension	18 (54.5)	10 (40)	
node visible in normal position	10 (30.3)	8 (32)	
Palpation finding, n (%)			
not palpable	0 (0)	0 (0)	–
palpable	33 (100)	25 (100)	
Node consistency, n (%)			
firm	19 (57.6)	21 (84)	0.031
elastic	14 (42.4)	4 (16)	
Node mobility, n (%)			
not mobile	0 (0)	3 (12)	0.041
mobile	33 (100)	22 (88)	
fT4 (pmol/L), mean ± SD	12.8 ± 3.3	11.6 ± 2.9	0.171
TSH (pmol/L), mean ± SD	1.6 ± 1	2 ± 0.8	0.129
Thyroglobulin (Tg), mean ± SD	77.7 ± 97.7	314 ± 553.6	0.045
Anti TPO Ab (IU/mL), mean ± SD	4.3 ± 5.8	24.3 ± 75.1	0.131
Anti Tg Ab (IU/mL), mean ± SD	3.1 ± 7.0	4.8 ± 10.9	0.486
Calcitonin > 2 nmol/L, n (%)	1 (3.0)	1 (4.0)	1.000

Note: **Data presented as n (%) were analyzed with the χ^2-test; data presented with arithmetic mean and standard deviation (SD) were analyzed with the Student's *t*-test.**
fT4 – free thyroxine; TSH – thyroid stimulating hormone (thyrotropin);
TPO Ab – thyroid peroxidase antibodies; Tg Ab – thyroglobulin antibodies.

lowing parameters were monitored: inspection and palpatory findings, consistency, nodules mobility, and the levels of TSH, fT4, Tg, calcitonin, TPOAb and TGAb. The inspection findings did not differ significantly between DTC and FA groups. All nodes were palpable in both groups of patients. Observing the parameters that were evaluated by clinical examination and laboratory analyses, significant difference between groups was found in the nodules of firmer consistency, immobile nodules and values of Tg (Table 2).

The nodules with a firmer consistency were significantly more present in the DTC group of patients. In the DTC group, there were 84% of patients with firmer consistency nodules and only 16% of elastic nodules. In the FA group there were more elastic nodules (42.42%). Fixed (non mobile) nodules were statistically significantly more present in the DTC group (12%). There were no fixed nodes in the FA group.

Tg levels were significantly higher in the DTC group of patients. The levels of fT4, TSH, TPOAb, and TGAb did not differ between the DTC and FA groups. Calcitonin levels > 2 nmol/L were present in one patient of each group, so there was no statistically significant difference between the DTC and FA groups.

Ultrasound characteristics and measurements of examined nodules are presented in Table 3. The distribution of anatomical localization of the nodules in patients did not differ significantly between the DTC and FA groups. Irregular edges were more common in patients with DTC, and the difference was near the level of statistical significance. The presence of hypoechogenic halo was more common in the DTC group, but the difference between the DTC and FA groups was not significant. Solid nodules were significantly more present in the patients with DTC. Namely, 88% of patients from the DTC group had solid nodules, while 12% of them were cystic. In the FA group, only 33.33% of patients had solid nodules. The homogeneity and echogenicity of the nodules did not differ significantly between the two groups. Micro- and macro-calcifications were significantly more present in the group of patients with DTC. Namely, 97% of the patients in the FA group did not have calcifications. In the

Table 3
Ultrasound characteristics of the disease in patients with follicular adenoma (FA) and patients with differentiated thyroid carcinoma (DTC)

Characteristics	FA (n = 33)	DTC (n = 25)	*p*
Localization, n (%)			
central	25 (75.8)	20 (80)	0.701
peripheral	8 (24.2)	5 (20)	
Anatomic localization, n (%)			
cranial	3 (9.1)	4 (16)	
medial	25 (75.8)	20 (80)	0.316
the caudal third of the lobe	5 (15.2)	1 (4)	
The regularity of the edges, n (%)			
absent	3 (9.1)	7 (28)	0.059
present	30 (90.9)	18 (72)	
Presence of hypoechogenic halo, n (%)			
absent	26 (78.8)	24 (96)	0.121
present	7 (21.2)	1 (4)	
The appearance of the node tissue, n (%)			
solid	11 (33.3)	22 (88)	
mixed	22 (66.7)	3 (12)	< 0.001
cystic	0 (0)	0 (0)	
Homogeneity, n (%)			
homogeneous	5 (15.2)	6 (24)	0.504
not homogeneous	28 (84.8)	19 (76)	
Echogenicity			
dominantly hypoechogenic, n (%)	29 (87.9)	24 (96)	
iso echogenic	3 (9.1)	0 (0)	0.386
hyper echogenic	1 (3)	1 (4)	
Presence of the calcifications, n (%)			
absent	32 (97)	10 (40)	
present, micro	0 (0)	13 (52)	< 0.001
present, macro	1 (3)	2 (8)	
Dimensions (mm), mean ± SD	25.6 ± 6.3	25.0 ± 5.0	0.719
Mobility (present), n (%)	20 (60.6)	21 (84.0)	0.080
Calcification of the edges, n (%)			
absent	29 (87.9)	25 (100)	0.126
present	4 (12.1)	0 (0)	

***Note*: Data presented as n (%) were analyzed with the chi-square (χ^2) test and these presented as arithmetic mean and standard deviation (SD) were analyzed with Student *t*-test.**

DTC group, 52% of the patients had microcalcifications and 8% macrocalcifications. The dimensions of the nodules and calcifications of the edges were not significantly different between the groups. More pronounced vascularization was more prevalent in the group of patients with DTC, and the difference was close to the conventional level of statistical significance (not shown).

The parameters registered with ultrasound elastography in the FA and DTC groups of patients are shown in Table 3. The elastography scores 2 and 3 were significantly more frequent in the group of patients with FA, whereas elastography score 4 was only present in the group of patients with malignant lesions (DTC). 17 patients (68%) in the DTC group had score over 4, whereas in the FA group none of the patients had the score 4. In the DTC group, no patient had the score of lower than 3. The sensitivity of Fukunara score 4 was 68%, and the specificity was 100% for the diagnosis of DTC. The elasticity score, SR > 2.5, was present in almost all patients in the examined groups, but there were no statistically significant differences between them. The SR > 2.5 did not prove to be sensitive sufficiently and specific enough for the diagnosis of DTC, therefore only numerical values were analyzed. The area under the ROC curve (AVC) was statistically significant (Figure 1). The SR ≥ 4.35 was 92% sensitive and 70% specific for the diagnosis of DTC in the examined patients. The results of the cytological analyzes obtained by FNAB showed that FNAB findings in patients with benign lesions (FA) were false positive, which makes this method insufficiently specific for the diagnosis of differentiated thyroid carcinoma.

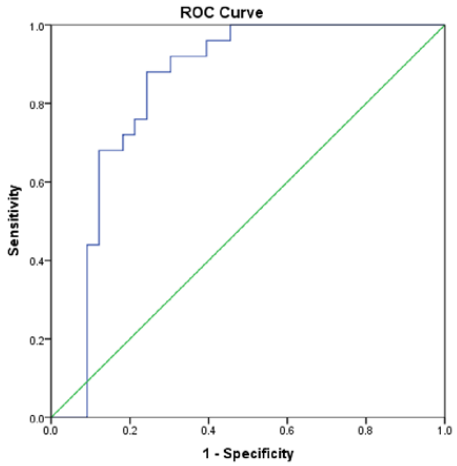

Fig. 1 – Receiver operating characteristic (ROC) curve of specificity and sensitivity of strain ratio in the diagnosis of differentiated thyroid carcinoma (DTC). Scala under ROC curve (95% confidence interval): 0.84. (0.731–0.949); $p < 0.001$.

Discussion

Increasing prevalence of primary thyroid tumors, benign or malignant, have been detected by over the past twenty years. In the general population, the prevalence is about 20–70% [1, 2, 38, 39]. The fact that about 5% of the thyroid nodules are malignant, imposes a need for a more precise detection and preoperative diagnosis of thyroid carcinomas [5, 6]. Regardless of all diagnostic possibilities, the definitive diagnosis of differentiated malignant tumors is made exclusively by the histopathological and immunohistochemical analyses. It is a constant professional need to timely diagnose and cure malignant thyroid gland disease, but also to reduce the number of operated benign nodules. Detailed clinical examination, biohumoral, echosonography, scintigraphy, and cytological analyses are often insufficient to clearly assess and establish an indication for surgical treatment. A high quality elastography measurements could indeed provide the necessary tool for this kind of decision. Therefore, the study was conducted in which 33 patients with FA and 25 patients with DTC were involved.

Both men and women have the lowest incidence of malignant nodules in the 70s. A typical risk of 5% malignancy of thyroid gland nodules varies by age and gender [40]. Patients of the male gender, younger age with individual nodules, have a higher risk of developing DTC. Therefore, the age and gender of a patient should be considered when DTC is suspected in patients with thyroid nodules [41]. However, according to age and gender, the incidence of malignant (DTC) and benign (FA) lesions did not differ significantly in this study.

When it comes to clinical and bio-humoral characteristics, there is a lot of contradictory data in the literature. A physical examination may be limited by the patient's physical characteristics and the subjectivisms of the physician [42]. In this study, a statistically significant difference between the DTC and FA groups was observed only for consistency and elasticity of the thyroid nodules. Namely, nodules of firm consistency and fixed nodules were more common in the group of patients with malignant lesions (DTCs). These data are only partly in line with the other published data. According to these results, an increased risk of thyroid gland cancer include nodules that are larger than 4 cm (19.3% risk of malignancy), palpation with firm consistency, nodules that are fixed to the surrounding tissues, lymphadenopathy of the neck and voice change (hoarseness) [43].

In this study, Tg levels were significantly higher in the group of patients with DTC, which is in correlation with the already published data. A preoperative interpretation of Tg level is often a complex task as they can be elevated in both benign and malignant thyroid diseases. Therefore, Tg use as a diagnostic marker for thyroid gland carcinoma is quite limited [44]. Tg is considered a good marker for follow-up monitoring after surgery and ablative therapy with radioactive iodine (RAI) of well differentiated follicular cell carcinoma. When Tg is not detected in the serum, it can be assumed that the disease is no longer present [45]. So, Tg has a great predictive value in the postoperative period after the RAI ablative therapy. Still, there are postoperative recurrences of diseases that do not go with an increased serum levels of Tg. Some autors [46] pointed out that the preoperative determination of serum Tg is important for identifying patients with a possible relapse of the disease in which there is no higher levels of Tg. Although the results of our research do not show a statistically significant difference between the DTC and FA

groups for TSH, fT4, anti-TPOAb, and anti-TGAb values, there are papers with opposite claims. McLeod et al. [46] have concluded that a higher TSH serum concentration is associated with a higher incidence of cancer in existing thyroid gland nodules. Boelaert et al.[45] noted that with the increasing TSH levels, the risk of malignancy in thyroid nodules increases. Jonklaas et al. [44] suggested that TSH suppression in high-risk patients would affect the reduction of mortality. TSH is an important factor that affects the growth of the thyroid gland and may have a clinical application in the future for the diagnosis of thyroid cancer.

Ultrasound examination is significant in assessing the nature of the nodules, determining their number and size, assessing the existence of lymphadenopathy, but also for ultrasound-guided aspiration biopsies [47–49]. Ultrasound characteristics of malignant nodules include their hypoechogenicity, irregular edges, microcalcifications, intranodal hypervascularity and regional lymphadenopathy. The combination of these characteristics can have a high malignancy prediction value [50, 51]. The characteristics which indicate the presence of benign nodules are completely cystic nodes (< 2% of all nodus), microcystic nodules with over 50% of cystic nodules volume (99.7% benign nature) [52]. The ultrasound characteristics of nodules in this study had a different degree of significance. Namely, a significant difference between the DTC and FA groups was registered only for the presence or absence of calcifications and the nodules appearances. The solid nodules and calcifications were significantly more frequent in the group of patients with malignant lesions (DTC). The sizes of nodules and the frequency of the marginal calcifications did not differ significantly between the two groups. Cappelli et al. [56] pointed out that malignancy is associated with a higher nodules size (especially 30 mm and larger nodes). Frates et al. [53] and Moon et al. [57] claimed that the size of nodules is not a useful category for distinguishing the malignant from benign nodules. In some studies, microcalcifications, but also rough, marginal calcifications, were designated as clear malignant predictors [58]. In other studies, the significance of microcalcification was noted [59] while the correlation of malignancy with rough and marginal calcifications is still a matter of debate. Ramundo et al. [54] have shown that there is a significantly higher risk of thyroid carcinoma in nodules located in the central part of the lobes of the thyroid gland, especially in cases of solid nodules. It was concluded that the nodules of such localization are an independent risk factor for malignancy, regardless of the ultrasound characteristics [53–55].

In our study, nodules localization had no predictive value. Nodules were most often localized in the central part of the thyroid gland (medially). The results of many studies suggested that hypoechogenic nodules and the ones with irregular edges are much more frequent in malignant lesions, i.e. they are considered independent predictors for malignancy of the nodules [54]. However, several studies showed the opposite results. In this study, the irregular edges were more frequent in the patients with DTC, although the difference was not statistically significant. The presence of hypoechogenic halo was also more common in the DTC group, but the difference was not significant statistically. The homogeneity and echogenicity of the nodules did not differ significantly between the two examined groups. Many studies have found that increased blood flow in nodules was a malignancy prediction [55–57], while some consider vascularization levelas a non-specific factor for it. Here, we found that an increased vascularization was more frequent in the group of patients with DTC, but the difference was significant statistically, probably due to lower number of patients involved.

Using the elastography method, qualitative and quantitative resistance of the nodules and the surrounding tissues was evaluated. We used the Fukunari sore [35]. Malignant nodules had a smaller score (3–4), while benign nodules had a greater elasticity (score 1–2). The same score system was used by Wang et al. [60], while the Ueno classification was used by Ciledag et al. [61] and Itoh et al. [62]. Our results indicated that significantly more frequent elastography score 2 and 3 were present in the FA group, while the elastography score 4 was only present in the group of patients with malignant lesions (DTC). The sensitivity of the Fukunara score 4 was 68% and the specificity was 100% for the diagnosis of DTC. In the paper of Rago et al. [63] it was shown that the elastography score 4 has a high predictive value for malignancy, with a sensitivity of 97% and a specificity of 100%, and that the method has a high potential for diagnosing thyroid cancer. Cantisani et al. [64] have shown that the sensitivity and specificity of the elastography are 94% and 81%, respectively, and the accuracy of the method is 83.7%, meaning that it is a promising technique for the detection of malignant thyroid nodules, especially in combination with echosonography. Rago et al. [63] showed that elastography score 1 had a high predictability for benign nodules, as it could be found in 102 of total 111 benign nodules, and only in one of 31 cases of DTC ($p < 0.0001$). It has also been shown that ultrasonic elastography is useful for the selection of patients for surgical intervention, especially in those with unclear cytological appearance [63]. Additionally, it was shown that elastography, especially when performed by the experts, is excellent for detection of malignant changes in the thyroid gland if combined with echosonography [67, 68].

In our study the elasticity score (SR > 2.5) was present in almost all patients in the FA and DTC groups, and there was no statistically significant difference between the two groups. In 12 patients with the SR > 2.5, it was not shown that the method was sufficiently sensitive or specific for the diagnosis of DTC. The SR ≥ 4.35 had 92% sensitivity and 70% specificity for the diagnosis of DTC in both patient groups. Unlike elastographic scoring, the determination of the SR index calculated by software provided quantitative measures of elasticity, therefore more reliable information [18–21]. In our study, the SR values ≥ 4.35 were thus highly specific for making the diagnosis of DTC. Lyshchik et al. [20] have suggested that SR ≥ 4.0 is a strong predictor of nodules malignancy, with a sensitivity of 82% and a specificity of 96%. Kagoya et al. [66] used the SR > 1.5 as a sign of nodules malignancy, with a sensitivity of 90% and a specificity of 50% [65]. When performing this technique, it should be taken into account that the depth of the tissue, in which the elasticity between the nodule and the

normal tissue is compared, is the same or at least similar, as well as that the elasticity estimation is performed on the longitudinal display of the thyroid gland, because on that occasion, a sufficiently large part of normal tissue is used for comparison and calculation of SR index [66]. Rago et al. [21] have shown that the size of the nodules does not affect the SR index and the predictability of elastography. However, other researchers suggested that the size of the nodule can affect this index, so they included the nodules which were up to a maximum size of 3 cm [61]. Other studies included all nodules up to 4 cm [64–66]. In our study we included all nodules up to a maximum of 3 cm. For nodules larger than 3 cm, the pressure applied during elastography cannot be the same in all parts of the nodule so the results of the SR index would be inadequate. For now, there is no reliable information on what the minimum size of the nodule should be before this kind of measurement. Some studies have suggested that when performing this method, one has to take into consideration the position of the carotid artery, since the pacing of the carotid artery may impair the proper interpretation of the elastography image [60, 61]. Our study did not include patients whose nodules were close to the carotid artery, and the method was performed by external compression.

Conclusion

Detailed clinical, biohumoral, echosonography, scintigraphy and cytological examinations are often not sufficient for a safe assessment and a clear indication for definitive surgical treatment of differentiated malignant thyroid tumors, which imposes the need for more precise preoperative evaluation of these patients. On the other hand, there is a constant need to reduce the number of operated on benign lesions. Here we showed that ultrasound elastography represents a new non-invasive method that could have an important role in the detection of thyroid carcinoma, especially in association with findings of other diagnostic procedures. By combining only a few echosonography and elastography characteristics, the sensitivity and specificity of the findings are significantly increased. The presence of at least one of them (solid tissue appearance, presence of calcificates, the Fukunara score ≥ 4, the strain ratio ≥ 4.35) has sensitivity of 100% and specificity of 43.8% for the diagnosis of DTC. The presence of at least two of the listed has 96% sensitivity and 87.9% specificity. The presence of 3 and more of the listed characteristics has 100% sensitivity and 89.2% specificity.

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Significance of myeloid-derived suppressor cells (MDSCs) like CD14+B7-H4 cells frequency in blood and tumor microcirculation of lung cancer patients

Značaj učestalosti populacije CD14$^+$B7-H4$^+$ ćelija koje odgovaraju mijeloidnim supresivnim ćelijama (MDSC) u krvi i tumorskoj mikrocirkulaciji bolesnika sa karcinomom pluća

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Abstract

Background/Aim. Myeloid-derived suppressor cells (MDSCs) suppress immune responses *via* a series of inhibitory mechanisms, which ultimately could lead to tumor growth. B7-H4 expression is significantly associated with poor outcome and promotion of tumor cell proliferation, invasion and migration in patients with various cancers. Data concerning B7-H4 expression in lung cancers (LC), either on tumor or immunological cells, are still sporadic. The aim was to estimate and correlate the number of CD14$^+$B7-H4$^+$MDSCs in blood and lung tumor microcirculation with clinical stage, histology type of tumor, tumor node metastasis (TNM) stadium, nodal status and disease outspread. **Methods.** The study included 44 lung cancer patients (III and IV clinical stage) and 30 healthy controls. CD14$^+$B7-H4$^+$ MDSC number was estimated by flow cytometry in blood and tumor microcirculation samples of each patient. **Results.** CD14$^+$B7-H4$^+$ MDSCs number was significantly higher in patients' samples compared to controls. CD14$^+$B7-H4$^+$ MDSC number was significantly increased in tumor compared to blood sample of the same patient. Clinical stage III patients had the increased number of the CD14$^+$B7-H4$^+$ MDSC compared to stage IV, in both types of samples. According to histology, small cell lung cancer (SCLC) patients had the highest average CD14$^+$B7-H4$^+$ MDSCs number, significantly increased compared to patients with squamous and large cell LC histology type. Tumor size was directly associated with the number of the CD14$^+$B7-H4$^+$ MDSC, both in blood and tumor samples. Furthermore, nodal involvement was associated with the gradual increase of the CD14$^+$B7-H4$^+$ MDSC number, being the highest in the N3 group, again both in blood and tumor samples. Finally, we detected higher CD14$^+$B7-H4$^+$ MDSCs number in the samples of patients without metastases. **Conclusion.** CD14$^+$B7-H4$^+$ MDSCs number in LC patients is significantly associated with tumor histology type, lymph node involvement, disease extent degree and tumor size. Concerning their large number in LC tumor microenvironment together with immunosuppressive capacities, CD14$^+$B7-H4$^+$ MDSCs could represent important tumor promoting factor in LC pathophysiology.

Key words:
lung neoplasms; myeloid-derived suppressor cells; immunologic factors; neoplasm metastasis; flow cytometry; histology.

Apstrakt

Uvod/Cilj. Mijeloidne supresorske ćelije [*myeolid derived suppressor cells* (MDSCs)] negativno regulišu imunski odgovor nizom inhibitornih mehanizama koji konačno omogućavaju rast tumora. Ispoljavanje B7-H4 je značajno povezano sa lošim ishodom, kao i promocijom proliferacije, invazije i migracije ćelija tumora kod bolesnika sa različitim tipovima

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karcinoma. Podaci koji prikazuju ispoljavanje B7-H4 u tumorima pluća, na tumorskim ili imunskim ćelijama, su i dalje retki. Cilj rada bio je utvrditi i korelirati zastupljenost $CD14^+B7\text{-}H4^+$ ćelija sličnih MDSCs ($CD14^+B7\text{-}H4^+$ MDSCs) u krvi i mikrocirkulaciji tumora pluća sa kliničkim stadijumom, histološkim tipom tumora, tumor nodus metastaza (TNM) stadijumom, nodalnim statusom i raširenošću bolesti. **Metode.** U studiju je bilo uključeno 44 bolesnika sa karcinomom pluća (III i IV klinički stadijum) i 30 zdravih osoba. Zastupljenost (%) $CD14^+B7\text{-}H4^+$ MDSCs je bila utvrđena protočnom citometrijom u krvi i mikrocirkulaciji tumora svakog bolesnika. **Rezultati.** Zastupljenost $CD14^+B7\text{-}H4^+$ MDSCs bila je značajno veća u uzorcima bolesnika u odnosu na kontrole. $CD14^+B7\text{-}H4^+$ MDSCs u uzorku tumora su bile značajno brojnije u odnosu na uzorak krvi istog bolesnika. Bolesnici III kliničkog stadijuma imali su povećane vrednosti $CD14^+B7\text{-}H4^+$ MDSCs u odnosu na one u IV stadijumu, u obe vrste uzoraka. Prema histološkom tipu, bolesnici sa sitnoćelijskim karcinomom pluća imali su najveće vrednosti $CD14^+B7\text{-}H4^+$ MDSCs, značajno povećane u odnosu na bolesnike sa skvamoznim ili giganto-ćelijskim tipom tumora. Veličina tumora bila je direktno udružena sa brojem $CD14^+B7\text{-}H4^+$ MDSCs, u obe vrste uzoraka. Zahvaćenost limfnih žlezda bila je udružena sa postepenim povećanjem vrednosti $CD14^+B7\text{-}H4^+$ MDSCs, sa najvećim vrednostima u N3 grupi, u obe vrste uzoraka. Na kraju, detektovali smo veće vrednosti $CD14^+B7\text{-}H4^+$MDSCs u uzorcima bolesnika bez metastaza. **Zaključak.** Vrednosti $CD14^+B7\text{-}H4^+$ MDSCs kod bolesnika sa karcinomom pluća značajno su povezane sa histološkim tipom tumora, zahvatanjem limfnih čvorova, stepenom raširenosti bolesti i veličinom tumora. S obzirom na visoke vrednosti u mikrookruženju tumora pluća zajedno sa njihovim imunosupresivnim kapacitetima, $CD14^+B7\text{-}H4^+$ MDSCs mogu predstavljati važan promovišući faktor u patofiziologiji karcinoma pluća.

Ključne reči:
pluća, neoplazme; ćelije supresori; imunološki faktori; neoplazme, metastaze; citometrija, protočna; histologija.

Introduction

Lung cancer (LC) is the most common carcinoma in men while in women it is the fourth most common malignancy, and the second by lethal outcome [1]. At the moment of diagnosis, more than 50% of patients are in a locally advanced stage or have distant metastases, with poor five-year survival even in patients with localized disease.

Focus of contemporary immunotherapy is tumor-mediated immune suppression and modulation of tumor specific T lymphocyte activity by acting on checkpoint immune inhibitors. Beside programmed death (PD-1), PD-1 ligand and CTLA-4, new data describe other members of B7 accessory molecules that critically regulate activation or suppression of T lymphocytes [2]. Among them, B7-H4 (B7S1, B7x, Vtcn1) is a strong inhibitor of T cell activity [3]. Investigation of B7-H4 mRNA demonstrated broad presence in human non-lymphoid tissues. Expression of B7-H4 is documented in various solid malignant tumors, and its presence on tumor cells is associated with the increased rate of proliferation, metastasis and unfavorable outcome in patients with kidney cancer, oral and esophageal squamous carcinoma, gastric and LC [2]. Sica et al. [3] and Prasad et al. [4] were among first that demonstrated that B7-H4 expression is not restricted to tumor cells. They described that *in vitro* stimulation induced B7-H4 expression on population of T lymphocytes, majority of B lymphocytes and monocytes/macrophages. Macrophages isolated from ovarian cancer or from ascites of patients with ovarian cancer demonstrated significant expression of B7-H4 and potently inhibited *in vitro* T lymphocyte activation [5]. Furthermore, B7-H4 expression and suppressive capacity could be stimulated on macrophages after incubation with interleukin (IL)-10 and IL-6 [6]. Recently, these suppressive population of B7-H4 macrophages have been demonstrated in patients with glioma [7]. Li et al. [8] investigated possible mechanisms involved in generation of exhausted $CD8^+$ tumor infiltrating T lymphocytes in patients with hepatocellular carcinoma. They showed that the expression of B7-H4 on myeloid cells in tumor is in direct correlation with inhibition of $CD8^+$ T lymphocytes activity.

Myeloid-derived suppressive cells (MDSCs) represent significant force that supports tumor in survival, proliferation and metastasis. Experimental models strongly support significant role of MDSCs in immunosuppressive balance that favors survival and growth of lung tumors [9]. Inefficiency of recent trials with inhibitors of immune checkpoint in patients with non-small cell lung carcinoma (NSC-LC) is explained to be a consequence of significant immunosuppressive state caused by MDSCs activity [10]. Patients with NSC-LC demonstrate high numbers of both monocytic and polymorphonuclear MDSCs in peripheral circulation and tumor tissue [11]. MDSCs number directly correlate with inflammatory cytokines concentration, both locally and systemically, and the majority of MDSCs express PD-L1 as phenotypic marker of high immunosuppressive capacity. Yamauchi et al. [11] demonstrated that frequency of MDSCs is significantly correlated with the disease course and outcome in their cohort of NSC-LC patients. PD-L1 is not the only immunosuppressive molecule detected on MDSCs in LC patients. Zhang et al. [12] identified two populations of MDSCs in tumor tissue of NSC-LC patients according to the presence of inhibitory B7-H3 molecule. Monocyte like MDSCs population ($HLA\text{-}DR^{-/low}$, $CD14^+$MDSC) that expresses B7-H3 is highly immunosuppressive, produces significant amounts of IL-10 and tumor necrosis factor (TNF)-α and the increase of their number is significantly associated with short disease free interval. Considering all these data, in our study we wanted to investigate MDSCs population that express B7-H4 in LC patients, another inhibitory molecule, and to analyze association of their value with pathological and clinical parameters.

Methods

Patients

The study enrolled 44 patients with diagnosed LC (33 males 11 females, 62 ± 8 years) and 30 healthy controls (22 males, 8 females, 57 ± 14 years). Patients were diagnosed and treated at the Clinic for Pulmonology, Military Medical Academy in Belgrade, Serbia, in 18-month-long period. All necessary diagnostic procedures (laboratory, radiological, bronchoscopy and histological) were carried out at the Military Medical Academy in Belgrade, Serbia. All the patients as well as healthy controls signed the Informed consent for participation in the research. This study was approved by the Ethics Committee of the Military Medical Academy in Belgrade, Serbia (12-02/2015).

Samples

Blood samples were taken from the cubital vein upon admission to the hospital, while tumor microcirculation samples were taken by needle biopsy from available pathological tumor blood vessels in the course of diagnostic bronchoscopy. Samples were taken in vacutainer tubes with K_2EDTA and erythrocytes removed using the lysing buffer (NH_4Cl, EDTA, $KHCO_3$) for 10 min with constant mixing. The remaining nucleated cells were washed two times with the RPMI 1640 culture medium complemented with 5% of normal human serum, centrifuged, resuspended, enumerated (Beckman Coulter ACT differ blood counter) and concentration was corrected to final suspension of 1×10^6 cells/100 µL *per* test tube.

Cells immunophenotyping

Final cell suspensions were stained with coctail of monoclonal antibodies, as Stanojević et al. [13] did in the previous study. Multicolor analysis was performed with different combinations of CD15-FITC or PECy7, CD33-PE or PECy7, CD45-ECD or PECy5, HLA-DR PE/Cy5, CD14-FITC, CD16-PE, CD11b-PE, CD10-PECy7, CD3-FITC, CD19-FITC, CD56-FITC, B7-H4-PECy7 (Biolegend, USA). The flow cytometry was performed using a Beckman Coulter FC 500 flow cytometer with CXP analysis software. MDSCs subpopulation was identified from the initial $CD45^+$/Side Scatter cell population, which was negative for T, B and NK antigens. This triple negative population of every sample was further gated on a CD11b versus HLA-DR dot plot histograme, and MDSCs were analyzed as lineage triple negative ($CD3^-$, $CD19^-$, $CD56^-$), $CD45^+$, HLA-$DR^{-/low}$, $CD11b^+$ and $CD33^{low}$ population. After further classification of this population according to the expression of CD15 or CD14, $CD14^+$ MDSCs population was further investigated for B7-H4 expression. The value of MDSCs $CD14^+$ B7-$H4^+$ cell population was expressed as % of all $CD45^+$ analyzed cells, as we did in our previous work [13].

Statistical analysis

Data analysis was performed using the GraphPad Prism 5 software. Comparison between multiple groups was done with nonparametric Kruskal–Wallis test, while identification of differences was performed with Dunn`s multiple comparison test. Difference between average values of two investigated groups was analysed with Mann Whitney (MW) test, while statistical significance of serial samples (blood/tumor microcirculation) of patients was analysed with Wilcoxon (W) matched pairs test.

Results

Lung cancer patients had significantly higher number of the $CD14^+$ B7-$H4^+$MDSCs than controls

A statistically significant number of $CD14^+$B7-$H4^+$ MDSCs was detected in samples of peripheral blood of LC patients as well as in tumor microcirculation compared to peripheral blood samples of control patients ($p = 0.0417$, MW test). Generally, all patients (excluding 3 that had values as healthy controls) demonstrated significantly higher number of $CD14^+$B7-$H4^+$ MDSCs in tumor microcirculation samples compared to their blood values, either after comparison of average number ($p = 0.0002$, MW test) or when assessing particular tumor/blood samples of each patient ($p = 0.0000$, W test) (Table 1).

Clinical stage III patients had insignificantly increased the number of the $CD14^+B7$-$H4^+$ MDSCs compared to clinical stage IV patients

Comparison of patients between the III and the IV clinical stage demonstrated no significant difference, either in blood or tumor microcirculation samples (Table 1). Again, all patients, in both clinical stages had significantly more of the $CD14^+$B7-$H4^+$ MDSCs in their correspondive tumor microcirculation than blood samples (Table 2).

Patients with small cell LC histology demonstrated the highest average CD14+ B7-H4+MDSC values in tumor microcirculation samples

Stratification of patients in groups related to histological type of the tumor revealed differences in $CD14^+$B7-$H4^+$ MDSCs number according to different histology. Although all groups had more $CD14^+$B7-$H4^+$ MDSCs in their tumor compartment compared to blood, these differences reached statistical significance for small cell LC, adenocarcinoma and squamous LC, but not for patients with large cell LC (Table 2, W test). Furthermore, analysis of the average $CD14^+$B7-$H4^+$ MDSCs number in tumor/blood compartment demonstrated significant increase only in tumor microcirculation of small cell LC and squamous cell LC groups (Table 2, MW test). Comparison between groups of patients with different lung cancer histology type in blood samples revealed no statistical significance, although, again, almost all patients had values higher than normal controls. But, analysis of tumor microcirculation

samples demonstrated that patients with small cell LC had the highest average $CD14^+B7\text{-}H4^+$ MDSC number, significantly increased compared to patients with squamous and large cell LC histology (Table 3, MW test).

Smallest tumors are significantly associated with the lowest $CD14^+B7\text{-}H4^+$ MDSC number

Blood samples of the LC patients with the smallest tumors (T1) demonstrated significantly low $CD14^+B7\text{-}H4^+$ MDSCs value, close to the number of healthy controls (Table 1). The increment of the tumor was significantly associated with the increase of $CD14^+B7\text{-}H4^+$ MDSCs number, compared to this group (T2 > T1, T3 > T1, T4 > T1, Table 3, blood, MW test). Interestingly, patients with T3 tumors had significantly increased number of the $CD14^+B7\text{-}H4^+$ MDSCs compared to those with the largest tumors (T3 > T4, Table 3, MW test). Analysis of tumor microcirculation compartment demonstrated almost the same relations as in blood, meaning that the patients with

Table 1

Presentation of $CD14^+B7\text{-}H4^+$ MDSCs in blood and tumor microcirculation samples of lung cancer (LC) patients according to clinical status and tumor characteristics

Parameter	MDSCs (% of total $CD45^+$ cells), mean ± SD	
	blood	tumor microcirculation
Clinical stage		
III (n = 27)	5.96 ±5.42	26.93 ± 22.03
IV (n = 17)	5.38 ± 4.72	23.94 ± 21.28
Histology type of LC		
Ad NSCLC (n = 13)	4.40 ± 4.88	24.10 ± 30.05
Sq NSCLC (n = 11)	7.50 ± 5.63	26.83 ± 20.31
Lc NSCLC (n = 10)	6.00 ± 5.15	9.40 ± 9.86
SCLC (n = 10)	6.10 ± 5.78	41.56 ± 9.45
Tumor size		
T1 (n = 11)	1.46 ± 1.37	6.82 ± 7.14
T2 (n = 13)	7.07 ± 5.44	30.43 ± 23.20
T3 (n = 13)	9.29 ± 6.15	28.57 ± 22.62
T4 (n = 7)	4.38 ± 2.45	27.00 ± 16.83
Nodal status		
N0 (n = 11)	3.00 ± 2.68	7.55 ± 7.83
N1 (n = 10)	4.50 ± 2.64	14.50 ± 13.79
N2 (n = 13)	7.15 ± 5.98	29.54 ± 21.62
N3 (n = 10)	14.67 ± 4.39	33.44 ± 16.27
Metastases		
M0 (n = 27)	27	20.43 ± 11.63
M1 (n = 17)	17	17.50 ± 3.97

***Note*: MDSCs value in blood samples of the control group (n = 30) was 1.04 ± 0.32. MDSCs – myeloid-derived suppressive cells; NSCLC – non small cell lung carcinoma; Ad – adenocarcinoma; Sq – squamose; Lc – large cell; SCLC– small cell lung carcinoma.**

Table 2

Comparison of $CD14^+B7\text{-}H4^+$ MDSCs presentation between blood and tumor microcirculation compartments in lung cancer (LC) patients according to clinical status and timor characteristics

Parameter	Compartment		Statistical tests	
	blood	tumor microcirculation	Mann Whitney	Wilcoxon
Clinical stage	III	III	$p = 0.0027$	$p = 0.0000$
	IV	IV	$p = 0.0122$	$p = 0.0007$
Histology type	SCLC	SCLC	$p = 0.0003$	$p = 0.0088$
	Ad NSCL	Ad NSCLC	ns	$p = 0.0213$
	Sq NSCLC	Sq NSCLC	$p = 0.0025$	$p = 0.0005$
	Lc NSCLC	LC NSCLC	ns	ns
Tumor size	T1	T1	$p = 0.0238$	$p = 0.0089$
	T2	T2	$p = 0.0011$	$p = 0.0038$
	T3	T3	$p = 0.0049$	$p = 0.0037$
	T4	T4	$p = 0.0021$	$p = 0.0156$
Nodal status	N0	N0	$p = 0.0362$	ns
	N1	N1	$p = 0.0141$	$p = 0.0112$
	N2	N2	$p = 0.0037$	$p = 0.0126$
	N3	N3	$p = 0.0091$	$p = 0.0017$
Metastases	M0	M0	ns	$p = 0.0028$
	M1	M1	$p = 0.0000$	$p = 0.0025$

For abbreviations see under Table 1.

the smallest tumors had significantly less $CD14^+B7\text{-}H4^+$ MDSCs compared to all other groups (T2 > T1, T3 > T1, T4 > T1, Table 3, tumor microcirculation, MW test).

N3 nodal status was associated with the highest $CD14^+B7\text{-}H4^+$ MDSCs values

Patients without nodal involvement demonstrated the lowest number of $CD14^+B7\text{-}H4^+$ MDSCs both in the blood and tumor microcirculation compartment. Furthermore, nodal involvement was associated with the gradual increase of the $CD14^+B7\text{-}H4^+$ MDSCs number, being the highest in the N3 group (Table 1). There was a significant increase in tumor microcirculation number of the $CD14^+B7\text{-}H4^+$ MDSCs compared to that in the blood, either when analysed as a group (Table 2, MW test) or in particular tumor/blood samples of each patient (Table 2, W test). Analysis among groups with different nodal involvement demonstrated that N3 group had significantly more $CD14^+B7\text{-}H4^+$ MDSCs than any other group, either in blood or tumor (N3 > N0, N3 > N1, N3 > N2) (Table 3). Additionally, the N2 group also demonstrated a significant increase of the $CD14^+B7\text{-}H4^+$ MDSCs number compared to the N0 group, both in blood and tumor microcirculation (N2 > N0) (Table 3).

Higher $CD14^+B7\text{-}H4^+$ MDSCs values detected in the group M0

Although we detected higher average $CD14^+B7\text{-}H4^+$ MDSCs value in the M0 group (Table 1), a comparison of patients between the M0 and the M1 group demonstrated no significant difference, either in blood or tumor microcirculation samples (Table 3). Comparison of blood/tumor average $CD14^+B7\text{-}H4^+$ MDSCs values demonstrated only significant differences in the M1 group (Table 2 MW test). Analysis of particular tumor/blood samples of each patient demonstrated significant increase of the $CD14^+B7\text{-}H4^+$ MDSCs number in tumor microcirculation (Table 2, W test).

Discussion

Expression of immunosuppressive B7-H4 molecule was widely demonstrated in samples of tumor tissue from patients with gynecological malignancies (ovary, uterus), as well as in patients suffering from colon and pancreas carcinoma [14, 15]. In physiological condition, B7-H4 is absent from the surface of normal cells [16]. Beside malignant cells, B7-H4 is extensively detected on the surface of tumor infiltrating macrophages with up to 2/3 of the tumor ascites

Table 3

Statistical analysis of difference between groups of patients according to clinical stage and tumor characteristics (Mann Whitney test)

Group	vs.	Group	Blood	Tumor
Histology type				
SCLC	/	Ad NSCLC	ns	ns
SCLC	/	Sq NSCLC	ns	0.0180
SCLC	/	LC NSCLC	ns	0.0010
Ad NSCLC	/	Sq NSCLC	ns	ns
Ad NSCLC	/	LC NSCLC	ns	ns
Sq NSCLC	/	LC NS-LC	ns	ns
Tumor size				
T1	/	T2	0.0029	0.0036
T1	/	T3	0.0004	0.0056
T1	/	T4	0.0040	0.0023
T2	/	T3	ns	ns
T2	/	T4	ns	ns
T3	/	T4	0.0442	ns
Nodal status				
N0	/	N1	ns	ns
N0	/	N2	0.0452	0.0042
N0	/	N3	0.0000	0.0002
N1	/	N2	ns	ns
N1	/	N3	0.0000	0.0137
N2	/	N3	0.0044	ns
Metastases				
M0	/	M1	ns	ns
Clinical stage				
III	/	IV	ns	ns

ns – no significant.
For other abbreviations see under Table 1.

$CD14^+$ macrophages being also B7-$H4^+$ [17, 18].

More than 10 years ago Ilona Kreyczek group demonstrated the importance of B7-$H4^+$ macrophages in human ovarian carcinoma [5, 6]. They investigated $CD14^+$ monocytes, B7-$H4^-$ and B7-$H4^+$ macrophages as well as regulatory T lymphocytes isolated from fresh tumor specimens, tumor induced ascites and blood of 103 patients with ovarian carcinoma. Although tumor cells also expressed B7-H4 intracellularly, only tumor infiltrating macrophages demonstrated surface B7-$H4^+$ expression. This B7-$H4^+$ expression was highly inducible, since it was possible to transform peripheral blood monocytes with tumor ascites or addition of IL-6 and IL-10. Interestingly, IL-4 and granulocyte-macrophage colony-stimaluting factors (GM-CSF) negatively regulate *ex vivo* and *in vitro* B7-$H4^+$ expression on macrophages. Authors initially concluded that the change of local factors concentration, with high IL-6 and IL-10, and low IL-4 and GM-CSF resulted in the transformation of tumor-associated macrophages toward B7-$H4^+$ cells. More importantly, Kreyczek et al. [18] further demonstrated that B7-$H4^+$ macrophages suppressed *in vitro* activity of T lymphocytes specific for HER2/Neu antigen, in a way independent of B7H1 mechanism, arginase or iNOS activity. Considering a high number of B7-$H4^+$ macrophages detected in tumor-associated ascites, which largely outnumbered regulatory T lymphocytes (30% vs. 5%), authors concluded that these cells could be principal immunosuppressive force, resulting in tumor promotion.

Matsunaga et al. [19] investigated association of blood and tumor $CD14^+B7H1^+$ or $CD14^+$B7-$H4^+$ cells with clinical and tumor characteristics in the patients with gastric cancer. Firstly, they demonstrated that level of $B7H1^+$ or B7-$H4^+$ expression is significantly increased on monocytes from gastric cancer patients, compared to healthy controls. They also found that tumor isolated monocytes expressed significantly more $B7H1^+$ or B7-$H4^+$ compared to blood monocytes from the same patient, and that the expression of these suppressive molecules is directly correlated. Contrary to our study, the expression of B7-$H4^+$ directly followed HLA-DR expression level on $CD14^+$ cells. Differences come from different gating strategies, since our goal was to investigate MDSCs which are HLA-$DR^{-/low}$ by definition. Anyway, these authors demonstrated significant immunosuppressive capacity of $CD14^+$ B7-$H4^+$ cells *in vitro* (reduction of interferon (IFN)-γ secretion by T lymphocytes), and also demonstrated that surgical removal of tumor resulted in decreased B7-$H4^+$ on circulating $CD14^+$ cells. They demonstrated differences in blood and tumor $CD14^+B7H1^+$ or $CD14^+$B7-$H4^+$ number according to histopathology type of gastric cancer, invasion depth, tumor size, node involvement, clinical stage and a level of lymphovascular invasion. Their data is in accordance with the data obtained by our study, indicating that the tumor size and the degree of the disease spread is directly associated with the number of suppressive $CD14^+$B7-$H4^+$ number. Interestingly, their patients in the earlier clinical stage also demonstrated insignificantly higher $CD14^+$B7-$H4^+$ number compared to later stages.

Data concerning MDSCs role in patients with lung cancer are still limited, especially investigations of B7-$H4^+$ MDSCs.

Investigation of prognostic B7-H4 value in patients with NSC-LC conducted as a meta-study by Tan and Shen [20] indicated significant association of B7-H4 overexpression with tumor size, node involvement and the presence of metastasis, but without the impact of tumor histology and other epidemiological factors. They concluded that B7-H4 expression is a negative prognostic factor for NSC-LC patients. Unfortunately, their study included one big imprecision, since in their selection criteria (criteria Nº 2) they included studies with B7-H4 expression detected with any method, which implies that they did not differentiate expression on malignant tissue or tissue infiltrating leukocytes.

Chen et al. [21] investigated $CD14^+$HLA-$DR^{-/low}$ MDSCs frequency in blood samples of almost 80 patients with squamous type of LC (Sq NSC-LC). They also demonstrated that LC patients had significanlty increased number of these cells compared to healthy controls, and that MDSCs number gradually rise in those patients with higher TNM stage. In favor of immunosuppressive MDSCs impact, they showed significantly reduced number of blood $CD4^+$ T and $CD8^+$ T lymphocytes in patient samples, as well as the impairment of T lymphocyte cytokine secretion *in vitro*.

Dendritic cells (DC) isolated from resected tumor tissue of NSC-LC patients express other immunosupressive molecules, also from B7 family, as B7H3 [22]. Those DC demonstrated severely impaired costimulatory activity towards autologous T lymphocytes, produced significantly more IL-10 and less IL-12 than controls.

Huang et al. [23] demonstrated that circulating $CD14^+$HLA-$DR^{-/low}$ MDSC are modulators of antitumor immune response and were associated with tumor metastasis and impaired response to treatment in NSCLC patients [23]. They evaluated 89 patients with advanced NSCLC. The ratio of the $CD14^+$HLA-$DR^{-/low}$ MDSCs, as a percent of total $CD14^+$ cells, was significantly higher in NSCLC patients compared to healthy controls, and it was proportional to clinical stage. Monocytic MDSCs also significantly negatively correlated with median progression-free survival ($p < 0.01$). Both the absolute number and percentage of $CD14^+$HLA-$DR^{-/low}$ cells were increased in NSCLC patients with metastasis, confirming their role in the disease progression. Huang et al. [23] also demonstrated that the role of $CD14^+$HLA-$DR^{-/low}$ cells in inhibition of T cell function of NSCLC patients was mediated by a functional NADPH oxidase, as shown by the expression of the oxidase component gp91phox and reactive oxygen species (ROS) production by these monocytic MDSCs.

Ex vivo study showed that circulating tumor cell lines from SCLC patients induced transformation of peripheral blood mononuclear cells (PBMNC) toward diferentiaton of monocytes in $CD14^+$ $CD163^{low}$ $CD68^+$ B7-$H4^+$ tumor associated macrophages [24]. Feng et al. [25] underlined the significance of MDSCs in patients with adenocarcinoma LC (Ad NSC-LC) positive for epidermal growth for receptor

(EGFR) mutation. They also showed that MDSCs number ($S100A^+CD68^+$) was increased in patient blood samples compared to healthy controls. Patients with a poor therapy response as well as patients with short progression free interval had increased MDSCs number compared to others. Again, as in our study, the number of MDSCs was much higher in tumor samples compared to matched blood ones. A recent study of blood MDSCs subpopulations in the NSC-LC patients demonstrated that they were increased in number compared to healthy controls, but also showed the increased number in a subgroup of COPD as second controls [11]. The same study showed that MDSCs were more frequent in resected tumor tissue than in blood samples of these NSC-LC patients. Data from that study demonstrated fine differences between LC with different histology. Patients with squamous type LC showed the increased number of granylocyte like MDSCs compared to the adenocarcinoma group, but without differences in monocyte like MDSCs. This was in concordance with our data, since we showed no significant differences in $CD14^+$ MDSCs frequency between NSC-LC patients, but only between small cell LC group versus others. On the other hand, Yamauchi et al. [11] showed that adenocarcinoma LC group had significantly more $CD14^+B7H1^+$ MDSCs and $CD15^+B7H1^+$ MDSCs compared to squamous cell LC patients. In our previous study we have demonstrated that different histology type of LC is significantly associated with particular cytokine profile, either in blood or tumor microcirculation samples [26].

In the study with limited number of NSC-LC patients, Pogoda et al. [27] demonstrated that $CD14^+HLA\text{-}DR^{-/low}$ MDSCs are not the only population that takes part in tumor induced immunosuppression. Beyond MDSCs, they showed that $CD14^+HLA\text{-}DR^+$ monocyte population secreted significant amounts of IL-10 in lymph node samples, and IL-1β and TNF in peripheral blood, lymph nodes and tumor tissue. Heuvers et al. [28] investigated MDSCs frequency in blood specimens of 185 NSC-LC patients demonstrating the increased number of MDSCs, especially granulocyte like, in LC patients compared to healthy controls. They also showed that the suppression capacity of MDSCs is significantly associated with arginase -1 activity.

The majority of published papers reflect investigations in NSC-LC patient population. In a study that involved 42 SC-LC patients, Tian et al. [29] demonstrated that the absolute number and frequency of blood $CD14^+HLA\text{-}DR^{-/low}$ MDSCs were significantly increased in SCLC patients compared with those in controls and that the MDSCs frequency correlated with tumor stage, serum lactate dehydrogenase (LDH) value and shorter overall survival. SCLC patients from our study demonstrated the highest average number of $CD14^+HLA\text{-}DR^{-/low}$ MDSCs, but in tumor microcirculation samples.

Conclusion

We demonstrated a significant association between the number of $CD14^+B7\text{-}H4^+$ MDSCs and the tumor size and lymph node involvement. We also showed that LCs of different histology dramatically differ in their capacity to induce $CD14^+B7\text{-}H4^+$ MDSCs number, which could be interpreted as different immunosuppression potential. We found that tumor microcirculation samples are easily available for analysis and more important than blood samples, offering more sensitive and informative data, more precisely reflecting the local balance between tumor and immune response.

Conflict of interest

Authors declare no conflict of interest.

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Risk factors for potential drug-drug interactions in a general neurology ward

Faktori rizika od potencijalnih interakcija između lekova kod bolesnika hospitalizovanih na neurološkom odeljenju

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Abstract

Bacground/Aim. Treatment of neurological diseases usually requires polypharmacy, and it is crucial to detect potential drug-drug interactions (DDIs) and recognize risk factors on time, as consequences of DDIs could be serious. The aim of the study was to analyze risk factors for the occurrence and the number of potential DDIs among patients in a general neurological ward. **Methods.** This study was conducted with 144 inpatients in a general-care neurological department of a tertiary care hospital. The effects of risk factors for potential DDIs were evaluated by multiple linear regression. The study had retrospective cohort design. Frequencies of various types of potential DDIs (according to severity) were discovered by Medscape, Epocrates and Micromedex online interaction checkers. **Results.** The number of prescribed drugs, age of a patient, value of the Charlson comorbidity index and prescription of an antidepressant increase risk of potential DDIs in a general neurology ward. On the other hand, being paralyzed, number of prescribers for a single patient, being bedridden for at least one day of hospitalization decreased the number of potential DDIs *per* patient. Number of prescribed drugs *per* patient [odds ratio (OR) = 1.466 ± 0.250; $p = 0.000$) and age (OR = 1.027 ± 0.026; $p = 0.041$)] increased, and number of prescribers *per* patient (OR = 0.056 ± 0.028; $p = 0.016$), especially if the patients were paralyzed (OR = 0.214 ± 0.294; $p = 0.007$), decreased the risk of contraindicated, serious, "use alternative" or major potential DDIs. Antidepressants increased the risk of absolute number of all monitor/modify potential DDIs (OR = 1.257 ± 0.726; $p = 0.035$). **Conclusion.** Frequency of potential DDIs among neurological patients is considerable and influenced to the largest extent by advanced age, comorbidities, total number of prescribed drugs *per* patient and concomitant use of antidepressants.

Key words:
nervous system, diseases; combination drug therapy; drugs, interactions; risk factors.

Apstrakt

Uvod/Cilj. Lečenje neuroloških bolesti obično zahteva polifarmaciju, pa je važno otkriti potencijalne interakcije između lekova i prepoznati rizik na vreme jer posledice po bolesnika mogu biti ozbiljne. Cilj ove studije bio je da analizira faktore rizika od pojave, kao i broj potencijalnih interakcija između lekova. **Metode.** U studiju su bila uključena 144 bolesnika hospitalizovana na Odeljenju opšte neurologije Kliničkog centra Kragujevac. Faktori rizika od interakcija ispitivani su multiplom linearnom regresijom. Studija je imala retrospektivni kohortni dizajn. Frakvencija različitih tipova interakcija bila je prepoznata uz pomoć internet proveravača interakcija (Medscape, Epocrates i Micromedex). **Rezultati.** Broj propisanih lekova, starost bolesnika, vrednost Charlsonove skale komorbiditeta i propisivanje antidepresiva povećavali su rizik od interakcija na Odeljenju neurologije. Sa druge strane, paralizovanost, broj lekara koji su propisivali lekove po bolesniku, vezanost za postelju na jedan dan hospitalizacije snižavala je verovatnoću za pojavu interakcija između lekova. Broj propisanih lekova po bolesnku [*odds ratio* (OR) = 1,466 ± 0,250; $p = 0,000$)] i starost bolesnika (OR = 1,027 ± 0,026; $p = 0,041$) su povećavali, a broj propisivača po bolesniku (OR = 0,056 ± 0,028; $p = 0,016$), posebno kod paralizovanih bolesnika (OR = 0,214 ± 0,294; $p = 0,007$), su smanjili rizik od kontraindikovanih, ozbiljnih, 'koristi alternativu' ili velikih potencijalnih interakcija. Primena antidepresiva povećavala je rizik od nastanka "prati/promeni" interakcija (OR = 1,257 ± 0,726; $p = 0.035$). **Zaključak.** Učestalost potencijalnih interakcija između lekova kod neuroloških bolesnika je značajna i povezana je sa godinama života bolesnika, komorbiditetima, brojem propisanih lekova po bolesniku i istovremenom upotrebom antidepresiva.

Ključne reči:
nervni sistem, bolesti; lečenje kombinovanjem lekova; lekovi, interakcije; faktori rizika.

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Introduction

Drug-drug interaction (DDI) could be defined as a change of a drug action when it is taken together with another drug/drugs, in terms of intensity of action, pharmacokinetic attributes or occurrence of adverse drug effects [1]. Early recognition of potential DDIs gives an opportunity to prevent them, which is significant not only from the healthcare provider′s but also from the patient′s point of view. Since polypharmacy is unavoidable in modern management of many diseases, creating an environment for the occurrence of DDIs, there is a growing concern that interactions will lead to the increased utilization of healthcare resources (e.g. outpatient visits, number and length of hospitalizations, etc.) within a healthcare system, accompanied with increased costs [2, 3]. It has already been shown that DDIs have a significant role in increased morbidity and mortality among hospitalized patients [4]. Discovery of potential DDIs is nowadays much easier with the use of online or offline interaction checkers which classify interactions according to severity, like Medscape [5], Epocrates [6] and Micromedex [7].

Drug-drug interactions on Medscape could be characterized as: contraindicated – which means that this combination of drugs should not be used due to high risk for dangerous interaction; serious – which indicates that this combination of drugs has potential for serious interaction and regular monitoring by doctor is required or alternate medication may be needed; then significant – which indicates that potential for significant interaction is high and monitoring by doctor is likely required, and minor – where interaction is unlikely, minor, or nonsignificant [8].

On Epocrates platform, DDIs are organized according to clinical management and involve different categories such as "Contraindicated," "Avoid/Use Alternative," "Monitor/Modify Therapy," and "Caution Advised". These categories are not intended to point the severity of proposed interactions, so all described interactions, even “Caution Advised” ones, may have serious clinical consequences and should not be dismissed categorically [9].

IBM Micromedex Complete Drug Interaction defines DDIs as: contraindicated – meaning drugs are contraindicated for concurrent use; major DDIs – indicating that interaction may be life-threatening and/or require medical intervention in order to minimize or prevent serious adverse reaction; moderate DDIs – implying that the exacerbation of the patient’s condition may be developed and the alteration of therapy is required; minor DDIs – where interactions would have limited clinical effects and generally not require a major alteration in therapy, and unknown DDIs. All described interactions are fully synopsized and referenced with excellent, good, fair or unknown level of documentation [10].

These online platforms encompass results regarding DDIs derived from different sources such as handbooks, textbooks, data from manufacturer and Internet sources. The main advantage of these checkers is their accessibility which enables doctor′s and pharmacist′s prompt reaction especially in the presence of harmful DDIs which decrease clinical outcomes or increase severity of patients′ status. In spite of these facts, interaction checkers differ in sensitivity or specificity, so, due to inconsistencies, health professionals should use more than one of them in practice. Also, some of these applications are free of charge, but some of them require payment which may limit their wide use [11].

The incidence of DDIs positively correlates with multiple, concurrent use of drugs and varies from 3–5%, if a patient takes a few drugs, to 20% in patients taking more than 10 drugs [12, 13]. Other risk factors which significantly contribute to the occurrence of DDIs are advanced age, comorbidities, weak coordination of healthcare for individual patients among health professionals of various specialties, non-adherence of patients, etc. [14]. Neurological patients are not an exception in terms of the occurrence of DDIs. The treatment of neurological diseases usually requires polypharmacy, and it is crucial to recognize risk factors, detect potential DDIs on time and prevent additional deterioration of health in these complex patients [15]. It has been shown recently that the advanced age of patients and the number of prescribed drugs are risk factors for the occurrence of DDIs in neurological patients [16]. Neurological diseases are among the most common reasons for hospitalization in modern society. A study in Italy showed that the prevalence of DDIs in a neurology ward was very similar to that in an internal medicine ward [16]. Dementia, for example, is a disease in expansion due to longer human life nowadays [17]. Also, there has been the result that nonvascular disease such as epilepsy increases the risk of DDIs in patients in a neurology ward [15]. It is known that antiepileptic drugs have a lot of behavioral side effects including depression, aberrant behaviors, and the development or worsening of irritability, impulsivity, anger, hostility, and aggression [18]. Neurological patients are frequently disabled, sometimes bedridden or out of control of their sphincters. When hospitalized, they frequently develop urinary or respiratory tract infections, requiring the prescription of antibiotics, which increases overall medication burden and predisposes to DDIs. All these give a certain specificity to DDIs problem in a neurology ward.

The aim of our study was to analyze risk factors for the occurrence and number of potential DDIs among the patients admitted to a general-care neurological department of a tertiary care hospital.

Methods

The study was approved by the Ethics Committee of Clinical Center Kragujevac (number of approval: N - 0 l /14886). Our study was a retrospective analysis of a patient cohort treated at the General Ward of the Clinic

for Neurological Disorders (GW-CND), Clinical Center Kragujevac, a public tertiary care hospital situated in Kragujevac, capital of the Šumadija region, Serbia. The study included patients who were admitted to the Clinic during two months in 2017 (September and October) and two months in 2018 (March and April); the files of the patients admitted during the period in-between were not available to the investigators due to technical reasons. Inclusion criteria were: neurological diagnosis on admission, complete data in the patient′s file and age over 18 years. Exclusion criteria were: not being prescribed drug therapy, being prescribed less than two drugs and emergency admission. The study sample was consecutive, ie. all patients admitted to the Clinic during the above mentioned four-month period were included in the study.

The data were extracted from the patients′ histories. The outcome variables were potential DDIs discovered by the three interaction checkers (Medscape, Epocrates and Micromedex), classified according to the severity. Since the number of contraindicated and serious DDIs *per* patient was mostly zero or 1, we made composite outcomes for the purpose of this study consisting of potential contraindicated and/or "serious/use alternative" DDI occurrence according to the Medscape checker, contraindicated and/or "use alternative" potential DDI occurrence based on the Epocrates checker, and contraindicated and/or major potential DDI occurrence discovered by Micromedex interaction checker. Other outcomes were median number of the potential "monitor closely" DDIs according to the Medscape checker, median number of "monitor/modify" DDIs according to the Epocrates, and median number of moderate DDIs revealed by Micromedex interaction checker.

Predictor variables taken into account were derived from socio-demographic data, data about pharmacotherapy and clinical data believed to have certain influence on DDIs. Socio-demographic data were limited to the age and gender of patients, while the data about pharmacotherapy included: names of drugs which were prescribed, total number of prescribed drugs, number of prescribers for a single patient, Anatomical Therapeutic Chemical (ATC) classification code of the prescribed drugs, number of different pharmacological/therapeutic subgroups prescribed, prescribing events involving anticoagulants, anticonvulsants, antidepressants, anti-arrhythmic or antiplatelet drugs. The following data about clinical condition of patients were included: main diagnosis on admission, total length of hospitalization, transfer from intensive care unit or other department to the GW-CND, being paralyzed, being bedridden for at least one day during hospitalization and comorbidities like dementia, delirium, renal failure, liver cirrhosis, diabetes mellitus, bronchial asthma, chronic obstructive pulmonary disease, hypertension, heart failure, etc. (also summated by Charlson Comorbidity Index) [19].

The data collected in our study were analyzed using descriptive statistics. Continuous numeric variables were described by mean and standard deviation if the data were normally distributed or by median and range if the normality of data distribution was not reached. Values of categorical variables were presented as rates or percentages.

The influence of potential risk factors on the number of potential DDIs *per* patient was evaluated by multiple linear regression analysis. Statistical validity of the regression model was checked by analysis of variance (F value), percentage of outcome (number of DDIs *per* patient) variability explained (coefficient of determination, R^2) and by variance inflation factor (VIF) which should take values below 10. Extent of influence of potential risk factors on number of DDIs *per* patient was assessed by their B coefficients within the regression equation, including confidence intervals (CIs).

The influence of potential risk factors on the occurrence of potential contraindicated/serious/major DDIs was estimated by binary logistic regression analysis. Validity of the logistic regression model was checked by the Cox and Snell R^2, Nagelkerke's R^2 and Hoshmer Lemeshow test. The strength of influence of potential risk factors on the occurrence of potential contraindicated/serious/major DDIs was assessed by adjusted odds ratio (OR), including CIs. All calculations were performed by the Statistical Program for Social Sciences (SPSS version 18).

Results

In total, there were 144 inpatients who participated in the study. Mean age of hospitalized patients was 59.0 ± 1.4, and median number of prescribed drugs *per* patient was 7 (2–20). The most common comorbidity was hypertension (55.6%). The most common reason for admission was diagnostic evaluation of patients with neurological symptoms like headache or vertigo (34.7%). The outcome of hospitalization for the majority of patients (89.6%) was discharge for further treatment at home. According to the Medscape interaction checker, potential contraindicated and serious-use alternative DDIs were found in 49.5% of patients, while median number of potential "monitor closely" DDIs *per* patient was 3 (0–26). Epocrates interaction checker discovered potential contraindicated or "use alternative" DDIs in more than half of the patients (53.5%), and median number of potential monitor/modify DDIs *per* patient was 1 (0–22). Finally, according to the Micromedex interaction checker, 59% of patients had potential contraindicated or major DDIs, and median number of potential moderate DDIs *per* patient was 1 (0–16). Detailed characteristics of the study sample are shown in Table 1.

The results of multivariate analysis for the outcomes of the presence of potential contraindicated, serious, "use alternative" or major DDIs and absolute number of "monitor closely", "monitor/modify" or moderate DDIs are presented in Tables 2 and 3, respectively. Variables included in both logistic and multiple linear regression

Table 1

Characteristics of the study sample

Variable	Values
Age (years), mean ± SD	59.0 ± 1.4
Gender, n (%)	
male	74 (51.4)
female	70 (48.6)
Clinical data, n (%)	
degenerative diseases	33 (22.9)
neurological symptoms	50 (34.7)
epilepsy	4 (2.8)
brain tumor	5 (3.5)
cerebrovascular diseases	46 (31.5)
autoimmune diseases	6 (4.2)
Length of hospitalization (days), median (range)	10 (1–40)
Transfer from another ward, n (%)	5 (3.5)
Transfer from emergency department, n (%)	5 (3.5)
Patients bedridden for at least one day of hospitalization, n (%)	34 (23.6)
Paralyzed patients, n (%)	24 (16.7)
Delirium/dementia, n (%)	9 (6.3)
Renal failure, n (%)	24 (16)
Liver cirrhosis, n (%)	1 (0.7)
Diabetes mellitus, n (%)	27(18.8)
Asthma, n (%)	4 (2.8)
Chronic obstructive pulmonary disease, n (%)	6 (4.2)
Hypertension, n (%)	80 (55.6)
Heart failure, n (%)	13 (9)
Charlson Comorbidity Index, median (range)	2.5 (0–11)
Outcome of hospitalization, n (%)	
Discharged for treatment at home, n (%)	129 (89.6)
Transfer to another ward, n (%)	13 (22.9)
Death, n (%)	2 (1.4)
Information about drugs, median (range)	
number of prescribed drugs	7 (2–20)
number of pharmacological/therapeutic subgroups prescribed (2nd level of ATC classification)	6 (2–14)
number of prescribers for a single patient	1 (1–6)
Anticoagulant therapy, n (%)	30 (20.8)
Anticonvulsants, n (%)	25 (17.4)
Antidepressants, n (%)	19 (13.2)
Antiarrhythmic drugs, n (%)	50 (34.7)
Angiotensin-converting enzyme inhibitors, n (%)	52 (35)
Non steroidal anti-inflammatory drugs, n (%)	80 (55)
Dual anti-aggregation therapy, n (%)	4 (2.8)
Drug allergy, n (%)	10 (6.9)

n (%) – number (percentage) of patients; ATC – Anatomical, Therapeutic, Clinical; SD – standard deviation.

were: the age of patients, gender, length of hospitalization, main diagnosis on admission, number of prescribed drugs, number of pharmacological/therapeutic subgroups prescribed (2nd level of ATC classification), number of prescribers for a single patient, cognitive incompetence (delirium or dementia), Charlson comorbidity index, paralysis, being bedridden for at least one day of hospitalization, and receiving anticonvulsants, antidepressants or anticoagulants.

The analysis showed that the number of prescribed drugs *per* patient increased and being paralyzed decreased the likelihood of both contraindicated/serious/major and moderate/monitor closely potential DDIs. Only contraindicated/serious/major potential DDIs were influenced by age, which increased, and by the number of physicians who prescribed drugs to a single patient, which decreased their likelihood. On the other hand, Charlson Comorbidity Index and prescribing antidepressants increased the number of moderate/monitor closely potential DDIs, while being bedridden for at least one day of hospitalization decreased the number of this type of potential DDIs.

There were 80 patients having the diagnosis of hypertension in our sample, and among them we found 50 patients (ten with mild to moderate renal failure) with contraindicated or serious-use alternative/major potential DDIs. Frequency of contraindicated potential DDIs were 10% [between two different non-steroidal anti-inflammatory drugs (NSAID)] while frequency of the serious-use alternative/major potential DDIs were 96% [between NSAIDs and angiotensin-converting enzyme inhibitors (ACEI)], Table 4.

Table 2

Multivariate regression analysis (binary logistic regression) for the outcome presence of "contraindicated", "serious", "use alternative" or "major" potential drug-drug-interactions (DDIs)

Interaction checker	Combination of two types of DDIs	Cox and Snell R^2	Nagelkerke's R^2	Hoshmer Lemeshow test	Significant variables	Adjusted OR (95% CI)	*p*
Medscape	Contraindicated and serious/ Use alternative	0.230	0.307	0.416	Number of prescribed drugs	1.466 (1.237–1.738)	0.000
					Age	1.027 (1.001–1.053)	0.041
					Number of prescribers *per* patient	0.556 (0.345–0.895)	0.016
Epocrates	Contraindicated and Use alternative	0.295	0.395	0.221	Number of prescribed drugs *per* patient	1.499 (1.253–1.793)	0.000
Micromedex	Contraindicated and Major	0.126	0.291	0.917	Number of prescribed drugs *per* patient/	1.573 (1.313–1.886)	0.000
					paralyzed patient	0.214 (0.069–0.656)	0.007

***Note*: Variables included in the last step of the model – "Contraindicated and Serious/Use Alternative" detected by Medscape (number of prescribed drugs *per* patient, age, number of prescribers for a single patient); "Contraindicated and Use Alternative" detected by Epocrates (number of prescribed drugs *per* patient); "Contraindicated and major" detected by Micromedex (number of prescribed drugs *per* patient, paralyzed patient).**
R – coefficient of determination; OR – odds ratio; CI – confidence interval.

Table 3

Multivariate regression analysis for the outcome of absolute number of "significant", "monitor/modify" or "moderate" potential drug-drug interactions

Interaction checker	Outcomes	R^2	F (*p*)	Number of excluded variables	Significant variables	B	95% CI	VIF
Medscape	Significant	0.545	2.195 (0.141)	13	Number of prescribed drugs *per* patient/	1.120	(0.902–1.109)	1.109
					paralyzed patient	-1.900	(0.884–1.131)	1.131
Epocrates	Monitor/ modify	0.509	1.370 (0.244)	12	Number of prescribed drugs *per* patient/	0.657	(0.737–1.356)	1.356
					paralyzed patient	-1.160	(0.734–1.363)	1.363
					Antidepressants	1.257	(0.885–1.130)	1.130
Micromedex	Moderate	0.386	1.135 (0.289)	12	Number of prescribed drugs *per* patient	0.385	(0.737–1.365)	1.256
					Charlson Comorbidity Index	0.185	(0.687–1.445)	1.455
					Patients bedridden for at least one day of hospitalization	1.140	(0.885–1.130)	1.363

***Note*: Variables included in the last step of the model – "Significant or Monitor closely" interactions detected by Medscape (number of prescribed drugs, immobile patients); "Monitor/modify" interactions detected by Epocrates (number of prescribed drugs, immobile patients, antidepressants); "Moderate" interactions' detected by Micromedex (number of prescribed drugs, Charlson Comorbidity Index, immobile patients at least for one day of hospitalization).**
R^2 – coefficient of determination; F (*p*) – value of F-test (probability of null hypothesis); B – unstandardized coefficient; CI – confidence interval; VIF – Variance Inflation Factor.

Table 4

The most common drug-drug interactions (DDIs) in a general neurology ward

Drugs	Type of DDIs by interaction checker		
	Medscape	Epocrates	Micromedex
Acetylsaclycilic acid and ketorolac	Contraindicated	Contraindicated	Contraindicated
Diclofenac and ketorolac	Contraindicated	Avoid-UA	Contraindicated
Acetylsaclycilic acid and fosinopril	Serious UA	Monitor/Modify	Moderate
Acetylsaclycilic acid and enalapril	Serious UA	Monitor/Modify	Moderate
Acetylsaclycilic acid and ramipril	Serious UA	Monitor/Modify	Moderate
Acetylsaclycilic acid and ibuprofen	Serious UA	Avoid-UA	Major
Ketorolac and ramipril	Serious UA	Monitor/Modify	Moderate
Ketorolac and perindopril	Serious UA	Monitor/Modify	Moderate
Diclofenac and enalapril	Serious UA	Monitor/Modify	Moderate
Diclofenac and quinapril	Serious UA	Monitor/Modify	Moderate
Ibuprofen and ramipril	Serious UA	Monitor/Modify	Moderate

UA – use alternative.

Discussion

The results of our study showed that the number of prescribed drugs, the age of a patient, the value of the Charlson comorbidity index and the prescription of an antidepressant increase the risk of potential DDIs in a general neurology ward. On the other hand, being paralyzed, a number of prescribers for a single patient, being bedridden for at least one day of hospitalization decrease the number of potential DDIs *per* patient. There are differences in sensitivity and specificity of available interaction checkers. Micromedex is rated as the most specific in general and the most sensitive for serious potential DDIs, while Epocrates and Medscape share the second place [11]. Accordingly, we revealed in our study the largest number contraindicated, serious, "use alternative" or major potential DDIs using Micromedex while the Medscape pointed to the largest number of "monitor closely", "monitor/modify" or moderate potential DDIs. The routine use of these interaction checkers could provide better care of these patients especially due to prompt access to these Internet platforms. On the other hand, complete set of information about consequences of potential DDIs and final desicion about final outcome of therapy could be summarized in the presence of other information derived as from medical and practical knowledge as well as from medical records of patients [20, 21].

Neurological disorders are one of the most common reasons for treatment in hospital facilities in modern society [16]. It is known that neurological diseases have chronic and progressive clinical course and due to these reasons patients in the neurological ward needed to be treated mostly with more than one drug. Polypharmacy increases the risk of development of potential DDIs which can contribute to the deterioration of primary medical condition of neurological patients [15]. Our results showed that the most frequently prescribed drugs were anticonvulsants, anticoagulant drugs, antidepressants, antiarrhythmic medicines, NSAIDs and ACEIs. These different groups of drugs have ability to increase the possibility of the development of clinically relevant DDIs and due to this reason neurological patients are more vulnerable population regarding DDIs, and require special concern in detecting potential DDIs on time and preventing additional deterioration of health [15–18].

Antidepressants have many indications and are frequently prescribed to neurological patients [22]. It is not surprising that patients who have been prescribed antidepressants have a higher risk of potential DDIs since the majority of antidepressant drugs are substrates for one or more of the cytochrome P-450 isozymes. Co-medication with inducers (eg. carbamazepine or phenytoin) or inhibitors (eg. valproate or imidazoles) may decrease or increase (50–60%), respectively, serum concentrations of antidepressants such as amitriptyline and nortriptyline, affecting their efficacy and safety [23]. On the other hand, selective inhibitors of serotonin reuptake may interact pharmacodynamically with anticoagulant and antiplatelet drugs increasing the risk of bleeding [24].

The number of prescribers *per* patient was a mitigating factor for a number of interactions not only in our study but also in the study conducted in the Netherlands by Vingerhoets et al. [25]. Nevertheless, in one more study within the settings of general practice, Andersson et al. [26] have established that potential DDIs in primary health care arise more often when multiple prescribers are involved in the treatment of a single patient. This difference could be explained by the settings themselves, as in a hospital, physicians communicate with each other directly while caring for patients, while in the primary care, they work mostly in shifts and rarely meet next to the patient to discuss the therapy.

It is not surprising that advanced age bears the higher risk for the development of potential DDIs, as there are numerous reasons. Aging is associated with important changes in the metabolism of drugs: biochemical composition of tissues is different, renal clearance is frequently reduced, the hepatic function is decreased, the physiological capacity of many organs is progressively diminished, and susceptibility to disease enhanced with increased vulnerability. Polypharmacy is of great concern in elderly patients as it increases the frequency of adverse drug reactions, new hospital admissions and prolongs actual hospitalization [27]. From Table 2 we can see that advanced

age increased the risk of occurrence of contraindicated and serious-use alternative potential DDIs. These findings confirmed once again that elderly patients require more attention of prescribing physicians, especially during hospital treatment, in order to limit prescribing to only absolutely essential drugs and recognize earlier potential DDIs.

Large number of prescribed drugs *per* neurological patient and numerous comorbidities in our study increased the risk of moderate and serious potential DDIs. Concurrent prescription of many drugs is frequently found in patients with multimorbidity and suffering from complex neurological diseases. Majority of other studies also found a relationship between the number of prescribed drugs per patient and the number of potential DDIs [15], as chances of DDIs statistically increase with the multiplication of prescribed drugs. It is important that physicians systematically search for potential DDIs when faced with polypharmacy (if necessary with the help of a clinical pharmacist), as majority of DDIs are preventable.

Interestingly, in our study, the occurrence of serious or moderate potential DDIs was less frequent in paralyzed patients and in patients bedridden for at least one day of hospitalization. The protective role of these medical conditions was not described previously, but it could be explained by clinicians paying more attention to patients unable to get out of the bed, which includes search for potential DDIs [25].

Despite the results of other studies, where the length of hospitalizations was a favorable factor for the occurrence of potential DDIs as in a neurology as in other wards [28, 29], that was not shown in our study. These differences could be observed in light of modest sample size in our study, but choosing a General Ward of Neurology Department we tried to provide representative population which could compare to other cohorts.

A lot of patients from our study sample were taking combination of NSAIDs and ACEIs. NSAIDs inhibit cyclooxygenase, thus decreasing prostaglandin production; as a consequence, blood flow through renal afferent arteriole decreases and glomerular filtration rate falls. On the other hand, inhibition of angiotensin II synthesis decreases vasoconstriction and blood pressure, which impairs renal blood flow and glomerular filtration rate. The ultimate result of combining ACEIs and NSAIDs is the decrease of glomerular filtration rate [30]. Although this potential DDI was less frequent in our study than in the Romanian one [31], a significant proportion of patients may experience a decrease in renal function due to the impaired perfusion of the kidneys. Simultaneous administration of two NSAIDs is also a matter of concern, since every year, about 1% to 1.5% of patients taking NSAIDs experience severe gastrointestinal side effects like perforation, ulcer or bleeding [31].

Our study has several limitations related to the modest sample size, the use of just three instead of full battery of interaction checkers and technical availability of medical records of patients in only certain periods of year (two months in the spring, and two months in the fall). This could induce omitting some significant factors which also contribute to the occurrence of potential DDIs. Also, using these checkers we found a lot of potential DDIs which were only theoretically defined and their clinical importance was not verified. The strength of our study was a detailed, in-depth analysis of the patients′ files, with the validation of extracted data, which increases the reliability of the results.

Conclusion

The frequency of potential DDIs among neurological patients is considerable and influenced to the largest extent by advanced age, comorbidities, total number of prescribed drugs *per* patient and concomitant use of antidepressants. More prescribers *per* patient and medical conditions that make patients bedridden protect from the occurrence of potential DDIs. However, both physicians and pharmacists in hospitals should pay more attention to potential DDIs in order to early detect them, which could contribute not only to the prevention of the serious and/or irreversible consequences, but also preclude the prolongation of hospital admission and additional increase in the treatment costs of these patients.

Acknowledgement

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Conflicts of interest

None.

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Antitumor effect of mifepristone on human endometrial stromal cell line

Antitumorski efekat mifepristona na stromalnu ćelijsku liniju humanog endometrijuma

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Abstract

Background/Aim. The main cause for development of endometrial hyperplasia is unopposed effect of estrogen on endometrial cells. The aim of our study was to investigate and compare cytotoxic and apoptotic effects of mifepristone on human endometrial stromal cell line for the first time. Both percentage of cytotoxic and apoptotic cells were determined after 24 h treatment with different doses of mifepristone. **Methods.** The percentage of cytotoxic cell was evaluated by viability assay while the percentage of apoptotic cells was determined using flow cytometry. Determination of apoptotic effects was confirmed using immunofluorescence method determining expression and localization of active Bax and Bcl-2 proteins. **Results.** Our results indicated that mifepristone induced cytotoxic and apoptotic effect on human endometrial stromal cell line (ThESC) through changes in expression level of Bcl-2 and active Bax proteins. **Conclusion.** Cytotoxic and pro-apoptotic effects of mifepristone on human endometrial stromal cell line *in vitro* was investigated in this study for the first time. It is crucial to point out that mifepristone expressed both cytotoxic and pro-apoptotic effect on ThESC cell line. Our results may contribute to determination of localization and expression level of the crucial proteins involved in apoptosis in ThESC cell line after the treatment with the lowest cytotoxic doses of mifepristone.

Key words:
endometrial hyperplasia; mifepristone; apoptosis; cell death; carcinoma.

Apstrakt

Uvod/Cilj. Glavni uzrok razvoja hiperplazije endometrijuma je neoponirani efekat estrogena na endometrijalne ćelije. Cilj naše studije bio je da se prvi put istraže i uporede citotoksični i apoptotični efekti mifepristona na stromalnoj liniji humanog endometrijuma. Procenat citotoksičnih i apoptotičnih ćelija je određivan nakon tretmana različitim dozama mifepristona u periodu od 24 časa. **Metode.** Procenat citotoksičnih ćelija bio je procenjen korišćenjem testa za ispitivanje vijabilnosti ćelija, dok je procenat apoptotičnih ćelija bio određen korišćenjem protočne citometrije. Utvrđivanje apoptotičnog efekta je potvrđeno određivanjem ekspresije i lokalizacije aktivnog Bax proteina i Bcl-2 proteina. **Rezultati.** Naši rezultati su pokazali da mifepriston ispoljava citotoksični i apoptotični efekat na humanu endometrijalnu ćelijsku liniju (ThESC) putem promene nivoa ekspresije aktivnog Bax i Bcl-2 proteina. **Zaključak.** U ovoj studiji smo prvi put ispitali citotoksični i pro-apoptotični efekat mifepristona na humanu stromalnu ćelijsku liniju endometrijuma *in vitro*. Od suštinskog značaja je nalaz da je mifepriston ispoljio i citotoksični i pro-apoptotični efekat na ThESC ćelijsku liniju. Naši rezulatati ukazuju na moguć nivo lokalizacije i ekspresije ključnih proteina apoptoze u ThESC ćelijama nakon tretmana sa najnižim citotoksičnim dozama mifepristona.

Ključne reči:
endometrijum, hiperplazija; mifepriston; apoptoza; ćelija, smrt; karcinom.

Introduction

Different types of morphological and physiological endometrial cell disorders may lead to development of endometrial diseases. One of them is presented in a form of endometrial hyperplasia. Uterine cells changes morfologicaly and physiologicaly during endometrial hyperplasia. These changes are represented as excessive glandular and stromal endo

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metrial cells proliferation associated with different degrees of cellular atypia and morphological abnormalities [1, 2]. These changes include cystic dilatation of endometrial glands without secretion, anovulatory cycles and presence of abnormal bleeding [3]. As a result of ongoing cell changes during endometrial hyperplasia, endometrium switches from normal towards tumorous tissue [2]. In women under the age of 30, endometrial hyperplasia is rare; however in age group of 50–54 years, increasing of incidence has been recorded. During aging, incidence of atypical endometrial hyperplasia with cytological changes increases as a prelude to a precancerous condition [4]. Treatment of endometrial hyperplasia is restricted to two types of approach. First treatment type is represented in a form of progestin (hormone) therapy, while second type of treatment is represented in surgical approach (hysterectomy) [5]. Progestin therapy approach is oriented towards one of the main problems leading to the development of endometrial hyperplasia – lack of progesterone component; while operative approach constitutes the last used technique considering complete removal of uterus and ovary. Mifepristone (RU-486, RU-38486) belongs to the class of progesterone and glucocorticoid receptor antagonist [6, 7]. This drug has various applications in different types of diseases. It has been showed that mifepristone can be suitable for patients with hyperglycemia as a secondary disorder in the Cushing's syndrome [8], it induces miscarriage and it is used to oppose proliferative effect of estrogen on endometrium, treatment of endometriosis, leiomyoma, breast cancer, and meningioma [9, 10]. Application of low doses of mifepristone express antiproliferative effects in various cancer of reproductive and non-reproductive organs [7, 9, 11, 12]. These data were confirmed in *in vitro* study performed by Goyenche et al. [13]. In this study, mifepristone exhibited inhibitory effect on ovarian cancer cell (SK-OV-3, Caov-3, OV2008, and IGROV-1) growth *in vitro*. In research that was conducted by Li et al. [12] it was shown that mifepristone induced apoptosis in Ishikawa cell lines through Bax translocation and caspase 3 activation; Some authors also confirmed the role of mifepristone on apoptosis induction in endometrial cancer cell lines (Hec-1A, KLE, and RL95-2) that involved changes in Bax and Bcl-2 regulation [11].

The aim of our study was to investigate the effects of single application of mifepristone on induction of apoptosis using human endometrial stromal cell line (ThESC cell line).

Methods

Cell line

In our study we used human endometrial stromal cell line (ThESC cell line – ATCC® CRL-4003) obtained from an adult woman with myoma. In our experiment, the passage of ThESC cells 8–10 was used and this cell line was telomerase reverse transcriptase (hTERT) immortalized. Cells were grown in complete Dulbeceo's Modified Eagle Mwedia (DMEM) medium containing glucose 4.5 g/L, 2% L-glutamine (2 mM), 1% penicilyn/streptomycin, 1% of non-essential amino acids, 1% of insulin transferrin supplement and 10% fetal bovine serum (FBS) in control environment at 37C° and 5% CO_2. The cells were divided into control group and cells treated with different doses of mifepristone (10, 20, 40, 60 and 80 μM) during 24 hour period. Cells were seeded in 96 well plates and treatment with investigated drugs was performed after reaching 85% of cell confluence.

MTT assay

Using a viability assay (MTT assay) cytotoxic effects of mifepristone was evaluated. MTT assay is based on the possibility of the metabolically active cell to perform the reduction of yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5 diphenyltetrazolium bromide) by the action of dehydrogenase enzymes in order to generate reducing equivalents such as $NADH+H^+$ and $NADPH+H^+$. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. Both control and experimental cells were resuspended in complete medium (1.8×10^4 cells/200 μL medium), seeded in 96 well micro titer plate and treated with investigated drugs. After 24 h period, supernatant was extracted and cells were incubated with MTT solution (5 mg/mL MTT dissolved in PBS) for 4 h (37°C, 5% CO_2). After incubation period for 4 hours, MTT solution was removed and cells were resuspended with 200 μL DMSO (Sigma Chemical, ST. Lois, Mo.) per well and incubated for 30 min on a shaker at room temperature. The absorbance was measured at wavelength 595 nm (multimode micro plate detector, Zenith 3100).

Apoptosis detection – flow cytometry

Type of cell death induced with investigated substances was determined with Annexin V-FITC/PI staining using flow cytometry. ThESC cells were seeded in 24-well plate (1×10^5 cells/well) and treated with different doses of mifepristone during 24 h period. At the end of incubation period cells were collected and washed 3 times with PBS, resuspended in 100 μL of ice-cold 1× binding buffer, stained with 10 μL of Annexin V-FITC and 20 μL of PI and incubated in the dark for 15 min at +4ºC. Finally, 400 μL binding buffer was added and the cells were analyzed by flow cytometer Cytomics FC500 (Beckman Coulter, USA). Data were analyzed using Flowing Software 2 and presented by bar charts.

Immunofluorescence assay

Following the determination of cytotoxic effect of investigated drugs, in our next experiment we treated the cells with the lowest cytotoxic doses of investigated substances. In order evaluate both the expression level and localization of two key members of Bcl-2 protein family, with pro- and anti-apoptotic activity [14], Bcl-2 and Bax, respectively, we used the immunofluorescence method. Incubation of the treated cells (1 h period; 1:50 dilution) was performed with different anti-rabbit primary antibodies: Bax (N20, sc-493, Santa Cruz Biotech. Inc), Bcl-2 (DC21, sc-783, Santa Cruz Biotech. Inc) and β-actin (A5316, Sigma Aldrich, Germany). Following the primary antibodies incubation, cells were washed three times in 1xPBS and incubated in the dark with specific secondary fluo-

rescent antibodies conjugated with Alexa 488 (11001, Invitrogen, USA) and Alexa 594 (gift from Dr Ljubica Ivanišević, Ottawa, Canada) (1:100) for 30 minutes. Cells were visualized by fluorescence microscopy at 100 × and 400 × magnifications on Olympus microscope (model BX51). In order to quantify fluorescence intensity of used specific primary and secondary antibodies, we used noncommercial software ImageJ 1.51j version. These values of fluorescence intensity obtained using ImageJ were transferred to Excel and used to produce bar charts that represented the intensity of fluorescence for specific antibodies which were used in these experiments.

Statistical analysis

All values were expressed as mean ± standard deviation (SD). Each experiment was performed in triplicate and conducted on every sample as described earlier. Commercial SPSS version 24.0 was used for statistical analysis. Statistical evaluation was performed using Student's *t*-test for paired observations, or one-way ANOVA depending on data distribution. *P* values that were less than 0.05 ($p \leq 0.05$) were considered as significant.

Results

Results of cytotoxic effects of different doses of mifepristone on ThESC cell line during 24 h period using MTT test, indicated that mifepristone expressed dose dependent cytotoxic effect on ThESC cell line in comparison to untreated cells (Figure 1). These results indicated that application of mifepristone in the highest dose (80 μM) caused cytotoxic effect in 72.72% loss viable of cells, while this percentage after 10 μM mifepristone treatment cytotoxic effect on the cells was 20.14 % (cytotoxicity was 3.61 times lower compared to cytotoxicity that was induced in the case of 8 times higher dose of mifepristone).

In order to evaluate the type of the cell death in mifepristone ThESC treated cells we used Annexin FITC/PI staining (Figure 2). Given the fact that higher doses of mife-

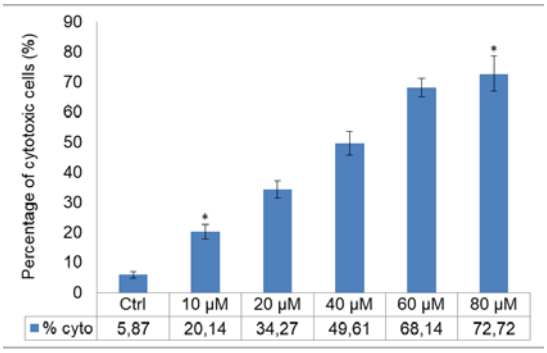

Fig. 1 – Cytotoxic effect of different mifepristone doses on human endometrial stromal cell line (ThESC) cells during 24 h period (Ctrl – control).

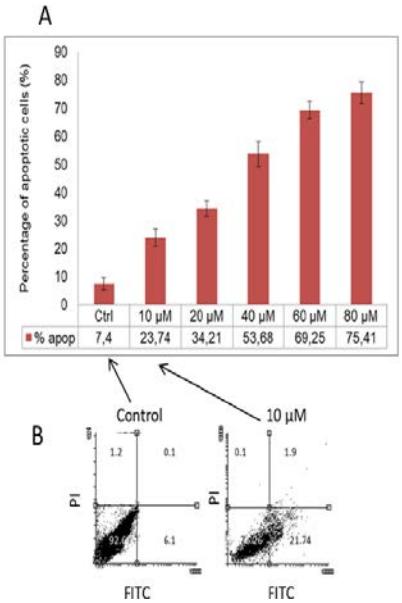

Fig. 2 – A) Human endometrial stromal cells (ThESC) were treated with different concentrations of mifepristone (10, 20, 40, 60, and 80 μM) for 24 h, cell apoptosis was analyzed by Annexin V FITC/PI staining (bar chart);
B) Mifepristone promoted ThESC cells apoptosis in a dose-dependent manner (Dot plot analysis: upper left quadrant – necrotic cells, upper right – late stage apoptotic cells, lower right – early stage apoptotic cells and lower left – live cells).
Bar charts analysis was given for control and cells treated with 10 μM mifepristone.

pristone induced statistically significant percentage of apoptotic cells (from 34.21% for 20 μM to 75.41% for 80 μM), higher doses of mifepristone (from 20 to 80 μM) ($p \leq 0.05$) were excluded from our further experiments.

Fluorescence-activated cell sorting (FACS) analysis showed that ThESC treatment with mifepristone (10 μM) resulted in 3-fold increase of apoptotic (23.74%) ThESC cells compared to control (7.4%) after 24 h period. The percentage of treated cells in early and late stage of apoptosis were 21.74% and 1.9%, respectively while in control cells percentage of early apoptotic cells was 4 times lower (6.1%). Results of Annexin V FITC/PI staining were in direct correlation with the result obtained by MTT test. Based on the results obtained with MTT assay and FACS analysis, in our next experiment we applied the immunofluorescence method in order to determine the mechanism of apoptosis induced with mifepristone. Our first step in determination of the apoptotic mechanism was to investigate and compare the level of expression of the anti-apoptotic Bcl-2 protein in treated and control cells. Immunofluorescence assay revealed the highest level of Bcl-2 expression of 96.1% in untreated, control cells (Figure 3). However, expression level of Bcl-2 protein in mifepristone treated cells (50.4%) was significantly lower with 1.9-fold decrease compared to the control (96.1%). Decreased level of the Bcl-2 protein expression after mifepristone treatment led us to investigate the effects of mifepristone treatment on the expression level of N terminal, active, mitochondrial Bax protein.

These data we used in order to show that changes in level of the Bax protein expression after mifepristone treatment directly induced apoptosis due to ongoing changes affecting mitochondrial membrane integrity. Our data confirmed these findings (Figure 3). The expression level of active Bax protein was lowest in the case of control cells (2.3%) while in the case of mifepristone treatment, expression level of Bax protein was statistically significantly higher compared to the control cells (29.9%) (Figure 3). Eleven-fold increase in expression level of Bax protein in mifepristone treated cells was statistically significant compared to the control cells. Our overall results indicate that mifepristone induced apoptosis in ThESC with the same mechanism involving inner, mitochondrial pathway.

Discussion

The benign changes in uterus that arise as the effect of prolonged and unopposed estrogen stimulation are dangerous for several reasons. First of all, extremely proliferated endometrial tissue derived as a result of estrogen effect on endometrium, cannot be adequately nourished by blood vessels, ultimately resulting in endometrial shedding followed by prolonged and irregular discharge that is not relevant to menstrual cycle [15]. Second, accumulation of replication/transcriptional errors, high frequencies of chromosomal aberrations and gene rearrangements and amplifications results in formation of the tumor [16]. These changes affecting endometrial cells are accompanied by others factors such as: obesity, inadequate hormonal balance, genetics, age, life style, early age menarche [17] lead to infertility and imminent onset of endometrial cancer. Treatment approaches for endometrial cancer include surgery and different types of drugs. One of those drugs is mifepristone which is registered for termination of early pregnancy. However, different data are still arising from new studies indicating mifepristone to be applicable for other diseases. Besides its original approval, mifepristone inhibited growth of cancer cells of reproductive and non-reproductive origin regardless of progesterone receptor expression [18]. In these experiments mifepristone induced maximum cell growth inhibition of MDA-MB-231 and MCF-7 breast cancer cell line at dose of 5 μM (90%), while in the case of higher dose (40 μM), cell growth inhibition percentage for both cell lines was 25%. However, when mifepristone was administered in ovarian cancer cell line, results indicated that higher doses of mifepristone (40 μM) induced cell growth inhibition of OVCAR-3 and SK-OV-3 cell line for 40% and 15%, respectively [19]. These data indicate that cancer cell growth inhibition of mifepristone directly depends on the cell line type. In the treatment of endometrial

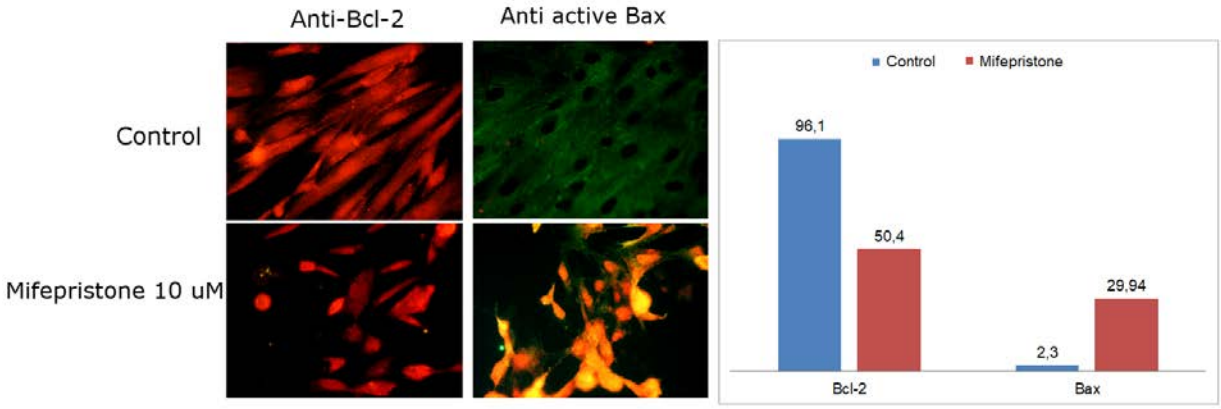

Fig. 3 – Expression of Bcl-2 and active Bax protein in human endometrial stromal cell line (ThESC) cells after treatment by mifepristone during 24 h period. Expression level of Bcl-2 protein in control cells was statistically higher compared to the cells treated with mifepristone (down-regulation of Bcl-2 protein in mifepristone treated cells). Active Bax is mainly localized in cytosol compared to mifepristone treated cells which show redistribution of active Bax to the mitochondria. Intensity of Bcl-2 and active Bax was calculated using ImageJ software (active Bax = 100 – mean of measured fluorescence).

hyperplasia, mifepristone openness' effects of estrogen on endometrial tissue. In studies conducted both on Ishikawa and human endometrial cell line (HEC1A) it was showed that mifepristone inhibited cell growth and induced apoptosis through caspase-3 activation [19–21]. This study indicated that IC_{50} values of mifepristone for HEC-1-A and Ishikawa were 16 and 19 μg/mL. Our results correspond to the previously published data [19–21], mifepristone applied in 10 μM during 24 h period caused down regulation of Bcl-2 and overexpression of active Bax protein in mifepristone treated ThESC cells, indicating direct involvement of mitochondrial apoptotic pathway. In our experiment we obtained similar results. Mifepristone expressed cytotoxic and apoptotic effect on human endometrial stromal cell line during a 24 h period. However, our findings indicated that mifepristone applied at a dose of 10 μM caused cytotoxic effect on 20.14% of the investigated cell population. When we compared the applied doses of mifepristone to the percentage of cytotoxic cells, results indicated that highest mifepristone dose resulted in nearly 2-fold increase in the percentage of cytotoxic cells compared to the effect that was induced with the lowest investigated dose of mifepristone. Our data are in correlation with the result that was obtained in different studies [12, 22]. Adequate dose of mifepristone compared to its cytotoxic effect on ThESC cell line was 10 μM. In study conducted by Ørbo et al. [21], researchers investigated effects of different doses of mifepristone on growth inhibition and apoptosis induction on Ishikawa cell line. Their results indicate that mifepristone caused cell cycle arrest in G1/G0 phase and induced apoptosis. In experiments conducted on Ishikawa, EM42, KLE, RL95-2 and HEC-1-A cell line, results also indicate that mifepristone promote apoptosis by overexpressing Bax and down-regulating Bcl-2 protein [12]. Along with C terminal mouth of Bax which is responsible for channel formation, N terminal mouth of Bax affects the permeability of the outer mitochondrial membrane and preserves its apoptotic capacity [22–24]. Translocation of active Bax towards outer mitochondrial membrane followed with pore formation, causes release of cytochrome c, formation of apoptosome and cleavage of executioner caspases. This type of pathway is called inner apoptotic pathway. Our results showed that mifepristone causes down-regulation of Bcl-2 protein and overexpression of active Bax toward outer mitochondrial membrane, thus indicating that mifepristone apoptotic mechanism of action in ThESC cells is orchestrated via inner, mitochondrial pathway.

Conclusion

Cytotoxic and pro-apoptotic effects of mifepristone on human endometrial stromal cell line *in vitro* was investigated in this study for the first time. Our results showed that mifepristone treatment statistically and significantly changed the expression levels of analyzed proteins involved in apoptosis, in opposite manner. It is important to point out that mifepristone expressed both cytotoxic and apoptotic effect on ThESC cell line. Our results indicate to a possible localization and expression level of the crucial proteins involved in apoptosis in ThESC cell line after the treatment with the lowest cytotoxic doses of mifepristone.

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Conflict of interest

Authors wish to declare that there is no conflict of interest.

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Bone and cartilage metaplasia in calcific aortic stenosis

Koštana i hrskavičava metaplazija u kalcifikantnoj aortnoj stenozi

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Abstract

Background/Aim. Calcification is a frequent change in aortic cusps and the most frequent cause for the occurrence of aortic valve stenosis. Recent studies have shown that the process of aortic calcification is often active and closely related to the formation of bone tissue in calcific aortic stenosis. The aim was to analyze the demographic characteristics of patients with calcific aortic stenosis, the most common symptoms, comorbidities and risk factors, incidence of bone and cartilage metaplasia and its possible association with the present comorbidities, as well as its clinical importance. **Methods.** We retrospectively analyzed the medical records of 115 patients referred to the University Hospital from January 2013 to December 2015. **Results.** Calcific aortic stenosis occurred more frequently in males. The average age of patients was 67.3 years. The majority of patients were non-smokers, overweight. The most common clinical symptoms were fatigue, shortness of breath and chest pain. Eighteen (15.6%) patients had no symptoms. Seventeen (14.8%) patients had cartilaginous and osseous metaplasia. Gender, age, smoking and body mass index (BMI) had the same distribution among patients with and without metaplasia. Metaplasia was equally prevalent among patients with moderate, severe and critical aortic stenosis. **Conclusion.** Age, sex, smoking, BMI and blood pressure values are not risk factors neither for osseous nor for cartilaginous metaplasia.

Key words:
aortic value stenosis; aortic valve calcification; metaplasia; risk factors.

Apstrakt

Uvod/Cilj. Kalcifikacija je česta promena aortnog kuspisa i najčešći uzrok nastanka stenoze aortne valvule. Prema novijim istraživanjima, ona predstavlja aktivan proces, usko povezan sa stvaranjem koštanog tkiva u kalcifikantno obolelim kuspisima. Cilj istraživanja je bio analizirati demografske karakteristike bolesnika sa kalcifikantnom aortnom stenozom, najčešće simptome bolesti, prisutne komorbiditete i faktore rizika, kao i učestalost javljanja koštane i hrskavičave metaplazije i njenu udruženost sa komorbiditetima, ali i njen klinički značaj. **Metode.** Retrospektivno je analizirana medicinska dokumentacija 115 bolesnika kojima je u periodu od januara 2013. do decembra 2015. godine na Univerzitetskoj klinici postavljena dijagnoza kalcifikantne aortne stenoze. **Rezultati.** Kalcifikantna aortna stenoza češće se javljala kod muškog pola. Prosečna starost bolesnika bila je 67,3 godine. Najveći broj bolesnika bili su nepušači, prekomerne telesne mase. Najčešći klinički simptomi bolesti bili su zamaranje, gušenje i bolovi u grudima. Osamnaest (15,6%) bolesnika nije imalo simptome. Kod sedamnaest (14,8%) bolesnika patohistološki su nađene koštana i hrskavičava metaplazija. Pol, starost, pušenje, indeks telesne mase (BMI) imali su istu raspodelu među bolesnicima sa i bez patohistološki potvrđene metaplazije. Metaplazija je bila podjednako zastupljena kod bolesnika sa umerenom, teškom i kritičnom aortnom stenozom. **Zaključak.** Godine, pol, pušenje, BMI i vrednosti krvnog pritiska nisu faktori rizika ni za koštanu, ni za hrskavičavu metaplaziju.

Ključne reči:
zalistak aortni, stenoza; zalistak aortni, kalcifikacija; metaplazija; faktori rizika.

Introduction

As the life expectancy is getting longer, there is an increasing number of patients with calcific aortic stenosis. It is estimated that the number of patients is even greater than the known statistical estimates, since the patients only report in the late stage of the disease, when the symptoms are pronounced, and the prognosis is worse. Knowing the possible risks and mechanisms for the occurrence of the disease would allow early diagnosis and treatment of these

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patients, possibly non-surgical. The question arises whether the development of bone and cartilage tissue is a common process or occurs in the predisposed patients.

The aim of our work was to analyze the demographic characteristics of patients with calcific aortic stenosis, the most common symptoms of the disease, the presence of comorbidities and risk factors, as well as to examine the frequency of bone and cartilage metaplasia in calcific aortic valve stenosis and its possible association with the present comorbidities. We also aimed to discover a clinical importance of metaplasia in calcific aortic stenosis.

Methods

The study included 115 patients with the replacement of the aortic valve during the period from January 2013 to December 2015 at the University Hospital due to the diagnosed calcific aortic stenosis. Furthermore, in some of the patients, revascularization coronary artery bypass grafting (CABG), thrombanderterectomy (TEA), replacement of the ascending aorta, mitral and tricuspid valve anuloplasty, patch plastics of the interstitial septum or atrial septal defect (ASD) closure were performed in addition to aortic valve replacement (AVR). The material for histopathological (HP) analysis was processed and analyzed in the Pathology Center, and demographic and clinical-morphological data included in the research were: age, sex, existing comorbidities, risk factors, disease symptoms, aortic valve area (AVA) obtained by Doppler and measured in cm^2, and histological type of metaplasia in aortic valve.

The only criteria for inclusion in the study were surgery: aortic valve replacement or myocardial revascularization with aortic valve replacement. Data for all patients were from the history of the disease and HP referrals. The exclusion criterion was a possible lack of data from the medical records.

Treatment of tissue samples for histopathological analysis

Aortic valve tissue taken during the operation was processed for a standard HP analysis, which involved fixing in 10% neutral formalin, paraffin molding, and microtome cutting to tissue sections of 4 micron thickness, followed by staining with hematoxylin-eosin (HE) method.

Statistical data processing

The data obtained by the research were entered into a separately created database of Microsoft Excel packages. The data were displayed as frequency distribution. The relationship between variables and determining the statistical significance of the results were calculated in the SPSS program using *t*-test, χ^2-test, Mann–Whitney *U* test, Kruskal–Wallis *H* test, correlation and regression analysis. The results are shown in tables and/or graphically.

Results

The study covered 115 patients with diagnosed calcific aortic stenosis.

Age and gender distribution

Patients were aged 24 to 83 years. The average age of the examined patients was 67.3 years (Figure 1). Out of 115 patients, 71 (61.7%) were male, while 44 (38.3%) were female.

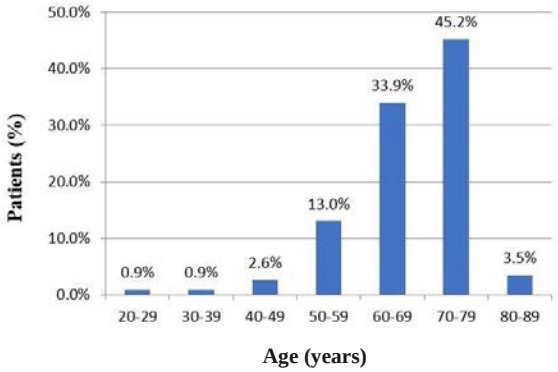

Fig. 1 – Age distribution among patients.

Other risk factor distribution

Of the 115 patients, 38 (33%) were smokers or former smokers, while 77 (67%) of patients stated that they never smoked. The highest value of systolic blood pressure among the patients was 190 mm Hg and diastolic 120 mmHg. The lowest systolic blood pressure was 90 mmHg and diastolic 60 mmHg. The average blood pressure among patients was 140/83 mmHg. We also analyzed body mass index (BMI) among the affected patients (Table 1).

Table 1

Histopathological findings among patients with metaplasia

Pathological findings	Patients		
	n	yes (%)	no (%)
Inflammation	6	35.3	64.7
Cartilaginous metaplasia	8	47.1	52.9
Red bone marrow	8	47.1	52.9
Yellow bone marrow	12	70.6	29.4
Osseous metaplasia	13	76.5	23.5
Neovascularization	14	82.4	17.6
Fibrosis	17	100.0	0.0
Myxomatous degeneration	17	100.0	0.0

Symptoms

Of the 115 patients, 18 (15.6%) did not have symptoms. Among symptomatic patients, 64 (55.7%) patients reported fatigue, 51 (44.3%) patients hard breathing, 37 (32.2%) chest pain, 14 (12.2%) dizziness or faint, 8 (7%) arrhythmia and 7 (6.1%) swelling of the legs, while 6 (5.2%) patients reported a history of the loss of consciousness.

Aortic valve area

Patients were classified in four groups following the American Heart Association (AHA) guidlines for the severity of aortic stenosis. None of them was in the group of mild aortic stenosis (AVA 1.5–2 cm^2). Sixty two (54%) patients were classified as those with severe aortic stenosis (AVA 0.6–1.0 cm^2), 38 (33%) as critical (AVA < 0.6 cm^2) and 15 (13%) as moderate aortic stenosis (AVA 1.0–1.5 cm^2). We used χ^2-test and Kruskal–Wallis *H* test to identify a possible association between the age and AVA, but no statistically significant association was found. The χ^2-test and Mann–Whitney *U* test showed that there was no statistically significant association between smoking and the severity of aortic stenosis. The ANOVA test showed that there was no statistically significant association between BMI and AVA, and the correlation analysis showed very low correlation (0.087) between these two variables. Using multinominal and linear regression analysis, the predictability of AVA values based on BMI and age was very low, namely 3.7–4.4% of variance was explained by the model. The Mann–Whitney *U* test showed that there was no statistically significant association between the presence of metaplasia and the severity of aortic stenosis.

Presence of metaplasia

Of all patients with calcific aortic stenosis, in 17 (14.8%) patients, the presence of bone and/or cartilage tissue in aortic cusps was found (Table 2, Figures 2, 3 and 4).

Table 2

Body mass index (BMI) of patients with calcific aortic stenosis

BMI (kg/m^2)	Patients, n (%)
< 18.5 (malnutrition)	0 (0)
18.5–24.9 (normal body mass)	32 (27.8)
25.0–29.9 (overweight)	52 (45.2)
≥ 30.0 (obesity)	31 (27)

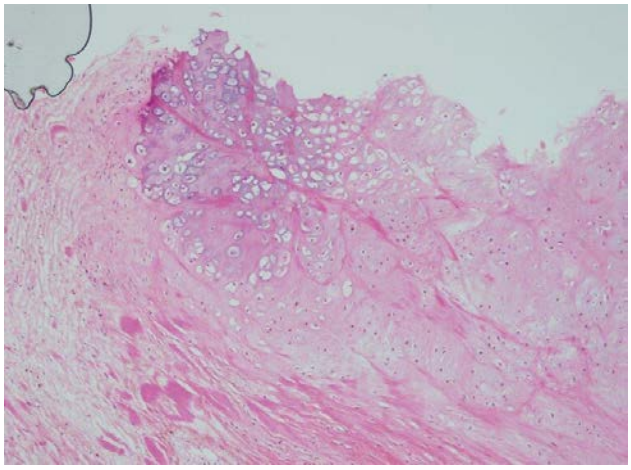

Fig. 2 – Hyaline cartilage in the aortic valve tissue [hematoxylin-eosin (HE), ×100].

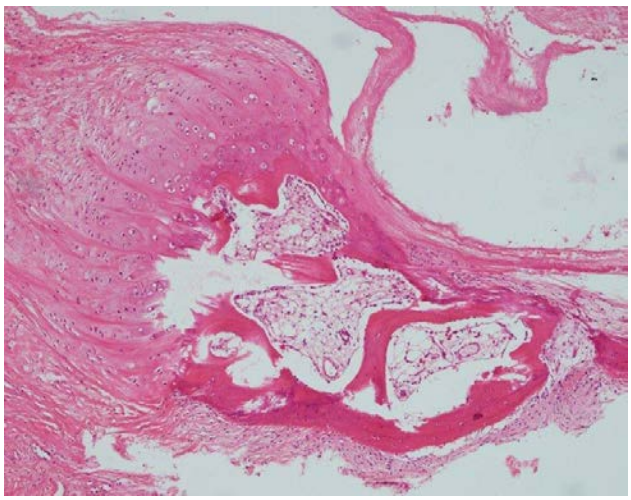

Fig. 3 – Endochondral ossification in the aortic valve [hematoxylin-eosin (HE), ×100].

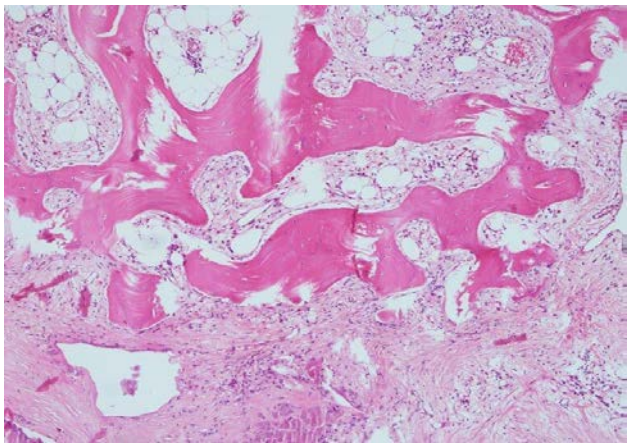

Fig. 4 – Yellow and red bone marrow in the aortic valve [hematoxylin-eosin (HE),×100].

Of the 17 patients with metaplasia, 8 (47.1%) of the patients belonged to the age group from 60 to 69 years, 6 (35.3%) of the age group from 70 to 79 years, while 3 (17.6%) patients were in the age group of 50 to 59 years. The average age of these patients was 66.8 years. Eleven (64.7%) patients were male, while 6 (35.3%) were female. Seven (41.2%) patients were overweight, while 6 of them (35.3%) were normally fed. Nine (52.9%) patients were non-smokers, while 8 (47.1%) were smokers.

The χ^2-test and *t*-test showed that there was no statistically significant association between the age of patients, sex, BMI and smoking, and the occurrence of metaplasia in calcified degenerate aortic cusps. Logistic regression analysis showed no statistically significant model when BMI and age were used as predictors, and only 0.8%–1.3% of variance of the occurrence of metaplasia could be explained by this model.

Treatment

Aortic valve replacement (AVR) was performed in all 115 patients. Of the 115 patients, 28 (24.3%) were revascularized – coronary artery bypass surgery (single,

double, triple or quadruple), in 6 (5.2%) patients the replacement of the ascending aorta had been done, in 4 (3.5%) thrombandarterectomy, in 3 (2.6%) mitral and tricuspid valve annuloplasty, and in one (0.9%) patient patch plastics of interstitial septum and atrial septal defect (ASD) suture.

Discussion

Calcification is a frequent change in aortic cusps and the most common cause of aortic valve stenosis. Possible initiators of this pathological process are abnormal haemodynamic forces in hypertension and increased stretching of cusps that initiate the remodeling process and inflammation, further leading to calcification and valvular ossification [1, 2].

Research on aortic swine valves showed that endothelial cells from the aortic side express different molecules from those on the ventricular side [5]. This suggests the possibility that endothelial cells of the aortic side participate in the calcification and oscillation process, especially when taking into account that these changes are almost always on the aortic side. The importance of endothelial cells in pathogenesis of valvular disease is also shown in studies on cloned endothelial cells of maturated sheep that showed the possibility of endothelial mesenchymal transformation in *in vitro* conditions. It seems that this process, otherwise decisive in the occurrence of specific cusp structure in embryonic period, can be re-initiated in adults, possibly creating osteoprogenitor cells [6].

Recent research defines five phenotypes that best present a family of valvular interstitial cells, since each subgroup has different roles in physiological and pathological conditions. These are embryonic progenitor endothelium/mesenchymal cells, resting interstitial cells (qVICs), activated interstitial cells (aVICs), progenitor (pVICs) and osteoblast interstitial cells (obVICs). They have the ability to cross one into another [7]. Resting interstitial cells allow the maintenance of the valvular structure and function. They express certain transmembrane proteins that are supposed to serve for their mutual communication.

In case of damage to the valve, certain cytokines that recruit bone marrow or blood progenitors are released, and these cells reach the site of damage by identifying specific ligands. Studies in mice show that hematopoietic stem cells inserted into the heart valve of the recipient differ in the direction of cells that are morphologically similar to the native interstitial cells of the recipient [8].

Mechanical stress and damage lead to the transition of resting into activated valvular interstitial cells. Activated cells exhibit the increased expression of α-smooth muscle actin (α-SMA) as well as the increased contractility, all for the purpose of repairing the damage to the valve. The disorder of this complex process leads to fibrosis, calcification and angiogenesis leading to the clinical manifestation of a valvular disease.

Valvular interstitial cells can create cartilage or mature bone. Calcificated nodules do not occur spontaneously in the culture of valvular interstitial cells. The calcification process has been proven to be dependent on alkaline phosphatase activity [9]. The presence of hydroxyapatite, osteopontin, bone sialoprotein, bone morphognetic protein-2 (BMP-2) and osteocalcin has been detected in calcified valves, suggesting that calcification is not a passive degenerative process, but an active process that implies the existence of an osteoblast cellular phenotype [4].

There are some similarities between the process of calcification and atherosclerosis. It has been proven that the patients with aortic sclerosis have a 40% higher risk of myocardial infarction and a 50% greater chance of sudden cardiac death. A possible explanation is that aortic sclerosis is an indicator of the developing process of atherosclerosis in the body, namely, atherosclerosis and aortic sclerosis are the two manifestations of one and the same disease. According to literature data, age, male sex, smoking, hypertension and hyperlipidemia are risk factors for both aortic sclerosis and atherosclerosis – which supports this theory [10, 11]. Our results are in line with the literature, given that calcific aortic stenosis in our patients was more common among men, most often in the seventh decade of life. The highest value of systolic blood pressure among our patients was 190 mm Hg and diastolic 120 mm Hg. The average blood pressure was 140/83 mm Hg. Blood pressure was measured at the moment of hospital admission. Among our patients, as opposed to literature, there were more non-smokers. This has to be interpreted bearing in mind the fact that the most people in Serbia are non-smokers, and not in direction of excluding smoking as a risk factor.

Low density lipoprotein (LDL), angiotensin converting enzyme (ACE) and its product-angiotensin II18 were found in the interstititium of calcified cusps, and even in the macrophages themselves [12]. Although there is a possibility that part of the ACE is produced in the cusps itself, most of it is still extracellular and occurs in the presence of apolipoprotein B, suggesting the possibility that ACE in the lesion has been linked to LDL particles [12]. The results of our research have shown that most of the affected patients are overweight. Since obesity, as a part of the metabolic syndrome, is associated with the increased production of LDL particles and increased production of inflammatory cytokines, our results support the assumptions that these events are likely to be a part of the pathomechanism that leads to calcific aortic stenosis [13–15]. However, in our study BMI had the same distribution among patients with moderate, severe and critical aortic stenosis. Also, high blood glucose level enhances valve interstitial cell (VIC) matrix calcium deposition [15].

An interesting fact is that diabetes mellitus reduces the occurrence of ossification in calcified altered valves [16].

Recent studies have shown a high association of warfarin therapy and the occurrence of aortic valvular calcification [17, 18]. Warfarin leads to calcification influencing the synthesis and function of the matrix Gla-protein, which is otherwise an inhibitor of the calcification process [16, 18].

Taking everything mentioned together, it might be that the haemodynamic and mechanical forces along with

oxidized lipids and exogenous substances (*eg*, bacterial lipopolysaccharides) transform calm valve interstitial cells (VICs) into activated VICs [19]. Activated VICs may be subjected to osteogenic transdifferentiation. Mechanical forces affecting cusps lead to the activation of the atrioventricular (AV) endothelium resulting in the increased expression of vascular cell adhesive molecule (VCAM), intercellular adhesive molecule (ICAM), bone morphogenetic protein 4 (BMP-4, proinflammatory osteogenic morphogen), transforming growth factor beta (TGF-beta) as well remodeling an extracellular matrix leading to an increased stiffness of cusps [20, 21]. The increased stiffness of cusps increases the effect of mechanical force on the cusps. The activation of the Toll-like receptor on VICs, possibly under the action of the liberated double-stranded RNA from injured cells, increases the bone morphogenic protein-2 (BMP-2) expression on VICs. The increased expression of BMP-2 on VICs leads to an increase in Runx2 protein levels, the factor of transcription of osteoblast gene expression, which is necessary for the formation of an improved bone and helps regulate the differentiation of chondrocyte and osteoblasts [22, 23]. BMP-2 also induces Sox9, the second factor of transcription that stimulates chondrogenesis [19]. VCAM and ICAM expression stimulate the infiltration of cusps with inflammatory cells [24, 25]. Chronic inflammation stimulates angiogenesis that is essential for bone formation and the release of TNF-alpha from activated leukocytes [26]. VIC-exposed TNF-alpha increase the expression of BMP-2 [27]. TGF-beta mediates extracellular matrix (EMC) remodeling and stimulates the production of reactive oxygen radicals [28]. Oxidative stress that is particularly high in calcification areas can lead to the transformation of activated VICs into osteoblasts, possibly via Wnt3a signaling [29, 30]. The occurrence of metaplasia in cusps can also have a genetic basis. Notch1 mutation results in the greater expression of BMP-2 by VICs [31].

According to literature, aortic stenosis is most often clinically manifested with the symptoms of choking, chest pain, dyspnea, or syncope [32, 33]. According to our results, the triassic symptoms in the affected people include fatigue, choking and chest pains, while fainting as a clinical symptom predominated in 14 (12.2%) patients, and only 6 (5.2%) patients were reduced to loss.

In 17 (14.8%) of our patients, the presence of bone and/or cartilage metaplasia was pathohistologically shown. In a study of Torre et al. [34] metaplasia appeared in 11.5% of the patients with tricuspid aortic valve. This study also showed more frequent reporting of metaplasia in males, which is in line with our results.

In our study, in patients with pathohistologically proven metaplasia, the presence of yellow bone marrow was more common, which corresponds to the results of other studies on this topic [35].

Metaplasia was equally prevalent among patients with moderate, severe and critical aortic valve stenosis, which may point out that metaplasia has no influence on the severity of aortic valve stenosis. It is still a question whether metaplasia is just a pathological finding or it has some influence on the clinical course of the disease. However, a greater sample is required to discover it.

To this date, no medical therapy has been proven to prevent or to stop the progression of aortic valve stenosis. Although there have been attempts to find another, the only therapeutic approach for now is aortic valve replacement [36, 37]. In order to develop the new treatment strategies, we have to discover the complex pathophysiological pathway of this disease.

Conclusion

Age, sex, smoking, body mass index and blood pressure values show the same distribution among patients with and without histopathologically proven metaplasia, which may point out that these are not risk factors for metaplasia among the patients with calcific aortic stenosis. Metaplasia shows no impact on the severity of the disease. Howewer, further studies are required to show whether there are specific risk factors that lead to metaplasia and whether it is connected with the late stage and worse outcome of the disease.

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Colorectal carcinoma: evaluation of systemic values of interleukin-1 and interleukin-33 in patients with and without thrombocytosis

Kolorektalni karcinom: procena sistemskih vrednosti interleukina-1 i interleukina-33 kod bolesnika sa i bez trombocitoze

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Abstract

Background/Aim. Reactive thrombocytosis, as a paraneoplastic syndrome, is often observed in cancer patients. A variety of tumor-related humoral factors and cytokines contribute to tumor-stimulated thrombopoiesis. However, the exact role of these cytokines in the pathogenesis of thrombocytosis remains unclear. The aim of this study was to analyze systemic values of cytokines and clinical-pathological characteristics in colorectal carcinoma (CRC) patients with and without thrombocytosis. **Methods.** Fifty nine CRC patients were involved in this study and divided into two groups according to the number of platelets. We recorded and analyzed the data about: age, gender, size of the cancer, localization, metastasis, vascular or lymph vessel invasion, nuclear grade, histological differentiation rate, tumor, nodus, metastasis (TNM) stage and concentration of cytokines [interleukin (IL)-1, IL-33, IL-12, IL-17 and interferon (IFN)-γ] in both groups. **Results.** CRC patients with thrombocytosis had significantly higher nuclear grade of the cancer ($p = 0.002$); higher percentage of detectable metastatic lesions in the liver ($p = 0.002$), lung ($p = 0.001$), peritoneal carcinomatosis ($p = 0.001$), detectable invasion of blood ($p = 0.012$) and lymph vessels ($p = 0.010$). Concentrations of tumor markers [alpha-fetoprotein (AFP), carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA19-9)] and serum values of IL-1 and IL-33 were significantly higher in CRC patients with thrombocytosis. IL-1/IL-12 ($p = 0.016$), IL-1/IFN-γ ($p = 0.007$), IL-1/IL-17 ($p = 0.006$), IL-33/IL-12 ($p = 0.001$), IL-33/IFN-γ ($p = 0.001$), IL-33/IL-17 ($p = 0.002$), and IL-33/IL-1 ($p = 0.006$) ratios were significantly higher in CRC patients with thrombocytosis in comparison to CRC patients without thrombocytosis. Analysis of Receiver Operating Characteristic (ROC) curves showed that values of IL-1 [area under curve (AUC) = 0.718; 95% confidence interval (CI): 0.567–0.868; sensitivity 69.2%, specificity 62.9%] and IL-33 (AUC = 0.763; 95% CI: 0.614–0.911; sensitivity 84.6%, specificity 65.7%)], could be serve as possible markers for paraneoplastic thrombocytosis in CRC patients. **Conclusion.** IL-1 and IL-33 significantly correlated to high thrombocyte number in patients with more aggressive CRC.

Key words:
colorectal neoplasms; thrombocytosis; cytokines; interleukins.

Apstrakt

Uvod/Cilj. Reaktivna trombocitoza, kao paraneoplastični sindrom, često se sreće kod obolelih od karcinoma. Različiti humoralni faktori i citokini povezani sa tumorom doprinose povećanom stvaranju trombocita. Međutim, tačna uloga ovih citokina u patogenezi trombocitoze nije potpuno jasna. Cilj studije je bio da se analiziraju sistemske vrednosti citokina i kliničko-patološke karakteristike kod obolelih od kolorektalnog karcinoma (CRC) sa i bez trombocitoze. **Metode**. U istraživanje je bilo uključeno 59 bolesnika sa CRC, podeljenih u dve grupe u zavisnosti od broja trombocita. Analizirani su podaci o: starosti, polu, veličini tumora, lokalizaciji, metastazama, invaziji krvnih ili limfnih sudova, nuklearnom

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gradusu, stepenu histološke diferencijacije, tumor, nodus, metastaza (TNM) stadijumu i serumskim koncentracijama citokina [interleukina (IL)-1, IL-33, IL-12, IL-17 i interferona (IFN)-γ] kod obe grupe ispitanika. **Rezultati**. Oboleli od CRC sa trombocitozom imali su značajno veći nuklearni gradus karcinoma ($p = 0,002$); veći procenat detektabilnih metastatskih lezija u jetri ($p = 0,002$), plućima ($p = 0,001$), karcinomatoza peritoneuma ($p = 0,001$), detektibilnih invazija krvnih ($p = 0,012$) i limfnih sudova ($p = 0,010$). Takođe, kod obolelih od CRC sa trombocitozom zabeležene su veće koncentracije tumorskih markera [alfafetoproteina (AFP), karcinoembrionalnog antigena (CEA) i karcinomskog antigena 19-9 (CA 19-9)] i serumskih vrednosti IL-1 i IL-33. IL-1/IL-12 ($p = 0,016$), IL-1/IFN-γ ($p = 0,007$), IL-1/IL-17 ($p = 0,006$), IL-33/IL-12 ($p = 0,001$), IL-33/IFN-γ ($p = 0,001$), IL-33/IL-17 ($p = 0,002$), and IL-33/IL-1 ($p = 0,006$) odnosi bili su značajno veći kod obolelih od CRC sa trombocitozom u odnosu na obolele od CRC bez trombocitoze. Analiza *Receiver Operating Characteristic* (ROC) krivulje pokazuje da se IL-1 (AUC = 0,718; 95% Cl: 0,567–0,868); osetljivost 69,2%, specifičnost 62,9% i IL-33 (AUC = 0,763; 95% Cl: 0,614–0,911); osetljivost 84,6%, specifičnost 65,7%, mogu koristiti kao potencijalni markeri paraneoplastične trombocitoze kod obolelih od CRC. **Zaključak.** IL-1 i IL-33 značajno koreliraju sa brojem trombocita kod bolesnika sa agresivnijom formom kolorektalnog karcinoma.

Ključne reči:
kolorektalne neoplazme; trombocitoza; citokini; interleukini.

Introduction

Colorectal cancer (CRC) is among the leading causes of mortality and morbidity throughout the world [1]. Overall, CRC ranks third in terms of incidence but second in terms of mortality [2]. The incidence of CRC is increasing due to ageing and unhealthy life style [3]. Although the distribution of CRC varies widely (worldwide incidence and mortality, 10.2% and 9.2% of all cancers, respectively), more than two-thirds of all cases and more than half of all deaths happen in countries with high human development index (HDI) [2]. Consumption of red or processed meat, alcohol drinks, and body fatness frequently increase the risk of CRC, whereas physical activity is protective [4–6].

Thrombocytosis, a paraneoplastic syndrome, frequently accompanies cancer growth and metastatic dissemination, and is observed in as many as 10–57% of cancer patients [7, 8]. A pathogenic feedback loop may be operative between platelets and tumor cells, with reciprocal interactions between tumor growth/metastasis and thrombocytosis/platelet activation [7]. A variety of tumor-related humoral factors and cytokines such as granulocyte colony-stimulating factor (G-CSF), granulocyte macrophage colony-stimulating factor (GMCSF), thrombopoietin (TPO) [8], interleukins (IL)-1, IL-3, IL-6, IL-11 influence thrombopoiesis in cancer and contribute to tumor-stimulated thrombopoiesis [8, 9].

Several recent studies have reported that thrombocytosis may be associated with the poor prognosis of CRC [10]. Thrombocytosis is associated with shorter overall, disease-free and cancer-specific survival. Overall survival is reduced in patients with thrombocytosis regardless of their clinical tumor stage, and ethnicity. Also, thrombocytosis is significantly related to female patients, colon tumor location, T3–4 stage, lymph node positivity, metastasis, undifferentiated histology and lymphatic involvement [11, 12].

IL-33 and IL-1 family member play an essential role in the regulation of immune response after cellular stress or damage [13, 14]. A recent study revealed that IL-33 by biding to its receptor ST2 inhibits host anti-tumor immunity, remodels tumor stroma and enhances angiogenesis, thereby promoting the development of CRC [15]. Patients with metastatic CRC, with higher expression of IL-33 in cancer tissues were significantly associated with poorer survival [14, 16]. IL-1 is secreted by different cell types, such as myeloid cells. Previous studies confirmed the importance of IL-1 in different processes, such as tumorigenesis, invasiveness and progression of tumor cells, as well as invasiveness of CRC, activation or inhibition of anti-tumor immune response [17]. However, the exact role of these cytokines in the pathogenesis of reactive paraneoplastic thrombocytosis remains unclear.

The aim of this study was to analyze clinical-pathological characteristics of the disease and systemic values of IL-1 and IL-33, as well as their correlation with pro-inflammatory cytokines [IL-12, interferon (IFN)-γ and IL-17], in CRC patients with and without thrombocytosis.

Methods

Patients

Fifty nine CRC patients were involved in this study, after confirmed diagnosis of CRC by means of endoscopic and histopathological examination. Exclusion criteria for patients were: not well-defined pathology, inadequate clinical document available or diagnosed CRC, previously treated with chemotherapy or radiation, active bleeding. After being included, all patients signed informed consent. In the study, we recorded and analyzed data about: age, gender, size of the cancer, localization, metastasis, vascular or lymph vessel invasion and clinical tumor, nodus, metastasis (TNM) stage [17]. In addition, pathological features (nuclear grade and histological differentiation rate) were analyzed in accordance with the American Joint Committee on Cancer (AJCC, 2010) classification. Thrombocytosis was defined as platelet count more or equal than 450×10^9/L, similar to the related study [18].

Ethical approvals

Study was performed at the Center for Gastroenterology, Clinical Center of Kragujevac and Center for Molecular Medicine and Stem Cell Research, Faculty of Medical Sciences, University of Kragujevac, Serbia. The researches were allowed by both institutions by obtaining ethical approvals

and made according to the Declaration of Helsinki and the Principle of Good Clinical Practice.

Measurement of cytokine concentration in serum

One blood sample in the volume of 10 mL was taken preoperatively, before surgery, from each patient. Serum was separated by centrifugation and stored at -80 °C until testing. The test was performed using commercial sensitive enzyme-linked immunosorbent assay kits (ELISA, R&D Systems, Minneapolis, MN, USA) particularly for human cytokines, such as IL-1, IL-33, IL-12, IL-17 and IFN-γ. The micro titer plates (MTP), with 96-wells, were coated with 100 μL of capture antibody in carbonate/bicarbonate buffer (pH 9.6) at the recommended concentrations, overnight. MTP were washed with washing buffer (0.05% Tween-20 in phosphate buffered saline – PBS). Briefly, standard recombinant IL-1, IL-12, IL-17, IL-33, IFN-γ or serum samples were incubated in plates for 2 hours before adding 100 μL of detection antibody and incubating for 1 hour at room temperature covered with an adhesive sealing film. We washed MTP and added 100 μL of streptavidin peroxidase for 1 hour, and then 100 μL of substrate reagent (prepared by mixing equal volumes of Color A and Color B reagents) for 20 minutes. The reaction was stopped with 50 μL of stop solution (4 mol/L sulfuric acid) Using a microplate reader (Biochrom, Anthos Zenyth 200, UK), we read the absorbance at 450 nm. As stated in the instructions of the manufacturer, concentrations of cytokines were estimated by interpolation of a standard curve (made from a series of previously established concentrations of cytokine) and were presented as pg/mL of sera. All measurements were performed in duplicate and in accordance with the manufacturer's recommendations.

Statistical analysis

SPSS software version 20.0 was applied for statistical analyses. All values were reported as means ± standard error of the means (SEM). The Student's *t*-test, Mann-Whitney *U*-test or Kruskal-Wallis test were used in order to assess statistical significance of differences between the means of two groups. Associations between thrombocytosis and tumor characteristics were evaluated using the chi-square (χ^2) test. Possible correlation between the markers of interest and thrombocytosis in CRC patients were analyzed with the Pearson's or Spearman's tests, as appropriate, and were determined as weak, moderate or strong (0.1–0.3, 0.3–0.5, 0.5–1.0, respectively), positive or negative. *P*-value ≤ 0.05 was considered significant.

Results

Fifty nine adult patients (n = 59) with diagnosed CRC were recruited in this study. Patients with diagnosed CRC were divided into two groups according to the number of platelets. The first group includes 35 CRC patients with the number of platelets < 450 × 10^9/L (group without thrombocytosis), while the second group included 24 CRC patients with the number of platelets ≥ 450 × 10^9/L [18, 19] (group with thrombocytosis). Clinical and pathologic characteristics of these patients are presented in Table 1.

There were no differences in age or gender distribution between two groups. Concentrations of tumor markers, alpha fetoprotein (AFP), carcinoembryonic antigen (CEA) and cancer antigen 19-9 (CA 19-9) were significantly higher in CRC patients with thrombocytosis compared to CRC patients without thrombocytosis (Table 1).

Evaluation of nuclear grade of CRC was also made in patients with the presence or absence of thrombocytosis. Nuclear grade was based on evaluation of the size and shape of the nucleus in cells of colorectal cancer and the percentage of tumor cells that are in the process of dividing or growing [20]. Results revealed that CRC patients with thrombocytosis had significantly higher nuclear grade of colorectal cancer in comparison to patients with normal number of platelets (*p* = 0.002; Figure 1A). Next, CRC patients with/without thrombocytosis were analyzed regarded detection of lymph and blood vessels invasion. Significantly higher percentage of CRC patients with diagnosed thrombocytosis had detectable invasion of blood

Table 1

Demographic, clinical and pathological characteristics of patients with colorectal carcinoma (CRC)

Characteristics	CRC patients		*p*
	without thrombocytosis	with thrombocytosis	
Gender (male/female), n (%)	23/12 (65.71/34.29)	12/12 (50/50)	0.487
Age (years), mean (range)	64.82 (50–82)	65.14 (56–80)	0.938
Platelets (× 10^9/L), mean ± SD	307.14 ± 12.36	555.28 ± 27.13	0.001
Histological differentiation rate, (well/moderate), n (%)	10/25 (28.57/71.43)	8/16 (33.34/66.67)	0.568
AFP (ug/mL), mean ± SD	4.97 ± 5.56	402.83 ± 212.03	0.047
CEA (ug/mL), mean ± SD	16.89 ± 5.19	402.18 ± 178.54	0.002
CA 19-9 (U/mL), mean ± SD	12.71 ± 2.27	814.26 ± 306.96	0.001

AFP – alpha fetoprotein; CEA – carcinoembryonic antigen; CA – cancer antigen; SD – standard deviation.

and lymph vessels in comparison to CRC patients without thrombocytosis (p = 0.012 and p = 0.010; Figures 1B and 1C, respectively). CRC patients with thrombocytosis had advanced TNM stage (III or IV), while CRC patients without thrombocytosis mostly had TNM stage I or II, but this difference did not reach statistical significance (data not shown). Further, CRC patients with/without thrombocytosis were analyzed on the basis of detection metastatic lesions in the liver and lung as well as peritoneal carcinomatosis. Significantly higher percentage of CRC patients with thrombocytosis had detectable metastatic lesions in the liver and lung as well as peritoneal carcinomatosis, in comparison to CRC patients without thrombocytosis (p = 0.002, p = 0.001, and p = 0.001, respectively; Figures 1D, 1E, and 1F, respectively).

Serum concentrations of cytokines of interest were measured in CRC patients with/without thrombocytosis. Results revealed that CRC patients with diagnosed thrombocytosis had significantly higher serum concentration of IL-1 in comparison to CRC patients with normal number of platelets (p = 0.022) (Figure 2A). Serum levels of IL-33 were significantly increased in CRC patients with diagnosed thrombocytosis compared to CRC patients without thrombocytosis (p = 0.001) (Figure 2B). Moreover, strong positive correlation was detected between serum values of IL-1 and IL-33 (r = 0.879, p = 0.001) (Figure 2C).

Further, we analyzed ratios of IL-1 and IL-33 and different pro-inflammatory cytokines. IL-1/IL-12 (p = 0.016), IL-1/IFN-γ (p = 0.007) and IL-1/IL-17 (p = 0.006) ratios were significantly higher in CRC patients with thrombocytosis compared to CRC group of patients with normal number of platelets (Figures 3A, 3B, and 3C, respectively). CRC patients with thrombocytosis had significantly higher ratios of IL-33/IL-12 (p = 0.001), IL-33/IFN-γ (p = 0.001) as well as IL-33/IL-17 (p = 0.002), in comparison to CRC group of patients without thrombocytosis (Figures 3D, 3E, and 3F, respectively). Additionally, IL-33/IL-1 ratio was significantly increased in CRC patients with thrombocytosis compared to CRC group of patients with the normal number of platelets (p = 0.006) (Figure 3G).

Finally, we analyzed the sensitivity and specificity of IL-1 and IL-33 in order to confirm whether these cytokines could predict paraneoplastic thrombocytosis in CRC patients. Analysis of receiver operating characteristic (ROC) curves showed that IL-1 [area under curve (AUC) = 0.718; 95% confidence interval (CI): 0.567–0.868; sensitivity 69.2%, specificity 62.9%] and IL-33 (AUC = 0.763; 95% CI: 0.614–0.911; sensitivity 84.6%, specificity 65.7%), could serve as possible markers for diagnosing paraneoplastic thrombocytosis in CRC patients (Figures 4A an, 4B, rspectively). The optimal cut-off values estimated for detection of paraneoplastic thrombocytosis in

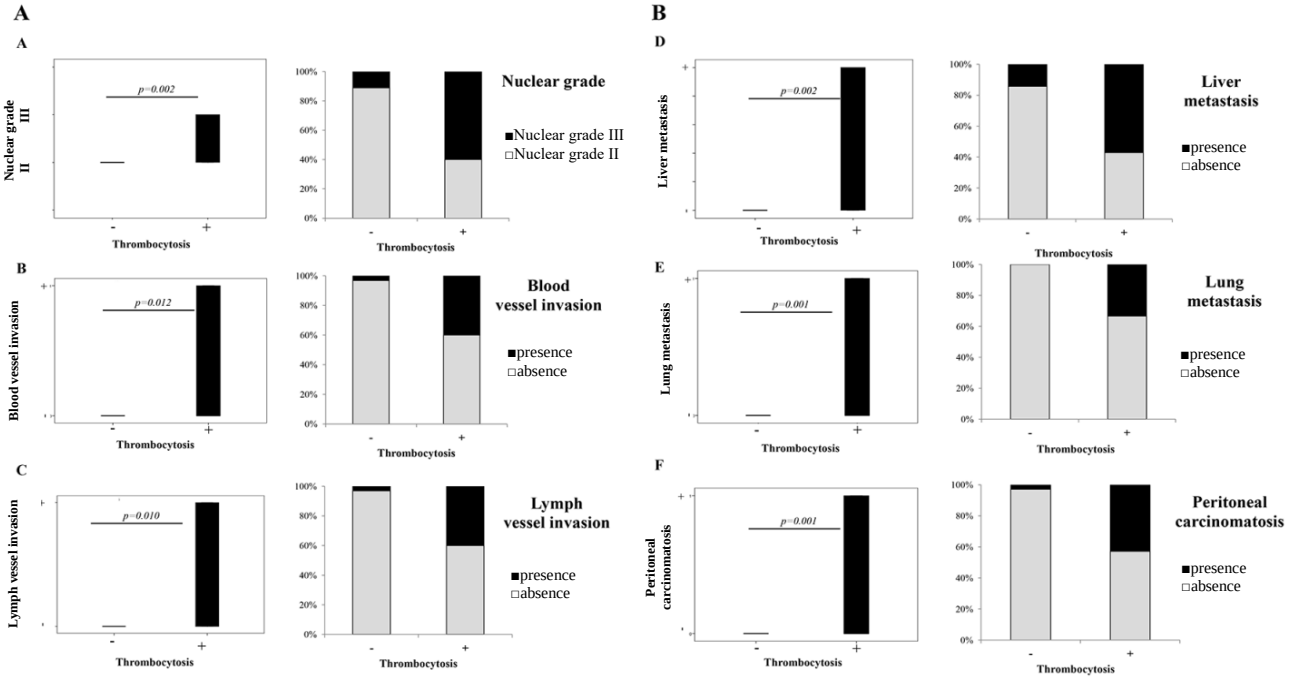

Fig. 1 – Colorectal cancer (CRC) patients with thrombocytosis have severe and advanced disease.

Panel A: Higher nuclear grade of CRC in patients with thrombocytosis in comparison to CRC patients without thrombocytosis (p = 0.002); Significantly higher percentage of CRC patients with diagnosed thrombocytosis had detectable invasion of blood (p = 0.012) and lymph vessels (p = 0.010) in comparison to CRC patients without thrombocytosis.

Panel B: Higher percentage of CRC patients with thrombocytosis had detectable metastatic lesions in the liver (p = 0.002) and the lung (p = 0.001) as well as peritoneal carcinomatosis (p = 0.001) in comparison to CRC patients without thrombocytosis.

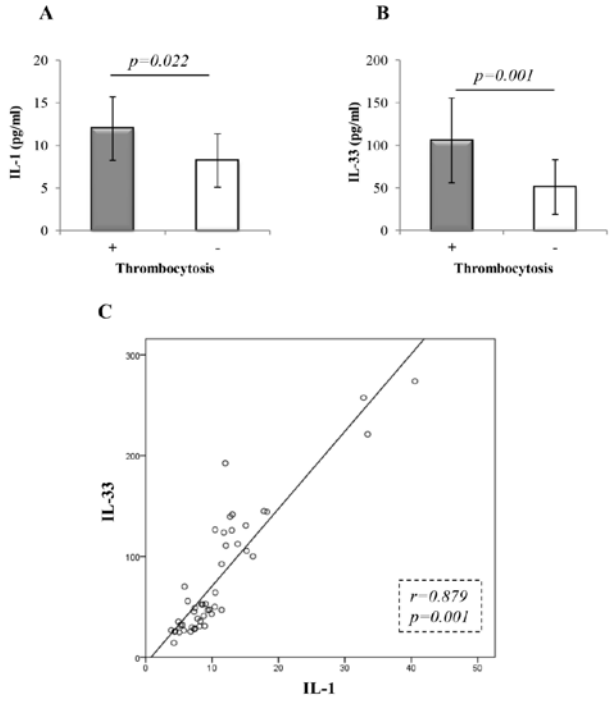

Fig. 2 – Increased serum values of interleukin (IL)-33 and IL-1 in colorectal carcinoma (CRC) patients with thrombocytosis.

(A) CRC patients with diagnosed thrombocytosis (+) had significantly higher serum concentration of IL-1 in comparison to CRC patients with normal number platelets count (–) (p = 0.022);

(B) Serum levels of IL-33 were also significantly increased in CRC patients with diagnosed thrombocytosis (+) compared to CRC patients without thrombocytosis (–) (p = 0.001);

(C) Strong positive correlation was detected between serum values of IL-1 and IL-33 (r = 0.879; p = 0.001).

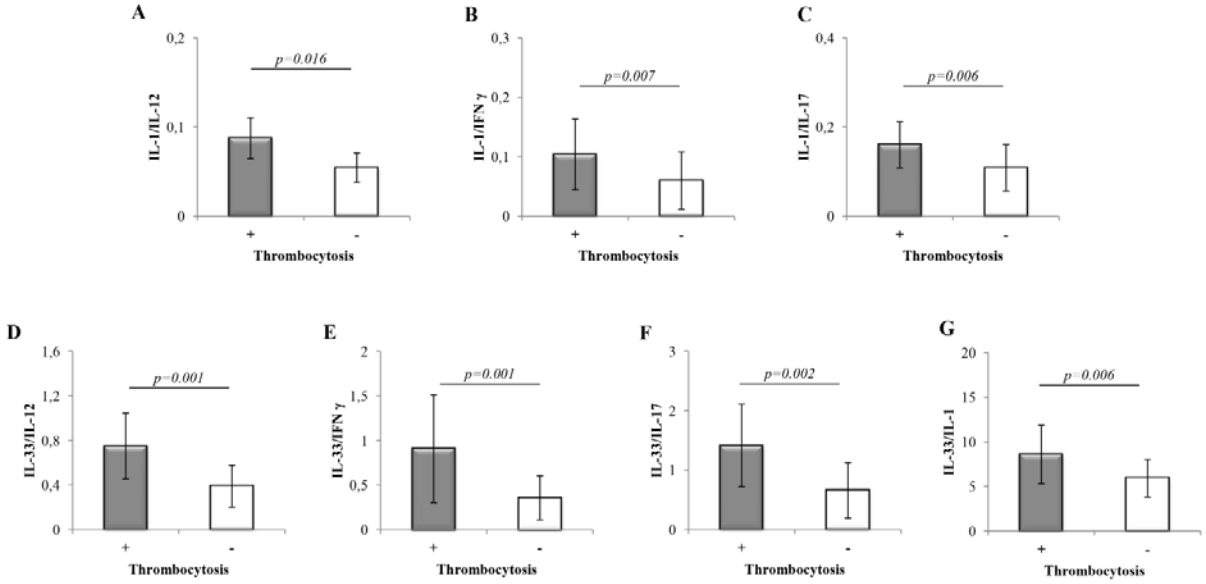

Fig. 3 – Predomination of interleukin (IL)-1 and IL-33 over IL-12, interferon (IFN)-γ and IL-17 in serum of colorectal carcinoma (CRC) patients with thrombocytosis.

CRC patients with thrombocytosis (+) compared to CRC group of patients with normal number of platelets (–) had significantly higher ratios of: (A) IL-1/IL-12 (p = 0.016); (B) IL-1/IFN-γ (p = 0.007); (C) IL-1/IL-17 (p = 0.006). CRC patients with thrombocytosis (+) in comparison to CRC group of patients without thrombocytosis (–) had significantly higher ratios of: (D) IL-33/IL-12 (p = 0.001); (E) IL-33/IFN-γ (p = 0.001); (F) IL-33/IL-17 (p = 0.002). (G) IL-33/IL-1 ratio was significantly increased in CRC patients with thrombocytosis (+) compared to CRC patients without thrombocytosis (–) (p = 0.006).

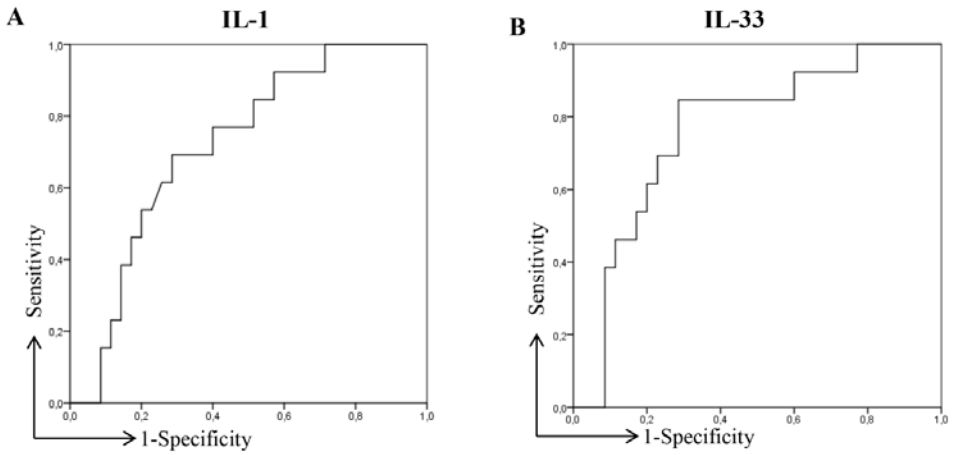

Fig. 4 – Receiver operating characteristic (ROC) curves analyses of serum interleukin (IL)-1 and IL-33 levels for prediction of paraneoplastic thrombocytosis in colorectal carcinoma (CRC) patients.
Sensitivity and specificity of IL-1 and IL-33 as possible markers for conformation of paraneoplastic thrombocytosis in CRC patients: Analysis of ROC curves illustrate sensitivity and specificity for (A) IL-1 [area under curve (AUC) = 0.718; 95% confidence interval (CI): 0.567–0.868, sensitivity 69.2%; specificity 62.9%], and (B) IL-33 (AUC = 0.763; 95% CI: 0.614–0.911; sensitivity 84.6%, specificity 65.7%).

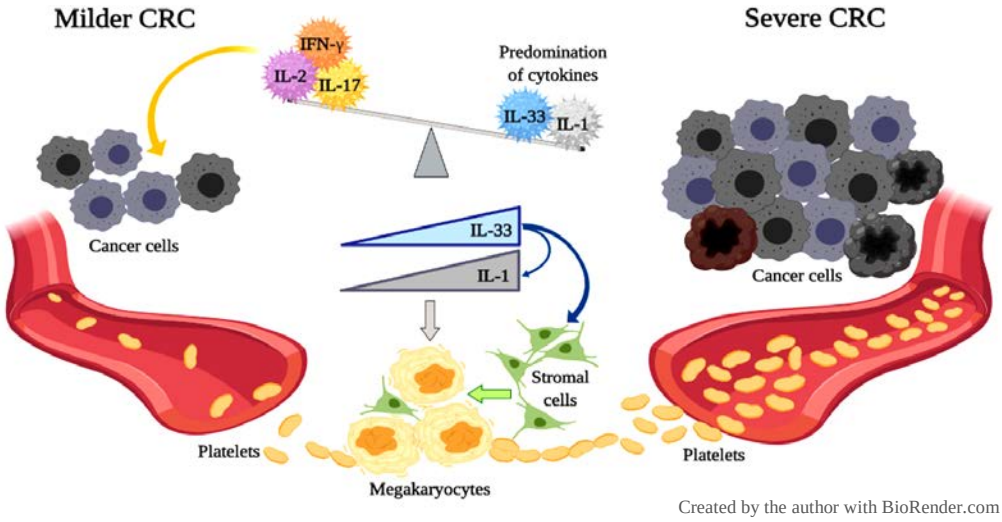

Fig. 5 – Potential united effect of interleukin (IL)-1 and IL-33 in pathogenesis of thrombocytosis in colorectal carcinoma (CRC) patients.
Schematic diagram showing direct stimulative effect of IL-1 on megakaryocytes, as well as, indirect stimulative effect of IL-33 on megakaryocytes through the stromal cells in bone marrow. Both cytokines (IL-1 and IL-33) play a potential simultaneous role in pathogenesis of thrombocytosis in CRC patients. Moreover, it is shown predomination of IL-1 and IL-33 over pro-inflammatory cytokines [IL-12, IL-17 and interferon (IFN)-γ] in severe form of CRC.

CRC patients were 9.47 pg/mL for IL-1 and 49.5 pg/mL for IL-33.

Potential united effect of IL-1 and IL-33 in pathogenesis of thrombocytosis in CRC patients is given in Figure 5.

Discussion

In the present study, we showed that CRC patients with diagnosed thrombocytosis had significantly higher nuclear grade compared to CRC patients with normal number of platelets. Significantly higher percentage of CRC patients with thrombocytosis had detectable metastatic lesions in the lung and liver and peritoneal carcinomatosis, as well as blood and lymph vessels invasion compared to CRC patients without thrombocytosis. Higher concentrations of tumor markers, AFP, CEA, CA 19-9, were detected in CRC patients with diagnosed thrombocytosis. In line with the revelation of previous studies that increased concentrations of tumor markers in patients with CRC indicate on more aggressive type of cancer and can be treated as poor prognostic factor, presented data indicate on more severe form of CRC in patients with thrombocytosis [21].

Reactive thrombocytosis is an elevated platelet count ($\geq 450 \times 10^9$/L) develops secondary to another disorder [19]. Previous studies investigated relation between cancer and thrombocytosis [10–12, 20–24]. Sasaki et al. [22] have shown that cancer-specific survival of CRC patients with thrombocyto-

sis was significantly shorter compared to CRC patients without diagnosed thrombocytosis. Moreover, thrombocytosis indicates adverse prognosis in CRC and also may serve as clinically useful, cost-effective, noninvasive marker to facilitate risk assessment and guide postoperative management [11, 12]. The platelet count is also treated as valuable prognostic marker for the survival in patients with metastasis of colorectal cancer [23]. Furthermore, pretreatment hematologic abnormalities, such as anemia and thrombocytosis, can also be considered as useful prognostic markers in patients with colorectal cancer [24]. In line with these studies, the presented data implicate on severe and more progressive form of CRC in patients with diagnosed thrombocytosis.

Several factors such as iron deficiency, acute infection and chronic inflammatory disorders can be causes of reactive thrombocytosis [19]. Lately, cancer is more often associated with paraneoplastic thrombocytosis [7]. A variety of tumor-related humoral factors and cytokines influence thrombopoiesis in patients with tumor and contribute to tumor-stimulated thrombopoiesis [8, 9]. In order to investigate the potential role of soluble molecules on thrombocytosis development, we further analyzed systemic concentrations of cytokines of interest. IL-1 is potent cytokine involved in variety of pro-inflammatory processes, but also in tumorigenesis and tumor progression [25]. Besides direct stimulating effect on tumor cell proliferation, it has also been shown that colon cancer cell–derived IL-1α may upregulate angiogenesis by modulating stromal cells within the tumor microenvironment [15]. In this study, significantly higher systemic level of IL-1 was measured in CRC patients with diagnosed thrombocytosis compared to CRC patients with normal number of platelets. Previous studies investigate the importance of IL-1 in thrombocytopoiesis [26–28]. Yang et al. [26] revealed a stimulative effect of IL-1β on megakaryocyte colony forming units production. Moreover, they confirmed that megakaryocytic cells have IL-1 receptors on their surface. The other study has shown that a single dose of IL-1 was able to stimulate an increase in platelet production for 3 weeks [27]. Also, administration of recombinant human IL-1β to C57B1/6 male mice consequently induced a remarkable thrombocytosis, about 2.3 times higher in IL-1β treated mice than in control mice [28]. These results are in line with our finding, suggesting an important role of IL-1 in thrombocytosis development.

IL-33, member of IL-1 family is mostly expressed by mucosal epithelial cells [29]. Previous studies confirmed important role of IL-33 in CRC pathogenesis [13, 14]. It is known that IL-33 activates core stem cell genes, recruits macrophages into the cancer microenvironment and stimulates them to produce prostaglandin E2, which all promote carcinogenesis of CRC and metastasis of cancers [16, 30]. To our knowledge, this is the first study describing significant increment of IL-33 in sera of CRC patients with diagnosed thrombocytosis compared to CRC patients without thrombocytosis. In line with our result are different animal and clinical studies indicating the importance of IL-33 in pathogenesis of thrombocytosis. Talabot-Ayer et al. [31] described that CMV/IL33 mice with IL-33 overexpression, characterized as increased local or systemic levels of pro–inflammatory mediators such as IL-1b, C-X-C Motof Chemokine hig and 1 (Cxcl-1), granulocyte colony – stimulating factor (G-CSF), and IL-6, also suffer from anemia, thrombocytosis, and dysregulation of myelopoiesis [31]. The other study has shown that ST2, membrane receptor for IL-33 is expressed in bone marrow mainly on endothelial, mesenchymal, and early myeloid cells. Activation of IL-33/ST2 signaling pathway on these cells stimulates the production of different soluble molecules that further promote development and proliferation of myeloid cells [32]. Previous studies have confirmed that thrombocytosis correlates with a shorter overall survival (OS) and poorer disease free survival (DFS) [33, 34]. Except platelet number, other platelet-associated indicators, such as plateletcrit (PCT), mean platelet volume (MPV) and platelet distribution width (PDW), may also correlate with poorer OS of CRC patient and can be used as potentional prognostic factors [35]. To our knowledge, some studies revealed that development and progression of CRC is associated with alterations in serum IL-1 level but its significance is not well defined [36, 37], while the significance of serum IL-33 as marker of CRC survival has not been explored yet.

Moreover, our study showed predomination of IL-1 and IL-33 over pro-inflammatory cytokines IL-12, IFN-γ and IL-17, known for their crucial role in antitumor immune response [38]. Interesting fact is that IL-33 also predominates over IL-1 in sera of CRC patients with thrombocytosis. Predomination of IL-1 and IL-33 over mediators of potent antitumor immunity in patients with thrombocytosis can partially explain more severe and progressive disease diagnosed in these patients.

Strong positive correlation detected between serum values of IL-1 and IL-33 indicated a potentially united effect of these cytokines in pathogenesis of thrombocytosis in CRC patients. This simultaneous effect can be realized in at least two manners: 1) IL-1 may directly affect megakaryocytes and stimulate production of platelets; 2) IL-33 may indirectly stimulate stromal cells in the bone marrow to produce mediators that can further stimulate thrombopoiesis in CRC patients.

This proposed mechanism was supported by analysis of ROC curves of IL-33 and IL-1 that revealed that these cytokines could be used as possible markers of paraneoplastic thrombocytosis in CRC patients.

Conclusion

Presented data revealed that IL-1 and IL-33 significantly correlated to high thrombocyte number in patients with more aggressive CRC.

Conflict of interest

The authors declare that they have no competing interests.

Acknowledgement

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Gender differences in stress intensity and coping strategies among students, future emergency relief specialists

Rodne razlike u intenzitetu stresa i mehanizama za kontrolu stresa kod studenata, budućih stručnjaka za pomoć u hitnim slučajevima

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Abstract

Background/Aim. Assisting students face high academic demands which, together with interpersonal, intrapersonal and professional requirements, can be a significant source of stress. The aim of the study was to examine the intensity and frequency of the source of stress, coping strategies and identify gender differences among students, future assisting professionals. **Methods**. An observational, cross-sectional study was conducted amongst the students of the University of the Belgrade Faculty of Security (Serbia) who, after graduation, will acquire the title of a security manager responsible for human resources in the civil sector. The data were collected in the period October-November 2018. The authorized questionnaire SSM-30 by Jović (Stress scale for the young – 30) was used, which enables students to assess the stress situations intensity on a scale from 1 (minimum) to 10 (maximum intensity). The SSM-30 questionnaire is a combination of the standard Life Events Scale – Holmes Rashe Life Events Scale, also known as the Social Readjustment Rating Scale and life events characteristic for the student population. The questionnaire also included the sample demographic characteristics – gender, and a year of study. The SSM-30 scale includes a list of stressful events and stress coping mechanisms shown in the results. **Results.** The most common sources of stress in both genders were social and academic ones: death in the family, critical illness in the family, an accident of a person they care about, unwanted pregnancy, lies from close people, disagreement with parents, loss of a study year, crisis, uncertainty after graduation and partner's infidelity. The most frequently used mechanisms for controlling and overcoming stress were mostly social: talking with friends, listening to music, family support, frequent walks, socializing and going out, using the Internet, frequent sleep, intense physical activity, crying and relaxation. Statistically significant differences between the genders were confirmed – female students demonstrated self-worth of higher intensity during the majority of stressful situations, as they use different stress coping mechanisms from their male students. **Conclusion.** The results obtained with regard to the assessment of stressors and the use of specific mechanisms for coping point to the need of additional education of students in this field in order to be more focused and open for free professional help, when necessary.

Key words:
sex factors; stress, psychological; students; surveys and questionnaires.

Apstrakt

Uvod/Cilj. Pred studente koji studiraju se obučavaju za *helper* profesije postavljeni su visoki akademski zahtevi koji uz interpersonalne, intrapersonalne i profesionalne zahteve mogu predstavljati značajan izvor stresa. Cilj istraživanja je bio da se ispita intenzitet i učestalost izvora stresa, mehanizmi prevladavanja stresa i utvrde rodne razlike kod studenata, budućih *helper* profesionalaca. **Metode**. Sprovedena je opservaciona studija preseka među studentima Fakulteta bezbednosti Univerziteta u Beogradu (Srbija) koji završetkom studija stiču naziv menadžera bezbednosti, odgovornih za ljudske resurse u civilnom sektoru. Podaci su prikupljeni u periodu oktobar-novembar 2018. Korišćen je autorizovani upitnik Skala stresa kod mladih-30 (SSM-30) po Joviću, koji omogućava da studenti ocene intenzitet stresnih situacija na skali od 1 (minimalni) do 10 (maksimalni intenzitet). Upitnik SSM-30 je kombinacija Standardne skale životnih događaja – *Holmes Rashe Life Events Scale*, takođe poznate i kao *Social Readjusment Rating Scale* i životnih događaja karakterističnih za studentsku populaciju. Upitnik je uključivao i demografske karakteristike uzorka – pol i godinu studiranja. Skala SSM-30 obuhvata listu stresnih događaja i mehanizme za prevladavanje stresa koji su prikazani u rezultatima. **Rezultati**. Najčešće navođeni izvori stresa kod oba pola bili su socijalni i akademski: smrt u porodici, teža bolest u porodici, nesreća kod osobe koju volim, neželjena trudnoća, laž od strane bliskih osoba, neslaganje sa roditeljima, gubitak godine studija, besparica, ekonomska kriza, neizvesnost

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nakon završetka studija i neverstvo partnera. Najčešće korišćeni mehanizmi kontrole i prevladavanja stresa su bili uglavnom socijalni: razgovor sa prijateljima, slušanje muzike, podrška porodice, česte šetnje, druženje i izlasci, upotreba interneta, a često i dugo spavanje, intenzivna fizička aktivnost, plakanje i relaksacija. Dokazane su statistički značajne razlike između polova sa većim intenzitetom samovrednovanja većine stresnih sutuacija kod studentkinja koje koriste drugačije mehanizme odbrane od studenata muškog pola. **Zaključak**. Dobijeni rezultati u vezi procene stresora i korišćenja specičnih mehanizama za suočavanje sa stresom, ukazuju na potrebu dodatne edukacije studenata u ovoj oblasti, kako bi bili više usmereni i slobodnije tražili stručnu profesionalnu pomoć kada je ona neophodna.

Ključne reči:
pol, faktor; stres, psihološki; studenti; ankete i upitnici.

Introduction

Young people's development during the transition period to adulthood is accompanied by numerous emotions and involves adaptation to many new life situations, and young people who have decided to study are exposed to particular challenges. Complex academic and living conditions create such an atmosphere that in this period students are often exposed to numerous sources of stress, so that studying can have both positive and extremely negative impact and consequences on students' mental health if it is not managed well [1–3]. The stress that students experience during the study was defined by Lazarus and Folkman, viewed as part of the student experience as "a particular relationship between the person and the environment that is appraised by the person as taxing or exceeding his or her resources and endangering his or her wellbeing" [4]. Previous studies in the area of stress sources in students identified the following stressors: interpersonal stress, intrapersonal stressors and academic stressors [5–7]. It was found that the stress symptoms are expressed through a series of somatic symptoms, such as energy loss, high blood pressure, appetite and sleep disorders [8–12], then hormone disorder [13], high prevalence of musculoskeletal pain, where shoulder pain is the most common one [14]. When these disorders take hold, the individual becomes disorganized, disoriented, and therefore less able to deal with everyday challenges, resulting in stress-related health problems [15, 16].

Studies confirm high exposure to stress of students trained for assisting professions, future members of professional services (medicine, nursing, social work, dentistry, law, psychology, law enforcement, educational institutions) where responsibility for clients' health, life and safety is expected [17–19]. The existence of gender differences in stress perception, assessment of its intensity and control mechanisms have been confirmed in previous studies, while higher levels of perceived stress and posttraumatic stress symptoms have been found in female students compared to male students, but also generally in the female part of the population compared to men [5, 15, 20, 21].

Given that increased student exposure to stress can damage their mental and physical health and affect their capacity to adequately meet the needs of users, in their future nursing professional practice [22, 23], it was interesting to examine stress intensity among non-medical students educated for assisting profession in our population, which is rarely described in the available literature.

The aim of the study was to examine the intensity and frequency of the source of stress, the mechanisms for coping and to identify gender differences among students, future assisting professionals.

Methods

An observational, cross-sectional study was conducted amongst the students of the University of Belgrade, Security Faculty, Serbia who, at the end of the study, will acquire the title of a security manager responsible for human resources in the civil sector. The data were collected in the period October-November 2018.

Nature and purpose of the examination were explained to the students who completed questionnaires in the classrooms immediately after the end of classes. Out of a total of 923 students, 831 students completed the questionnaires. The study protocol was approved by the Ethics Commission of the Faculty of Security and before testing, all subjects signed an informed consent.

The authorized questionnaire Stress scale for the young – 30 [Skala stresa kod mladih-30 (SSM-30) in Serbian] by Jović was used, which enables students to assess the stress situations intensity on a scale from 1 (minimum) to 10 (maximum intensity). The SSM-30 questionnaire is a combination of the standard Life Events Scale – Holmes Rashe Life Events Scale, also known as the Social Readjustment Rating Scale – PRS [21] of life events which students pointed out in previous research by the same author as stressful and specific to their population. The questionnaire also included the sample demographic characteristics – gender, and a year of study. SSM-30 by Jović was previously tested on a sample of 1,273 students of the Faculty of Medicine in Niš, Serbia from 1996 to 2006 and 269 students of medicine at the Faculty of Medicine, East Sarajevo (Foča, Republic of Srpska, Bosnia and Herzegovina in the period from 2007 to 2010 [21, 24]. The questionnaire contains 30 stressful events, most frequently cited in the abovementioned previous studies, evaluated by students according to the intensity using grades from 1 (minimum) to 10 (maximum). The second part of the questionnaire referred to the mechanisms for overcoming stress and offered students 19 stress relief mechanisms, cited in the study of the same author [21, 24] where students stated whether or not they used such mechanisms of stress defense.

Statistical analysis was done using the SPSS software package version 20.0. For comparison of statistical significance, gender differences were used from nonparametric Pirson's quadratic square frequency test, and from parametric Student's *t*-test

for numerical features, taking $p < 0.05$ as the level of statistical significance.

To verify the validity of both questionnaires used, the Kronbach coefficient was used.

Results

Out of the total of 831 students surveyed, there were 188 young men (22.6%) and 643 young women (77.4%); 46.5% of

Table 1

Estimation of stress intensity on the Stress scale for the young-30 in students

Life events	Total		Men		Women		*t*	*p*
	rank	mean ± SD	rank	mean ± SD	rank	mean ± SD		
Death in a family	1	9.47 ± 1.70	1	9.22 ± 2.02	1	9.54 ± 1.60	2.926	0.022
Critical illness in a family	2	8.94 ± 1.81	2	8.50 ± 2.08	2	9.07 ± 1.70	3.809	0.000
An accident of a beloved person	3	8.07 ± 2.08	3	7.46 ± 2.21	3	8.24 ± 2.02	4.525	0.000
Unwanted pregnancy	4	7.47 ± 2.91	8	6.22 ± 2.20	4	7.82 ± 2.72	6.682	0.000
Lies by close people	5	7.20 ± 2.37	4	6.65 ± 2.43	5	7.36 ± 2.34	3.587	0.000
Disagreement with parents	6	7.11 ± 2.45	6	6.37 ± 2.63	6	7.33 ± 2.36	4.751	0.000
Loss of a study year	7	6.92 ± 2.72	9	6.06 ± 2.98	7	7.17 ± 2.58	4.964	0.000
Lack of money, economic crisis	8	6.82 ± 2.56	7	6.28 ± 2.68	8	6.97 ± 2.51	3.271	0.001
Uncertainty after graduation	9	6.67 ± 2.57	12	5.73 ± 2.69	9	6.94 ± 2.47	5.778	0.000
Partner'sinfidelity	10	6.60 ± 2.88	5	6.53 ± 2.69	11	6.62 ± 2.86	0.346	0.729
Exams and grading	11	6.48 ± 2.60	16	5.37 ± 2.64	10	6.80 ± 2.51	6.794	0.000**
Separation from the family	12	6.35 ± 2.83	13	5.46 ± 2.94	12	6.61 ± 2.73	4.945	0.000**
Permanent loss of a friend	13	6.26 ± 2.63	10	5.98 ± 2.67	13	6.34 ± 2.62	1.641	0.101
Great material loss	14	6.20 ± 2.53	11	5.96 ± 2.74	15	6.27 ± 2.47	1.467	0.143
Lack of time for fun	15	6.11 ± 2.57	15	5.45 ± 2.57	14	6.31 ± 2.54	4.042	0.000
Separation from the loved person	16	6.00 ± 2.65	14	5.46 ± 2.57	16	6.16 ± 2.67	3.185	0.002
Care whether a student meets the requirements of classes	17	5.77 ± 2.71	19	4.77 ± 2.49	17	6.07 ± 2.71	5.866	0.000
Burden of obligations	18	5.73 ± 2.62	18	4.87 ± 2.71	18	5.98 ± 2.55	5.179	0.000
Feeling of unsafety	19	5.47 ± 2.01	24	4.26 ± 2.95	19	5.83 ± 2.94	6.417	0.000
Poor communication with staff at professional practice	20	5.32 ± 2.53	20	4.69 ± 2.39	20	5.50 ± 2.55	3.906	0.000**
Belief in one's own efficiency	21	5.14 ± 2.75	27	4.06 ± 2.79	21	5.45 ± 2.67	6.212	0.000**
Administrative jobs on the faculty	22	5.09 ± 2.93	21	4.41 ± 2.89	22	5.29 ± 2.91	3.641	0.000**
Physical conflict with someone	23	5.00 ± 2.89	17	5.32 ± 2.94	27	4.90 ± 2.87	1.976	0.049*
Request for the perfect performance of professional skills	24	4.92 ± 2.55	22	4.36 ± 2.35	24	5.09 ± 2.58	3.435	0.001**
Availability of literature for the preparation of exams	25	4.84 ± 2.72	26	4.08 ± 2.69	25	5.06 ± 2.70	4.384	0.000**
Organization of classes and practical work	26	4.82 ± 2.90	28	3.91 ± 2.71	23	5.10 ± 2.91	4.957	0.000**
Teachers' and associates' behavior	27	4.81 ± 2.55	25	4.07 ± 2.57	26	5.02 ± 2.50	4.522	0.000**
Practical work environment	28	4.61 ± 2.61	29	3.69 ± 2.24	28	4.88 ± 2.65	5.582	0.000**
Excessive weight	29	4.50 ± 2.10	30	3.59 ± 1.67	29	4.76 ± 2.77	4.580	0.000**
Watching a game where a team is loosing	30	3.20 ± 2.84	23	4.35 ± 2.32	30	2.86 ± 1.62	6.464	0.000**

SD – standard deviation.

respondents were at the second year of study, 37.5% at the third year, and 14.3% at the fourth year, while the least number of included respondents were at the first year (1.7%), because at the time of the study they were not having lectures.

Reliability of questionnaires on stress factors was extremely high ($\alpha = 0.910$), meaning that the questionnaire was well conceived, as well as that the scoring was excellent. It is interesting that the elimination of any issue did not change significantly the value of the Kronbach coefficient, so the conclusion of this analysis was that all the questions in the questionnaire should remain and that scoring should be the same in the future work.

The reliability of the second part of the questionnaire, the mechanisms for overcoming stress, was medium ($\alpha = 0.516$), meanings that the questionnaire was well conceived. It is interesting that the elimination of any issue did not change the significant value of the Kronbach coefficient, and the conclusion of this analysis was that all the questions in the questionnaire should remain there, and the biggest loss would be to remove the issue of using the Internet and the greatest gain to eliminate the issue of intense physical activities.

An analysis of the intensity of stressful events/situations in a complete sample of students was performed and gender differences were examined (Table 1).

First, the high-ranked situations in both genders were: 1. Death in the family, 2. Critical illness in the family, and 3. Accident of a beloved person. The list of the other analysed life events and difference in their perception by gender is shown in Table 1.

The analysis of stress by gender (Table 1), from the 4th place onwards, shows a different self-assessment of the intensity of stress in some situations, regarding the student's gender. So, at the high 4th place with the female students is the Unwanted pregnancy, while with the male students only at the 8th place. For all items, the average score was higher for female students, except questions 28 and 29 (Watching the favorite team's game when losing and a Physical conflict with someone, respectively) where the scores were greater among the young men.

Comparison of the average scores from the questionnaire on stress factors in relation to gender showed that the difference was statistically significant for all questions, except for questions 5, 19 and 26 (Breaking Friendship, Great material loss and Partner's infidelity, respectively).

Further, the frequency of various mechanisms for overcoming stress in the whole sample of students, as well as gender differences, were examined (Table 2).

The most commonly used mechanisms for overcoming stress were: 1. Conversation with friends, 2. Listening to music, 3. Family support, 4. Frequent walks, 5. Socializing and going out, 6. Internet usage, 7. Frequent and long sleep, 8. Intensive physical activity, 9. Crying and 10. Relaxation. The first five mechanisms involve the use of social support (family, relatives, friends) or self-help. Matching the frequency of students' responses with the questionnaire on stress factors by gender and the mechanism of defense showed that young women statistically significantly more frequently used certain ways of overcoming stress: Talking with friends (79.0% vs. 70.2%), Family support (67.8% vs. 52.1%), Frequent walks (63.1% vs. 46.3%), Frequent outings and socializing (51.0% vs. 43.6%), Listening to music (74.3% vs. 62.2%) Reading books and magazines (30.6% vs. 16.0%), Using sedatives (4.2% vs. 1.6) and Frequent crying (42.3% vs. 5.9%), where statistically significance of gender differences was convincingly the biggest.

Young men used the following mechanisms for overcoming stress more often than young women: Intense physical activity (55.3% vs. 30.2%), Frequent relaxations (38.3% vs. 30.9%), Frequent TV viewing (37.2 % vs. 28.0%), the

Table 2

Frequency of coping mechanisms in students and gender differences

Coping mechanism	Total (yes)		Men (yes)		Women (yes)		χ^2	*p*
	rank	n (%)	rank	n (%)	rank	n (%)		
Conversation with friends	1	640 (77.0)	1	132 (70.2)	1	508 (79.0)	6.352	0.024
Listening to music	2	595 (71.6)	2	117 (62.2)	2	478 (74.3)	10.483	0.000
Family support	3	534 (64.3)	4	98 (52.1)	3	436 (67.8)	15.572	0.000
Frequent walks	4	493 (59.3)	5	87 (46.3)	4	406 (63.1)	17.146	0.000
Socializing and going out	5	410 (49.3)	6	82 (43.6)	5	328 (51.0)	3.498	0.048
Using the Internet	6	361 (43.4)	7	79 (42.0)	6	282 (43.9)	0.199	0.884
Frequent and long sleeping	7	330 (39.7)	8	75 (39.9)	8	255 (39.8)	0.003	0.991
Intense physical activity	8	298 (35.9)	3	104 (55.3)	11	194 (30.2)	39.998	0.000
Crying	9	283 (34.1)	16	11 (5.9)	7	272 (42.3)	86.062	0.000
Relaxation	10	271 (32.6)	9	72 (38.3)	9	199 (30.9)	3.575	0.038
Watching TV	11	250 (30.1)	10	70 (37.2)	12	180 (28.0)	5.905	0.034
Reading books and magazines	12	227 (27.3)	13	30 (16.0)	10	197 (30.6)	15.790	0.000
Religion, faith (prayer)	13	191 (23.0)	12	46 (24.5)	13	145 (22.6)	0.302	0.887
Using alcohol	14	128 (15.4)	11	48 (25.5)	15	80 (12.4)	19.129	0.000
Smoking cigarettes	15	123 (14.8)	14	22 (11.7)	14	101 (15.7)	1.851	0.109
Shouting and quarreling	16	100 (12.0)	15	21 (11.2)	16	79 (12.3)	0.171	0.910
Professional help (psychologist)	17	37 (4.5)	18	5 (2.7)	17	32 (5.0)	1.836	0.201
Using sedatives	18	30 (3.6)	19	3 (1.6)	18	27 (4.2)	4.011	0.044
Using drugs	19	15 (1.8)	17	7 (3.7)	19	8 (1.2)	5.045	0.039

Use of alcohol (25.5% vs. 12.4%) and the Use of illegal drugs (3.7% vs.1.2%), which statistically significant gender differences were confirmed (Table 2).

Statistically significant difference in gender was not defined with regard to the following ways of overcoming stress: Religious motives, Shouting and quarreling, Frequent and long sleeping, Tobacco or cigarette use, Internet use, and seeking help from an expert, which students of both genders are extremely rarely using (Table 2).

Discussion

The conducted research is a study of the perception of stressful life situations and the impact of gender differences on experiencing stress in the population of the Faculty of Security, University of Belgrade, who are studying for the position of security managers responsible for the protection of human resources safety and health. The survey included respondents of all four years of study, with a female population dominating the sample (77.4% vs. 22.6%), which is in line with data from other surveys on prevalent female students at most faculties in our country and in the world educating assisting professionals (assistant professions) [21, 24, 25]. Reference data show that feminine gender is a significant independent predictor of stress perception, that is, a higher stress response [5, 24, 26, 27], which means that these gender differences are not specific to students who are educated for future emergency care specialists [6, 20, 21].

Most situations of high-ranking stress levels arise from nonacademic sources, mainly from family relationships, relationships with people important to students (friends, family members, partners) and socioeconomic problems.

Our study showed that young women evaluated the majority of stressful situations on the SSM-30 scale statistically significantly more intensively than young men (in 27 out of 30 items, with the exception of three items: Breaking Friendship, Great material loss and Partner's infidelity) (Table 1). A possible explanation for the differences found is that it is easier for a feminine gender to express their feelings related to stressful situations, unlike young men, and it seems that young women express their emotions more turbulently [26]. Blanch et al. [27] in the revised literature review of gender differences among students in the US in terms of self-confidence find that female students have a lower level of self-confidence and a higher level of anxiety with relation to male students, which can also be one of the reasons for a more turbulent response to stress. Gender-specific approach to programs for cognitive-behavioral stress management [28] is also based on these findings.

Students who are studying for assisting professions must meet high academic requirements which, together with interpersonal, intrapersonal and professional requirements, can be an important source of stress.

Interpersonal stresses include: insufficient interest in a particular field, subject or task, negative thoughts arising from the review of their own behavior, feelings related to changes of their own bodies and dissatisfaction with their own appearance [5], relationships with the roommates, unwanted pregnancy of female students, sexual problems, relationships with the opposite sex [5, 29]. These stressors also include divorce, unemployment, illness or death of parents, excessive expectations from parents, friends and close relatives, or insufficient social support, which ultimately can lead to disappointment or lead to depression and change in interpersonal relationships [11]. Intrapersonal stressors are related to public appearances, changes in eating habits, new way of managing finances and often lack of money [30–31]. Our results have shown that they are highly ranked on the scale of stress. Social stressors are Death in a family, Critical illness in a family, Accident of a beloved person. Unwanted pregnancy, Lies from close persons, Disagreement with parents, Lack of money, Economic crisis, and Partner's infidelity. Sreeramareddy et al. [33] state that the most significant and most frequently cited psychosocial sources of stress for medical students were family separation and dwelling in a students' dormitory, overly high expectations from parents, a transient curriculum, and a lack of time and conditions for fun. Situations of an Accident of a beloved person and Partner's infidelity are on the 3rd and 10th place among the students in our research, and are also highly quoted in the research of Muirhead and Locker [34], where 60% of students stated that they were under stress due to problems in relations with the opposite sex.

Academic sources of stress are also high on the list of stressful life events of faculty students educated for assisting professionals [6, 25]. Academic stressors include: change of the educational environment [35], the way of organizing obligations during the semester [11, 36], inadequate material for the preparation of the exam [37], unclear tasks and uncomfortable classrooms, relationships with faculty employees and time pressures that can also be a sources of stress [16] as well as the need for constant self-control and the development of better thinking skills, including specific techniques/learning methods. Students under stress show signs of emotional suffering, aggressive behavior, shyness, social phobia, depression, anxiety, suicidal thoughts, concentration drop and often lack of interest in common activities. Additionally, the stressors can include the obligation to pay tuition fees, as well as potential doing business (employment) while complying with student obligations [38], and taking care of an unclear future [6, 39]. Of the academic stressors in our research, the highly ranked are a Loss of study year, Uncertainty after graduation and Exams and grading (Table 1), and similar results are often cited in literature [24, 40, 41]. The main stressors for students more often related to professional training, individual learning, progress during the year, achievements and availability of literature, than to personal problems [40]. In addition to these situations, the studies from the available literature state that intensive stress for students is also associated with the following situations: pressure to perfectly perform skills related to working with clients, obligations overload, belief in their own efficiency at work [6, 18], day filled with obligations and lack of free time for relaxation [41]; double obligation – the role of a student and the role of a spouse at the same time [34], which the respondents in our study did not cite as a significant source of stress, would be among the top 15 on the list of life events.

The most frequently used mechanisms for stress control by students in our study were: Conversation with friends, Listening to music, Family support, Frequent walks, Socializing and going out, or using social support mechanisms (Table 2), which is in line with research by other authors [1, 24, 29, 42]. It is therefore important to promote social support among students, especially among those with a low level of support. Students without social support find alternate support as a protective factor in order to build resilience and face the stress more efficiently. Peer support especially reduces stress and is advocated as a valid method of stress management among students. However, this strategy is just one aspect of a wider solution and it is necessary to comprehensively examine the problem at the institutional level. What is an alarming result of our study is that an extremely small number of students addresses an expert (psychologist or psychiatrist) to seek professional help, and that a significant percentage of them, primarily male students, use ineffective and harmful health mechanisms, such as the use of alcohol, tobacco and illegal drugs, which can also be a socio-cultural feature of the social milieu.

Stressors during study can affect the quality of life and satisfaction with life, as well as the results of exams, and later the reduced efficiency in their future assisting profession [16, 22, 23, 43] therefore, the implementation of preventive measures in this area is extremely important, based on stress assessment and stress coping mechanisms.

The significance and contribution of the study to the investigated problem is that in our country, as far as the authors are informed, no research in stress and coping mechanisms has been conducted so far, with the examination of gender differences in nonmedical students for the assisting profession.

It is recommended to students with discovered high overall stress levels to complete standardized questionnaires for the diagnosis of anxiety and depression, for the purpose of selecting a category of students requiring expert assistance in coping with psychological problems.

The limitations of the study are related to the fact that this is a cross sectional study carried out at one faculty. It would be useful to conduct a prospective study, as well as to compare self-assessments of stress among medical and nonmedical assisting professionals in order to plan specific education and preventive measures for certain types of assisting professions. Another research limitation was the uneven number of students by year of study – fewer first-year students (1.4%) and fourth-year students (14.3%), which affected the research results. This information is significant for future research.

Conclusion

The results of this study showed the high frequency and intensity of self-assessment of stress among the examined students. The most prominent were social stressors, followed by the academic ones. The most frequently used mechanisms of stress management by students in our study were social support mechanisms: Conversation with friends, Listening to music, Family support, Frequent walks, Hanging out and going out. The results obtained with regard to the assessment of stressors and the use of specific mechanisms of coping point to the need of additional education of students in this field in order to be more focused and free to seek professional help, when necessary.

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CT appearance in the 330 patients with coronavirus disease 2019 (COVID-19) in Serbia

CT karakteristike bolesti koronavirus 2019 (COVID-19) kod 330 bolesnika lečenih u Srbiji

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Abstract

Background/Aim. The corona virus disease 2019 (COVID-19) primarily affects the respiratory system, so radiological diagnosis has been shown to be necessary. Chest computed tomography (CT) is to be shown the best modality in suspected COVID-19 cases for initial evaluation because CT findings may be present before the onset of symptoms. The aim of this study was to show different CT imaging features or patterns in COVID-19 patients with a different time course and disease severity. **Methods.** This prospective cohort study analysed 330 patients (the average age was 52.37 ± 15.36) with confirmed COVID-19 via laboratory testing. During hospitalization, all patients included in the study underwent chest CT in order to assess the extent of changes in their lungs. COVID-19 patients, with a different time course and disease severity, were classified into four categories: lung, bronchial, pleural and mediastinal changes. Based on the time interval between the onset of symptoms and performed CT scan, all patients were divided into three groups: group 1 (CT scans done ≤ 1 week after symptom onset); group 2 (from > 1 to 2 weeks after symptom onset); group 3 (from > 2 weeks after symptom onset). In order to monitor the distribution of changes in the lungs more accurately, bilateral lungs were divided into 12 'lung' zones. Each zone was assigned a CT score. Total severity score was calculated by summing the scores for each zone. **Results.** In 93.6% patients with COVID-19, the CT findings were positive. About 92.1% patients had multiple lesions. The lesions were bilateral in 87.6% of patients, localized both peripheral and centrally in 63.3% of patients, and occurred more frequently in posterior areas (93%), as well as in lower lung zones (91.2%). The average total severity score was 11.00 (7.00–16.00). The most common CT findings in all patients were the ground-glass opacities (97.7%), reticular pattern (91.3%), consolidation (71.5%) and fibrotic streaks (63.8%). In the group 1, changes on CT were found in 80.0%, in the group 2 in 95.0%, and in the group 3 in 99.4% of the patients. **Conclusion.** CT is proven to be a very important diagnostic method in COVID-19 patients, and together with clinical and laboratory findings, gives a complete picture of the patient's condition and contributes significantly to decision-making for further treatment.

Key words:
covid-19; lung; diagnosis; organ dysfunction scores; pneumonia; severity of illness index; tomography, x-ray computed

Apstrakt

Uvod/Cilj. Bolest koronavirus 2019 (COVID-19) primarno zahvata respiratorni sistem, tako da se radiološka dijagnostika pokazala neophodnom. Kompjuterizovana tomografija (CT) grudnog koša pokazala se kao najbolji modalitet za inicijalnu procenu osoba sumnjivih na COVID-19, pošto CT nalaz može biti pozitivan i pre pojave simptoma. Cilj ove studije bio je da se prikažu različite CT karakteristike obolelih od COVID-19, sa različitim vremenskim tokom i težinom bolesti. **Metode.** Sprovedena je prospektivna kohortna studija na 330 bolesnika (prosečne starosti 52,37 ± 15,36) sa dijagnozom COVID-19, potvrđenom putem laboratorijskog testiranja. Tokom hospitalizacije, svim bolesnicima uključenim u studiju urađen je CT pregled grudnog koša u cilju procene proširenosti promena u plućima. Promene na CT snimcima podeljene su u četiri kategorije: plućne, bronhijalne, pleuralne i medijastinalne promene. Na osnovu vremenskog intervala od pojave simptoma do CT pregleda, bolesnici su bili podeljeni u tri grupe: prva grupa (≤ 7 dana); druga grupa (7–14 dana); treća grupa (> 14 dana). U cilju što preciznijeg praćenja distribucije

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lezija u plućima, pluća su podeljena na 12 'plućnih' zona. Svakoj zoni je dodeljen CT skor. *Total severity score* bio je izračunat kao suma skorova pojedinih zona. **Rezultati.** Kod 93,6% obolelih od COVID-19, CT nalaz je bio pozitivan. Oko 92,1% bolesnika imalo je više lezija. Lezije su bile bilateralne kod 87,6% bolesnika, periferno i centralno lokalizovane kod 63,3% bolesnika, a češće su se javljale u posteriornim segmentima (93%), kao i u donjim plućnim zonama (91,2%). Prosečan *Total severity score* bio je 11,00 (7,00–16,00). Najčešći CT nalaz je bila opacifikacija po tipu „mlečnog stakla" (97,7%), retikularna šara (91,3%), konsolidacija (71,5%) i fibrozne trake (63,8%). Promene na CT snimku nađene su kod 80.0% bolesnika iz prve grupe, kod 95.0% iz druge grupe i kod 99.4% iz treće grupe. **Zaključak.** CT se pokazao kao veoma važna dijagnostička metoda kod bolesnika sa COVID-19 i zajedno sa kliničkim i laboratorijskim nalazima daje potpunu sliku stanja bolesnika čime značajno utiče na donošenje odluka o daljem lečenju.

Ključne reči:
covid-19; pluća; dijagnoza; skorovi, disfunkcija organa; pneumonija; bolest, indeks težine; tomografija, kompjuterizovana, rendgenska

Introduction

Several cases of pneumonia with an unidentified origin occurring in Wuhan City, the capital of Central China's Hubei Province were reported to the World Health Organization (WHO) on December 31, 2019. The cases are epidemiologically related to Huanan Seafood Wholesale Market [1]. The viral origin of the mentioned pneumonias was soon determined, and on January 7th, 2020 the causative agent was identified as the 2019 novel coronavirus (2019-nCoV), later this was renamed to severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [2]. The WHO declared the outbreak of a global health emergency on January 30th, 2020 [3]. On 12th February 2020, WHO named the disease caused by SARS-CoV-2 as coronavirus disease 2019 (COVID-19). Finally, on 11th March 2020, WHO formally announced that COVID-19 was a pandemic [4]. By the end of October 2020, the disease has spread to almost all countries with about 50,000,000 patients and about 1,000,000 deaths and the number of patients and deaths are still increasing.

The real- time reverse transcription polymerase chain reaction (rRT-PCR) of viral nucleic acid is regarded as a reference standard in COVID-19 diagnosis. The rRT-PCR test for COVID-19 is believed to have high specificity but the sensitivity has been reported to be as low as 60–70% [5, 6].

The disease primarily affects the respiratory system, so radiological diagnosis has been shown to be necessary. Chest radiographs are the first-line imaging test for identifying pneumonia, but are of little diagnostic value in the early stages. In the intermediate to advanced stages of COVID-19 chest radiographs may show a progression of radiological features. At the same time chest computed tomography (CT) is to be shown the best modality in suspected COVID-19 cases for initial evaluation because CT findings may be present before the onset of symptoms [7, 8]. In a number of cases with an initial false-negative rRT-PCR test, some authors have reported CT findings that have proved to diagnose with sensitivity of 98% [5]. Chest CT has also significance in monitoring disease progression and therapeutic efficacy. The predominant CT findings of COVID-19 are bilateral, peripheral, posterior and basal predominant ground-glass opacity (GGO) with or without consolidation [9, 10].

Studies so far suggest a possible association between CT findings and the severity of the clinical features [11]. The problem is still asymptomatic patients and ones with an atypical clinical features. Adequate interpretation of CT findings, typical and atypical CT characteristics, as well as the evolution of CT findings are essential for effective patient management and treatment. Therefore, the aim of this study was present a wide range of CT features in patients with COVID-19, all with the aim of a better understanding of them.

Methods

The principles of the International Conference on Harmonization (ICH) Good Clinical Practice, the Declaration of Helsinki and national and international ethical guidelines were strictly followed during this study. The approval from the Ethics Committee of the University Hospital "Dr. Dragiša Mišović – Dedinje", Belgrade, Serbia was obtained (Nº 01-7661).

This prospective cohort study analysed 330 patients (consecutive sampling) with confirmed COVID- 19 via laboratory testing with rRT-PCR of respiratory secretions obtained by nasopharyngeal or oropharyngeal swabs, which were treated in the University Hospital "Dr. Dragiša Mišović – Dedinje", Belgrade, Serbia, between March and May 2020. During hospitalization, all patients included in the study underwent chest CT in order to assess the extent of changes in their lungs. The patient and clinical data including age, gender, comorbidities, symptoms, date of onset of symptoms, laboratory examination results were collected for the entire group and analysed.

CT protocol

All CT scans were obtained using the Canon (former Toshiba), Aquillion One (TSX-301C), 320 row MDCT System (Canon, Tokyo, Japan). Scans acquisition were done from the level of the thoracic entrance to the inferior level of the costophrenic angle with patient in a supine, arms raised, head forward position and with breath-holding manner during end inspiration. Unenhanced CT scans were obtained for all patients. The following parameters were used: tube voltage 120 kV with automatic tube current modulation, slice

thickness 1.0 mm. In pregnat women, a radiation shield was used over the gravid uterus.

Chest CT image analysis

All CT images were reviewed by two experienced radiologists with extensive experience in thoracic imaging, on a diagnostic workstation (Vitrea extend-Vital, Canon, Tokyo, Japan) with multiplanar reconstruction (MPR) tools. The images were viewed in the lung window settings (width, 1,600 HU; level, 400 HU) and mediastinal (soft tissue) window settings of width, 380 HU; level, 40 HU. All discrepancies were resolved by consensus.

Since chest CT images could show different imaging features or patterns in COVID-19 patients with a different time course and disease severity [12–15], we classified them into four categories: lung, bronchial, pleural and mediastinal changes (Figures 1 and 2).

Based on the time interval between the onset of symptoms and the CT scan, all patients were divided into three groups: group 1 (CT scans done ≤ 1 week after symptom onset); group 2 (CT scans done > 1 to 2 weeks after symptom onset); group 3 (CT scans done > 2 weeks after symptom onset).

In order to monitor the distribution of changes in the lungs more accurately, bilateral lungs were divided into 12 'lung' zones, as follows: each side of the lung was divided into two areas – the anterior and posterior area (the boundary between these two regions was represented by an imaginary vertical line passing through the middle of the diaphragm in the sagittal plane); after that each area was divided into three zones – above the carina is the upper zone, the middle zone is from the carina to the inferior pulmonary vein, and below the inferior pulmonary vein is the lower zone. Each zone was assigned a CT score which was based on the following criteria: no involvement – score 0, 1% to less than 25% – score 1, 25% to less than 50% – score 2, 50% to less than 75% – score 3 and more than 75% – score 4. Total severity score was calculated by summing the scores for each zone, with maximum possible score of 48.

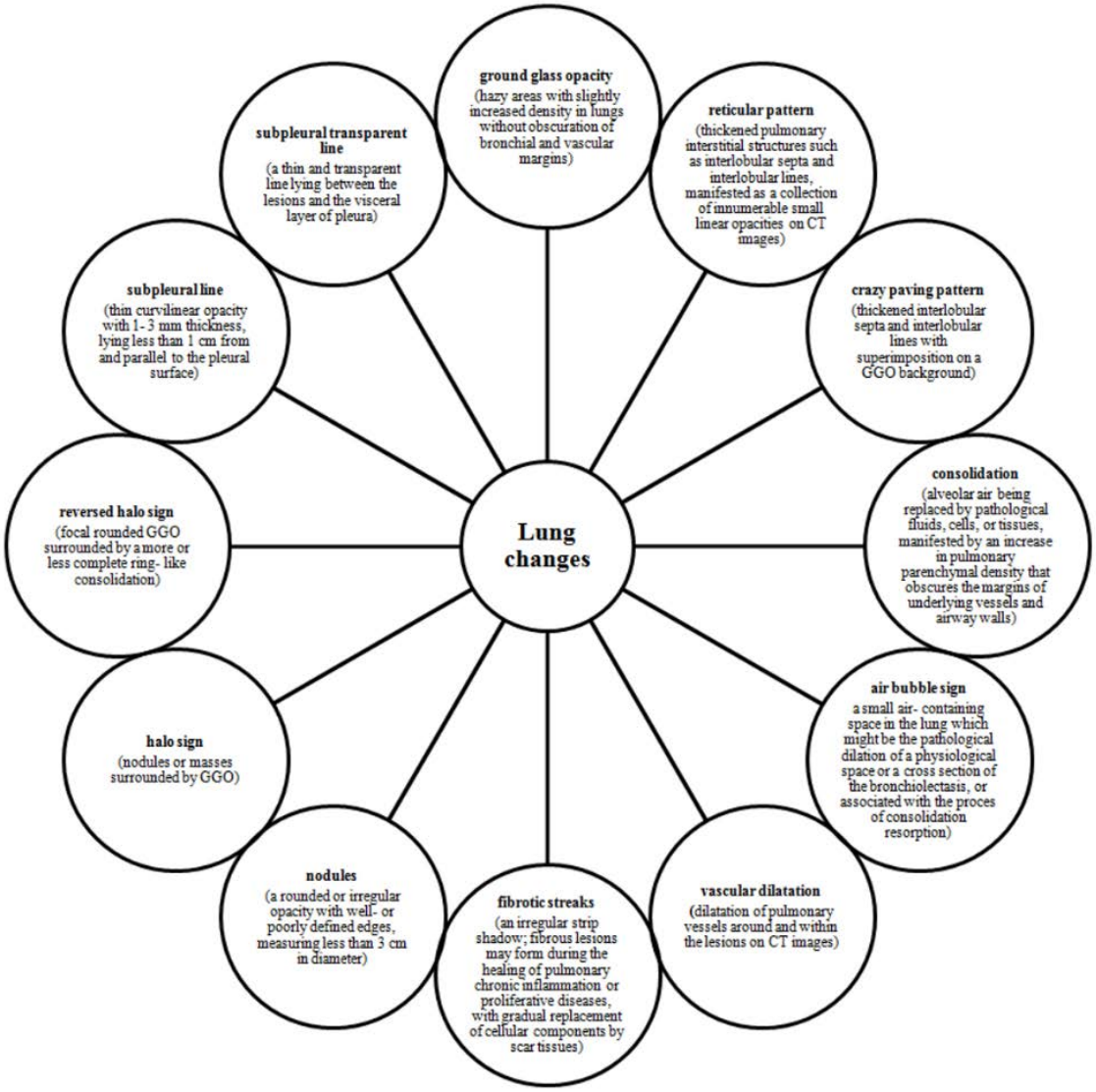

Fig. 1 – Lung changes on chest computed tomography (CT) in coronavirus disease 2019 (COVID-19) patients. GGO – ground-glass opacity.

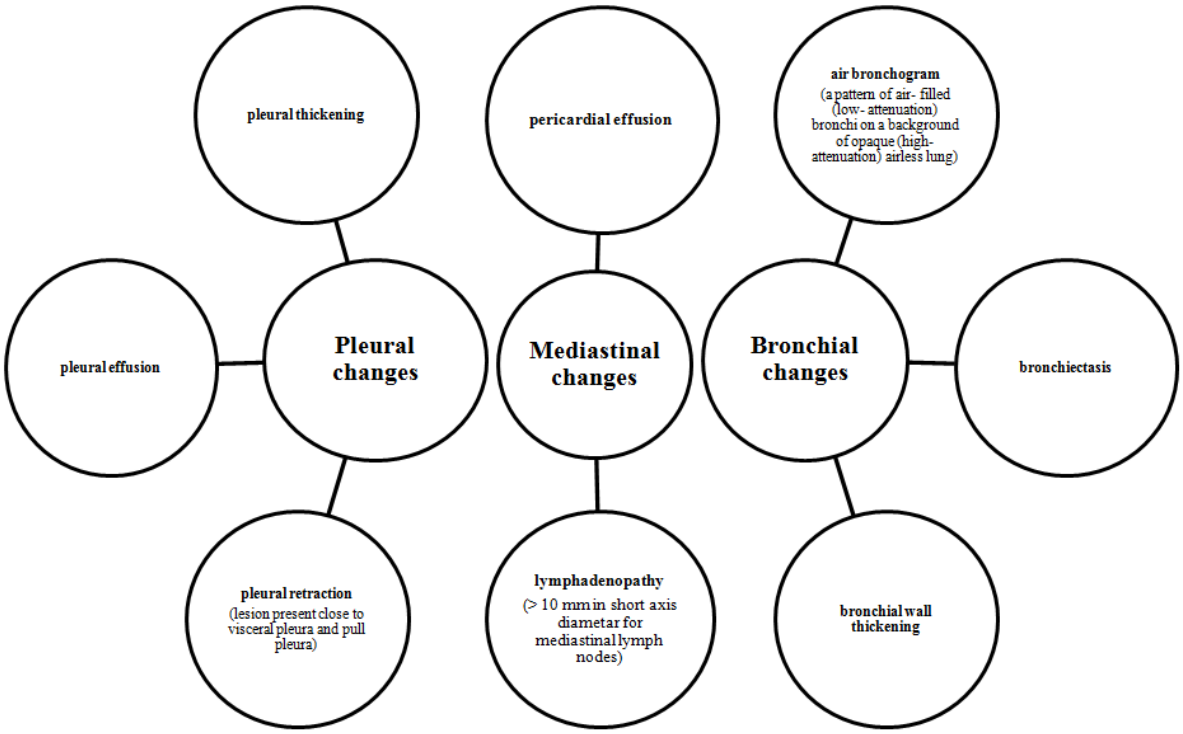

Fig. 2 – Pleural, bronchial and mediastinal changes on chest computed tomography (CT) in coronavirus disease 2019 (COVID-19) patients.

Statistical analysis

Based on standard statistical parameters [study power 80% (0.85), probability of α error 0.05, two-way testing, equal group sizes], to find a significant difference in the value of CT score between different parts of lungs in patients with COVID-19 infection (CT score was 3.0 ± 3.4 in the upper lung, and 4.5 ± 3.8 in the middle lung) [15], the required sample size was calculated by *t*-test for independent samples, using G*Power 3.1, from 103 patients per group (effect size 0.42). However, it was planned to include a total of 110 patients in each of the three groups, as a possible loss of 10% per group was included.

Statistical analysis was conducted using an IBM SPSS Statistics 19.0 computer program (IBM, USA, 2011). All continuous variables were described in the form of the mean ± standard deviation or median [interquartile range (IQR)] values. The categorical variables were expressed as percentages and examined using the χ^2-test; the Yates's correction for continuity was with a 2 × 2 contingency table. Comparisons of parametric variables between 2 groups were performed by independent samples *t*-test and non-parametric variables were tested with the Mann-Whitney *U* test. Comparisons of non-parametric variables between more than 3 groups were performed with the Kruskal-Wallis test. The normality distribution of data was tested by the Kolmogorov-Smirnov test. All the analyses were evaluated at the level of statistical significance of $p < 0.05$. Missing or incomplete data were not used in the statistical analysis.

Results

In our study the total number of patients with COVID-19 was 330, male sex was dominant (Table 1). The average age of all patients was 52.37 ± 15.36 (male: 52.76 ± 15.11; female: 51.60 ± 15.87; Independent samples *t*-test, $p = 0.518$). All patients had symptoms at the onset of the disease, of which the most common was fever, present in 86.4% patients. Arterial hypertension was the most common comorbidity (37.6% patients). Also, we treated 9 (2.7%) pregnant women, all in late fetal period (over 25 weeks of gestation).

Blood tests were performed for all 330 patients (Table 1). The main haematological findings we found in our patients were a decreased erythrocytes count (20.3%) and a decreased haemoglobin (21.8%). The largest number of patients had a normal leukocyte count, and we found an increased monocyte count in 67.9% of patients. As for the other laboratory findings, increased ferritin was found in 70.9%.

The CT finding was positive in 309 out of 330 patients (93.6%) with COVID-19. About 6.5% of the COVID-19 patients with clinical positive findings had no

Table 1

Demographic and clinical characteristics of the patients with coronavirus disease 2019 (COVID-19)

Characteristic	Patients
Gender	
male	219 (66.4)
female	111 (33.6)
Age (years)	52.37 ± 15.36
Symptom	
fever	285 (86.4)
fatigue	225 (68.2)
shortness of breath	128 (38.8)
coughing and sputum	244 (73.9)
muscle pain	181 (54.8)
digestive symptoms (diarrhea, abdominal pain, vomiting)	51 (15.5)
other (chest pain, headache, disorientation, taste and smell disorder, pain in throat)	72 (21.8)
Comorbidities	
arterial hypertension	124 (37.6)
other heart diseases	16 (4.8)
obesity	98 (29.7)
diabetes	32 (9.7)
chronic renal failure	22 (6.7)
stroke	13 (3.9)
other neurological disease	3 (0.9)
pulmonary diseases (asthma, chronic obstructive pulmonary disease, chronic bronchitis)	22 (6.7)
other (anemia, cholangitis, benign hypertension of prostate, dementia, Hashimoto thyroiditis, depression, carcinomas, sarcoidosis etc.)	90 (27.3)
Other conditions	
pregnancy	9 (2.7)
Selected laboratory test findings	
decreased erythrocytes	67 (20.3)
decreased hemoglobin	72 (21.8)
leukocyte count: decreased / increased	30 (9.1)/22 (6.7)
lymphocyte count: decreased / increased	115 (34.8)/18 (5.5)
neutrophil count: decreased / increased	35 (10.6)/95 (28.8)
eosinophil count: decreased / increased	117 (35.5)/ 6 (7.9)
monocyte count: decreased / increased	1 (0.3)/224 (67.9)
increased erythrocyte sedimentation rate	117 (35.5)
increased serum procalcitonin	19 (5.8)
increased c- reactive protein	208 (63.0)
increased lactate dehydrogenase	124 (37.6)
increased ferritin	234 (70.9)

Results are expressed as number (%) or mean ± standard deviation (SD).

CT pathological findings. About 92.1% had multiple lesions and lesions were most often bilateral (Table 2, Figures 3–5). Most patients had both a peripheral and central localization of lesions at the same time (63.3%) (Figures 3 and 4). If we look at the distribution of lesions by lung zones, then we see that the posterior areas were more often affected (93%). It is also noticeable that changes occurred more often in lower lung zones (91.2%) (Table 2).

The average Total severity score in patients, in whom CT changes were found in the lungs (309), was 11.00 (7.00–16.00). The average Total severity score was significantly higher in patients with multiple rather than with single lesions, as well as in bilateral versus unilateral lesion distribution. Also, the Total severity score was higher in patients with peripheral and central lesion distribution, compared with patients with only peripheral or central lesion distribution (Table 2).

In relation to the time period after the onset of symptoms, the scan was usually done after 14 days (47% of the patients). Slightly less was done between the seventh and 14th day (30.3%) and least amount in the first 7 days following the onset of the disease symptoms (22.7%). When the time interval was monitored between onset of symptoms and the CT scan, it was noticed that in the group 1 changes on CT were found in 80.0% of the patients, in the group 2 in 95.0% of the patients, and in the group 3 in 99.4% of the patients. It should be noted that significant differences were found in the time distribution of individual CT signs. As the most common CT finding in all patients, ground-glass opacities (GGO) was observed in 97.7%, reticular pattern in 91.3%,

Table 2

Distribution of computed tomography (CT) pathological findings (lesion distribution) in the patients with coronavirus disease 2019 (COVID-19)

Parametres	Number of patients	CT score	*p*-value
Number of lesions	309 (93.6)	11.00 (7.00–16.00)	
single	5 (1.5)	1.00 (1.00–1.00)	< 0.001*
multiple	304 (92.1)	11.00 (7.00–16.00)	
Lesion distribution			
peripheral	98 (29.7)	6.00 (3.00–9.00)	
central	2 (0.6)	3.50 (1.00-/)	< 0.001#
peripheral and central	209 (63.3)	14.00 (10.00–19.00)	
Side distribution			
unilateral	20 (6.1)	1.00 (1.00–3.00)	< 0.001*
bilateral	289 (87.6)	12.00 (8.00–16.50)	
Lung			
left	13 (3.9)	1.00 (1.00–3.00)	
right	7 (2.1)	1.00 (1.00–3.00)	< 0.001#
left and right	289 (87.6)	12.00 (8.00–16.50)	
Lung zone[1]			
left upper anterior	205 (62.1)	1.00 (0.00–1.00)	
left upper posterior	219 (66.6)	1.00 (0.00–1.00)	
left middle anterior	222 (67.3)	1.00 (0.00–1.00)	
left middle posterior	237 (71.8)	1.00 (1.00–2.00)	
left lower anterior	230 (69.7)	1.00 (0.00–1.00)	
left lower posterior	278 (84.2)	1.00 (1.00–2.00)	0.887#
right upper anterior	191 (57.9)	1.00 (0.00–1.00)	
right upper posterior	208 (63.0)	1.00 (0.00–1.00)	
right middle anterior	214 (64.8)	1.00 (0.00–1.00)	
right middle posterior	241 (73.0)	1.00 (1.00–2.00)	
right lower anterior	222 (67.3)	1.00 (0.00–1.00)	
right lower posterior	286 (86.7)	1.00 (1.00–2.00)	
Lung zone[1]			
upper	277 (83.9)	3.00 (1.50–5.00)	
middle	234 (70.9)	4.00 (2.00–6.00)	< 0.001#
lower	301 (91.2)	4.00 (3.00–6.00)	
Lung area[1]			
anterior	278 (84.2)	5.00 (3.00–7.00)	< 0.001*
posterior	307 (93.0)	6.00 (4.00–9.00)	
Lung[1]			
left	302 (97.7)	6.00 (3.00–8.00)	0.982*
right	296 (95.8)	5.00 (3.00–8.00)	

Results are expressed as number (%) or median (IQR- interquartile range: 25–75th percentile); [1]Cumulative percentage; *Mann-Whitney test; #Kruskal-Wallis test.

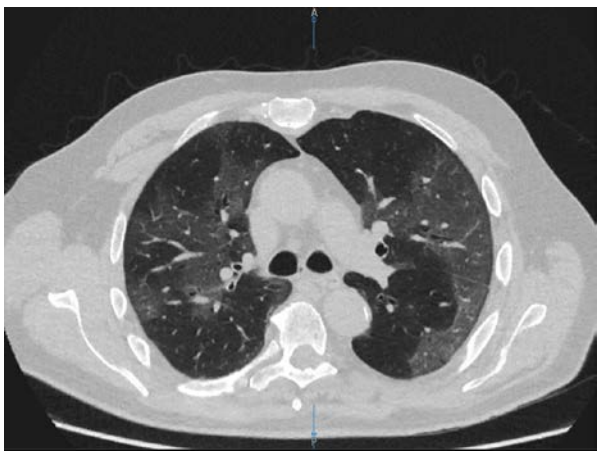

Fig. 3 – Axial thin-section unenhanced computed tomography (CT) scan shows bilateral ground-glass opacities (GGO) with reticulation, bronchiectasis and bronchovascular thickening with central and peripheral distribution.

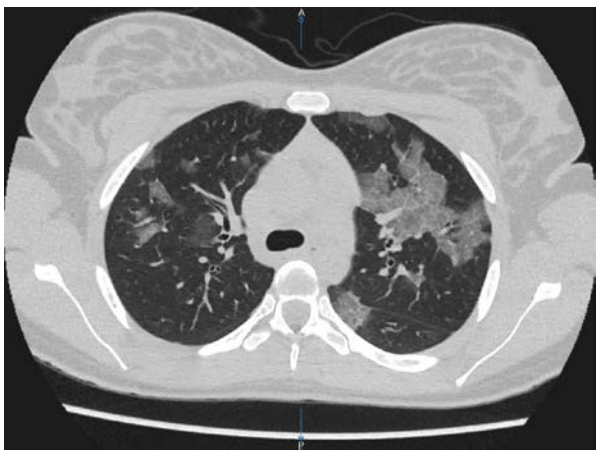

Fig. 4 – Axial thin-section unenhanced computed tomography (CT) shows bilateral crazy paving pattern with vascular enlargement in the upper and lower left lobe and in the upper right lobe with central and peripheral distribution .

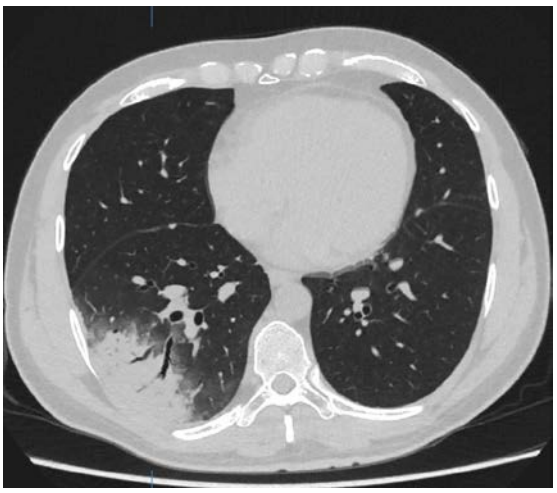

Fig. 5 – Axial thin-section unenhanced computed tomography (CT) scan shows right unilateral consolidation with air bronchogram with peripheral distribution in the right lower lobe.

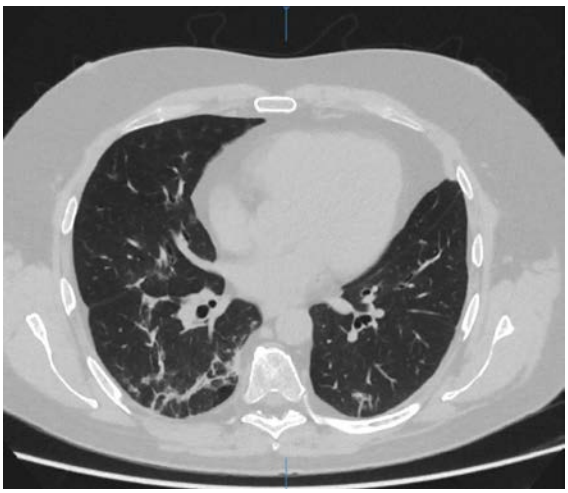

Fig. 6 – Axial thin-section unenhanced computed tomography (CT) scan shows left pleural thickening with bilateral subpleural lines and fibrous stripes in the posterior segments of the lower lobes.

Table 3

Computed tomography (CT) features in the patients with coronavirus disease 2019 (COVID-19) according to time of CT screening from onset of the disease

CT feature	All patients	Group 1 < 7 days	Group 2 7–14 days	Group 3 > 14 days	*p*-value*
Total number of patients	330 (100)	75 (22.7)	100 (30.3)	155 (47.0)	
CT positive findings	309 (93.6)	60 (80.0)	95 (95.0)	154 (99.4)	< 0.001
ground-glass opacities	302 (97.7)	58 (96.7)	92 (96.8)	152 (98.7)	0.522
reticular pattern	282 (91.3)	55 (91.7)	89 (93.7)	138 (89.6)	0.538
crazy paving pattern	65 (21.0)	22 (36.7)	28 (29.5)	15 (9.7)	< 0.001
consolidation	221 (71.5)	44 (73.3)	75 (78.9)	101 (66.2)	0.203
air bubble sign	21 (6.8)	7 (11.7)	7 (7.4)	7 (4.5)	0.171
vascular dilatation	147 (47.6)	39 (65.0)	48 (50.5)	60 (39.0)	0.002
fibrotic streaks	197 (63.8)	15 (25.0)	59 (62.1)	123 (79.9)	< 0.001
nodules	32 (10.4)	5 (8.3)	15 (15.8)	12 (7.8)	0.112
halo sign	71 (23.0)	15 (25.0)	31 (32.6)	25 (16.2)	0.011
reversed halo sign	29 (9.4)	4 (6.7)	11 (11.6)	14 (9.1)	0.584
subpleural line	76 (24.6)	2 (3.3)	19 (20.0)	55 (35.7)	< 0.001
subpleural transparent line	63 (20.4)	5 (8.3)	17 (17.9)	41 (26.6)	0.009
air bronchogram	49 (15.9)	10 (16.7)	18 (18.9)	21 (13.6)	0.528
bronchiectasis	194 (62.8)	39 (65.0)	66 (69.5)	89 (57.8)	0.166
thickening of the bronchial wall	195 (63.1)	40 (66.7)	66 (69.5)	89 (57.8)	0.146
pleural retraction	12 (3.9)	0	1 (1.1)	11 (7.1)	0.012
pleural effusion	29 (9.4)	5 (8.3)	10 (10.5)	14 (9.1)	0.887
pleural thickening	64 (20.7)	14 (23.3)	17 (17.9)	33 (21.4)	0.684
lymphadenopathy	9 (2.9)	0	3 (3.2)	6 (3.9)	0.307
Pericardial effusion	12 (3.9)	3 (5.0)	4 (4.2)	5 (3.2)	0.821

Results are expressed as number (%); *χ^2 test .

consolidation in 71.5%, fibrotic streaks in 63.8%, thickening of the bronchial wall in 63.1%, bronchiectasis in 62.8% and vascular dilatation in 47.6% (Table 3, Figures 3–6).

Discussion

In this study we examined the demographics, clinical characteristics and chest CT findings for 330 COVID-19 patients which were treated in our hospital with more detailed analysis of he most common findings.

Of the total number of patients, the majority (66.4%) were men, and the average age of patients was 52.37 ± 15.36, which is in accordance with previous studies [6, 15, 16].

As for symptoms, as in other available studies [15, 16], they were dominated by fever (86.4%), coughing and sputum (73.9%), fatigue (68.2%), muscle pain (54.8%), shortness of breath (38.8%), which correlates with the fact that the disease primarily affects the respiratory system. A significant number of patients (15.5%) also had digestive symptoms such as diarrhoea, abdominal pain and vomiting, as has been reported by other authors [15] and may confirm the hypothesis

of an association between the gastrointestinal symptoms with the pathogenesis of the COVID-19 infection and the involvement of angiotensin-converting enzyme 2 receptor (ACE-2) which shows a high expression in the gastrointestinal tract as well.

The most common comorbidities in our patients were arterial hypertension (37.6%), obesity (29.7%), diabetes (9.7%), other lung diseases and chronic renal failure (both 6.7%), which, in some published papers have shown, correlate with the severity of the clinical features [17].

Considering the study included nine pregnant women, it is important to note that the CT scan is essential for evaluation of the clinical conditions in these patients and the risk benefit ratio of the diagnostic procedure is acceptable [18]. Radiation exposure through CT scan is at a dose much lower than the exposure associated with fetal harm [19]. According to the American College of Radiology and American College of Obstetricians and Gynecologists, when a pregnant woman undergoes a single chest CT scan, the radiation dose to the fetus is 0.01– 0.66 mGy [19].

As for laboratory findings, lymphopenia is considered by some authors [20] as a cardinal laboratory finding, with prognostic potential. A recent meta-analysis noted that 35–75% of COBID-19 patients developed lymphopenia [21], and that percentage in our study was 34.8%. An increased monocyte count was also found in a significant (67.99%) number of patients which also supports other studies published and whose authors go on to say that monocytes are primarily involved in triggering the hyperinflammatory immune response [22, 23]. Blood parameters, such as increased ferritin (70.9%), C-reactive protein (CRP) (63.0%), lactate dehydrogenase (LDH) (37.6%), erythrocyte sedimentation rate (35.5%) have also emerged as poor prognostic factors [20, 21], although ferritin is referred to as a key mediator of immune dysregulation [24].

As for CT findings, almost all patients included in the study (93.6%) had changes in their CT, with only 1.5% having a single lesion and 92.1% having multiple lesions, but it should be noted that a normal CT finding was found in a number of COVID-19 patients. Some studies [12, 15] correlated the extent of lesions with the time that elapsed from the onset of symptoms to the CT examination and found that in patients with less prevalence and single lesions, this time was significantly shorter and these patients underwent CT examination at a significantly earlier stage of the disease. In that sense, our study showed that in patients examined in the first week after the onset of symptoms (group 1), CT signs were found in 80.0% of patients, in group 2 in 95.0% of patients, and in group 3 in 99.4% of patients.

When it comes to the distribution of lesions, it can be observed that the majority were bilateral lesions (87.6%) and combined peripheral and centrally localized lesions (63.3%). These most often affected the posterior (93%) and lower zones (91.2%). These results also do not deviate from other studies, and can be partly explained by the anatomy of the tracheobronchial tree, which does not branch evenly and symmetrically and, especially in the earlier stages of the disease, may allow the virus to infect individual branches more frequently, leading to an uneven distribution [15, 25].

When we analyse an individual CT feature, GGO was the most common CT finding (97.7%), which correlates with the results of other studies [14, 26, 27]. By frequency, the reticular pattern results closely followed GGO (91.3%), and can be related to interstitial lymphocyte infiltration which leads to interlobular septal thickening [14, 27, 28]. There was no significant difference in any of the 3 groups of patients when GGO and reticular patterns occur (GGO: 96.7%–98.7% and reticular pattern: 89.6%–93.7%).

The crazy paving pattern which can be defined as a combined GGO and reticular pattern in the same location and as a result of alveolar oedema and interstitial inflammation of an acute lung injury did not occur as frequently (21%) as each of the components individually [13, 14]. However, unlike GGO and reticular pattern, crazy paving pattern showed a significant difference in time distribution, with this most evident in the group 1 (36.7%) and least often in the group 3 (9.7%), which is in line with the assumption that this indicates acute damaged lungs.

Consolidation, according to our study, is a common finding (71.5%) in patients with COVID-19, although some previous studies show a wide frequency range of 2– 64% [14]. Consolidation can be linked to cellular fibromyxoid exudates in alveoli [29], and can be considered a predictor of disease progression. Recent studies have shown that changes in the lungs progress to consolidation up to about two weeks after the onset of symptoms [13], and these findings are confirmed by our results which showed that consolidation was more common in the groups 1 and 2 (73.3% and 78.9%, respectively), compared to the group 3 (66.2%), but without a statistically significant difference among groups.

We observed vascular dilatation in 47.6% of the patients, with this occured significantly more often in the group 1 (65%) compared to the group 3 (39%), which could be attributed to thickening and damage of the walls of the blood vessels caused by proinflammatory factors [14] in the early phase of the disease, compared to the advanced phase.

Bronchial changes, primarily bronchiectasis and bronchial wall thickening were found in our study in 62.8% and 63.1% of the patients, with no significant difference in time distribution. This is in contrast to other authors who reported these manifestations in smaller percentage of cases, bronchiectasis up to 11% [12], and bronchial wall thickening up to 23% [26]. Regardless bronchial changes are the consequence of bronchial obstruction and inflammatory damage of the bronchial wall, with consequent fibrotic changes, Li et al. [26] showed a correlation of bronchial changes with the severity of the clinical picture in a sample of 83 COVID-19 patients.

Some changes occurred significantly more often in patients from the group 3, and here we primarily mean fibrotic streaks (79.9%), subpleural line (35.7%), subpleural transparent line (26.6%) and pleural retraction (7.1%). These changes are characteristic of the advanced phase of the disease and they may correspond to repair changes.

Conclusion

CT has proven to be a very important diagnostic method, and its ability to show the distribution, shape, degree of involvement, as well as typical radiological characteristics accurately, gives it a key role in diagnosing and monitoring patients with COVID-19 pneumonia. However, CT findings in COVID-19 patients have been shown to give a variety of patterns, most commonly involving lung and bronchial changes, which require a deep understanding by radiologists, but further research, especially in terms of time distribution is necessary. Together with clinical and laboratory findings, CT gives a complete picture of the patient's condition and contributes significantly to decision-making on further treatment.

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High level of interleukin-10 in serum after therapy is characteristic of prostate carcinoma patients with high Gleason score, high tumor volume and present peritumoral infiltration

Visok nivo interleukina-10 u serumu nakon terapije karakterističan je za bolesnike sa karcinomom prostate koji imaju visok Gleason gradus, veliki volumen tumora i prisutnu peritumorsku infiltraciju

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Abstract

Background/Aim. Recent data imply the significance of certain cytokines in the appearance and development of prostate cancer (PC), as well as their association with pathohistological and/or clinical characteristics of PC. The aim of this study was to examine the relationship between the IL-10 concentration with histopathological and clinical characteristics of PC patients. **Methods.** IL-10 concentration was determined in serum, urine and prostate massage secret (pms) samples of 88 CP patients (initially and after therapy), 20 benign prostatic hyperplasia (BPH) patients and 15 healthy controls. **Results.** Compared to BPH and control groups, PC patients had the highest average serum IL-10 concentration. Interestingly, BPH patients demonstrated the highest concentration of IL-6 in urine and pms samples. Also, patients with G3 gradus and with the highest Gleason score (4 + 4) demonstrated the highest IL-10 serum level. PC patients without any histopathological sign of tumor invasion had a significantly increased serum IL-10, either before or after the therapy, compared to the patient group with evident invasion of tumor cells. The therapy induced different IL-10 profile in serum and urine samples. After the therapy, there was a clear significant IL-10 increase in serum of patients with unfavorable Gleason score (4 + 4), with present infiltration of tumor cells in peritumoral tissue (lymphatic, vascular and combined) and in patients with high tumor volume. **Conclusion.** PC patients without any histopathological signs of tumor invasion before the therapy have significantly increased serum IL-10 concentration compared to those with the signs of tumor invasion. There is a clear dissociation of IL-10 values between a serum sample and local, urine and pms samples from a particular patient. After the therapy, high IL-10 serum concentration is present only in patients with high Gleason score, present infiltration of peritumoral tissue and high tumor volume.

Key words:
interleukin-10; interleukins; neoplasm grading; prostate neoplasms; prostatic hyperplasia.

Apstrakt

Background/Aim. Skorašnji podaci ukazuju na značaj pojedinih citokina u pojavi i razvoju karcinoma prostate (KP), kao i na njihovu udruženost sa patohistološkim i/ili kliničkim karakteristikama KP. Cilj studije bio je da se ispita udruženost koncentracije interleukina-10 (IL-10) sa patohistološkim i kliničkim karakteristikama bolesti kod bolesnika sa KP. **Metode.** Koncentracija IL-10 određivana je u uzorcima seruma, urina i *prostate massage secret* (pms) kod 88 bolesnika sa KP (inicijalno i posle terapije), 20 bolesnika sa benignom hiperplazijom prostate (BHP) i 15 zdravih osoba (kontrolna grupa). **Rezultati.** U poređenju sa bolesnicima sa BPH i kontrolnom grupom, bolesnici sa KP imali su najveće serumske vrednosti IL-10. Interesantno, bolesnici sa BHP imali su najviše koncentracije IL-10 u urinu i uzorcima pms. Takođe, bolesnici sa G3 gradusom i najvećim Gleason skorom (4 + 4) imali su najveće serumske vrednosti IL-10. Bolesnici sa KP bez ikakvog patohistološkog znaka invazije tumora, u odnosu na bolesnike sa evidentnom invazijom tumorskih ćelija, imali su značajno povećane serumske vrednosti IL-10, kako pre, tako i posle terapije. Terapija je indukovala različite profile IL-10 u uzorcima seruma i urina. Nakon terapije detektovali smo značajno povišenje serumskih vrednosti

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IL-10 kod bolesnika sa nepovoljnim Gleason skorom (4 + 4), prisutnom infitracijom tumorskih ćelija u peritumorskom tkivu (limfnom, vaskularnom ili kombinovano) i u grupi bolesnika sa visokim volumenom tumora. **Zaključak.** Bolesnici sa KP bez patohistoloških znakova invazije tumora pre terapije imaju značajno povišene vrednosti serumskog IL-10 u odnosu na grupu bolesnika sa dokazanom invazijom. U našem istraživanju postoji jasna disocijacija vrednosti IL-10 u uzorcima seruma, urina i pms pojedinačnog bolesnika. Posle terapije, visoka koncentracija IL-10 u serumu prisutna je samo kod bolesnika sa visokim Gleason skorom, prisutnom infiltracijom peritumorskog tkiva i visokim volumenom tumora.

Ključne reči:
interleukin-10; interleukini; neoplazme, određivanje stadijuma; prostata, neoplazme; prostata, hipertrofija.

Introduction

Prostate cancer (PC) is one of the most common malignant tumors in male population, and the most frequent among the population above the age of 50 [1]. Prostate tissue samples of the PC patients often demonstrate signs of chronic inflammation, with abundant inflammatory cellular infiltrate composed of T lymphocytes, B lymphocytes, macrophages, neutrophils and mastocytes. Epithelial prostate cells (both androgen-dependent and independent) influence the growth and differentiation of both normal and cancer cells. It has been shown that the epithelial prostate cells produce proinflammatory cytokines [2], which suggests that cytokines are associated with the pathophysiology of the prostate cancer [3]. In healthy people, immune system components specifically recognize and eliminate tumor cells. Tumor infiltration with T lymphocytes, natural killer (NK) cells and/or NKT cells should represent favourable response, i.e. a positive outcome of the treatment of a malignant disease [4]. However, chronic, alternative and uncontrollable activation of immune cells (macrophages, neutrophils and mastocytes) has a protumoral role rather than a defensive one, thus enabling the tumor to avoid the protective immune response, to further grow and to metastasize [5, 6]. Cytokines produced from tumorous cells and infiltrating immune cells are important factors which enable the survival of tumorous cells and their growth in spite of the existence of tumor specific response. So far, several studies focused on cytokines determined in the blood samples, prostate exprimate and urine of the patients with PC [3, 7–11]. The results of these studies pointed to the possible association of cytokines interleukin (IL)-4, IL-1α, IL-1β, IL-6, tumor necrosis factor alpha (TNF)-α, granulocyte-macrophage colony-stimulating factor (GM-CSF), interferon- γ (IFN-γ), IL-2, IL-8, IL-10, IL-12, IL-1RA, IL-4, IL-12, MIC1, IL-5, hepatocyte growth factor (HGF), IL18BP, intercellular adhesion molecule 1 (ICAM-1), IL-17, NT-3, GITR and epithelial neutrophil activating peptide-78 (ENA-78) with the appearance, growth, histopathological and/or clinical characteristics of the PC. The aim of our study was to analyze the IL-10 concentration in the PC patient samples [serum, urine, prostate massage secret (pms)] and to estimate their association with clinical and histopathological parameters of the disease.

Methods

The research was conducted at the Clinic for Urology, Institute of Radiology, and the Institute for Medical Research at the Military Medically Academy in Belgrade, after it had been approved by the Military Medical Academy Ethical Committee. Eighty eight men above 18 years of age were included in this prospective clinical observational case-control study. All patients were confirmed as PC after the initial screening of infiltrative change in prostate [digit rectal examination of prostate (DRP), elevated prostate-specific antigen (PSA)] and positive histopathological confirmation of tissue sample taken with diagnostic biopsy. Control groups consisted of 20 men with benign prostatic hyperplasia (BPH) and 15 healthy men (regular physical examination) that had never suffered from active/chronic genitourinary disease or any malignant disease. Tumorous tissue samples for further histopathological analysis were obtained during the surgical procedure of the PC patients who had undergone the surgery. Blood and urine and samples were obtained at the control examinations of these patients (before and two months after the surgery) Pms samples were obtained after the prostate massage during DRP. The existence of other malignant diseases, autoimmune diseases, kidney diseases, bladder diseases and the consumption of medications which affect hematopoiesis were the criteria for the exclusion from the research. All the subjects included in the study signed an informed consent form, approved by the Military Medical Academy Ethical Committee. According to the National Guidelines for Good Clinical Practice for PC, beyond DRP, all patients were submitted to bone scintigraphy. Histopathological analysis of the tumor tissue included determination of histological type, tumor volume, Gleason score [12], Gleason differentiation degree, histological grade, existence of lymphovascular and/or perineural tumor invasion and pathological tumor, nodus, metastasis (TNM) stage. The clinical stage of the disease was determined according to the 8th edition TNM classification [13].

PSA serum values were determined with a commercial test, ABBOTT ARCHITECT *i*System analyzer PSA assay. IL-10 cytokine concentration in all investigated samples was determined using a commercial kit for cytokine concentration determination (YSL flow multiplex cytokine test kit) with the flow cytometer (Beckman Coulter FC500).

All statistical analyses were done using GraphPad Prism 5 version 5.01 software. Unmatched comparisons between groups were done using Student's *t*-test and Mann-Whitney test. For unmatched comparison of more groups One-way ANOVA test with additional Bonferroni test was used. To estimate statistical significance of relationships between different parameters Pearson's and Spearman's correlation tests were used. The results were presented as mean ± standard deviation (SD).

Results

PC patients have the highest average serum IL-10 concentration

We have investigated IL-10 concentration in various samples of all groups, healthy control men (C), and the BPH and PC patients (Table 1). According to our investigation design, since there were no serial samples of the C and BPH groups, only initial values of the PC group (0th day) were taken into this analysis. Comparison of serum samples demonstrated the highest average IL-10 in the PC group (PC > BPH > C), but without significant difference compared to other participants.

Table 1

Concentration of interleukin-10 (pg/mL, mean ± SD) in various samples of the investigated groups at day 0

Group	serum	urine	pms
C	55 ± 14	36 ± 17 [a]	35 ± 12
BPH	72 ± 22	56 ± 15 [a,b]	49 ± 6
CP	77 ± 69	37 ± 17 [b]	40 ± 23

C – control; BPH – benign prostatic hyperplasia;
PC – prostate cancer;
pms – prostate massage secret;
SD – standard deviation.
[a]BPH/C, $p < 0.05$; [b]BPH/CP, $p < 0.01$.

Interestingly, IL-10 in urine samples demonstrated the highest average value in BPH patients, significantly increased compared to other two groups (BPH > C, $p < 0.05$; (BPH > PC, $p < 0.01$), with no difference between the C and PC groups.

Similarly, local IL-10 concentration found in pms had the highest value in the BPH group, but without significant differences compared to other two groups (BPH > PC > C).

Patients with G3 gradus demonstrated the highest average serum and urine IL-10 concentration

Our patients were divided in four groups according to pathological findings of tumor gradus. Interestingly, patients with gradus 4 (G4) demonstrated the lowest average IL-10 values in serum samples at both time intervals (Table 2). Patients from the G3 group had the highest average IL-10 values, significantly increased compared to the G2 and G4 groups (G3 > G2, 0th day, 6th day; (G3 > G4, 0th day, 60th day) (Table 3). After the therapy (60th day), the G3 group IL-10 serum concentrations were still significantly increased compared to those of the G2 and G4 patient groups (Table 3).

Urine IL-10 values reflected serum concentrations, again with G3 group patients having the highest average IL-10 concentration, with only significant difference between the G3 and G4 groups in the initial time interval (G3 > G4, 0d).

Table 2

Concentration of interleukin-10 (pg/mL, mean ± SD) in prostate cancer samples according to the investigated parameters.

Parameter	Group	Serum		Urine		Prostate massage secret	
		0th day	60th day	0th day	60th day	0th day	60th day
Gradus	G1	72 ± 47	89 ± 40	30 ± 7	37 ± 17	23 ± 9	22 ± 7
	G2	54 ± 37	55 ± 37	34 ± 16	33 ± 15	34 ± 19	30 ± 16
	G3	77 ± 34	89 ± 37	44 ± 19	42 ± 10	54 ± 24	42 ± 22
	G4	29 ± 11	50 ± 31	27 ± 7	30 ± 10	26 ± 5	24 ± 10
Gleason score	3 + 3	62 ± 46	62 ± 40	31 ± 13	37 ± 16	34 ± 22	32 ± 15
	3 + 4	50 ± 21	40 ± 17	56 ± 30	31 ± 11	31 ± 14	27 ± 13
	4 + 3	75 ± 43	71 ± 35	45 ± 21	41 ± 13	56 ± 26	41 ± 25
	4 + 4	42 ± 14	81 ± 32	33 ± 6	39 ± 9	29 ± 7	34 ± 11
Lymphatic invasion	no	72 ± 55	68 ± 46	35 ± 17	36 ± 13	35 ± 22	31 ± 15
	yes	66 ± 28	93 ± 43	43 ± 17	40 ± 13	51 ± 21	40 ± 22
Vascular invasion	no	72 ± 50	70 ± 38	37 ± 16	38 ± 12	39 ± 22	32 ± 15
	yes	53 ± 30	93 ± 75	38 ± 22	32 ± 18	43 ± 27	35 ± 27
Neural invasion	no	95 ± 62	89 ± 55	38 ± 17	39 ± 16	43 ± 27	34 ± 16
	yes	52 ± 24	54 ± 26	36 ± 18	35 ± 14	38 ± 22	32 ± 20
Invasion score	0	90 ± 67	93 ± 58	35 ± 16	37 ± 16	39 ± 26	34 ± 17
	1	51 ± 22	53 ± 26	39 ± 20	37 ± 9	36 ± 22	29 ± 13
	2	70 ± 31	78 ± 23	35 ± 3	42 ± 7	41 ± 13	36 ± 11
	3	53 ± 30	59 ± 38	38 ± 22	32 ± 18	43 ± 27	35 ± 27
Tumor volume (%)	> 15 (high)	48 ± 20	52 ± 10	34 ± 13	33 ± 17	40 ± 20	32 ± 18
	< 15 (low)	76 ± 46	81 ± 52	40 ± 19	36 ± 13	42 ± 25	34 ± 20
PSA (ng/mL)	> 10 (high)	64 ± 42	59 ± 31	38 ± 20	35 ± 14	35 ± 21	29 ± 15
	< 10 (low)	69 ± 46	87 ± 64	39 ± 15	38 ± 14	48 ± 16	33 ± 15
Therapy	RRP	45 ± 35	48 ± 20	33 ± 20	33 ± 15	34 ± 32	34 ± 24
	RTh	61 ± 22	70 ± 36	39 ± 15	38 ± 13	43 ± 15	33 ± 15

PSA – prostate specific antigen;RRP – retropubic radical prostatectomy; RTh – radiation therapy;
SD – standard deviation.

Table 3

Statistical analysis of the differences in interleukin-10 concentrations among investigated PC groups

Parameter	Group	Serum		Urine		Prostate massage secret	
		0th day	60th day	0th day	60th day	0th day	60th day
Gradus	G1/G2						
	G1/G3					***	**
	G1/G4	*					
	G2/G3	*	*			*	
	G2/G4						
	G3/G4	***	**	*		*	*
Gleason score	3 + 3 / 3 + 4			*		*	
	3 + 3 / 4 + 3						
	3 + 3 / 4 + 4						
	3 + 4 / 4 + 3		*			*	
	3 + 4 / 4 + 4		*	*			
	4 + 3 / 4 + 4					*	
Lymphatic invasion	no / yes		**			*	
Vascular invasion	no / yes						
Neural invasion	no / yes	***	**				
Invasion score	0/1	*	*				
	0/2						
	0/3						
	1/2						
	1/3						
	2/3						
Tumor volume (%)	< 15 / > 15	*	*				
PSA (ng/mL)	< 10 / > 10		*			*	
Therapy	RRP / RTh		**			*	

PC – prostate cancer; PSA – prostate specific antigen; RRP – retropubic radical prostatectomy; RTh – radiation therapy.

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

In urine samples taken before the therapy, prostate massage induced the highest IL-10 values in the G3 group, significantly increased compared to all others groups (G1, G2, G4) (Table 3). After the therapy (60th day), in voided urine there was no significant difference among the investigated groups, while prostate massage again induced a significant IL-10 concentration increase in patients of the G3 group compared to the G1 and G4 patients.

Different association of Gleason score with IL-10 concentration in urine and serum samples

Our patients were stratified in groups according to the updated Gleason score scale [12]. Interestingly, the analysis of serum samples before the therapy (0th day) demonstrated the highest IL-10 values in groups with the lowest Gleason score (3 + 3), together with 4 + 3 group. There were no significant differences between the groups. After the therapy, the highest IL-10 sera level was noticed in the group with the highest Gleason score (4 + 4), significantly higher compared to group 3 + 4.

Analysis of urine samples demonstrated different association of Gleason score and IL-10 concentration. Initially, the highest IL-10 values were detected in groups with intermediate Gleason score (3 + 4, 4 + 3) (Table 2).

At the same time interval, prostate massage induced a significant increase only in 4 + 3 group, at the level significantly elevated compared to all other groups (Tables 2 and 3).

The therapy induced no significant differences between the investigated groups either in urine or pms.

Tumor infiltration of lymphatic, vascular and neural structures and IL-10 concentration in urine and serum samples

Absence of perilymphatic infiltration was associated with insignificant serum IL-10 increase before the therapy (0th day). To the contrary, after the therapy (60th day), the invasion of lymph vessels was associated with a significant average IL-10 value increase compared to noninvasive finding (Tables 2 and 3). Urine IL-10 value was constantly higher in patients with verified tumor perilymphatic infiltration. Patients with positive perilymphatic infiltration had significantly more IL-10 in pms samples initially.

Serum IL-10 according to perivascular infiltration showed same pattern as in perilymphatic infiltration, with higher IL-10 initially in patients without infiltration and lower IL-10 value after the therapy in the same patient group. Again, urine IL-10 was increased in the samples of patients with perivascular infiltration, without statistical significance. IL-10 in pms samples demonstrated insignificant differences.

Perineural infiltration demonstrated completely different association to IL-10 levels. Namely, patients without per-

ineural tumor infiltration demonstrated higher IL-10 average concentration, both in serum and urine samples, and either before or after therapy (Table 2). This difference was significant for serum samples taken initially (0th day) and after the therapy (60th day). IL-10 value in urine and pms samples showed no differences in either group.

Finally, we analyzed our patients according to the sum of all investigated infiltration types (invasion score – Table 2). According to this organization, patients without any sign of tumor invasion had significantly increased serum IL-10, either before or after the therapy (Table 3). The investigation of urine and pms IL-10 concentrations did not demonstrate any significant differences.

Patients that had more than 15% of tumor in prostate tissue (high volume) demonstrated significantly increased average IL-10 concentration in serum samples, both initially (0th day) or at the second time interval (60th day) (Tables 2 and 3). The analysis of IL-10 concentrations in urine and pms samples showed insignificant differences between these two groups of patients.

Initial serum IL-10 concentration (0th day) demonstrated similar values in both groups of patients (Table 2), while 60 days after therapy significant IL-10 increment was noted in the high PSA group (Table 3). Both groups demonstrated similar IL-10 values in urine samples. The group with high serum PSA level showed increased average IL-10 in pms samples before the therapy.

The comparison of serial samples of the investigated PC patients after/before the therapy demonstrated increased average IL-10 values in serum according to all investigated parameters (60th/0th day, Table 4). There was a clear significant increase in sera of patients of unfavorable Gleason score (4 + 4), with present infiltration of tumor cells in peritumoral tissue (lymphatic, vascular and combined) and in patients with high tumor volume. Interestingly, local urine IL-10 demonstrated different profile. Among the PC patients, after the therapy, IL-10 was significantly increased only in the Gleason score 4 + 4 group and significantly decreased in the PC vascular and/or combined peritumor invasion, as well as in patients with high tumor volume. Similarly, pms samples demonstrated significant increase after the therapy only in the Gleason score 4 + 4 group and significant decrease in the G3 patients, those with present peritumor invasion (any kind), high tumor volume, high serum PSA and in patients with RTh.

Discussion

Role of IL-10 in benign prostatic inflammation is far from clear. Vignozzi et al. [14] investigated cytokine profile produced from primary human prostatic smooth muscle cell lines derived from six patients out of 42 investigated with BPH. Although all BPH patients demonstrated intraprostatic inflammation of some grade, they found clear indirect correlation between inflammation and testosterone level, with the most intensive inflammation in severe hypogonadal patients. *In vitro*, prostatic stromal cell lines produced spontaneously significant amount of IFN-γ, IL-12, IL-6, IL-8, CXCL-10,

Table 4

Comparison of interleukin-10 concentrations in serial samples of prostate cancer patients, 60th/0th day, according to the investigated parameters

Parameter	Group	Samples		
		serum	urine	pms
Gradus	G2	=	▼	▼▼
	G3	▲	▼	▼▼▼
Gleason score	3 + 3	=	▲	▼
	3 + 4	▲	▼	▼
	4 + 3	▼	▼	▼▼▼
	4 + 4	▲▲▲	▲▲	▲▲
Lymphatic invasion	absent	▼	▲	▼
	present	▲	▼	▼▼
Vascular invasion	absent	▼	▲	▼
	present	▲	▼▼▼	▼▼
Neural invasion	absent	▼	=	▼
	present	▲	▼	▼
Invasion score	absent	▼	▲	▼
	1	▲	▼	▼
	2	▲	▲	▼
	3	▲	▼▼▼	▼▼
Tumor	< 15% (Hi)	▼	▼	▼▼▼
Volume	> 15% (Low)	▲	▼	▼▼▼
PSA	< 10 ng/mL	▼	▼	▼
	> 10 ng/mL	▲	=	▼▼▼
Therapy	RRP	▲	=	=
	RTh	▲	▼	▼▼▼

PSA – prostate specific antigen; RRP – retropubic radical prostatectomy; RTh – radiation therapy.
▲ increased; ▼decreased; ▲$p < 0.05$; ▲▲$p < 0.01$; ▲▲▲$p < 0.001$; = not significant (Wilcoxon test).

monocyte chemoattractomil protein-1 (MCP-1) and basic fibroblast growth factor (bFGF). Simulation of infection or further inflammation, with exogenously added lipopolysaccharide (LPS) or TNF-α, dose dependently increased this production several times. Additionally, prostatic stromal cell lines exhibited an antigen presenting function, because their co-culture with $CD4^+$T cell clones induced significant increase of inflammatory mediators (TNF-α , IL-1β, MIP-1α, and MCP-1) and importantly, inhibited production of IL-10. Dihydrotestosterone treatment of prostatic stromal cell lines significantly reduced response to TNF-α or LPS stimulation, significantly reduced $CD4^+$T cell clones proliferation and production of proinflammatory mediators, but increased IL-10 production. Authors concluded that activation of prostate cell lines *via* androgen receptors attenuates inflammation and increase IL-10 production, diminishing autoimmune and inflammatory processes. Our BPH patients demonstrated high IL-10 values in all samples, serum, urine and prostate massage secret, with average urine IL-10 concentrations significantly higher than those in the C and PC groups. Since all our BPH patients had physiological testosterone level, it is attractive to speculate that locally produced high IL-10 found in urine and pms could be physiological, testosterone driven response aimed to control underlying prostatic inflammation.

Data concerning IL-10 in PC is controversial, indicating both pro- and antitumor activity. Considering its anti-inflammatory function, IL-10 is potent local and systemic negative regulator of T lymphocyte mediated response, enabling tumor cells with opportunity to escape from host immune surveillance. On the other hand, there are i*n vitro* and *ex vivo* data reporting that IL-10 exerts anti tumor activity by inhibiting tumor vessel formation and metastasis. Majority of data concerning IL-10 and PC came from genetic studies, based on the notion of significant genetic influence in PC and hypothesis that variations in genes that regulate inflammation could differentially affect risk inherited for this type of tumor. Practical significance of IL-10 gene polymorphism is represented by argument that particular genotype is associated with potential to produce less or more IL-10. Turner et al. [15] were the first who demonstrated that healthy persons that carry GG alleles for IL-10 at 1,082 position have the capacity to produce significantly more IL-10 than those who are carriers of AA allele. Namely, *in vitro* lymphocyte cultures from GG-1,082 persons stimulated with concanavalin A produced more IL-10 compared to AA-1,082 cultures. In different model (whole blood sample stimulated with LPS) IL10R2 / IL10G14 haplotype produced significantly more IL-10 than IL10R3 / IL10G7 haplotype [16].

Generally, the results of IL-10 polymorphism studies are still confusing, ranging from no significant association [17–24] to association of particular alleles in PC patients [15, 25–36]. Several of these studies that confirmed association of particular IL-10 polymorphism with PC reported also a significant correlation of GG-1082 gene alleles with low grade tumors [18, 26, 28, 32, 34, 37], absence of bone metastasis [30] or low recurrence rate [31]. Taken together, these data indicate that genetically determined low IL-10 producers among PC patients more frequently demonstrate high grade tumors, higher Gleason score or more aggressive disease course. In our patients, the G3 group demonstrated the highest average IL-10 value, either in serum, urine or pms sample, both before and after therapy. Surprisingly, patients with G4 grade showed the lowest average IL-10 concentration.

First data that associate Gleason score and IL-10 were from study conducted twenty years ago, from Stearns et al. [38] who investigated effects of IL-10 on carcinoma prostate cell lines on microvasculature formation. They established cell lines from PC patients either with high or low Gleason stage. Cell lines originating from more advanced PC (higher Gleason score) induced formation of significantly more microvessels from human bone marrow endothelial cells (HBMCE-1 cells) in three dimensional gel model. All cell lines produced significant amounts of MMP2 and MMP9, especially those originating from high Gleason tumors. They demonstrated that IL-10 treatment of cell lines significantly decreased MMP secretion and microvasculature formation, underlying the significance of locally produced IL-10 in PC. Generally, there are few studies that investigated Gleason score and IL-10 association in PC patients. In the study of 120 PC patients, Horvat et al. [37] demonstrated significant association of PC patients with IL-10 -1,082 AA haplotype and high Gleason score, more than 7. This haplotype is generally considered as low IL-10 producer. Similar findings came for study of Liu et al. [39] in Chinese population, that demonstrated significant association of gene alleles that are distinguished for high IL-10 production (G -1082, C -819, C -592) with low Gleason score. Immunohistochemical study of Cardillo and Ippoliti [40] demonstrated no association between Gleason score and IL-10 presence and positivity (in tumor stroma and epithelium), although they found correlation of IL-10 positivity with tumor grade and TNM scoring. Hu et al. [41] investigated presence of tumor associated macrophages (TAM) and alternatively activated macrophages (AAM) in PC tissue with pathological and clinical parameters. They found that $CD68^+$ TAM was significantly more present in tissue of PC patients with metastasis, that AAM were significantly more present in tumors of patients with higher grade, higher Gleason score, and serum PSA level. According to this group, these macrophages were the dominant cellular source of IL-10, indicating that patients with the highest Gleason score will have the highest IL10 value. In our PC patients stratified according to Gleason score, the lowest average IL-10 was in groups with the smallest (3 + 3) or the highest (4 + 4) Gleason score, in all kind of samples before therapy. Interestingly, when we analyzed samples from every particular patient after/before therapy (Table 4), only patients with the highest Gleason score (4 + 4) demonstrated significant increase, either in serum, urine or pms samples. In second sample of our patients, since tumor tissue was reduced by therapy, local IL-10 production in urine and pms samples probably originate from other cell type, most probably TAM, AAM and lymphocytes, as considered in study of Hu et al. [41].

Generally, increased IL-10 is associated with the higher capacity of tumor cells for migration and invasion, at least in

melanoma[42], neuroblastoma[43], hepatocellular carcinoma [44] and non-small-cell lung cancer [45]. Newer data suggest that increased expression of IL-10 could have even anti tumor effects, as documented in breast cancer patients, showing significant correlation with favorable clinical parameters as well as disease free interval [46]. Twenty years ago, Stearns and Wang [47] demonstrated anti tumor properties of IL-10 at prostate carcinoma, both experimentally and *in vitro*. They showed that IL-10 transfection of human PC cell line given to severe combined immunodeficient (SCID) mice suppressed tumor growth and formation of metastasis, and prolonged animal survival. In the next study, they demonstrated that IL-10 treatment significantly suppressed angiogenesis induced by of PC cell lines [38]. In recent study, Yu et al. [48] investigated pro- and antitumor effects of IL-3, IL-6, IL-11, IL-10 and IL-24 on testosterone sensitive and insensitive PC cell lines. Contrary to other tested cytokines, IL-10 and IL-24 suppressed proliferation, growth, migration and invasion, inhibited expression of CD44, SOX2 and ABCG2, and increased sensitivity to docetaxel treatment. Author concluded that IL-10 exert significant antitumor effects on PC cell lines. Analysis of IL-10 in our study demonstrated significantly increased average value only in those PC patients without any infiltration (lymphatic, vascular or neural), with the lowest infiltration score (Table 2) and only in serum samples both before and after therapy. In other words, high serum IL-10 value was significantly associated with absence of invasion in our PC patients. The simplest interpretation could be in line with antitumor effects presented by Yu et al. [48]. But, when we analyzed IL-10 in serial samples of particular patients (after/before therapy, Table 4), we found significantly increased serum concentrations of IL-10 after therapy in patients with lymphatic, vascular or combined infiltration. We can only speculate that this invasion associated IL-10 increment could be either a consequence of T lymphocytic activity as a attempt of tumor spread control, or reflection of TAM/AAM activity, as a pro tumor setting of further disease recurrence.

Among rare studies that associated PSA level to cytokines, Dwivedi et al. [49] investigated IL-18 and IL-10 serum concentrations together with PSA values in almost 150 PC patients, BPH and control subjects. They found that serum IL-10 concentration directly followed PSA value, being the highest in PC patients with T3 and T4 stage, and also in patients with evident clinical progression. Tazaki et al. [3] studied values of Th1 (IL-12, IFN-γ, IL-2), Th2 (IL-4, IL-5, IL-6, IL-10), inflammatory (IL-1β, TNF-α) cytokines and IL-8 in serum samples of PC patients, with limited, advanced or metastatic form of disease [51]. They found significant increase of IL-10 in patients with disseminated disease and with cachexia compared to patients with limited disease and controls. Although informative, these results must be taken with reserve because of small number of investigated patients.

Conclusion

We consider as our key result that PC patients without any signs of tumor invasion (lymphatic, vascular, neural) before therapy have significantly high serum IL-10 compared to those with signs of tumor invasion. There is a clear dissociation of IL-10 value between a serum sample and local, urine and pms samples from a particular patient. This is especially noted after therapy in patients with high grade and high Gleason score, present infiltration and high tumor volume, with increase of serum IL-10 and decrease of urine/pms IL-10. Average IL-10 concentration does not have needed specificity and sensitivity (ROC curves not shown), but evaluation of its value in serial serum samples (after/before therapy) demonstrated significant association of increased IL-10 with advanced disease.

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CASE REPORTS

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Topical timolol for superficial cutaneous infantile hemangiomas in very preterm infants

Lokalna primena timolola u lečenju površnih infantilnih hemangioma kod veoma rano prevremeno rođene dece

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Abstract

Introduction. Infantile hemangiomas (IHs) occur 3 to 4 times more frequently in preterm infants than in those born at term. Yet, data about efficacy and safety of topical therapy for IHs in preterm infants, especially in those born with very low gestational age (less than 33 gestational weeks) is very scarce. **Case report.** We reported five very preterm girls treated with topical timolol maleate 0.5% gel for superficial cutaneous IHs. In four infants topical timolol was applied on a single IH each, and in one infant two IHs were treated. Out of six treated IHs, one was located on the face, four on the trunk and one on the leg. They were regularly monitored for IH involution and potential adverse effects of timolol for 6–13 months of local treatment. Good therapeutic effect was achieved in all the presented infants, with no adverse effects related to topical administration of timolol-maleate. **Conclusion.** Topical timolol is an effective and safe therapy for superficial cutaneous infantile hemangiomas in very preterm infants. The treatment should be discussed in detail with parents and individualized management plan should be tailored for each infant in order to maximize the chances of a successful outcome, while avoiding adverse effects.

Key words:
hemangioma; skin neoplasms; timolol; administration, topical; child; infant, premature; treatment, outcome.

Apstrakt

Uvod. Infantilni hemangiomi (IH) se javljaju 3 do 4 puta češće kod prevremeno rođene dece nego kod one rođene u terminu. Ipak, podaci o efikasnosti i sigurnosti lokalne terapije IH kod prevremeno rođene dece, naročito one sa veoma niskom gestacijom (manje od 33 nedelje gestacije) su veoma oskudni. **Prikaz bolesnika**. U radu je prikazano 5 devojčica rođenih sa veoma niskom gestacijom koje su lečene lokalnom primenom 0,5% gela timolol maleata zbog površnih kutanih IH. Kod četvoro dece, timololom je bio tretiran po jedan IH, a kod jednog deteta su tretirana dva IH. Od ukupno šest tretiranih IH, jedan je bio lociran na licu, četiri na trupu i jedan na nozi. Tokom trajanja terapije (od 6 do 13 meseci), redovno je praćeno napredovanje involucije IH i eventualna pojava neželjenih efekata. Dobar terapijski efekat je postignut kod sve dece, a neželjeni efekti, koji bi se mogli povezati sa lokalnom primenom timolola, nisu zapaženi. **Zaključak.** Lokalna primena timolola predstavlja efikasnu i bezbednu terapijsku opciju za lečenje površnih kutanih infantilnih hemangioma kod dece rođene sa veoma niskom gestacijom. O terapiji se mora detaljno prodiskutovati sa roditeljima i treba sačiniti individualizovani terapijski plan za svako dete, kako bi se povećale šanse za terapijski uspeh, a izbegli neželjeni efekti.

Ključne reči:
hemangiom; koža, neoplazme; timolol; lokalna primena; deca; novorođenče, prevremeno; lečenje, ishod.

Introduction

Infantile hemangiomas (IHs) occur more frequently in preterm infants than in those born at term. There is a growing body of evidence that IHs frequency increases with decreasing birth weight and gestation length [1, 2], making preterm infants 3 to 4 times more likely to develop IH compared to term infant [3]. Yet, there are very few studies about the efficacy and safety of topical therapy for IHs in preterm infants, especially in those born with very low gestational age, i.e. those born before 33 gestational weeks. Topical therapy is even more important for treatment of IHs in this subpopula

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tion, since they are increasingly sensitive for potential adverse effects of systemic therapy.

Case reports

We present therapy effects in 5 very preterm infants with one or more superficial cutaneous IHs, who were treated with topical timolol maleate.

All the presented patients were very preterm girls, hospitalized at the tertiary level institution within the university hospital after birth for prematurity and different early morbidities. None of the presented patients had severe neonatal asphyxia, there was no need for intubation at birth, no episodes of hypoglycaemia nor hemodynamic instability during the birth and primary hospitalization. In all the presented patients, brain and abdomen ultrasound examination did not reveal any additional hemangiomas on internal organs. As high-risk infants for different medical and developmental adverse outcomes, due to their very low gestation at birth, each of the presented patients had regular follow-up examinations after discharge; those examinations were the opportunity for check on IHs too, which were done by neonatologist and/or by dermatologist. The photographs were taken at different points in time, before as well as during the usage of topical timolol therapy. The effect of timolol therapy was assessed using Hemangioma Activity Score (HAS) [4] at the starting point before initiating the therapy, as well as after 6–13 months of regular timolol application.

Timolol maleate was applied as a 0.5% gel, one drop twice a day rubbing directly on IH while avoiding surrounding skin. None of the adverse effects that could be related to timolol therapy were noticed in any of the presented patients. All the parents declared in later stage check-ups that they easily incorporated the local timolol therapy in the infant's routine daily care – therefore the daily use of timolol did not pose a burden to them.

The most important perinatal characteristics of the presented patients and their IHs are shown in Table 1.

Patient 1: At the age of five weeks (corrected age 37 weeks) we noted an oval superficial IH, 4 mm in diameter, touching the bottom edge of left mamilla. After four weeks, 3 more round IHs were also noted: one on the left forearm with diameter of 2 mm, and two punctiform IHs – one in the middle of the abdomen and the other in the right inguinal region; all three were at the level of the surrounding skin. At the age of 4 months (2 months corrected age) the near-mamilla IH was 16 × 10 mm, about 2 mm above the surrounding skin, with a granular surface; its HAS was 5. The forearm IH's longer diameter was 5 mm, and it was flat; the abdominal and inguinal IHs were about 2 mm, also flat (Figure 1a). Parents were acquainted with both management op-

Table 1

The most important perinatal characteristics of the presented very preterm infants and their superficial cutaneous infantile hemangiomas

Patient	Gender	Gestation (gestational weeks)	Birth weight (grams)	Early respiratory or cardiovascular morbidity	Number of IHs	Location of IHs	Number of IHs treated	Postnatal age at initiation of timolol (months)	Corrected age at initiation of timolol	Adverse effects of timolol
1	Girl	32 1/7	1450	Moderate RDS	4	Near the mamilla, forearm, abdomen, inguinal region	1	4	2 months	none
2	Girl	32	1370	Mild RDS	1	Abdomen	1	3	4 weeks	none
3	Girl	32 3/7	1620	Moderate RDS	1	Upper leg	1	3	4 weeks	none
4	Girl	29 4/7	1260	Moderate RDS	1	Abdomen	1	4.5	6 weeks	none
5	Girl	32 4/7	1960	PDA	2	Near the eye, abdomen	2	3	6 weeks	none

IHs – infantile hemangiomas; RDS – respiratory distress syndrome; PDA – patent ductus arteriosus.

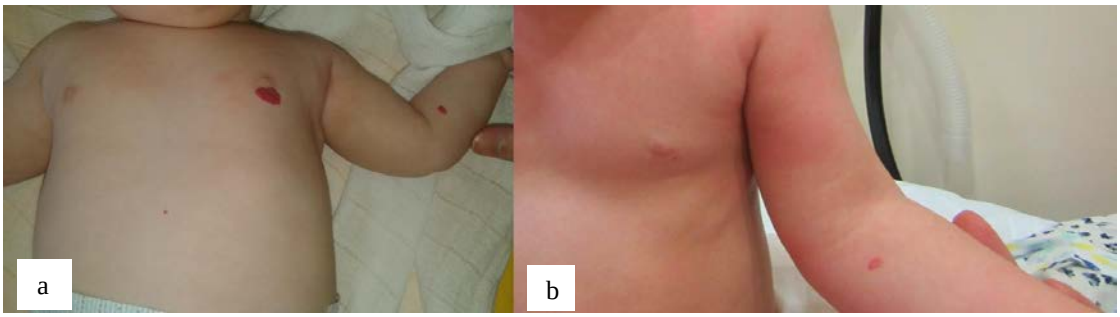

Fig. 1 – Superficial infantile hemangioma near the left mamilla and at the left forearm: a) at the age of 4 months (2 months corrected age), before topical timolol therapy was introduced; b) at the age of 15 months, after 11 months of timolol topical therapy on near-mamillar hemangioma, but without the treatment of left forearm hemangioma.

tions for near-mamilla IH: topical therapy with timolol maleate or oral with propranolol. They decided to use topical therapy. The other 3 IHs were small and flat, and there was no rationale for their treatment. The parents were advised to put baby oil on the left mamilla first, and then to rub timolol maleate on the IH avoiding mamillar ridge as much as possible. Two months later the treated IH was flattened almost to the level of the surrounding skin, and its surface was of greyish-red colour. Subsequent controls showed further gradual fading of the treated IH. At the age of 15 months, after 11 months of topical timolol usage, the near-mamilla IH was barely visible, with HAS 0. At the same time, the second largest IH at the left forearm was 7 mm in its longer diameter, light-red and still clearly visible (Figure 1b).

Patient 2: At the postnatal age of 3 weeks an IH was noticed on the left hemiabdomen. It had an irregular pentagon shape, size of 17 × 12 mm, 1 mm above the surrounding skin, with a granular surface. At the age of 3.5 months (1.5 months corrected age), IH has increased in size to 22 × 18 mm in diameter. It was 2 mm above the surrounding skin, with a red surface even more granulated than before (Figure 2a). Its assessed HAS was 5. Local therapy with 0.5% timolol maleate gel was introduced at this time. Subsequent follow-up showed that IH faded gradually starting from the middle (Figure 2b), with a more and more flattened surface. At the age of 16 months (12 months of timolol usage) the IH was completely flat and its whole surface was very pale (Figure 2c), similar in colour with the surrounding skin, and HAS score dropped to 0.

Patient 3: A superficial IH located on the front of the left upper leg has appeared during the third postnatal week. It was in the form of a horizontally elongated irregular rectangle, dark red in colour, almost flat, 9 × 4 mm in diameter (Figure 3a). At the age of almost 3 months (4 weeks corrected age) IH has grown to 18 × 10 mm in diameter (twice the size than on the onset) with a granular surface rising 2 mm above the surrounding skin (Figure 3b). Its HAS score was assessed at 3.5. At this point topical therapy with timolol maleate was advised. On subsequent follow-ups the colour of IH was more and more greyish-pale, evenly on the entire surface. At the age of 16 months, after 13 months of the regular usage of topical timolol, the entire surface of IH eventually became very pale, and only slightly elevated (Figure 3c), with HAS score of 0.

Patient 4: After discharge, at the postnatal age of 2.5 months (40 corrected weeks) parents noticed an oval flat IH

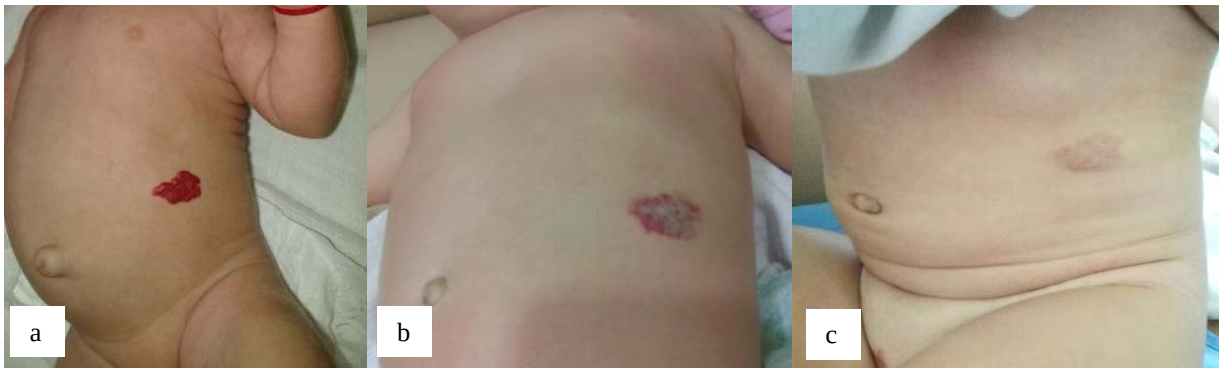

Fig. 2 – Superficial infantile hemangioma on the left front trunk: a) at the age of 3.5 months (1.5-month corrected age), before initiating of topical timolol; b) at the age of 6 months, after 3 months of topical timolol; c) at the age of 15.5 postnatal months, after 12 months of usage of topical timolol therapy.

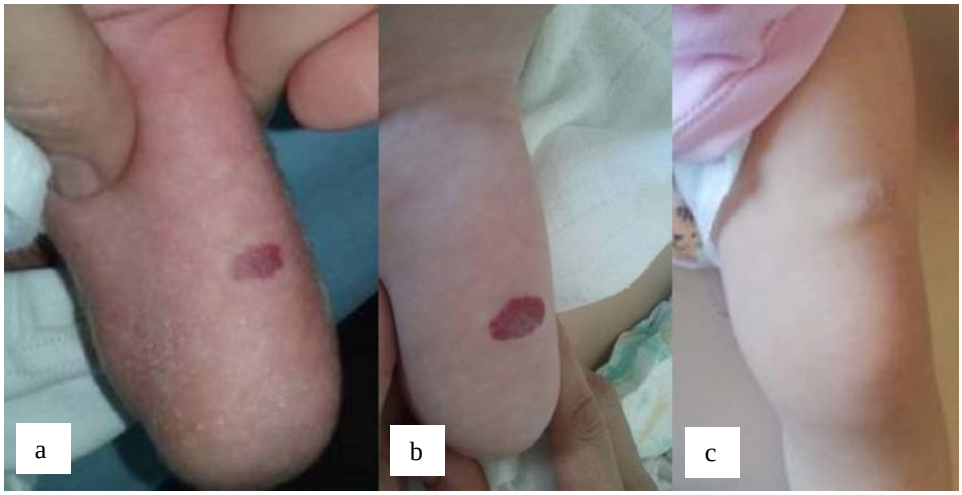

Fig. 3 – Superficial infantile hemangioma on the left upper leg: a) at the age of 3 postnatal weeks; b) at the age of 3 months, before the initiation of topical timolol; c) at the age of 16 postnatal months, after 13 months of topical timolol therapy.

on the left hemiabdomen. At the postnatal age of 4.5 months (6 weeks corrected age) the widest diameter of IH was 11 mm and it was rising above the level of the surrounding skin by 1 mm, with HAS score 5 (Figure 4a). Infant's parents were concerned about possible side-effects of IH, even though it was not very big nor on the functional or cosmetologically sensitive location. Topical timolol was advised. After 6 months of topical timolol, IH was at the level of the surrounding skin, pale red and 6 mm in diameter (Figure 4b), with HAS score of 1.

Patient 5. Within the 4th postnatal week (36 corrected weeks) an irregularly shaped IH appeared at the right hemiabdomen, and one week later another oval IH appeared near the outer corner of the right eye. At the age of 3 months (6 corrected weeks) the IH near the eye was oval, 9 × 6 mm in diameter, dark-red, and about 1 mm above the surrounding skin in its central part (HAS score 5). IH on the abdomen was in the shape of an irregular pentagon, 20 × 18 mm in diameter, elevated more than 1 mm above the skin, with a red, granulated surface (Figure 5a); HAS score was also 5. Infant's parents were concerned about IHs, especially the one near the eye, about their enlargement and the potential adverse medical and aesthetic effects. Since echocardiography showed that ductus arteriosus was still patent, we decided to start topical timolol therapy, with additional advice of eye protection when applying timolol on the facial IH. At the age of 6.5 months (4.5 months corrected age) we observed significant improvement of IHs, especially the one located near the eye. At that time echocardiographic exam revealed the spontaneous closure of ductus arteriosus. The possibility of changing the IH therapy from topical to oral propranolol was discussed with the parents. They, however, were satisfied with the progress accomplished with topical therapy and decided to continue with timolol. At the age of 9 months (7 corrected months), after 6 months of timolol therapy, the IH near the eye was reduced (6 × 4 mm), flat and had a pale bluish surface with HAS score 1 (Figure 5b). The involution of IH on the abdomen was slower; it was also reduced in size (20 × 17 mm), with further enlargement of the pale central part. Its surface almost became flat (Figure 5c), and HAS score decreased to 2.

Discussion

There are some important differences in the occurrence of IHs between preterm and term infants. The incidence of IHs increases with the decreasing gestation and birth weight [2]. Furthermore, the number of IHs per child also in-

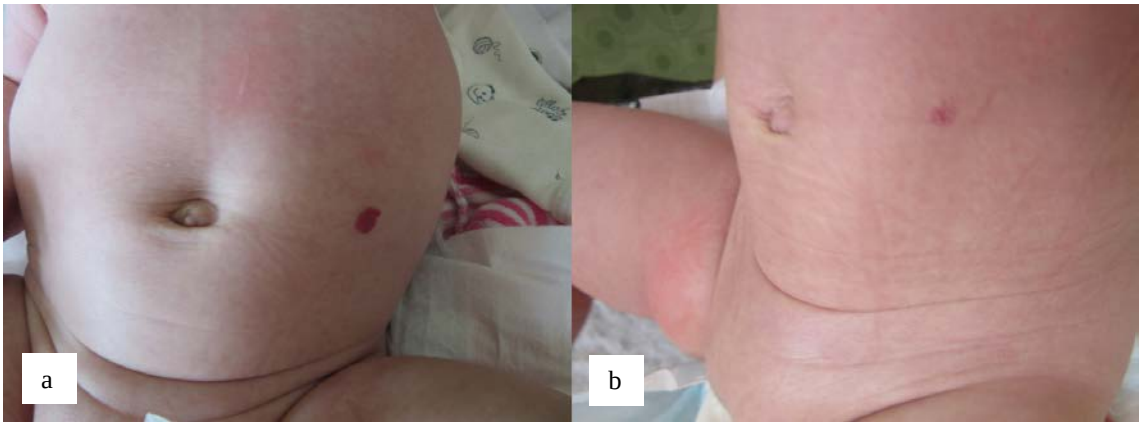

Fig. 4 – Superficial hemangioma of the left hemiabdomen: a) at the age of 4.5 months (6 corrected weeks), before timolol therapy; b) at the age of 9.5 months, after 6 months of topical timolol therapy.

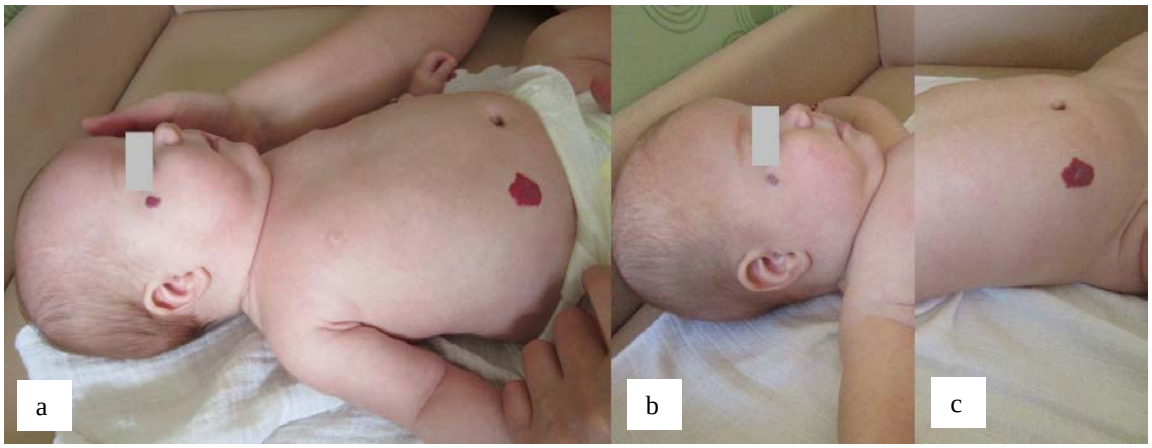

Fig. 5 – Superficial infantile hemangioma: a) near the right eye and on the right hemiabdomen at the age of 3 months, before the initiation of topical timolol; b) after 6 months of topical timolol therapy near the right eye; c) after 6 months of topical timolol therapy on the right hemiabdomen.

creases with decreasing gestation, with 2 to 5 IHs found in 40% of preterm *vs* 24.5% of term infants, and more than 5 IHs in 7.5% preterm *vs* 3% of term infants. These data indicate that preterm infants are especially prone to solitary and multiple IHs.

The introduction of beta-blockers in the therapy of IH – oral propranolol in 2008 [5], and topical timolol in 2010 [6], and, to a much lesser extent, some other oral or topical agents from the same pharmacological group (atenolol, nadolol, carteolol) revolutionized the treatment of the most common tumour in infancy. The management of IH in the era before beta-blockers consisted of different modalities (systemic or intralesional corticosteroids, interferon-alfa, vincristin, cryotherapy, laser, surgery), with an uncertain and often nonsatisfactory outcome, and with numerous, sometimes very serious adverse effects. Beta-blockers, oral as well as topical, turned out to be an effective and safe treatment for IH, as a number of studies demonstrated [7–15]. Their high level of efficacy and safety broadened the list of IHs indicated for the treatment, including those on the face, scalp, neck, hands, feet and intertriginous and perineal regions [16, 17].

Most of superficial cutaneous IHs are benign and harmless. However, significant proportion of them can be complicated by a central ulceration, bleeding and pain during their proliferative phase, as well as life-long functional and aesthetic consequences [17]. Naturally, critical IHs i.e. those which are life-threatening (in the airways), function-threatening (on the eyelids), ulcerated or with a high-risk thereof, or with a risk for acute or chronic disfigurement must be treated. But this list is not exhausted, since there is a growing body of evidence of a high proportion of residual deformity found in 69–88% of IHs, even with those that do not possess apparently aggressive properties [18, 19]. According to some authors, every IH over 8 mm in diameter can be predisposed to express residual effects after involution, hence it should be among those being considered for treatment [13]. Aesthetics is among rational reasons for IH treatment, because of the potential for mental pressure on the parents as well as an unpredictable psychological burden in the child [15]. This reason is especially pronounced in girls with IH. All our patients were girls, which might be the reason for the increased parents` concern for medical but also aesthetic long-term effects of the IHs on their infants. The reasons for the introduction of IH therapy were, besides aesthetics, the potential for disfigurement. In patient 1, the girl with IH near her left mamilla, there was a fear of significant residuals on the breast; even breast hypoplasia has been described in the literature [20], although with mixed IH. In patient 3 IH showed rather fast growth, and in patient 5 IH was on the face, near the eye.

Today beta-blockers are considered the first-line therapy for different types of IH, either in systemic or topical form. Systemic therapy, most frequently with oral propranolol, turned out to achieve faster and more complete involution of IH compared to topical beta-blockers[21]. But the associated adverse effects are more frequent with systemic beta-blockers compared to topical application. Adverse effects of oral propranolol in paediatric population are hypotension, bradycardia, pulmonary symptoms (bronchial obstruction, apneic episodes), hypoglycaemia, sleep disturbances, somnolence, cool or mottled extremities, and gastrointestinal symptoms (gastroesophageal reflux, diarrhoea) [17]. These side-effects are rare, but potentially dangerous, as some reports showed [22–24]. For these reasons in many hospitals, ours included, introduction of oral propranolol is performed in the hospital settings, with cardiological, respiratory and metabolic testing and monitoring. Besides, propranolol as a lipophilic molecule crosses the blood–brain barrier and may cause several effects in the central nervous system (CNS). In a small study, propranolol has been found to impair short- and long-term memory, psychomotor function, sleep quality, and affect mood [25]. On the other hand, some other authors did not confirm such an effect of propranolol [26]. Nevertheless, this possibility should not be neglected in very preterm infants, in whom psychomotor development is already affected by low gestation at birth. Special medical issues concerning therapy for superficial IH in very preterm infants are their comorbidities, as well as a greater potential for the manifestation of adverse reactions on any therapy. Although oral propranolol is, by today's knowledge, the first line therapy for IH, its application in preterm infants raises numerous questions about proper indication, timing of initiations and surveillance of potential adverse effects of this therapy mode.

Numerous studies confirmed that topical timolol was proven to be an agent with good therapeutic efficacy for the management of superficial and mixed IHs [11–14]. Among them is the study conducted by Yu et al. [15]. Out of 101 treated infants, in 12 of them the complete involution of IH was achieved in 4 months of topical timolol usage. The same study also showed significantly better effects in the subgroup of infants in which topical timolol was initiated at the age of 1–6 months, compared to those in which the same therapy was initiated at the age of 7–12 months. It is well known that the management of IHs should be initiated in the early proliferative phase – during first 2–3 months of life, for the maximum effect [27].

Bearing all of the above in mind, topical timolol seems to be a good alternative to systemically applied therapy in preterm infants, including those with very low gestation. It achieves quicker and more complete involution of IH than naturally, but with low potential for adverse events in especially vulnerable patients. Topical timolol therapy may have some adverse effects, although they occur very rarely: bradycardia, hypotension, apnea, sleep disturbances and hypothermia [28, 29]. In most studies dealing with the efficacy and safety of topical timolol for IHs these effects have not been recorded at all [13, 28]. In the study conducted on 103 infants treated with topical timolol for IHs, 22 infants were considered at high-risk for adverse effects for any reason: low age at the initiation of therapy (less than 4 weeks corrected age); those receiving more than two drops per day; application to a site with potential for high systemic absorption (mucosal, ulcerated IH, under occlusion). This high-risk group included 6 preterm infants born with 33–36 and one with 26 gestational weeks. Adverse effects were observed in two infants: brady-

cardia and hypothermia in 33-weeker, and bradycardia and apnea in 26-weeker [29]. The introduction of timolol in these infants started at the corrected age of 37 and 34 gestational weeks, respectively. In all our cases, infants were at the corrected age of 4 weeks or older at the initiation of timolol therapy. Also, in all our patients timolol was applied on the skin, not on mucosal surfaces, nor on ulcerated IH or in the diaper area. All the presented infants received just 2 drops of timolol per day, except patient 5, in which topical timolol was applied on 2 IHs simultaneously, that is – she received 4 drops of topical timolol per day. But even in this girl there were no adverse effects of therapy, even though she still had PDA at the time of topical therapy initiation. We think that not initiating topical timolol before 4 corrected weeks in preterm infants is the most important factor contributing to the avoidance of adverse therapy effects.

Discussing the management of IH with parents is very important. The therapeutic effects are most favourable in the early proliferative phase of the IH during the first 2–3 months of age, at the time when long-term residuals are still neither visible nor predictable. A detailed explanation and cooperation with parents is crucial in order to achieve good therapy compliance as well as to monitor possible side effects. On the other hand, parents of very preterm infants have already undergone an emotionally difficult period after the childbirth and have a high degree of concern for the health of their infant. They are less willing to use the therapy requiring hospitalization, as is the case with oral propranolol.

All the girls presented here had relatively small and uncomplicated IHs, which is a limiting factor in evaluating the efficacy and safety of local timolol therapy. Further studies that would include large and/or complicated IHs in preterm infants would be needed to better evaluate the effect of timolol in this vulnerable group of patients.

Conclusion

Topical timolol is an effective and safe therapy for superficial cutaneous IHs, in our case – a group of very preterm infants. Treatment modalities for IHs should be discussed in detail with parents. An individualized management plan should be tailored for each child, in order to maximize the chances of successful treatment, and avoid adverse effects.

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CASE REPORT

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Early massive gastrointestinal bleeding as a complication of left ventricular assist device implantation

Rano masivno gastrointestinalno krvarenje kao komplikacija ugradnje uređaja za mehaničku potporu rada leve komore

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Abstract

Introduction. Implantation of left ventricular assist device (LVAD) improves survival and quality of life in patients with end-stage heart failure. We reported a case of a severe gastrointestinal bleeding as a life threatening complication in early recovering postoperative period of continuous blood flow LVAD implantation. **Case report.** The patient had a history of heart failure due to ischemic cardiomyopathy with low systolic function, as an indication for LVAD implantation. The operation and the postoperative course were uneventful. On the 17th postoperative day, the patient developed severe anemia, which was followed by melena with blood clots. Endoscopic examinations revealed diffuse colonic bleeding. Oral anticoagulation therapy was discontinued, and fresh frozen plasma, K vitamin substitution, and human prothrombin complex were administered. The LVAD speed was reduced and subcutaneous administration of somatostatin analog octreotide was initiated. These measures successfully stopped the bleeding and the patient was stabilized. Due to a multidisciplinary team approach, the bleeding was successfully managed and the patient recovered. **Conclusion.** Acute gastrointestinal bleeding represents a serious, life-threatening condition that can develop after LVAD implantation, but with timely and appropriate measurements, it can be successfully treated.

Key words:
gastrointestinal hemorrhage; heart assist devices; heart failure; postoperative period; treatment outcome.

Apstrakt

Uvod. Implantacija pumpe za mehaničku potporu rada leve komore (LVAD) poboljšava preživljavanje i kvalitet života bolesnika sa terminalnom srčanom slabošću. Prikazan je slučaj teškog gastrointestinalnog krvarenja kao životnougrožavajuće komplikacije u ranom postoperativnom periodu nakon ugradnje LVAD uređaja sa kontinuiranim protokom krvi. **Prikaz bolesnika.** Dugogodišnjem bolesniku sa srčanom slabošću na terenu ishemijske kardiomiopatije sa sniženom sistolnom funkcijom, bila je indikovana ugradnja LVAD uređaja. Operacija i postoperativni tok su protekli uredno. Sedamnaestog postoperativnog dana došlo je do pojave teške anemije, koja je bila praćena pojavom melene sa prisutnim krvnim ugruščima. Endoskopskim pregledima otkriveno je difuzno gastrointestinalno krvarenje, pretežno iz debelog creva. Odmah je obustavljena oralna antikoagulantna terapija i primenjeni su sveže smrznuta plazma, supstitucija K vitamina i humani protrombinski kompleks. Brzina LVAD pumpe je smanjena i započeta je primena somatostatinskog analoga oktreotida. Preduzete mere dovele su do prestanka krvarenja i stabilizacije bolesnika. Zahvaljujući saradnji multidisciplinarnog tima krvarenje je uspešno zbrinuto i bolesnik se oporavio. **Zaključak.** Akutno gastrointestinalno krvarenje predstavlja ozbiljno, životno-ugrožavajuće stanje koje se može javiti nakon ugradnje LVAD uređaja, ali uz pravovremene i adekvatne mere može se uspešno izlečiti.

Ključne reči:
krvarenje, gastrointestinalno; srce, implantabilni mehanički aparati; srce, insuficijencija; postoperativni period; lečenje, ishod.

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Introduction

Left ventricular assist device (LVAD) implantation is one of the recommended treatment modalities used for end-stage heart failure. New generation continuous blood flow LVADs have improved durability and better survival for patients, but at the same time they are associated with an increased risk of gastrointestinal (GI) bleeding and other specific complications [1, 2].

In this article, we report a case of a severe life threatening GI bleeding, developed as an early complication of continuous blood flow LVAD implantation.

Case report

A sixty-year-old male patient was admitted for the management of chronic end-stage heart failure with the implantation of LVAD. The patient had a history of coronary artery disease and was first diagnosed seven years ago when percutaneous coronary intervention (PCI) was performed with the implantation of multiple stents into coronary arteries. In the following years, progressive decline in systolic function and heart failure developed. His symptoms included fatigue and shortness of breath (NYHA class 4, INTERMACS profile 5). The NT-proBNP value was 3,147 pg/mL. The only comorbidity was diabetes mellitus type 2. He was a long-time smoker with a positive family history of cardiovascular diseases and no prior GI disease.

Echocardiography showed ischemic dilatative cardiomyopathy with poor systolic function (Figures 1 A–D). The left atrium (LA 53 mm; LAVs 101 mL) and the left ventricle (LVIDs 52 mm, LVIDd 74 mm, EDVLV 294 mL, ESVLV 252 mL) were dilated and remodeled, with the ejection fraction of 15% (normal range: 50%–75%) by the Simpson method. The mitral annulus was also dilated (MADd 41 mm; MAAd 13.2 cm^2) with severe mitral regurgitation. Diastolic dysfunction with the restrictive filling pattern was also present (E/e' = 25). The dilated right atrium (RAVs 97 mL), dilated tricuspid annulus (TADd 38 mm, TAAd 11.34 cm^2) with moderate-severe tricuspid regurgitation and dilated right ventricle (RV1 51 mm, RV2 37 mm, RV3 96 mm) were registered as well. The right ventricular systolic function was also evaluated (TAPSE 17 mm, FAC 28%).

Right heart catheterization showed the following hemodynamic parameters: central venous pressure 23 mmHg (normal range: 8–12 mmHg), right ventricular systolic pressure 54 mmHg (normal range: 15–39 mmHg), pulmonary arterial pressure 53/8 mmHg (normal range: 15–39 mmHg), pulmonary arterial wedge pressure 25 mmHg (normal range: 2–14 mmHg), cardiac index 2.49 L/min/m^2 (normal range: 2.6–4.2 L/min/m^2), and systemic vascular resistance 2,279 dynes s/cm^{-5} (normal range: 700–1,500 dynes/s/cm^{-5}).

After detailed preoperative preparation and all the necessary examinations, the patient underwent the implantation of the Heartware LVAD through the standard open surgical approach with transesophageal

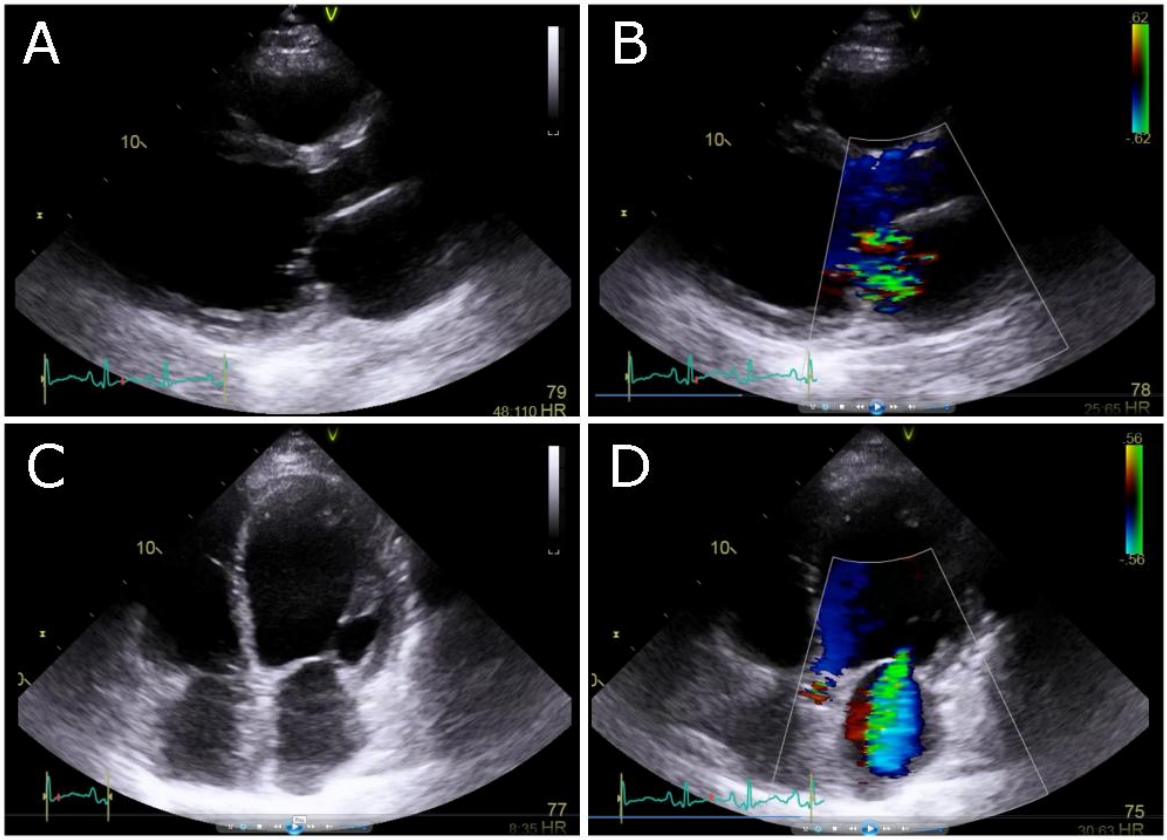

Fig. 1 – Transthoracic echocardiography prior to surgery: A) Parasternal long axis view; B) Parasternal long axis view – Doppler effect; C) Apical four-chamber view; D) Apical four-chamber view – Doppler effect.

echocardiography guidance, using general endotracheal anesthesia and extracorporeal circulation.

The immediate postoperative course was uneventful. The patient was hemodynamically stable with dobutamine (8 μg/kg/min) and noradrenaline (0.03–0.06 μg/kg/min) stimulation. The LVAD was programmed to work at 2,700 rpm, with the blood flow of 5.0 L/min.

Continuous heparin infusion was used for anticoagulation. Oral anticoagulation therapy using acenocoumarol was initiated on the second postoperative day after the removal of chest tubes, with target international normalized ratio (INR) values between 2.0–3.0.

Regular periodic echocardiography was performed according to the standard LVAD protocol. Due to the development of left-sided pleural effusion, the drainage of the left pleural cavity was performed resulting in the evacuation of 2,400 mL of serohemorrhagic fluid (Figures 2, A and B).

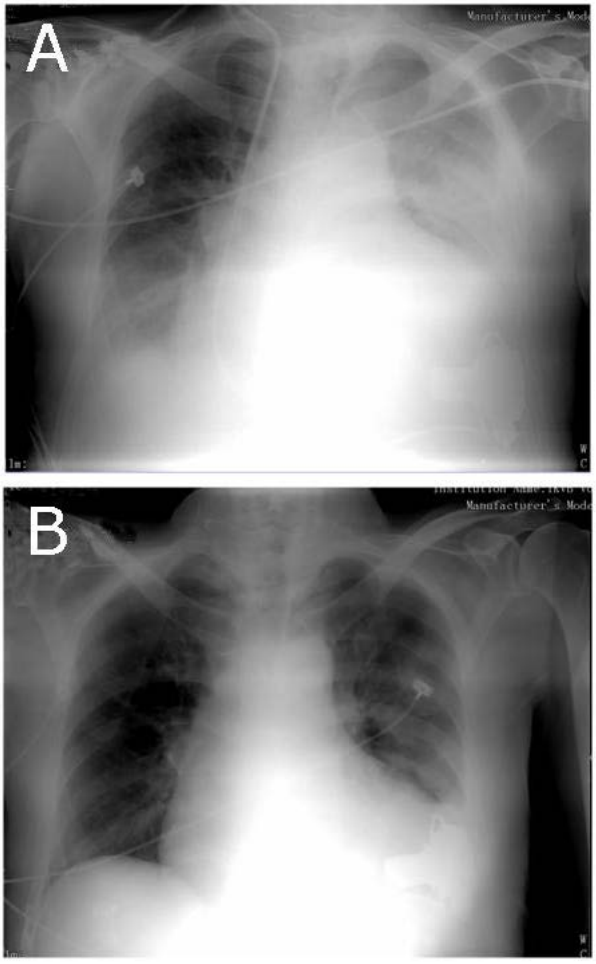

Fig. 2 – Chest X-ray: A) before, and B) after the drainage of the left pleural cavity and intensified diuretic therapy.

The patient was recovering well and all laboratory analyses were normal. Enteral nutrition was gradually introduced, normal intestinal peristalsis was established and the normal stool was formed. The patient was mobilized and intense physical rehabilitation followed.

On the 17th postoperative day, the patient started developing severe anemia. Blood hemoglobin dropped to 69 g/L. Analysis of the red blood cell corpuscular parameters revealed normocytic normochromic anemia. After a transfusion of packed red blood cells, a satisfactory hemoglobin value was established.

His stool was normal and digital rectal examination showed no signs of bleeding. The urine analysis excluded hematuria as a cause of anemia. In search of the source of bleeding, a computed tomography (CT) scan of chest and abdomen with intravenous contrast was performed (Figure 3), however no signs of active bleeding or pathological changes were identified.

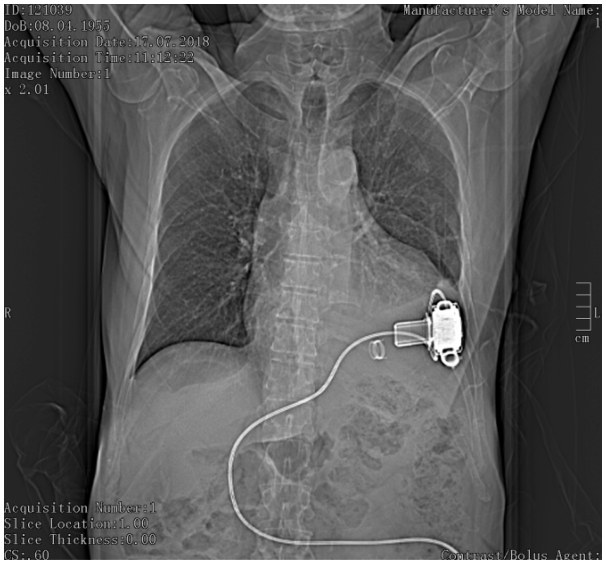

Fig. 3 – Left ventricular assist device implantation position on chest computed tomography.

After a few days, large amount of melena with blood clots appeared, so the endoscopic examination of the GI tract was indicated. Esophagogastroduodenoscopy revealed no signs of active bleeding. Following that, colonoscopy discovered a large amount of hemolyzed blood in the form of film that completely covered the colonic mucosa, stretching over its entire length. However, underlying colonic mucosa appeared to be normal, so the site of active bleeding could not be detected.

The abdomen and pelvis scintigraphy with radiolabeled erythrocytes also did not reveal any signs of active bleeding (Figure 4). Considering that melena with blood clots continued, an exploratory laparotomy was performed. The colon appeared to be filled with large number of parietal thrombi, however no visible pathological changes on the intestinal wall could be identified, so the conclusion was made that this was a case of diffuse colonic bleeding.

Along with the search for the bleeding site, substitution therapy for anemia with numerous packed red blood cells transfusions was applied. Occasionally, blood hemoglobin levels fell below 60 g/L and were partially improved after the transfusions.

At that point, therapeutic values of INR had already been reached, so the oral anticoagulation therapy with

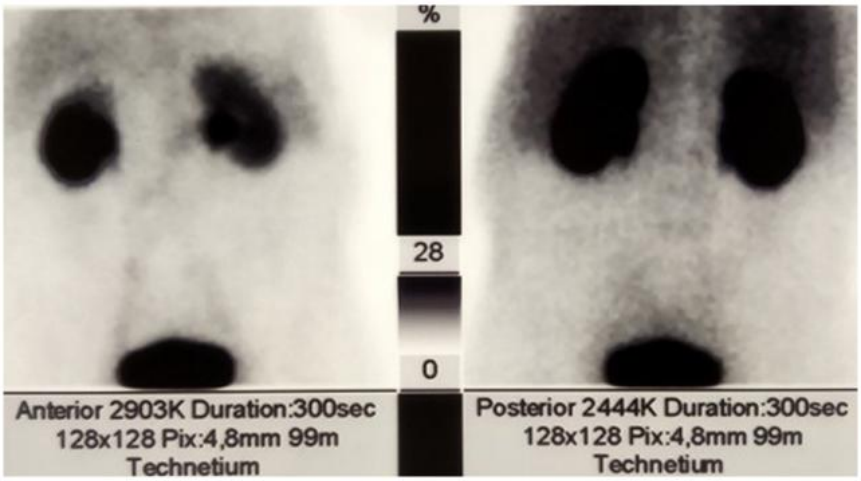

Fig. 4 – Abdomen and pelvis scintigraphy with radiolabeled erythrocytes: no signs of bleeding.

acenocoumarol had to be discontinued immediately. The patient was given fresh frozen plasma, K vitamin substitution, and human prothrombin complex. In order to prevent LVAD thrombosis, low molecular weight heparin was left in therapy. Additionally, subcutaneous administration of the somatostatin analog octreotide was initiated in a dose of 0.1 mg every 8 h. The LVAD speed was gradually reduced from 2,700 to 2,500 rpm, which resulted in blood flow of 4.0 L/min.

After the introduction of octreotide, the GI bleeding stopped, melena disappeared and blood hemoglobin level stabilized. After the patient's general condition improved and the laboratory findings normalized, acenocoumarol was again gradually introduced. LVAD speed was carefully increased to 2,600 rpm, resulting in a flow of 4.5 L/min.

The patient was discharged on the 45th postoperative day with a blood hemoglobin level of 90 g/L, INR values within the therapeutic range and no signs of bleeding. He was discharged with cardiac therapy, along with iron supplementation and mesalazine 2 g per day, as recommended by gastroenterologist.

Seven days after the discharge, the patient came for a check-up. He was feeling well, had no congestive symptoms, and could tolerate moderate physical activity. There were no symptoms and signs of GI bleeding. His blood hemoglobin level was 84 g/L. There was no record of the LVAD alarm activation. Control echocardiography revealed the aortic valve opening with every heartbeat.

Discussion

Bleeding is the most common complication after LVAD implantation and it commonly originates in the GI tract. Most recent data suggest that as much as one third (30–34%) of patients with LVAD exhibit at least one episode of GI bleeding during the first three years [3–5].

GI bleeding following the continuous blood flow LVAD implantation is not associated with any form of preexisting GI lesions, as well as antiplatelet or anticoagulation therapy. The main underlying mechanism responsible for the bleeding in this setting is hypothesized to be angiodysplasia [6].

Angiodysplasia develops due to a narrowed arterial pulse resulting from a decreased aortic valve opening that occurs after the LVAD implantation, causing the loss of normal cardiac cycle and pulsatile blood flow. For this reason, GI bleeding is observed more frequently in LVAD pumps with continuous blood flow in comparison with pulsatile one [2].

The loss of pulsatile blood flow produces dilatation of the intestinal capillaries and venules, as well as a decreased venous outflow [7]. An autopsy study that performed a microscopic analysis of the intestines in patients with continuous flow LVADs discovered a consistent form of angiodysplasia in all the observed samples and concluded that this could be classified as a distinct form of pathology [8].

High shear forces produced by the continuous blood flow result in the increased consumption of the von Willebrand factor, leading to a hemostatic disorder similar to the hereditary von Willebrand disease [9]. The consequent impaired hemostatic cascade and platelet aggregation further contribute to the bleeding risk. This condition, called the acquired von Willebrand syndrome, has been observed in patients with various cardiovascular diseases, such as aortic stenosis, hypertrophic obstructive cardiomyopathy, as well as various forms of mechanical circulatory support like extracorporeal membranous oxygenation (ECMO) and LVAD. Newest discoveries suggest that actually every single patient after LVAD implantation exhibits this disorder in some degree [10].

It seems that our patient was prone to GI bleeding. Some of his medical characteristics, such as the history of coronary artery disease, ischemic cardiomyopathy, high systemic vascular resistance and diabetes mellitus were identified as risk factors for GI bleeding in a recent study on 351 LVAD patients [3].

The bleeding location in our patient was colon, which is not typical. According to literature, the most common site of GI bleeding in LVAD patients is the upper GI tract in almost

half cases, whereas the colon is the source of bleeding in 22%, and small bowel in 15% [11].

The mainstay of the GI bleeding management in our patient was the reduction of the LVAD flow, as well as the introduction of octreotide in therapy. Reduction of the LVAD flow from 2,700 to 2,500 rpm, enabled aortic valve opening with every heart cycle, thus improving pulsatility in the peripheral circulation. This measure is considered as the main approach in the treatment of GI bleeding after LVAD implantation across literature [7, 12].

Octreotide is a somatostatin analogue that causes splanchnic vasoconstriction, improves platelet aggregation and increases vascular resistance [13]. Through these mechanisms, as well as the inhibition of angiogenesis, this drug is capable of stabilizing GI bleeding. The application of octreotide for the GI bleeding in patients with LVAD has been reported to have a notable success [14]. A recent study by Al Bawardy et al. [4] demonstrated that conservative therapy including octreotide is in fact superior to balloon-assisted enteroscopy for the management of LVAD associated GI bleeding.

Additional steps in the medical treatment of GI bleeding in LVAD patients include the ones generally recommended for any form of acute GI bleeding [7, 13, 15]. In our case, they included proton pump inhibitor, fluid replacement, packed red blood cells transfusions and discontinuation of anticoagulation and antiplatelet medication.

Conclusion

It should be noted that acute GI bleeding represents a serious, life-threatening condition that can develop after LVAD implantation. With a comprehensive literature search for the bleeding management strategy in LVAD implanted patients, as well as a remarkable collaboration of the multidisciplinary team, the bleeding was successfully managed and the patient survived.

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CASE REPORT

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Wells' syndrome associated with eosinophilic granulomatosis with polyangiitis − A case report

Velsov sindrom udružen sa eozinofilnom granulomatozom sa poliangiitisom

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Military Medical Academy, *Department of Dermatology and Venereology, ‡Department of Rheumatology, Belgrade, Serbia; †University of Defence, Faculty of Medicine of the Military Medical Academy, Belgrade, Serbia

Abstract

Introduction. Wells` syndrome (eosinophilic cellulitis) is a chronic, recurrent disease characterized by episodes of erythematous and edematous plaques or nodules with occasional development of hemorrhagic bullae on the trunk and limbs. Eosinophilic granulomatosis with polyangiitis, formerly known as Churg-Strauss syndrome, is a specific variant of the group of diseases characterized by necrotizing vasculitis of small to medium-sized blood vessels affecting multiple organ systems. The association of Wells` syndrome and eosinophilic granulomatosis with polyangiitis is very rare, and to our knowledge has been reported in only ten patients. **Case report**. We present a case of a 34-year-old woman with a 3-year history of periodical onset of erythematous plaques on the trunk and edematous plaques clinically resembling cellulitis on her lower limbs. The patient reported a one-year history of asthma, rhinosinusitis, and nasal polyposis. Skin biopsy revealed the presence of diffuse eosinophilic infiltrates in the dermis accompanied by characteristic "flame figures". Further investigation showed peripheral blood eosinophilia (22.6%), bilateral maxillary sinusitis, presence of eosinophil infiltrates and microabscesses in the bronchial wall, and pericapillary eosinophil infiltrates in the pulmonary interstitium shown by bronchoscopy and transbronchial biopsy, respectively. Treatment was started with methylprednisolone 0.5 mg/kg/day, and the dose was gradually tapered for the following twelve weeks. Complete remission of skin changes was achieved, but new lesions appeared in the past two years, which required repeated treatment. **Conclusion.** Association of these syndromes is unusual and may be based on the common pathogenetic background. We hypothesize that Wells` syndrome could be a stage preceding eosinophilic granulomatosis with polyangiitis, and that patients should be evaluated for eosinophilic granulomatosis with polyangiitis, since these two diseases overlap in clinical and laboratory findings.

Key words:
biopsy; churg-strauss syndrome; diagnosis; eosinophilia; wells syndrome; therapeutics; treatment outcome.

Apstrakt

Uvod. Velsov sindom (eozinofilni celulitis) je hronično rekurentno oboljenje koje odlikuju epizode eritematoznih i edematoznih plakova ili nodusa, uz povremenu pojavu hemoragičnih bula na trupu i ekstremitetima. Eozinofilna granulomatoza sa poliangiitisom, u ranijoj terminologiji Čarg-Štrausov sindrom, je specifična varijanta grupe bolesti koje se karakterišu nekrotizirajućim vaskulitisom malih i srednjih krvnih sudova. Prema našem saznanju, udruženost Velsovog sindoma i eozinofilne granulomatoze sa poliangiitisom je retka, do sada opisana kod deset bolesnika. **Prikaz bolesnika.** Prikazana je bolesnica, stara 34 godine, sa trogodišnjom istorijom periodične pojave eritematoznih plakova na trupu i edematoznih plakova nalik celulitisu na donjim ekstremitetima. U ličnoj anamnezi navela je astmu u prethodnih godinu dana, rinosinuzitis i nazalnu polipozu. Biopsijom kože uočeno je prisustvo difuznog eozinofilnog infiltrata u dermu sa karakterističnim "plamenim figurama". Daljim pretragama evidentirana je eozinofilija u perifernoj krvi (22,6%), obostrani maksilarni sinuzitis, eozinofilni infiltrati i mikroapcesi u bronhijalnom zidu i perikapilarni eozinofilni infiltrati u intersticijumu pluća, uočeni brohoskopijom, kao i transbronhijalnom biopsijom. Lečenje je započeto metilprednizolonom 0,5 mg/kg/dan uz postepeno snižavanje doze narednih 12 nedelja. Postignuta je kompletna remisija promena na koži, uz ponovnu pojavu u poslednje dve godine, što je zahtevalo ponavljanje terapije. **Zaključak.** Udruženost ova dva sindroma je neuobičajena, sa mogućom zajedničkom patogenetskom osnovom. Pretpostavljamo da Velsov sindrom može biti prethodni stadijum eozinofilne granulomatoze sa poli

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angiitisom i mišljenja smo da bolesnike treba ispitati u smislu postojanja eozinofilne granulomatoze sa poliangiitisom s obzirom na to da ove bolesti mogu imati klinička i laboratorijska preklapanja.

Ključne reči:
biopsija; angiitis, alergijski, granulomatozni; dijagnoza; eozinofilija; vels sindrom; lečenje; lečenje, ishod.

Introduction

Wells' syndrome (WS) or eosinophilic cellulitis, described by Wells in 1971, is a rare inflammatory dermatosis with episodes of erythematous urticarial plaques [1]. It may also manifest itself as the development of vesicles and bullae or nodules and granulomatous eosinophilic infiltrates in the dermis. WS is characterized by benign disease course with lesions usually located on the head, trunk and limbs. Seven clinical variants have been described [2]. The classic plaque- type variant is the most common in children, while the granuloma-like variant is more common in adults. To date, approximately 200 cases of WS have been reported.

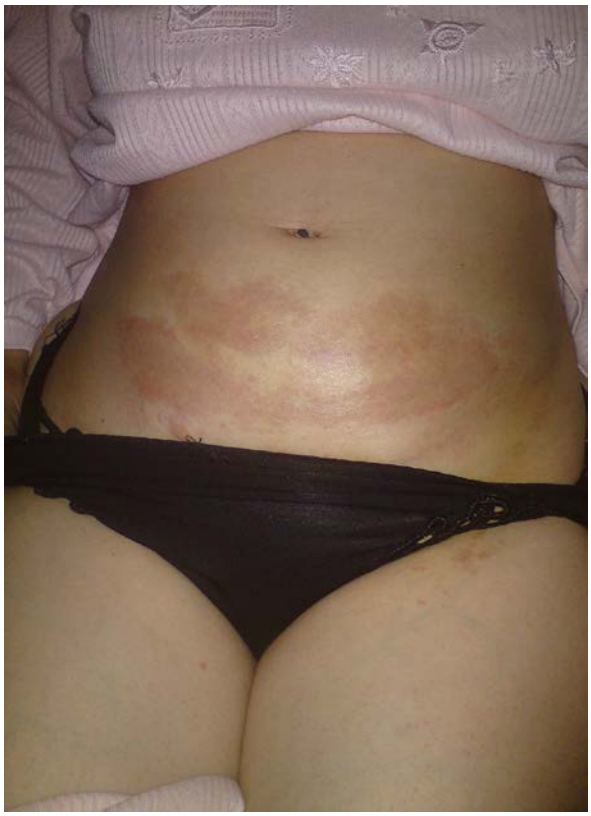
Eosinophilic granulomatosis with polyangiitis (EGPA), formerly known as Churg-Strauss syndrome (CSS), is a rare, systemic small to medium sized vasculitis associated with asthma and eosinophilia. The manifestation of the disease depends on the systems involved [3].

To our knowledge, the association between WS and EGPA has been reported in ten patients. These syndromes may have a common pathogenetic mechanism with hypersensitive reaction to the underlying cause including allergens, insect bites, infections, vaccinations, medications, and malignancy [4–12].

Case report

A 34-year-old woman with a 3-year history of periodical onset of erythematous plaques on the trunk and edematous plaques clinically resembling cellulitis on the lower limbs was admitted to our Department (Figure 1). At the time, the lesions were extremely pruritic and occasionally accompanied by burning sensation. Previously, the patient had been treated with antihistamines and occasionally with short-term administration of prednisone. However, she had had several exacerbations following each discontinuation of prednisone. The patient denied taking any additional medications, presence of fever, weight loss, or joint pain. She reported a one-year history of asthma, rhinosinusitis, and nasal polyposis. To treat asthma, she had been taking inhaled corticosteroids and beta-2-agonists.

Histopathologic examination of the skin lesions demonstrated abundant lymphocytic and eosinophilic infiltrates in the dermis, with characteristic eosinophilic staining in the form of "flame figures", which was consistent with the diagnosis of WS (Figure 2). Direct immunofluorescence microscopy did not reveal any deposits of immunoglobulin or complement in the skin biopsy.



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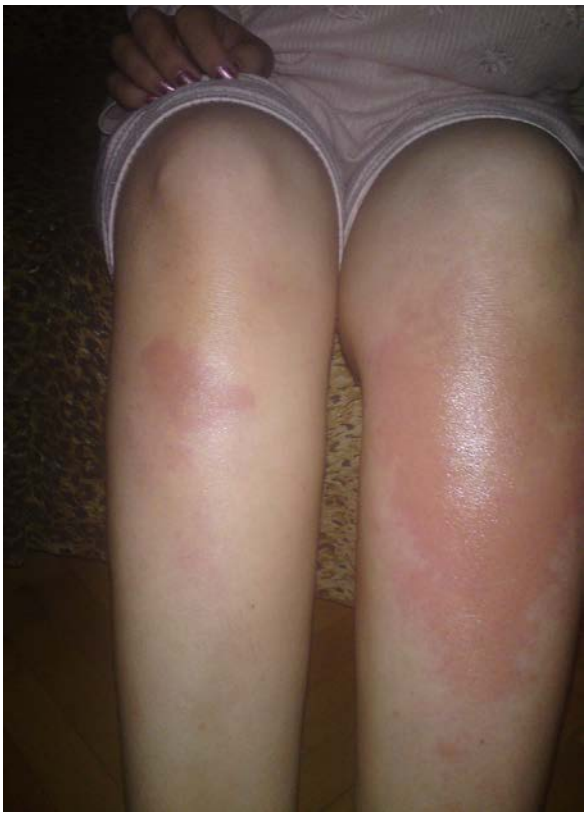
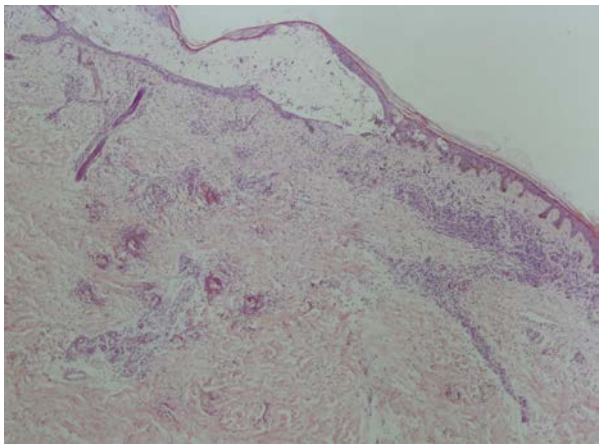


b)

Fig. 1 – a) Widespread erythematous plaques with sharp borders and central regression on the abdomen; b) Urticarial plaques on the lower extremities, clinically resembling cellulitis.



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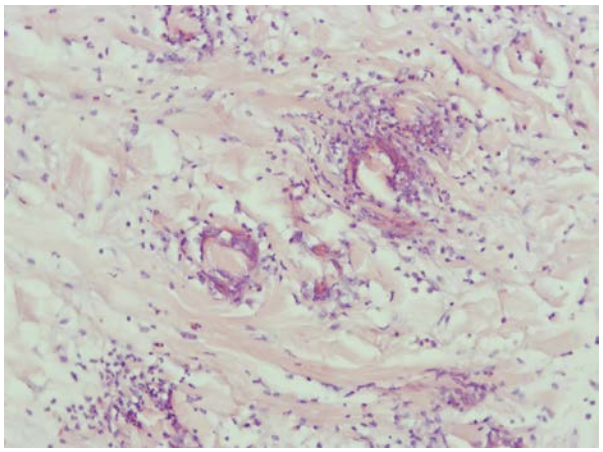

b)

Fig. 2 – a) Histology of the skin biopsy shows an intraepidermal vesicle in the spinous layer and abundant eosinophilic and lymphocytic infiltrates with degeneration of collagen surrounded by eosinophils in the dermis (hematoxylin and eosin, ×40); b) "Flame figures" with surrounding dense eosinophilic infiltration (hematoxylin and eosin, ×200).

Laboratory investigation showed eosinophilia (22.6%). Erythrocyte sedimentation rate, C-reactive protein, rheumatoid factor, liver and renal tests and immunoglobulin E (IgE) were within normal ranges. Cytoplasmic and perinuclear antineutrophil cytoplasmic antibody (c- and p-ANCA), antinuclear antibodies (ANA), ENA (extractable nuclear antigen) screening, cryoglobulins and immune complexes were negative. Enzyme-linked immunosorbent assay (ELISA) for *Toxocara*, *Toxoplasma*, *Echinococcus*, *Cysticercosis*, *Strongyloides*, anti HIV 1/2, HBsAg and HCV antibodies were negative. Other infective causes of eosinophilia were also ruled out: stool sample test for intestinal parasites, sputum for acid-resistant bacilli (ARB) direct examination and Löwenstein cultivation and bacterial swabs were negative. Prick tests with the standard battery of inhaled allergens were negative. Pulmonary function tests showed normal spirometry finding, transfer factor for carbon monoxide (70% normal range 50%–80%) and transfer coefficient (72%) were reduced; oxygen saturation was 97% (normal range ≥ 97%).

Radiological examination revealed bilateral maxillary sinusitis (Figure 3). The electromyoneurographic examination was performed and neuropathy was excluded. Peripheral blood smear and bone marrow biopsy ruled out a hematological disorder. Chest radiography and heart ultrasonography were normal. However, microscopic examination of bronchoalveolar lavage confirmed eosinophilia (63%). Histopathologic findings of bronchoscopic biopsy revealed fragments with abundant eosinophilic and lymphocytic infiltrates as well as the foci of eosinophilic microabscesses. Pericapillary eosinophilic infiltrates affecting the lung interstitial were observed with transbronchial biopsy which could be a histologic sign of vasculitis.

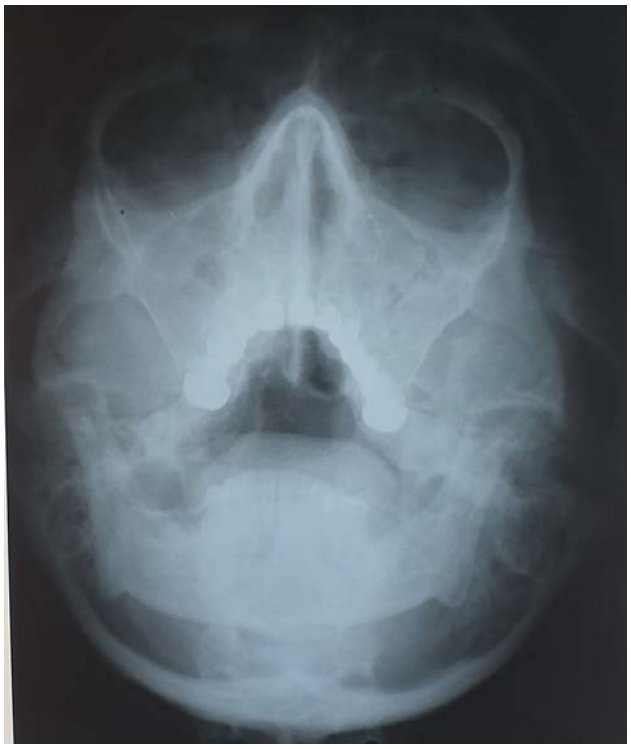

Fig. 3 – Radiological examination revealed bilateral maxillary sinusitis.

WS associated with EGPA was confirmed and the initial treatment with methylprednisolone was introduced at a dose of 0.5 mg/kg/day. For the following 12 weeks, the dose was gradually reduced to the maintenance dose of 5 mg every other day that was prescribed for the period of one year. The administered therapy was sufficient to manage the condition for the subsequent six years, after which the lesions appeared again and the treatment was reintroduced. Any attempt to discontinue the treatment led to reappearance of skin changes.

In the last two years skin lesions have reappeared, and methylprednisolone was reintroduced with tapering to 5 mg every other day, up until now. Nevertheless, any attempt to discontinue the drug led to reappearance of skin lesions that were mild compared to the initial presentation of the disease. Additional treatment with inhaled budesonide/formoterol and supplementation with calcium 1,000 mg/day and vitamin D at the dosage of 800 IU/day was prescribed.

Discussion

WS is a rare inflammatory dermatosis with spontaneous remission but frequent recurrence [1]. It is characterized by pruritic, urticarial plaques, vesicles, bullae or nodules during the acute phase, and indurated morphea-like lesions in later stages. Skin symptoms are accompanied with peripheral blood eosinophilia in almost half of the patients [1, 2, 4].

Indicative of this disease are the histopathologic "flame figures", which can also be observed in EGPA associated with WS, hypereosinophilic syndrome, cutaneous eosinophilic vasculitis, insect bite reactions, cutaneous parasitic infections, bullous pemphigoid and herpes gestationis [12, 13].

EGPA is a disorder that may affect multiple organ systems. It has been found that EGPA evolves through three phases. The prodromal phase, characterized by asthma and rhinosinusitis is followed by the eosinophilic phase with peripheral eosinophilia and eosinophil infiltrations in various organs, the lung, heart and gastrointestinal tract being most commonly affected. The third, vasculitic phase is marked by small vessel vasculitis, fever, fatigue, and improvement of asthma as a cardinal feature of this phase [3, 14]. Skin lesions are variable, appearing as palpable purpura, nodules, erythematous, maculopapular, and rarely as bullous lesions [15]. Although etiopathogenesis of the disease is still considered to be unclear, the infiltration by eosinophils and ANCA are likely the most important mechanisms [3]. WS and EGPA display involvement of abnormal Th2 cells, increased production of IL-5, and consequently, activated eosinophilic granulocytes driving nonspecific hypersensitivity response to exogenous or endogenous stimuli. The pathogenic T-helper cells present in skin lesions display memory Th cells phenotype (CD4+CD7-)[16], but it is still unclear what their activation mechanisms are. A correlation has been found between clinical parameters of EGPA disease activity (Birmingham Vasculitis Activity Score – BVAS, eosinophilia) and expression of IL4, IL5, IL10 and STAT5A [17]. Considering that myeloid cells, particularly dendritic cells (DC), are most potent Th polarizing cells which drive their differentiation towards Th2 or Th1717, it is of great importance to study the functions of DC from patients with WS and EGPA.

Patients with ANCA-positive EGPA more frequently had peripheral neuropathy, glomerulonephritis and palpable purpura due to small-vessel vasculitis [18, 19]. In contrast, patients with ANCA-negative EGPA experience erythematous plaques, urticarial lesions and eosinophilic infiltration of the lung, myocardial and gastrointestinal tissue, as in our case [3, 18, 19]. Since WS is characterized by eosinophilic infiltration without vasculitis, the authors point out that EGPA with WS would be ANCA-negative [20], which we confirmed, in contrast to three other reported cases [5, 8, 12]. This is the case of ANCA negative EGPA associated with WS.

The most commonly used criteria for diagnosing EGPA are defined by the American College of Rheumatology with 85% of sensitivity and 99.7% specificity [21]. The affected patients should meet four out of the following six criteria: asthma, eosinophilia of $>10\%$ in differential white blood cell counts, mononeuropathy or polyneuropathy, migratory or transient pulmonary infiltrates, paranasal sinus abnormalities on radiography, and extravascular eosinophil infiltration on biopsy findings [21]. In case of our patient, four criteria were met: the presence of eosinophilia, asthma, paranasal sinus abnormalities, and pericapillary eosinophilic infiltrates affecting the lung interstitium observed with transbronchial biopsy as the key histologic characteristic of vasculitis. The key histological characteristics of EGPA are eosinophilic tissue infiltration and/or vasculitis and/or extravascular eosinophilic granulomas. Mononeuropathy and migratory pulmonary infiltrates were not present.

In addition to the presence of distinct EGPA diagnostic criteria, the IgE levels were normal, ANCA and rheumatoid factor were negative, and immune complexes were undetectable, classifying the case of our patient in the group of ANCA-negative EGPA, which may be present in one third of the patients [3, 18, 19]. The association between these two diseases is unusual; Lee et al. [8] published a case where EGPA preceded WS, suggesting that EGPA may induce WS through the pathogenetic effect of eosinophils infiltration of the skin. In contrast, in our patient and other reported cases, WS was a prodromal manifestation and it developed after EGPA [11, 12]. Some authors also propose that WS should be considered in the differential diagnosis of patients with EGPA whose clinical presentation includes erythematous urticarial plaques [9].

These diseases share common features including blood and/or tissue eosinophilia, abnormal eosinophilic reactions to underlying factors, similar skin manifestations, and good therapeutic response to systemic corticosteroids [2, 3, 11]. The severity of the disease can be determined based on the presence of poor prognostic factors for EGPA according to French Vasculitis Study Group [22]. The presence of each factor is allocated 1 point. For patients with a score equal to or greater than 1, treatment with corticosteroids and cyclophosphamide is recommended. Our patient had no poor prognostic factors, no evidence of proteinuria, cardiomyopathy or involvement of the gastrointestinal tract or central nervous system, with normal values of serum creatinine; such cases are usually treated with glucocorticoids. Current treatment options for WS include topical and systemic corticosteroids, antihistamines, cyclosporine, azathioprine, griseofulvin, doxycycline, minocycline, antimalarials, oral tacrolimus/topical tacrolimus, sulfasalazine, interferon alpha and gamma, TNF-alpha inhibitors, colchicine and psoralen and ultraviolet A (PUVA) therapy [23]. Although multiple treatment modalities have been used with variable success rates, the first-line treatment option should be topical and/or systemic corticosteroids. Since long-term systemic corticosteroid therapy can have a wide range of side effects, careful monitoring and using appropriate preventive strategies may minimize them [24].

Regarding treatment, our patient had a favorable response to methylprednisolone, and remission was achieved and maintained for six years. However, due to relapse, additional treatment was necessary.

Conclusion

Association of these syndromes is unusual and may be based on the common pathogenetic background. We hypothesize that WS could be a prior stage of EGPA and that patients should be evaluated for EGPA since these two diseases overlap in clinical and laboratory findings. Corticosteroids are the first line of treatment and usually sufficient for patients who do not have severe organ involvement as in the case of our patient, where we achieved long-term remission. Additional immunosuppressive or biological agents may be necessary in cases of severe organ damage, treatment failure, and frequent relapses.

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CASE REPORT

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Development of Crohn´s disease in a patient with ankylosing spondylitis and essential thrombocythemia following etanercept therapy - A case report and the review of the literature

Pojava Kronove bolesti kod bolesnika sa ankilozirajućim spondilitisom i esencijalnom trombocitemijom tokom terapije etanerceptom

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Abstract

Introduction. The development of inflammatory bowel disease during the treatment with tumor necrosis factor-α inhibitors is seen in patients with ankylosing spondylitis. Crohn´s disease is the mainly developing form, and etanercept is the most frequently associated agent. Although thrombocytosis in patients with ankylosing spondylitis and inflammatory bowel diseases is often seen due to chronic inflammation, iron deficiency anemia or drug administration, presence of essential thrombocythemia is not common. To our knowledge, there is no published data of coexistence of these three diseases in one patient. **Case report.** We reported a 35-year-patient with simultaneous presentation of ankylosing spondylitis and essential thrombocythemia. Due to hepatotoxicity of initial treatment with sulfasalazine and metotrexate, tumor necrosis factor-α inhibitor (etanercept) was introduced. Both diseases were well controlled until Crohn´s disease emerged. Two years after switching from etanercept to adalimumab all three coexisting diseases were in remission. **Conclusion.** Treatment with tumor necrosis factor-α inhibitors significantly improved clinical outcome of patients with chronic inflammatory diseases. However, the appearance of adverse effects may cause a discontinuation or change of a drug. The existence of comorbidities additionally complicates the treatment of such patients.

Key words:
biological therapy; comorbidity; crohn`s disease; drug utilization; spondylitis, ankylosing; thrombocythemia, essential.

Apstrakt

Uvod. Tokom terapije inhibitorima faktora nekroze tumora alfa kod bolesnika sa ankilozirajućim spondilitisom može doći do nastanaka inflamatorne bolesti creva. Kronova bolest je najčešća forma, a etanercept je lek koji se najviše povezuje sa pojavom bolesti. Iako se trombocitoza često javlja kod bolesnika sa ankilozirajućim spondilitisom kao rezultat hronične inflamacije, sideropenijske anemije ili primene lekova, pojava esencijalne trombocitemije nije česta. Nema objavljenih radova o koegzistenciji ove tri bolesti kod jednog bolesnika. **Prikaz bolesnika.** Prikazali smo složen slučaj bolesnika starog 35 godina sa istovremenom pojavom ankilozirajućeg spondilitisa i esencijalne trombocitemije. S obzirom na hepatotoksičnost izazazvanu inicijalno uvedenim lekovima, sulfasalazinom i metotreksatom, započeto je lečenj etanerceptom, inhibotorom faktora nekroze tumora alfa. Obe bolesti su bile zadovoljavajuće kontrolisane sve dok nije dijagnostikovana Kronova bolest. Dve godine nakon zamene etanercepta adalimumabom, sve tri bolesti su bile u remisiji. **Zaključak.** Terapija inhibitorom faktora nekroze tumora alfa je značajno poboljšala klinički ishod lečenja bolesnika sa hroničnim inflamatornim bolestima. Ipak, pojava neželjenih efekata može usloviti prekid ili promenu leka. Prisustvo komorbiditeta dodatno komplikuje terapijski pristup tim bolesnicima.

Ključne reči:
biološka terapija; komorbiditet; kronova bolest; lekovi, korišćenje; spondilitis, ankilozirajući; trombocitemija, esencijalna.

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Introduction

Ankylosing spondylitis (AS), which is the most frequently occurring form of spondyloarthritis (SpA), is a chronic immunomediated inflammatory disease characterized by inflammation that predominantly affects the axial skeleton [1]. Inflammatory bowel diseases (IBD) [Crohn´s disease (CD) and ulcerative colitis (UC)] are the most frequent extra-articular manifestations of AS. Although the most significant genetic association for SpA is with the genes related to the MHC (HLA-B27), several polymorphisms outside the MHC were identified, including IL-23R, PSMG1, ERAP1/2 and TNFSF15 which are also established IBD loci [2]. The discovery of several inflammatory pathways in both AS and IBD led to the era of the biologic therapies, which meant a revolution in their treatment and prognosis. All tumor necrosis factor α inhibitors (TNF-α inhibitors) are efficacious in treating AS, but there are differences regarding IBD. Monoclonal antibodies [infliximab (INF), adalimumab (ADA), certolizumab-pegol (CPG), golimumab (GOL)] are efficacious in the treatment of IBD whereas etanercept (ETA) is not [3]. Paradoxal adverse events (PAEs) refer to the occurrence of pathological condition opposite to the effect which would normally be expected. The development of IBD during the treatment with TNF-α inhibitors is seen in patients with AS. CD is the mainly developing form of IBD, and ETA is the most frequently associated agent. Paradoxal IBD is generally well controlled by the interruption of the damaging by TNF-α inhibitor and switching to the monoclonal antibodies.

Essential trombocythemia (ET) is a Philadelphia chromosome (Ph)-negative myeloproliferative neoplasm (MPN) characterized by thrombocytosis and megakaryocytic hyperplasia of the bone marrow, with the presence of Janus kinase 2 valin 617 phenylalanine (JAK2V617F) mutation in 50%–60% of patients. ET can transform into myelofibrosis and acute myeloide leukemia in the minority of cases and, in general, life expectancy is considered not far from that of healthy population [4]. HLA-B27 has been suggested to be important in the pathogenesis of AS, furthermore; HLA-B27 seems to also raise the risk of hematological malignancies, notably myelodysplastic syndrome (MDS), acute leukemia and lymphoid malignancies but not Ph-negative MPN [5].

Case report

We reported a 35-year-old man presented with a 10-year history of morning pain and stiffness in the low back, buttocks and hips. He was positive for human leukocyte antigen (HLA-B27) and plain radiography showed bilateral sacroilitis. The patient was diagnosed with AS in 2005 according to the modified New York criteria [6]. He started with nonsteroidal anti-inflammatory drugs (NSAIDs), but after several months sulfasalazine (SSZ) 2 g/day was introduced due to right knee arthritis. Only after methotrexate (MTX) 17.5 mg/week was administered, the patient started to feel better.

Even before the patients was diagnosed with AS, in 2002, he was examined due to the symptoms of erythomelalgia and thrombocytosis. After bone marrow biopsy preliminary diagnosis of ET was made. The patient was advised to take antiplatelet drug and to undergo further evaluation. However, he decided to visit hematologist four years later, when erythomelalgia symptoms got worse. Further analysis proved JAK2V61F7 mutation, breakpoint cluster region – Abelson (BCR/ABL) rearrangement was negative and cytogenetic analyses were normal. Bone marrow aspiration and repeated bone marrow biopsies showed hypercellularity with dominant megakaryocytic hyperplasia. After the definite diagnosis of ET was made (November 2006), he started taking aspirin, but when anagrelide was introduced his platelet (PLT) count was below 1,000 cells/mm³ (normal range is 140,000–440,000 cells/min^3). At that time, the patient was taking SSZ and MTX for AS and the disease activity was mild [Bath Ankylosing Spondylitis Disease Activity Index (BASDAI) score was 3.7].

In July 2008, laboratory findings indicated hepatotoxicity, and the Roussel Uclaf Causality Assessment Method (RUCAM) [7] for estimation of drug-induced liver injury was performed for all medications that the patient was taking. MTX was assigned the highest RUCAM score at 6 (the probable cause of liver injury), SSA was 3 (possible cause) and anagrelide was 1 (unlikely cause). According to RUCAM scoring results, SSZ and MTX were suspended. Soon, his back pain worsened, BASDAI was 6.7 and MRI pointed at active sacroiliitis. His ET was satisfactory controlled (PLT below 500 cells/mm³). Regarding all of the above, we decided to induce TNF-α inhibitor in the therapy of AS and in August 2009 ETA was initiated sc with the dose of 50 mg/weekly. After four months of ETA therapy, the patient was much better, his BASDAI score dropped to 2.4, C-reactive protein (CRP) was 4.9 and his liver enzymes were in normal range. Anagrelide was terminated in November 2009 and aspirin was reintroduced. For the next six years the patient was stable, taking only ETA and aspirin.

In May 2016, the patient reported cramping abdominal and anal pain associated with diarrhea (4–5 movements/day) and fever. The patient's laboratory findings were as follows: CRP 41 mg/L (normal range < 3 mg/L), white blood cells count (WBC) 16.97 cells/mm³ (normal range 4,500–10,000 cells/mm^3 hemoglobin (Hb) 142 g/L (normal range 138–142 g/L), PLT 982 cells/mm³. ETA was discontinued. The patient was referred to a gastroenterologist and colonoscopy was performed. Colonoscopy and histological finding showed changes consistent with CD with perianal fistula. Pelvic magnetic resonance imaging (MRI) showed complex intersphincteric perianal fistulas with abscess. Abscess drainage and seton placement were performed with the use of antibiotics (ciprofloxacin, metronidazole) followed by prednisolone 40 mg/day. After three months, antibiotics were discontinued and the patient was on steroid tapering regimen. He was in clinical remission without fistula draining. But, in September 2016, his back pain returned, BASDAI was 6.2 and Ankylosing Spondylitis Disease Activity Score (ASDAS) 3.8, inflammatory markers were elevated, and colonoscopy revealed no flare of CD. In November 2016, second TNF-α inhibitor, ADA 40 mg every two weeks was

started. After only two months of ADA treatment, the patient started to feel better and the inflammation declined. The patient is currently taking only ADA and aspirin. He has no gastroenterological or musculoskeletal signs that implicate active disease. Laboratory findings from May 2019 were as follows: PLT 992 cells/mm³, hemoglobin 139 g/L, leukocytes 7.6 cells/mm³, CRP 2.3 mg/L.

Discussion

The association of AS with IBD has already been described and subclinical gut lesions resembling CD seen in up to 50% of patients, up to 10% which developed clinically overt IBD with time [8]. Data from the IBSEN study reported the prevalence of AS in IBD to be 3.7% (2.6% in UC; 6% in CD), compared to about 1% in the general population [9].

All available TNF-α inhibitors are similarly efficacious in the treatment of AS, whereas monoclonal antibodies are efficacious in the treatment of IBD and ETA is not [3]. Furthermore, ETA is the main TNF-α inhibitor associated with paradoxal IBD, predominantly CD. In order to analyze the incidence of flares and new onset of IBD in patients with AS treated with anti-TNF agents, Braun et al. [10] analyzed data from 9 separate trials. A history of IBD was reported in 76 (6.7%) out of 1,130 patients. The relative risk for flare of IBD or development of a new-onset IBD during ETN treatment was determined as 18 times higher compared to INF therapy, but with no significant difference for the placebo group [10]. O'Toole et al. [11] searched for cases of IBD provoked by ETA from an IBD Referral Center and Food and Drug Administration (FDA) in period between 1998 and 2014. A total of 443 cases (297 CD, 146 UC) were identified and data of 49 patients (44 CD, 5 UC) were complete. Number of AS patients who developed IBD following ETA treatment was 14 (11CD, 3UC). French series described 14 patients with AS and new-onset IBD under TNF-α inhibitor treatment (10 cases with ETA, 2 with INF). Most of the patients had CD and Crohn's-like disease (1 case with unclassified colitis), and all patients were successfully treated by switching the TNF-α inhibitor to INF or ADA [12]. A recent publication analyzing all adverse events regarding TNF-α inhibitors reported to the FDA described 158 cases of new-onset IBD, most of them involved ETA (105 cases) [13]. Paradoxal IBD is generally well controlled by the interruption of the damaging TNF-α inhibitor and switching to a monoclonal antibody. The mechanism underlying paradoxical events developed during ETA treatment remain unknown. A potential pathophysiological hypothesis might be that, in predisposed patients having certain genetic factors, the introduction of TNF-α inhibitor and notably ETA, modify the cytokine balance and lead to the circumstances for development of IBD. Apoptosis is an important cellular process involved in CD remission. Anti TNF-α monoclonal antibodies can induce apoptosis of peripheral blood cells and *lamina propria* T cells but not ETA [14]. In addition, ETA only partially respects the production of TNF-α and may induce the production of interferon-γ (IFN-γ), favoring the inflammation in the bowel mucosa and granuloma formation, while anti–TNF-α monoclonal antibodies inhibit IFN-γ release [12].

It stays unclear if development of the CD in our patient was paradoxal effect of ETA or mere occurrence of rather common extra-articular manifestation of AS, regarding the fact that ETA is not efficient in IBD. However, after ETA was discontinued from the therapy and ADA was introduced, successful control of both diseases was accomplished.

Although thrombocytosis in AS and IBD patients is often seen due to chronic inflammation, iron deficiency anemia or drug administration, developing of ET or other Ph-negative MPN is not a common condition. We have found only four reports on the association between AS and Ph-negative MPN, three of them emerge after TNF-α inhibitor was introduced. Caramaschi et al. [15] report a case of a 62-year-old Italian with AS and bone involvement due to polycytemia rubra vera (JAK2V617 positive). The case of a 69-year-old men with AS and ET who was treated with ETA and hydroxyurea and developed mantle cell lymphoma has been described [16]. Finally, the cases of a 34-year-old Korean man who developed ET following adalimumab therapy and a 31-year-old Italian who was treated with infliximab and developed PRV, both of whom shared similar genetic background (HLA-B27-positive, JAK2V617-negative), have been described [17, 18]. Our patient was diagnosed with ET years before ETA was introduced, so there was no association between the occurrence of the disease and TNF-α inhibitor therapy.

Today, 17 years after TNF-α inhibitors were approved for the use in AS, data from the real-world national registries demonstrated no increased risk of overall malignancies compared to both general population and patients with AS without TNF-α inhibitor treatment [19]. The risk we accepted introducing TNF-α inhibitors in the treatment of ET was significant, regarding the fact that there were little data about adverse effects back in 2010. Our patient was carefully observed by a hematologist, and there was no sign of ET transformation. On the contrary, ET was in remission.

To our knowledge, there is no literature reporting association between AS, ET and CD. Although the coexistence of these diseases in our patient is probably a pure coincidence, there is a possible bond. Janus kinase (JAK)/signal transducers and activators of transcription (STAT) pathway appear to have a pivotal role in the pathogenesis of many immune-mediated diseases by facilitating the signal transduction of many different cytokines and other molecules [20]. Evidence suggests that the inhibition of JAK-mediated pathways may be a promising approach for the treatment of patients with both CD and AS. The currently marketed drugs, tofacitinib (JAK1/3 inhibitor) and baricitinib (JAK1/2 inhibitor), show efficacy and acceptable safety in rheumatoid arthritis, UC and psoriatic arthritis, and there are encouraging results in the clinical trial of tofacitinib in AS and SpA [21]. The new selective JAK1 inhibitors that are close for FDA approval for both AS and CD are upadacitinib and filgotinib [22]. On the other hand, JAK2 is crucial for signal transduction downstream of the erythropoietin, thrombopoetin, and related receptors that control erythrocyte and megakar-

yocyte expansion. Following the discovery of JAK2V617F in 2005 as the driver mutation of the majority of Ph-negative MPNs, quest for JAK2 inhibitor began. So far, only one JAK2/JAK1 inhibitor (ruxolitinib) has been approved by the FDA in the treatment of intermediate to high-risk MP and hydroxyurea-resistant or intolerant PV. As for ET, the MAJIC trial showed the lack of superiority of ruxolitinib compared to current second-line therapies for these patients [23].

Conclusion

The recent investigations and studies have improved the understanding of the pathogenesis of chronic inflammatory diseases like AS and CD, and also facilitated the development of new treatment strategies. The existence of comorbidities additionally complicates the treatment of such patients. Therefore, an individual approach is essential for every physician.

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CASE REPORT

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Schwannoma of the abdominal wall – diagnostic challenge

Švanom u abdominalnom zidu – dijagnostički izazov

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Abstract

Introduction. Schwannoma or neurilemmoma is a benign tumor of the nerve sheath originating from the Schwann cells. Localization in abdominal wall is rare. Schwannomas usually manifest themselves as slow-growing asymptomatic tumors. Symptomatology depends on the location, the involved nerve and the size of the tumor. **Case report.** We present a 43-year-old female patient with a schwannoma localized in the right hypochondriac region. Diagnostic procedures included ultrasound with color Doppler (US), high-resolution magnetic resonance imaging (MRI), and histopathological examinations (HP). US showed a solid, well-circumscribed mass, with a whorled, echogenic internal architecture in the anterior abdominal wall. MRI revealed an oval, well-circumscribed, heterogeneous, 3 × 2.5 × 2.5 centimeters large fusiform mass. T1-weighted imaging presented low signal intensity, while T2-weighted image showed heterogeneously high signal intensity. The lesion was completely removed. After pathohistological analysis with standard and immunohistochemical methods of coloring, the diagnosis of the schwannoma was confirmed. **Conclusion.** Schwannoma as a slow-growing tumor, which is often without clinical manifestations, may cause a delay in diagnosis and treatment. Clinical presentation of a schwannoma is indolent and non-specific. Diagnosis of this tumor requires a multidisciplinary and interdisciplinary approach. MR is a useful and highly specific method for the verification of peripheral nerve sheath tumors. A HP analysis confirmed definitive diagnosis of the lesion.

Key words:
schwannoma; abdominal wall; diagnostic techniques and procedures; surgery; histology.

Apstrakt

Uvod. Švanom ili neurilemom je benigni tumor nervnog omotača koji potiče od Švanovih ćelija. Lokalizacija u abdominalnom zidu je retka. Švanomi se obično manifestuju kao sporo rastući asimptomatski tumori. Simptomatologija zavisi od lokacije, zahvaćenog nerva i veličine tumora. **Prikaz bolesnika.** Predstavljamo 43-godišnju bolesnicu sa švanomom lokalizovanim u desnom hipohondrijskom regionu. Dijagnostika je obuhvatala ultrazvuk sa kolor Doplerom (UZ), magnetnu rezonancu (MR) visoke rezolucije i patohistološka ispitivanja (PH). Ultrazvuk je pokazao čvrstu dobro ograničenu masu, ehogene unutrašnjosti u prednjem abdominalnom zidu. Magnetnom rezonancom ova promena je predstavljena kao ovalna, dobro orijentisana, heterogena, fusiformna masa, dijametra 3 × 2.5 × 2.5 centimetara. Na T1 sekvenci promena je imala nizak intenzitet signala, dok je na T2 sekvenci bila heterogeno visokog intenziteta signala. Lezija je u potpunosti uklonjena. Patohistološkom analizom, standardnim i imunohistohemijskim metodama bojenja, postavljena je dijagnoza švanoma. **Zaključak.** Švanom kao spororastući tumor, koji je često bez kliničkih simptoma, može ostati dugo nedijagnostikovan. Klinička prezentacija švanoma je blaga i nespecifična. Dijagnoza ovog tumora zahteva multidisciplinarni i interdisciplinarni pristup. Magnetna rezonanca je korisna i visokospecifična metoda za verifikaciju tumora perifernih nerva. Patohistološka analiza potvrdila je definitivnu dijagnozu lezije.

Ključne reči:
švanom; abdomen, zid; dijagnostičke tehnike i procedure; hirurgija; histologija.

Introduction

Schwannoma or neurilemmoma is a benign, slow-growing, encapsulated tumor of the nerve sheaths, which arises from the Schwann cells of the peripheral, cranial and autonomic nerves. Schwannoma can push the nerve laterally but without infiltrative potential [1]. Most schwannomas are solitary. Multiple schwannomas are usually associated with

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neurofibromatosis type 1 (von Recklinghausen disease) [2]. Depending on the location, involved nerve and the size of the tumor, schwannomas can give different symptomatology. Schwannomas usually manifest themselves as slow-growing tumors, which can exist from months to years without clinical manifestation. Malignant schwannomas are very rare and account for approximately 6% of all sarcomas [3–5].

The aim of this case report was to present the patient with the localization of the schwannoma in the abdominal wall. Discussion about diagnosis and therapeutic approach to these tumors will largely contribute to more efficient clinical management.

Case report

A 43-year-old female patient with a localized, painful mass in the abdominal wall of the right hypochondriac region was admitted for evaluation and surgical treatment. Tumor mass slowly enlarged over a period of 2 years, although the patient complained about symptomatology only two months before examination. There was no history evidence of weight loss, fever, anorexia, stress or trauma, and no family history of the similar symptomatology. On physical examination, in the right hypochondriac region there was present a 2–3 cm solid mass, oval-shaped, painful on the palpation and not fixed to the skin of the abdominal wall. Laboratory tests were in the range of normal values. After clinical examination, the first diagnostic procedure was high-resolution ultrasound performed with Linear Array 3–13 MHz on Siemens Acuson Antares ultrasound machine, which showed the solid heterogeneous mass, well circumscribed, with a whorled, echogenic internal architecture in the anterior abdominal wall. Color Doppler sonography showed no appreciable vascularity.

Magnetic resonance imaging (MRI) was performed on machine GE Signa 3.0T, in T2 SS (T2 Single Shot), T2SSFSE (T2 Single Shot Fast Spin Echo), T1 Dual, DWI 50 (Diffusion-Weighted Imaging), DWI 500 (Diffusion-Weighted Imaging), T2FRFSE (Fast Recovery Fast Spin Echo), FRFSE (Fast Recovery Fast Spin Echo) with fat suppression and dynamic T1 (LAVA) Liver acquisition with Volume acquisition images, in all three projections. A mass was revealed under the muscular layers of the right side and lateral at the height of the 6th segment of the liver. The revealed structure was oval, well-circumscribed, heterogeneous, fusiform 3 × 2.5 × 2.5 centimeters in diameter. Mass compressed the liver, without the signs of infiltration. In the surrounding muscle structures there was no sign of edema. After the application of contrast agent, there was a clear demarcation of the capsule. The central part of the lesion also showed intensive but heterogeneous post-contrast opacification.

On T2-weighted images this lesion was peripherally hyperintense and centrally with heterogeneous low and intermediate signal intensity (Figures 1–3). Unenhanced T1-weighted LAVA sequence showed lower signal intensity compared to the signal intensity of the surrounding muscles (Figure 4) and after contrast administration on T1-weighted LAVA sequence, the mass showed intense enhancement (Figure 5).

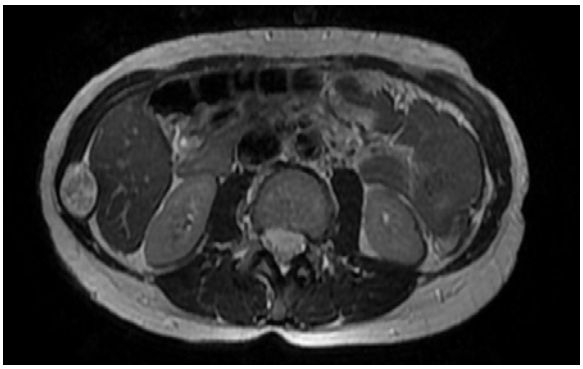

Fig. 1 – T2 axial, clearly demarked incapsulated lesion, peripherally with high signal intensity.

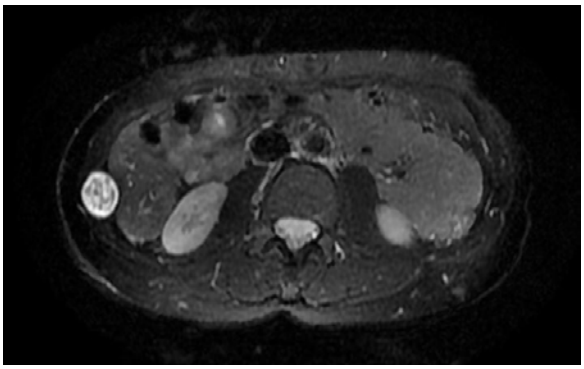

Fig. 2 – T2 fat sat axial lesion, still peripherally hyperintense, probably due to cystic degeneration.

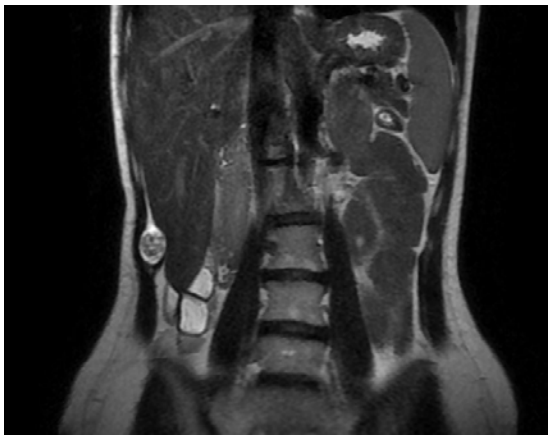

Fig. 3 – T2 core, central part is with "patchy" zones of signal hypointensity.

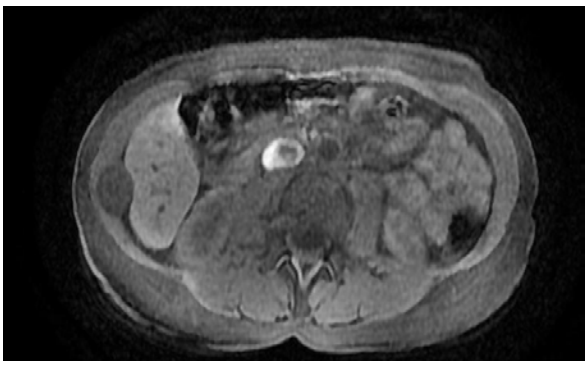

Fig. 4 – T1* LAVA native axial, lesion is hypointense.
*** LAVA – liver acquisition with volume acquisition.**

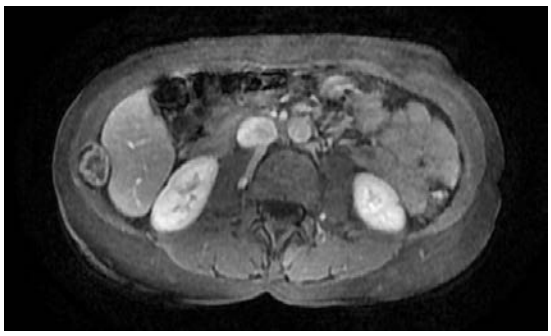

Fig. 5 – T1* LAVA +C axial, there is a smooth rim-like enhancement with central heterogeneous postcontrast enhancement.
*** LAVA – liver acquisition with volume acquisition.**

After all examinations, tumor excision was performed. Abdominal oblique muscles were separated and isolated. The lesion was identified between the right hypochondriac and lumbal region near costal cartilage and intercostal spaces. The lesion was 3 cm large, of ellipsoid shape and solid consistency. It was completely resected together with a capsule and sent for histopathological analysis.

Macroscopically, cystic formation had dimensions 30 × 20 × 10 mm, shiny smooth surfaces, and a narrow pedicel part, dimensions 10 × 1 mm. After cutting, there was a whitish yellow tissue, with partially pseudocystic appearance on the cross-section of the tissue.

Microscopically, the lesion was composed of an area of spindle cells with oval, extruded nuclei and palisading. Histiocytes, mast cells, collagen fibers were also present. Small number of blood vessels had a thickened wall surrounded by a myxomatous extracellular matrix (Figures 6–9).

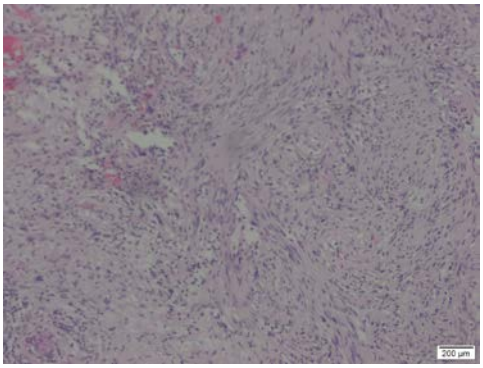

Fig. 6 – Schwannoma (H&E, ×20).

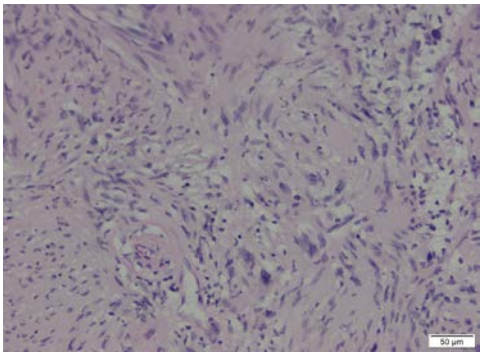

Fig. 7 – Schwannoma (H&E, ×40).

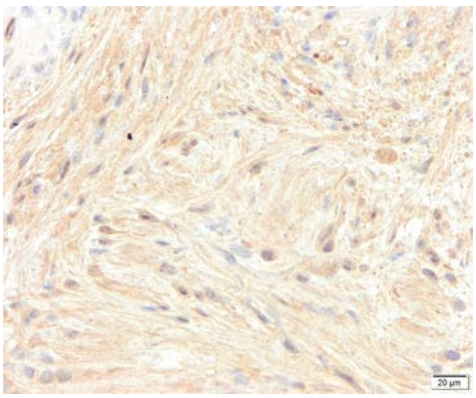

Fig. 8 – Immunohistochemical positivity (S100, ×40).

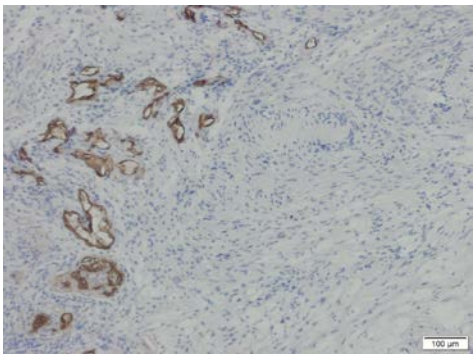

Fig. 9 – Pan Actin positivity only in the blood vessels without immunoreactivity in the Schwann cells (H&E, ×25).

On the histological examination, we found that the tumor cells were positive for S100 protein. The final diagnosis was a schwannoma. The postoperative process was regular.

Discussion

We can conclude the literature review by stating that only few cases of abdominal wall schwannomas were described. Schwannomas are the most common tumors of peripheral nerves with incidence 5% of all benign soft-tissue tumors. Schwannomas affect patients between the ages of 20–50, and with moderate predilection in females. It is commonly associated with neurofibromatosis type 2.

The most common localizations of schwannomas are retroperitoneum (32%), mediastinum (23%), head and neck (18%), and extremities (16%) [5]. There have been reports of their location in the *porta hepatis*, retroperitoneum, pelvis, adrenals, kidneys, vagina and a few in the abdominal wall [4]. Depending on the location, the involved nerve and the size of the tumor, schwannomas can present different symptomatology.

In the majority of cases, schwannomas arise from the nerve sheath of large peripheral nerves, usually asymptomatic and accidentally identified through physical examination or imaging. However, when they grow larger, they usually manifest the symptoms of the compression of the involved nerve [2, 4].

There are two groups of benign peripheral nerve sheath tumors that are usually present as a solitary lesion: schwan-

noma or neurilemmoma and neurofibroma. Schwannomas are encapsulated tumors. They are separated from the nerve with fibrotic capsule, unlike neurofibroma where the nerve is a part of a tumor and must be removed together with the tumor [5, 6]. Ancient schwannomas are a subtype of classic schwannomas and they usually display cellular degenerative changes, including nuclear atypia and pleomorphism, with a tendency to nuclear palisading. Histopathologically, they contain areas of relatively dense cellularity corresponding to Antoni A (AA) regions as well as loose, myxoid Antoni B (AB) regions [7, 8]. Immunohistochemical staining shows that schwannomas strongly react with S100 protein and can be used to differentiate them from malignant peripheral nerve sheath tumors [7–10].

Schvannomas have mostly uniform MR presentation which is T2 high signal intensity with centrally present zones of low and intermediate signal intensity that correspond to central fibrous components and peripheral myxomatous elements seen at pathologic analysis. On spin-echo T1-weighted MR images, the lesion is homogeneous and isointense relative to skeletal muscle [3].

There are certain imaging characteristics that may aid the radiologist in establishing a preoperative diagnosis of a peripheral nerve sheath tumor. These characteristics include association with a peripheral nerve, intermuscular location, and mostly specific MR image [3, 11, 12].

The opposite of schwannomas, malignant peripheral nerve sheath tumors as sarcomas have no specific imaging features, but aggressive biologic behavior may be suggested by indistinct margins, the infiltrative nature of the lesion within the nerve and adjacent structures. In many cases imaging characteristics help identify the neurogenic origin of a mass, but these patterns of signal intensity are neither specific for neural tumors, nor they allow differentiation between benign and malignant nerve sheath tumors. Ideally, at MR imaging we could make difference between a schwannoma and a neurofibroma [3].

The treatment is complete surgical excision, and prognosis is generally good. Incomplete excision can lead to recurrence of the tumor *in situ* or at a distant site after resection [4]. Our patient had good postoperative rehabilitation. We recommended clinical monitoring over a period of one year with shorter intervals between examinations; and during the second year with longer intervals between examinations. It included a review of the surgeon and radiological treatment. During the follow-up there was no evidence of recurrence found on MR images.

Conclusion

Schwannoma as a slow-growing tumor and without clinical manifestations may cause a delay in diagnosis and treatment. Clinical presentation of a schwannoma is indolent and non-specific. Diagnosis of this tumor requires a multidisciplinary approach. MR is a useful method for the verification of peripheral nerve sheath tumors with high sensitivity and specificity. Histopathological analysis confirmed definitive diagnosis of the observed lesion.

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Health care of convicts in penal institutions in the Principality and the Kingdom of Serbia

Zdravstvena zaštita osuđenika u kaznenim zavodima Kneževine i Kraljevine Srbije

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Key words:
disease; epidemics; history of medicine; hospitals; legislation; serbia; therapeutics.

Ključne reči:
bolest; epidemije; istorija medicine; bolnice; zakonodavstvo; srbija; lečenje.

Introduction

Expanding the area of application of the penalty of deprivation of freedom, initially by court practice and then based on the law, resulted in its domination within the penal system of Serbia in the second half of the 19th century. Competent state authorities awared that prison population, as a particularly endangered population in terms of health, represent a great risk to the health of the remaining population at the same time, and made efforts to carry out the health care measures of convicts within the reform of penitentiary system. This is why the medical treatment of convicts became the subject-matter of legal regulations very early. The concept of health care was not defined by the regulations (Law on establishing the national medical fund in 1879 and the Law on regulation of medical profession and protection of the national health in 1881) [1], only the medical bodies and institutions as well as their respective duties were determined. The main tasks were to deter the population from quackery by providing medical treatment according to the rules of "scientific medicine" and to overcome the problems occurring due to medical assistants impersonating medical doctors or masters of surgery, who in the lack of real medical doctors might have been hired as physicians [2].

In the Principality/Kingdom of Serbia, the penalty of deprivation of freedom was served in three penal institutions: in the Belgrade penitentiary with Topčider prison (1851) [3], in the Požarevac penitentiary (1865) [4] and in the Niš penitentiary (1878) [5]. The rules on distribution of convicts depending on gender, type of penalty and territory in these three institutions were changed several times. During the longest period of time, it was in Belgrade and Niš that the adult male convicts served their penalties of imprisonment and hard labor, and it was in Požarevac where their respective sentences were served by adult males convicted to imprisonment, women convicted to imprisonment and hard labor as well as juveniles.

Trying to organize that serving the penalty of deprivation of liberty was based on modern penological principles, the Defenders of the Constitution legally established the health care system for the convicts. First the title of the medical doctor was established in 1853, in the Belgrade penitentiary which housed the highest number of convicts [6]. After the Law on building and organization of hospitals (state, county, district, municipal and private) had been adopted (in 1865) [7], a shift was made also in legal regulation of health care of the convicts. The Minister of Justice Đorđe Cenić prescribed the Rules on domestic order of the penal institution in Požarevac (1868) [8], the Rules according to which the hospital of Belgrade penitentiary should be run (1869) [9], and which were also applied in all penitentiaries, and the Law on release of convicts on parole [10]. The rights and duties of penitentiary doctors were defined by the Law on penitentiary doctors (of 1883) [11].

The health care of convicts, in addition to the usual medical treatments of the sick people, which the doctors in infirmaries and hospitals of penal institutions were in charge of, included the preventive measures: mandatory physical examination before coming into the penitentiary aimed at early detection of a disease, vaccination and revaccination. Particularly bad living conditions had additional aggravating influence on the convicts' health. Insufficient and inadequate housing capacities for serving the sentence of deprivation of liberty for a longer period of time made the "prison issue" the burning penological issue.

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The penitentiary doctors made efforts through the Ministry of Justice to get the measures necessary to care for the convicts' health standardized, but their implementation was limited by the policy of government austerity measures. Creation of adequate living conditions and provision of medical supplies was connected with considerable financial costs, which resulted in forced decisions.

Penitentiary doctors

Health care of convicts was carried out in penitentiary infirmaries and hospitals, and also in county and town hospitals when required. The first significant step in the organization of health care of convicts was to establish the title of a special doctor who was in charge of monitoring the convicts and their hospital in the Topčider penitentiary (1853), at the time when only nine county towns in Serbia had their respective hospitals [6]. The largest number of convicts served their sentence in the Belgrade penitentiary which consisted of 16 dungeons in Belgrade and one building in Topčider [12]. The fact that the Belgrade penitentiary had its permanent doctor even before the Belgrade town hospital, where the town physicians treated patients until the appointment of Jovan Valenta for the head of the hospital in 1865, speaks about the awareness of the authorities that the prison population was a particularly risky population in terms of health. Continuous presence of a doctor, however, could not be provided in all penitentiaries due to the insufficient number of doctors in Serbia. Only the Belgrade penitentiary had its permanent doctor, while in other penitentiaries part-time doctors took care of the convicts' health.

According to the Rules (1869) [9], the duties of penitentiary doctors were: to have a detailed physical examination of the convicts when they are admitted to the penitentiary, to visit convicts regularly twice a day every day and additionally at any time of the day, particularly at times of epidemic, to treat sick convicts, to control the nutrition of the sick convicts (quality of food, dishes, water, milk and other drinks), to train nurses to take care of the sick, to control the work of hospital staff and hospital commissioner, to control implementation of protocols and statistical examinations of the sick, cured and dead convicts, the control of medicines used for medical treatment, food, bandages and medical supplies, to take care of the organization and maintenance of the hospital within the approved budget, to make and give regular medical reports to the Ministry of Justice (monthly and annual reports) on movement of the sick convicts and their health condition, to implement preventive measures in order to prevent spreading of infectious diseases, to take care of convict immunization and keeping records on vaccinated and revaccinated convicts, to prescribe doctor's orders on the diet and taking medicines by the sick convicts, to take care of serious injuries and training of nurses to take care of small injuries. The penitentiary doctor was also a member of the Consulting Committee of the penitentiary, who decided on the classification of the convicts into certain classes and made suggestions for their parole.

The penitentiary doctors, according to the 1883 Law on Penitentiary Doctors were appointed by the King's Executive Order at the proposal of the Minister of Justice [11]. The Law on Organization of Medical Profession and Preserving the National Health (1881) [2] prescribed that the penitentiary doctors had to be the doctors for the whole medicine or doctors of medicine and surgery, making them equal in terms of their rights, duties and salaries with county physicians.

It was the Belgrade Penitentiary that got its first doctor. In 1854 the master surgeon Jovan Siber was appointed a contractual doctor at the Topčider farm. Siber took care of the health of the convicts and the students of the Agricultural School at the same time, up until 1856. A county doctor and surgeon Josif Vardian was a county doctor twice (1856–1857, 1859–1860), while in the meantime this duty was performed by Bernhardt Kalmanj (1857–1858) and then the physician Milosav Pavlović (1861). Temporary penitentiary doctors were Jovan Kovač (1862–1864), Bogoljub Đorđević (1865–1867), Mladen Obradović (1868) and Pavle Stejić (1874–1875). From 1865 to 1873 and from 1876 to 1880, the position of the penitentiary doctor was empty [13]. After the Law on Organization of Medical Profession and Protection of National Health (1881) [2] had been adopted, the following permanent penitentiary doctors were appointed again: Milutin M. Popović (1881–1895), Đura Gavrić (1896–1899), Selimir Đorđević (1899) and Milan Vasić (1899–1914) [13].

In the Požarevac Penitentiary, the following contractual doctors took care of the convicts' health: Vladislav Jasnjevski (1884–1886), Viktor Skubica (1887–1888 and 1890–1892), Stanojlo Vukćević (in 1885 and 1892–1900), Milenko Đorić (1901–1904, in 1906 and in 1910), and Milovan Milovanović (1907–1909) [13]. In 1905, 1912 and 1914 respectively, the Požarevac penitentiary did not have a doctor at all.

In the Niš penitentiary the contractual doctors were: Andrija Janković (1884–1885), medical captain Borisav Pavlović (1896), Jovan Bogdanović (1897–1903), Stojadin Stojanović (1906–1909) and Milutin Kopte (1910) [13]. This penitentiary was also without a doctor in 1904, 1905, 1911 and 1914.

Penitentiary nurses

Due to the lack of qualified hospital personnel, both male and female convicts worked as nurses in the penitentiary hospitals. Since working engagement in a penitentiary hospital could result in parole or amnesty of the convicts [14], given the prescribed conditions had been fulfilled, it was prescribed nurses could be taken among the convicts, especially those "who are healthy, strong, who are not disgusted by the diseases or the sick people and those who are hard-working, as well as those who are sentenced to minor penalties, who are obedient and are of generally good behavior" [9].

The penitentiary nurses were classified into two categories, the first one including just the literate convicts, while the literacy was not mandatory for the second category. A first-class nurse was in charge of five seriously ill people or ten ordinary sick bedridden convicts or twenty recovering patients, while six second-class nurses were in charge of fifty

sick convicts with proportional increase (7 : 60, 8:70, etc.). The nurses had to treat the sick people humanely and fully observe the guidelines and recommendations of the doctors and the orders of the hospital commissioner. Their duties included taking care of regular diet and taking medicines by the patients, maintenance of hygiene of patients' clothing and bedding and bringing incense burner after washing up and tidying the room. They took special care of dying patients, and they had to inform both the doctor and the hospital commissioner immediately on the death of a convict. The first-class nurses had mandatory regular 24-h-on-call shifts with continuous presence of at least one nurse in every hospital room. The nurses on call controlled the presence of all patients at stationary treatment, registered every change in convict's health condition, took care of the order and hygiene and reported to the doctor in the morning. The second-class nurses took care of the hygiene of patient's rooms, washbasins and toilettes, of regular heating and lighting of all hospital premises and prepared the dead convicts for funeral [9].

Penitentiary hospitals

In Serbia in 1879, three out of 23 hospitals were intended for the treatment of convicts – in Topčider, Kragujevac and Požarevac, and from 1880 on in Niš. Penitentiary hospital did not necessarily mean a separate building. The Topčider hospital was connected with the Belgrade Penitentiary; the Niš Penitentiary had a separate building in the Fortress of Niš until 1903, while the Požarevac penitentiary consisted of individual convict's rooms. The sick convicts working in Kragujevac cannon foundry were treated in the county hospital.

Although Cenić considered that it would be best to build a separate building for the sick convicts within a penitentiary in order to prevent spreading a disease in case of some epidemic [15], due to the lack of money, he prescribed that two to three rooms should be assigned as penitentiary hospitals which are positioned at the healthiest and cleanest place in the building to be able to receive 20–30 sick convicts. Those who were sick with a dangerous infectious disease were to be immediately transferred from the penitentiary to the town hospital [15].

The convicts from 16 dungeons of the Belgrade Penitentiary were treated in the Topčider hospital. The hospital was under the same roof and directly connected with the penitentiary [16], which made it difficult to separate healthy and sick convicts and increased the risk of infectious diseases spreading. This is why those affected by infectious diseases were put into the rooms where prison guards lived. Since due to the increased inflow of convicts (at the end of 1897 it was 50 convicts a day), the number of prison guards increased as well, the accommodation of the convicts with infectious diseases became the most pressing issue. The penitentiary doctor Đura Gavrić asked the Ministry of Justice to have a separate building constructed for infectious diseases with six rooms or to make adaptation to the already existing building within the penitentiary complex. The new building could not be built due to the lack of money, and the attempt by the Minister of Justice to obtain the already existing building for that purpose [16] was without success since the management of the Topčider farm had already given its buildings to the army. After the morbilli epidemic, which in March 1898 affected Belgrade, and particularly Topčider where the proportions of the disease had been larger than in city dungeons (in Belgrade there were three infected persons per 110 convicts, while in Topčider this ratio was 700 : 20) [16], the convicts with infectious diseases were sent to the town hospital. In the Belgrade penitentiary hospital there was a hammam which was rarely used because it was difficult to purchase the wood for its heating and difficult to supply water which was brought in barrels from the Sava and the Danube [17].

The Niš hospital for the convicts was in a separate building of the Fortress of Niš until October 1903, when the penitentiary management ceded the building to the then town command [18]. The hospital building was demolished, and the material was used for erecting a new building for the army requirements. By reducing the accommodation capacity of the penitentiary the medical treatment of convicts became more difficult – 13 prison rooms could receive up to 500, and the mosque up to 200 convicts. In late 1904, through the Ministry of Justice, the penitentiary management asked the permission from the Minister of War to build a hospital for the convicts in the Fortress of Niš or at least the permission to use the buildings ceded to the army [19]. According to the approval of the Minister of War, Radomir Putnik, the penitentiary was given the stable and the mosque but not the sewing facility [18]. In addition to this, the penitentiary management also chose the three most comfortable dungeons for patient rooms [18]. The convicts with infectious diseases were sent to the town hospital.

The Požarevac hospital for the convicts consisted of three convict rooms – two for male and one for female patients. In the newly-built building of the women's prison (1874), one room was a hospital room. Due to limited hospital capacities and the lack of a permanent doctor, in addition to the convicts with infectious diseases, the penitentiary used to send other patients to the Požarevac general hospital. The condition partially improved by building two more additional premises to the hospital for men (1900) [20], and also in 1912 when the new juvenile penitentiary was built in Zabela with two hospital rooms [21].

By the 1869 Rules, hospital equipment was also prescribed. A hospital room had to have an iron bed for each patient with either a straw-mattress or a mattress, a sheet, a wool-filled pillow and a blanket, one long jacket and a linen cap and one pair of slippers. The hospital had to have a sufficient number of food and medicine dishes, bedpans and spittoons and one board for each bed to register the use of medicines. The reality was, however, different. The inventory in the prison hospitals was rather modest. In 1869, for equipping the Belgrade penitentiary hospital, the following was asked to be purchased as necessary: "30 spittoons; a necessary number of robes, caps and slippers, one tin funnel for the pharmacy; 60 wooden spoons for the patients, one water glass for each patient, one mortar to crash and compose medicines; one small scale with necessary units of mass to meas-

ure medicines for the patients and, in addition, one pharmaceutical pane to cook medicines, and one screen to strain the cooked medicines" [9]. In one inventory which was preserved for year 1889/1890 "listing the things in convicts' hospital", it can be seen that, in the category of medical supplies and equipment, the hospital also had various kinds of thermometers, douches, hygrometers, hernia trusses, as well as "jars with leeches" [22].

Health care measures for the convicts

The health care measures for the convicts included preventive doctor's examinations before sending them to the penitentiary, mandatory doctor's examinations when admitting the convicts into the penitentiary, immunization, hospital and infirmary treatment of the sick convicts, reducing the number of convicts by paroling them, as well as initiative by the penitentiary doctors to suspend wearing the shackles.

Mandatory doctor's examination of the convicts when sending them to penitentiaries was the most important preventive measure [9], and its frequent omission caused reaction by the penitentiary management [23]. In 1884, the Minister of Interior warned county police authorities to send only completely healthy convicts to penitentiaries. Some of them clearly continued to neglect this obligation, so in 1892 once again the Minister ordered the mandatory doctor's examination making it precise that "if it is established by the physical examination that the convict is seriously ill, and especially if he had an infectious disease, such as measles, typhoid, cholera, etc. he had to be sent to hospital and there he should be kept under care until healed, and only then sent to prison" [24].

The treatment of the sick convicts implied prescribing and application of the corresponding therapies by the penitentiary doctors in accordance with the professional rules and austerity measures, as well as a special diet and certain palliative measures. The same medicines were used to treat penitentiary convicts as for the treatment of the rest of the population. The diet of the convicts was, as for all other patients, dietary and individualized on a daily basis. The dietary regime of the sick convicts, proportionate to the illness and the health condition of a convict, implied restricted diet, half a diet and the whole diet.

Isolation of infectious patients was difficult because of insufficient accommodating capacities for all sick convicts. This is why they had to resort to forced solutions, such as to send those convicts to other penitentiaries until epidemic decreased or until the accommodation for all infected convicts was provided. Thus, for instance, when in 1906 in just one day seven typhoid-infected convicts were admitted to the Belgrade penitentiary hospital, the Minister of Justice ordered all first-instance courts not to send convicts into this penitentiary until March 1, 1907, [25] or until new shacks for accommodation of the sick people were built [26].

Infectious diseases were the greatest danger to convicts' health. The most serious disease and the most frequent cause of death of convicts was tuberculosis. Lymphatic gland tuberculosis was especially dangerous. It developed so fast in the population of young convicts after a long period of incarceration that they would die only two to three years later. In the period from 1900 to 1903, death rate from tuberculosis in convicts was 22% [27]. Prevention and suppression of tuberculosis in Serbian penitentiaries at the time was a part of general fight with this disease, for which according to the opinion of doctors it was necessary that "there was a harmony of: wise management, well prepared doctors and reasonable people" [28]. In addition to isolation, the measures of protection from tuberculosis spreading among the convicts were: to equip hospitals with the necessary number of white clay enameled spittoons which had to be filled with sublimate solution twice a day (up to 1/3 at 1: 1,000 ratio) and emptied twice a day into special pits disinfected with sublimate as well, to disinfect hospital beds, walls and floors surrounding them up to two meters in radius by sublimate solution [27, 29]. In 1910, doctor Vasić of the Belgrade Penitentiary, recommended relatively inexpensive prophylactic measures to suppress tuberculosis in prisoners and convicts: regular fortnight medical examinations of convicts and enabling relatives to bring them food and clean clothing, to spend 6–8 hours a day in fresh air, washing and scrubbing of cells at least once a month, washing the woodwork with hot water and disinfecting with sulfur and painting the walls every three months, bathing, shaving and cutting hair of convicts at least once a month [30].

Cholera was particularly dangerous for convict population. Since in the first half of the 19th century Serbia faced cholera epidemic for five times, the instructions, rules and directions of doctors regarding this disease were made public rather early. The convicts in penitentiaries were particularly endangered since non-hygienic conditions in which they lived were rather favorable for the outbreak and spreading of cholera. When during the Balkan wars the cholera epidemic was transferred into neighboring countries and in August 1913 it affected Niš, the Committee for national defense asked in a telegram to the Ministry of Justice to provide 50 convicts to help in the fight with the epidemic. The request was denied since cholera had already affected the penitentiary as well [31], taking the first victim at the beginning of September [32]. The penitentiary management managed to stop the spreading of the disease by isolating the sick people in cholera shacks near the "Red Cross" – twelve out of 40 convicts died, and the rest were returned to prison when they recovered [32].

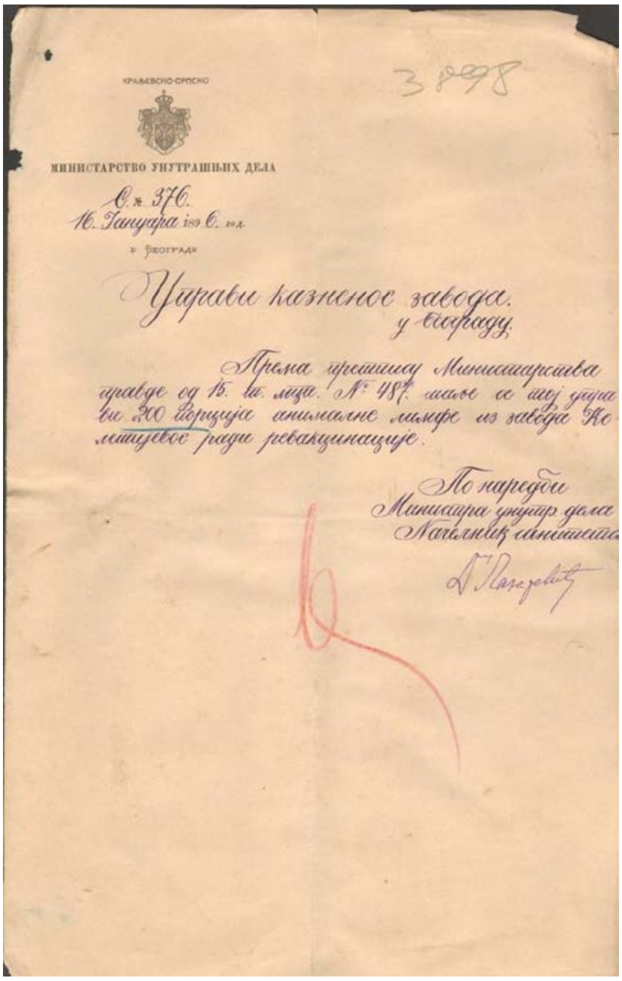
Scabies was almost regular company of the prison convicts. In addition to non-hygienic conditions in penitentiaries, the greatest problem in suppressing this disease was a huge daily inflow of those convicted to short-term imprisonment, who served their respective sentences in their own clothing – vagrants and beggars in dirt-filled "rags". In August 1908, in order to suppress scabies a separate building was built in the Belgrade penitentiary for disinfecting device which was moved from the town into the penitentiary complex as early as in 1900 [33].

Due to malnutrition, scurvy or "alkaline disease", as it was called, was also very spread among the convicts [34]. Although the positive effects of eating fresh vegetable and lemon juice in treating scurvy were known, their becoming a

part of convicts' diet was connected with too many difficulties, therefore only pepper and spinach and more inexpensive alternatives such as sour cabbage and nettle were used. Pepper juice, which as early as in 1864 was recommended by the military doctor Maksim Nikolić-Miškovićev as an efficient and inexpensive therapy in treating scurvy, was forgotten since the committee underestimated his findings. The quantity of 20 g of flour per day per convict, which was used to make roux for meals, was reduced to 5 g in 1898 at the proposal of the doctor of the Belgrade penitentiary [34]. At the end of the following year, doctor Vasić of the Belgrade Penitentiary supported the proposition of the management to give fatty food to the convicts two times a week instead of five times a week and that daily portion of bread would be reduced from 1,000 to 750 g, which would ultimately reduce the number of convicts "suffering from scurvy, the disease very rare and almost unknown beyond this prison" [34]. A longtime doctor of the same penitentiary, Popović, disagreed with Vasić, finding that "further reduction of already small amount of meat would not be advisable" [34]. Governed by the reasons of government austerity measures, the Ministry of Justice prescribed for the convicts to continue to get "1,000 g of wheat bread, well baked", and starting from January 1, 1900 to get 200 g of beef or sheep meat only on Sundays and Thursdays when they are not fasting" [34].

Immunization in Serbia was already practiced in the first half of the 19th century. The greatest threat was from the smallpox. Vaccination against measles was prescribed as early as in 1839 by the so called Pacek's Law – The Ordinances for District and Municipal Physicians [35], it was performed "from hand to hand" until 1886, when vaccination with animal lymph was introduced.

Measures of mandatory vaccination and revaccination of the sick convicts were consistently carried out, so that there were no epidemics in prisons but only individual cases of falling ill. For instance, in 1896 the Medical Division of the Ministry of Interior sent 200 portions of animal lymph to the Belgrade Penitentiary for revaccination of the convicts who were working in Dobričevo, with the instructions for those who might be sick to be sent to the Ćuprija hospital [36] (Figure 1). There was a smallpox outbreak in the Belgrade Penitentiary in 1886 after an infected prisoner came from the Niš Penitentiary [37], and the following year several convicts in the Niš Penitentiary also fell ill because Kruševac county superiors sent an infected convict without medical examination [38].


КРАЉЕВСКО-СРПСКО
МИНИСТАРСТВО УНУТРАШЊИХ ДЕЛА
С № 376.
16. Јануара 1896. год.
у Београду

Управи казненог завода.
у Београду.

Према тражењу Министарства правде од 15. ов. мес. № 487. шаље се тој управи 200 порција анималне лимфе из завода Кошутијевог ради ревакцинације.

По наредби
Министра унутр. дела
Начелник санитета

Fig. 1 – Medical division of the Ministry of Interior sends 200 portions of animal lymph for the revaccination of convicts (Historical Archives of Požarevac, raw archival material [36]).

One of the forced health care measures was to reduce the number of convicts in penitentiaries. Most frequently those who had fallen ill without the possibility to recover or the convicts with severe bodily defects, as well as those sick convicts who fulfilled certain conditions for parole and whose health would be aggravated by further prison service, were acquitted from further serving of their respective sentences. These measures were often initiated by the penitentiary doctors, guided not only by the need to unburden the accommodation capacities of prison hospitals but also by medical and human ethics: "Considering that in such cases it is more humane to make necessary exceptions and allow the convict – a man – to keep at least his life, I find that such patients need better living conditions for the sake of their own preservation" [39].

Serving the sentence of deprivation of liberty in shackles aggravated convicts' health additionally. Day-and-night wearing of shackles, both in summer and in winter, even during work caused leg, back and even chest pains in some convicts [40].

Older and sick convicts were particularly endangered. Acknowledging that "long-term wearing of iron mutilated many people in such a way that they could not recover at all since tendons under knees remained contracted and from the knees downward completely numb", the penitentiary doctors often proposed for such convicts to be released from shacks [17].

Conclusion

The absence of thorough reform of penitentiaries due to the lack of financial resources reflected on the range of the convicts' health care measures. Insufficient number of permanent doctors and nurses, limited capacities of penitentiary hospital and infirmaries, the lack of medicines and equipment, in addition to non-hygienic and inadequate accommodating conditions and disrespect of regulations on mandatory medical examination of convicts before sending them to prisons, were the greatest problems. The conditions for serving the sentence of deprivation of liberty in the penitentiaries in Serbia were certainly considerably worse than those in the developed European countries, and preventive health care of the convicts practically boiled down to mandatory medical examinations and mandatory immunization. As a part of general progress of medical conditions in Serbia in the second half of the 19th century, which occurred mainly thanks to the efforts made by doctors, there were certain shifts made even in the health care of the convicts.

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LETTER TO THE EDITOR
(RESEARCH LETTER)

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Anti-SARS-CoV-2 IgG seroprevalence study among blood donors in the Republic of Srpska: a 30-day survey

Studija seroprevalencije anti-SARS-CoV-2 IgG kod davalaca krvi u Republici Srpskoj: 30-dnevno ispitivanje

To the Editor:

In most cases, individuals with the COVID-19 infection have few or no symptoms and represent a significant source of transmission as well as a challenge for prevention of the spread of the infection. The key elements for understanding total prevalence and epidemic significance of this disease are assessment of prevalence and infectiousness of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) [1]. Determination of the prevalence of SARS-CoV-2 in the population of blood donors might enable the monitoring of the virus transmission among healthy people, as well as the implementation of transmission-reducing strategies, especially in the absence of global or nationwide seroprevalence surveys [2–3].

Between June 17th and July 16th, 2020, at the Transfusion Medicine Institute of the Republic of Srpska (Bosnia and Herzegovina), a total of 807 blood samples from apparently healthy blood donors, aged 18–64, were tested for the first time for the presence of anti-SARS-CoV-2 IgG antibodies. A two-step sandwich enzyme immunoassay with a final fluorescence detection (ELFA) was used. It is the assay principle for VIDAS SARS-CoV-2 IgG, produced by Biomerieux. All of the steps are performed automatically by the instrument and the intensity of the fluorescence is proportional to the level of antibodies in the tested sample. A test values are acquired as the results that are automatically calculated at the end of the assay by the instrument according to the S1 standard, stored in memory.

The results showed that a total of 22 (2.7%) blood donors were positive for the presence of anti-SARS-CoV-2 IgG antibodies. The Fisher's exact test determined there were no statistically significant differences in the number of the blood donors with anti-SARS-CoV-2 IgG antibodies regarding gender (p = 0.421), ABO blood groups (p = 0.538), and Rhesus blood groups (p = 0.781) (Table 1). What is clearly shown by the chi-squared test is that there are statistically significant differences in the number of donors with anti-SARS-CoV-2 antibodies and different age groups (χ^2 = 9.676, df = 2, p = 0.008). Anti-SARS-CoV-2 seroprevalence was significantly higher in blood donors aged 18-30 (5.1%, 15/296) compared with those aged 31–50 (1.4%, 6/427) (p = 0.017) (Table 1).

Table 1

Anti-SARS-CoV-2 IgG seroprevalence among blood donors in the Republic of Srpska according to gender, age, ABO blood types and Rhesus (Rh) blood groups

Variables	No. positive/No. tested (%)	p-value*
Gender		
male	16/643 (2.5)	0.421
female	6/164 (3.7)	
ABO blood type		
A	9/332 (2.7)	
B	2/142 (1.4)	0.538
O	8/271 (3)	
AB	3/62 (4.8)	
Rh blood groups		
positive	19/660 (3)	0.781
negative	3/147 (2)	
Age (years)		
18–30	15/296 (5.1)	
31–50	6 /427 (1.4)	0.008
51–65	1/84 (1.2)	

***A probability level of $p < 0.05$ was considered to indicate statistical significance. Frequencies were estimated by direct counting. Fisher's exact test or chi-squared test, as appropriate, were used to evaluate the differences between SARS-CoV-2-positive and SARS-CoV-2-negative blood donors with respect to different characteristics.**

The seroprevalence of antibodies to SARS-CoV-2 among blood donors in the state of Rio de Janeiro, Brazil, collected on 2,857 blood donors from April 14th to April 27th, 2020 was 4.0% [95% confidence interval (CI): 3.3–

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4.7%] [4]. However, this estimate is higher than 1.9% of seroducted in Denmark from April 6th to May 3rd, 2020 [5]. German authors found anti-SARS-CoV-2 IgG antibodies in 3,186 regular blood donors in three German federal states between March 9th and June 3rd, 2020. The overall seroprevalence of IgG was 0.91%, ranging from 0.94% in North Rhine-Westphalia to 1.22% in Lower-Saxony and 0.66% in the federal state of Hesse [6]. The overall seroprevalence of SARS-CoV-2 antibodies in Timis County, Romania, among 2,115 blood donors was 1.51% (32/2115; 95% CI: 1.07%-2.13%) [7]. The reason for the difference in seroprevalence of anti-SARS-CoV-2 antibodies among blood donors in different countries is explained by various levels of health infrastructure in each country, implementation of different asymptomatic vectors of infection and its most significant reservoir. The social habits and behavior of this part of the population present a significant epidemiological risk for the transmission and the speed of spread of coronavirus disease 2019 (COVID-19). In addition, Olariu et al. [7] observed higher infection rates in individuals over 60, together with higher infection rates in individuals under 30. Increased susceptibility of older individuals to SARS-CoV-2 infection is explained by changes in many cellular and molecular elements of both the innate and adaptive immune systems related to age, leaving older adults particularly prone to SARS-CoV-2 [9].

Our results regarding the anti-SARS-CoV-2 IgG antibodies frequency in asymptomatic blood donors, obtained at the Institute for Transfusion Medicine of Republic of Srpska, are preliminary. For a detailed analysis which could be useful for epidemiological studies, we need more information concerning a larger number of investigated blood donors, correlated with their gender, age groups, education level, place of residence, and donation site. Despite the lack of all these variables and the age limitation for blood donation (18 to 65 years), our results show that the population of healthy prevalence in a large survey of 20,640 blood donors con measures for epidemic prevention and control, variability of method sensitivity and specificity for the detection of anti-SARS CoV-2 antibodies, and different times when the studies were conducted [7, 8].

Our results are in accordance with previous reports showing that gender, ABO, and Rhesus blood groups are not associated with SARS-CoV-2 infection [5, 6]. However, anti-SARS-CoV-2 seroprevalence was significantly higher in our blood donors aged 18-30 compared to older age groups. This observation is in accordance with published findings by other authors, who observed infection rates in individuals under the age of 30 [4, 7]. This information is of particular importance because it confirms that young people are the dominant blood donors in the Republic of Srpska has a certain level of herd immunity, particularly in younger persons.

Conflict of interest

The authors of this paper declare no conflicts of interest, including financial interests, relationships, and/or affiliations relevant to the subject matter or materials included.

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