YU ISSN 0042-8450

ВОЈНОСАНИТЕТСКИ ПРЕГЛЕД

Часопис лекара и фармацеута Војске Србије

Military Medical and Pharmaceutical Journal of Serbia

Vojnosanitetski pregled

Vojnosanit Pregl 2016; October Vol. 73 (No. 10): p. 891–896.

YU ISSN 0042-8450 vol. 73, br. 10, 2016.

VOJNOSANITETSKI PREGLED

Prvi broj *Vojnosanitetskog pregleda* izašao je septembra meseca 1944. godine

Časopis nastavlja tradiciju *Vojno-sanitetskog glasnika*, koji je izlazio od 1930. do 1941. godine

IZDAVAČ
Uprava za vojno zdravstvo MO Srbije

Adresa redakcije: Vojnomedicinska akademija, Institut za naučne informacije, Crnotravska 17, poštanski fah 33–55, 11 040 Beograd, Srbija. Informacije o pretplati: Tel.: +381 11 3608 997. E-mail (redakcija): **vsp@vma.mod.gov.rs**

Radove objavljene u „Vojnosanitetskom pregledu“ indeksiraju: Science Citation Index Expanded (SCIE), Journal Citation Reports/Science Edition, Index Medicus (Medline), Excerpta Medica (EMBASE), EBSCO, Biomedicina Serbica. Sadržaje objavljuju Giornale di Medicine Militare i Revista de Medicina Militara. Prikaze originalnih radova i izvoda iz sadržaja objavljuje International Review of the Armed Forces Medical Services.

Časopis izlazi dvanaest puta godišnje. Pretplate: Žiro račun br. 840-314849-70 MO – Sredstva objedinjene naplate – VMA (za Vojnosanitetski pregled), poziv na broj 1227423129552141 5. Za pretplatu iz inostranstva obratiti se službi pretplate na tel. 3608 997. Godišnja pretplata: 5 000 dinara za građane Srbije, 10 000 dinara za ustanove iz Srbije i 150 € (u dinarskoj protivvrednosti na dan uplate) za pretplatnike iz inostranstva. Kopiju uplatnice dostaviti na gornju adresu.

Štampa Vojna štamparija, Beograd, Resavska 40 b.

YU ISSN 0042-8450 vol. 73, No. 10, 2016

VOJNOSANITETSKI PREGLED

The first issue of *Vojnosanitetski pregled* was published in September 1944
The Journal continues the tradition of *Vojno-sanitetski glasnik* which was published between 1930 and 1941

PUBLISHER
Military Health Department, Ministry of Defence, Belgrade, Serbia

Editorial Office: Military Medical Academy, Institute for Scientific Information, Crnotravska 17, PO Box 33–55, 11 040 Belgrade, Serbia. E-mail: **vsp@vma.mod.gov.rs**

Papers published in the Vojnosanitetski pregled are indexed in: Science Citation Index Expanded (SCIE), Journal Citation Reports/Science Edition, Index Medicus (Medline), Excerpta Medica (EMBASE), EBSCO, Biomedicina Serbica. Contents are published in Giornale di Medicine Militare and Revista de Medicina Militara. Reviews of original papers and abstracts of contents are published in International Review of the Armed Forces Medical Services.

The Journal is published monthly. Subscription: Giro Account No. 840-314849-70 Ministry of Defence – Total means of payment – VMA (for the Vojnosanitetski pregled), refer to number 12274231295521415. To subscribe from abroad phone to +381 11 3608 997. Subscription prices per year: individuals 5,000.00 RSD, institutions 10,000.00 RSD, and foreign subscribers 150 €.

Printed by: Vojna štamparija, Beograd, Resavska 40 b.

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SHORT COMMUNICATIONS / KRATKA SAOPŠTENJA

Dr. Laza Popović (Sremski Karlovci, July 1877 – Belgrade, May 1945), a physician, writer and social worker was the most important among founders of the Sokol movement (the system of physical training focused on physical and health education as well as cultural and national revival and unity of nation) in the Serbian poeple under the Austro-Hungarian rule. With his enthusiastic and devoted work, he made a significant contribution to the development and popularization of the Serbian and Yugoslav Sokol movement (see pp. 976 – 979).

Dr Laza Popović (Sremski Karlovci, 1877 – Beograd, 1945), lekar, pisac i društveni radnik, bio je najzaslužniji među osnivačima sokolskog pokreta (sistem telesnog vežbanja usmeren na fizičko i zdravstveno prosvećivanje, kao i kulturni i nacionalni preporod i jedinstvo naroda) kod srpekog naroda pod austro-ugarskom vlašću. Svojim neumornim i požrtvovanim radom dao je značajan doprinos razvoju i omasovljenju srpskog i jugoslovenskog sokolskog pokreta (vodi str. 976 – 979).

ORIGINAL ARTICLES

UDC: 616.314-76/-77
DOI: 10.2298/VSP150105089P

Measurement of the accuracy of dental working casts using a coordinate measuring machine

Ispitivanje preciznosti radnih modela pomoću koordinatne merne mašine u stomatologiji

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Abstract

Background/Aim. Dental impressions present a negative imprint of intraoral tissues of a patient which is, by pouring in gypsum, transferred extraorally on the working cast. Casting an accurate and precise working cast presents the first and very important step, since each of the following stages contributes to the overall error of the production process, which can lead to inadequately fitting dental restorations. The aim of this study was to promote and test a new model and technique for *in vitro* evaluation of the dental impression accuracy, as well as to asses the dimensional stability of impression material depending on the material bulk, and its effect on the accuracy of working casts. **Methods.** Impressions were made by the monophasic technique using the experimental master model. Custom trays with spacing of 1, 2 and 3 mm were constructed by rapid prototyping. The overall of 10 impressions were made with each custom tray. Working casts were made with gypsum type IV. Measurement of working casts was done 24 h later using a coordinate measuring machine. **Results.** The obtained results show that the working casts of all the three custom trays were in most cases significantly different in the transversal and sagittal planes in relation to the master model. The height of abutments was mainly unaffected. The degree of convergence showed certain significance in all the three custom trays, most pronounced in the tray with 3 mm spacing. **Conclusion.** The impression material bulk of 1–3 mm could provide accurate working casts when using the monophasic impression technique. The increase of the distance between abutment teeth influences the accuracy of working casts depending on the material bulk.

Key words:
denture bases; denture design; elastomers; computer-aided design; sensitivity and specificity.

Apstrakt

Uvod/Cilj. Otisak predstavlja negativ intraoralnih tkiva, čijim se izlivanjem u gipsu njihova morfologija prenosi ekstraoralno na budući radni model. Sa laboratorijskog aspekta izrade zubnih nadoknada, izlivanje tačnog i preciznog radnog modela predstavlja prvi i veoma bitan korak, pošto svaka sledeća faza doprinosi daljem povećanju greške tokom izrade, što za krajnji ishod može imati neodgovarajuću zubnu nadoknadu. Cilj istraživanja bio je da se ispitaju novi model i tehnika za *in vitro* procenu preciznosti zubnih otisaka, kao i da se odredi uticaj količine otisnog materijala na dimenzionu stabilnost otisaka i preciznost izrade radnih modela. **Metode.** Za uzimanje otisaka korišćena je monofazna tehnika otiskivanja. Individualne kašike sa međuprostorom od 1, 2 i 3 mm napravljene su aditivnom tehnologijom za brzu izradu prototipova. Sa svakom kašikom napravljeno je po 10 otisaka. Radni modeli izlivani su u gipsu tipa IV. Merenje radnih modela vršeno je nakon 24 sata na koordinatnoj mernoj mašini. **Rezultati.** Rezultati pokazuju da radni modeli napravljeni pomoću sve tri individulane kašike u transverzalnoj i sagitalnoj ravni značajno odstupaju od glavnog dela modela. Visina patrljaka je u većini slučajeva bila kao na glavnom modelu. Stepen konvergencije pokazao je određena odstupanja samo kod kašike sa međuprostorom od 3 mm. **Zaključak.** Monofazna tehnika otiskivanja i otisni materijal debljine od 1 do 3 mm obezbeđuju izradu preciznih radnih modela. Rastojanje između zubnih patrljaka utiče na preciznost izrade radnih modela u zavisnosti od količine otisnog materijala.

Ključne reči:
zubna proteza, baze; zubna proteza, oblikovanje; elastomeri; kompjutersko oblikovanje; osetljivost i specifičnost.

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Introduction

Dental impressions present a negative imprint of intraoral tissues of a patient which is, by pouring in gypsum, transferred extraorally on the working cast. From the laboratory perspective of dental restorations manufacturing, casting an accurate and precise working cast presents the first and very important step, since each of the following stages contribute to the overall error of the production process and can lead to inadequately fitting of dental restorations [1].

Making an impression is a clinically challenging procedure which is influenced by numerous factors of the oral environment, as well as the properties of the material itself. Examining the factors that influence the accuracy of dental impressions can be conducted in two ways, by direct mesurement of the impression, or by measurement of the appropriate working cast. Both methods have their advantages and limitations. The first method provides direct data about the condition of the impression material, thus avoiding superimposition of further errors. Limitations are related to the use of contactless measurement, which is affected by a small measurement field, software processing and adequate optical characteristics of the impression material [2–4]. The second method, measurement of the working casts, provides a wider range of possibilities regarding the measurement technique. Both contact and contacless measurement can be used, with ease of access for manipulation with measurement object [5]. Superimposition of errors, when casting a gypsum model, can be minimised by fixing the experimental conditions with equal casting protocols for all of the investigated dental impressions. Although making of the gypsum working cast prolongs the time and effort needed to obtain necessary data, it is a reference base for manufacturing of dental restorations and as such, provides better insight when assesing the discrepancies of future dental restorations.

Determination of accuracy of dental impressions requires a complex model that can replicate *in vivo* conditions of making of dental impressions, with the accuracy and precision of *in vitro* investigation. Earlier studies included a variety of models that consisted of custom blocks, cylinders, single or several abutment teeth and complete edentulous jaws of various materials [5–11]. Regarding this, there is the need for a reliable experimental model, which combined with a corresponding measurement instrument, could overcome the difficulties of data interpolation between laboratory conditions and clinical practice. The two should complement each other, forming a complex model for data acqusition. From this point of view, the use of a coordinate measuring machine (CMM) was chosen due to the possibility of the accurate and precise three-dimensional (3D) measurement [12, 13].

Previous studies that investigated the influence of material bulk on the accuracy of working casts were conducted using a measurement microscope and included only two dimensional measurements [14–16]. Also, the models used consisted of one to several abutment teeth, while as to our knowledge, the influence of material bulk on partially edentulous dental arch model has not been made [14–18]. A more detailed analysis requires a model more similar to the intraoral conditions, since the accuracy of impressions depends on the material bulk which changes with spacing between the remaining teeth [19]. The possibility of an independent 3D analysis by CMM for each of the segments of the working casts was considered to be an improvement compared to the previous studies.

Additionally to the previous studies, we aimed to incorporate another use of rapid prototyping (RP) technology in the field of dentistry. Making impressions with RP made custom trays is a new attempt to investigate the use of computer-aided design/computer-aided manufacturing (CAD/CAM) technology into a broader perspective of dental practice. The rapid prototyping technique was chosen with the aim of avoiding possible distortions of the material that can occur in standard acrylic custom trays, and providing a stable base for the impression material [20]. Thus, the aim of this study was to promote and test a new model and technique for *in vitro* evaluation of the dental impression accuracy, as well as to asses dimensional stability of elastomeric impression material in reference to the material bulk, by examining working casts with CMM.

Methods

Measurement of the experimental master model and construction of custom trays

For the purpose of this study, an experimental metal master model which consisted of six abutment teeth was constructed. It presented the upper jaw with two central incisors, canines and first molars (Figure 1). Dimensions of the teeth were taken from literature and reduced by the amount of tooth substance expected to be removed with grinding [21]. The taper was set at 6°. The master model consisted of an assembly with machined components. Geometry of the master model and generation of computer numerical control machines (CNC) code was designed by the CAD/CAM system.

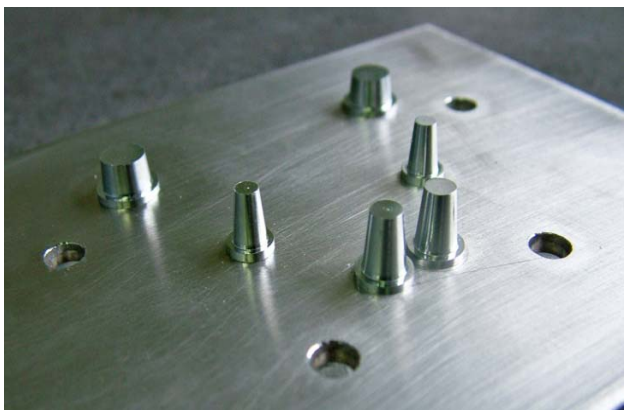

Fig. 1 – The experimental master model.

Measurement of the experimental master model was performed with the CMM (Contura G2, Carl Zeiss, Germany) equipped with a contact probe. Maximum permissible error for size measurement (MPE_E) of this CMM is 1.9 + L/330 μm. The master model was measured five times and

the resulting mean values of parameters of measurement were calculated. Nominal dimensions of the CAD model were corrected using mean values and were subsequently used to create a custom tray model as a negative of the previous CAD model. Physical models of custom trays with 1, 2 and 3 mm spacings for the impression material, were constructed by rapid prototyping (Z310 plus, 3D Systems, USA). The powder used was gypsum based (zp 131), with a binder (zb 60) and two component epoxy resin as the filler (s5000), presented at Figures 2 and 3.

Fig. 2 – Custom trays with 1, 2 and 3 mm spacing made by rapid prototyping.

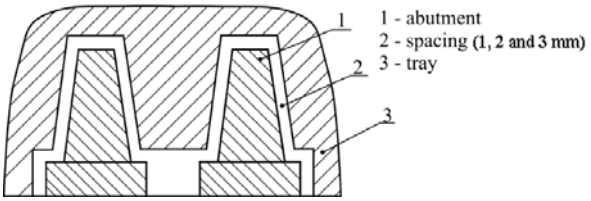

Fig. 3 – Custom tray seated on the top of the experimental master model.

Impression technique

Impressions were made using a monophasic technique with silicone addition. Due to the micromechanical retention of the impression material to a custom tray, which is the result of the successive layering technique of rapid prototyping, the tray adhesive was not used. The impression material was automixed (Elite Hd + regular, Zhermack, Italy), working time was set to 60 sec. Making of impressions was conducted at room temperature (23°C), retention of the tray was done with the weight of 1 kg. Setting time was set at 10 min, overall of 10 impressions were made with each custom tray. Due to the expected viscoelastic recovery of the impression material, pouring of gypsum was delayed by 30 min. Working casts were made with the gypsum type 4 (Elite rock, Zhermack, Italy) and were allowed to set for 1 h before the impression was removed. Measurement of the working casts was done 24 h later on CMM.

Measurement of working casts on a coordinate measuring machine

Measurement of working casts was conducted under the same conditions as those of the master model. Inspection was conducted conformant to the new generation of product geometry specification (GPS) [22]. This was based on the fact that the model consisted of geometric primitives (cones, cylinders and planes). Each geometric primitive was measured in a finite number of discrete points randomly arranged on the primitive surface. The measurement strategy of the cone (abutment) contained 100 measurement points, 50 points for measurement of cross-section plane between the cone and the cylinder (abutment and chamfer) and 50 for measurement of the cylinder (chamfer). The output of this measurement included the coordinates of all measurement points which were subsequently used to generate associative geometry of the primitives. Furthermore, the software analysis was used to determine all geometric characteristics (size, angle, form, orientation, location) according to the specification requirements.

Parameters, $X_{1\text{-}3}$ and $Y_{1\text{-}6}$, represented the axial distances which were derived features from the cone. The axial distances were observed on the cross-sectional plane which passed through the base of the abutments. Parameters denoted as $Z_{1\text{-}6}$, were determined as the distance between two planes which limited the abutment vertically, while $\alpha_{1\text{-}6}$ represented the degree of convergence and was directly derived from the abutment measurement (Figure 4).

The presence of measurement uncertainty was disregarded due to the fact that all working casts were measured in

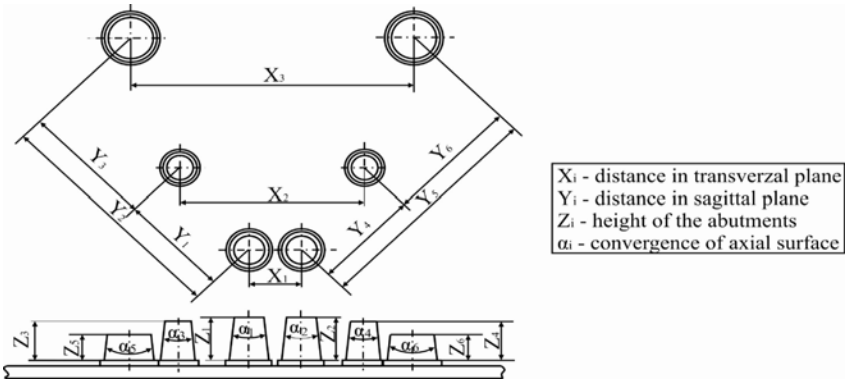

Fig. 4 – Parameters of measurement.

identical laboratory conditions: 24 h after casting, using the same position on machine table, by the same operator and using identical inspection strategies and stylus [23, 24].

Statistical analysis

Statistical analysis of the results obtained from the measurement of the working casts was conducted by the two-sample *t*-test, which determined whether there was a statistically significant difference between the designated distances of working casts compared to the master model. One-way analysis of variance (ANOVA) was also conducted to establish if there were any significant differences in the results obtained by the type of custom tray for the considered parameters.

Results

The results of the two-sample *t*-test, with a 95% confidence interval, show that the working casts of all three custom trays were in most cases significantly different in transversal and sagittal plane in relation to the experimental master model (X_{1-3}, Y_{1-6}). The height of the abutments (Z_{1-6}) was mainly unaffected, while the degree of convergence (α_{1-6}) increased, especially in the working casts made by custom trays with 3 mm spacing. The results are presented in Table 1.

Most notable deviations for the first tray (T_1) were measured in X_3 (20 μm) for the transversal plane, Y_2 (27 μm) for the sagittal plane, Z5 (-13 μm) for the height of the abutments and α1 (-0.09°) for the convergence of axial surface. The dimensions of Z_1–Z_4, Z_6 and α_3, α_4 and α_6 showed no statistical significance in relation to the experimental master model.

Most pronounced deviations for the second tray (T_2) were measured in X_3 (22 μm) for the transversal plane, Y2 (27 μm) for the sagittal plane, Z5 (-15 μm) for the height of the abutments and α1 (0.11°) for the convergence of axial surface. The dimensions of Y_1, Y_5, Z_1–Z_4 and α_6 showed no statistical significance in relation to the experimental master model.

The third tray (T_3) showed largest deviations in X_3 (36 μm) for the transversal plane, Y_2 (24 μm) for the sagittal plane, Z_5 (-7 μm) for the height of the abutments and α1 (-0.18°) for the convergence of axial surface. Dimensions of Y_1, Y_5, Y_6, Z_1–Z_6 showed no statistical significance in relation to the experimental master model.

Analysis of variance with a 95% confidence interval showed that the type of tray did not have any significant influence ($p > 0.05$) on the parameters of measurement, except for the X_3 and α1-6. ($p < 0.05$) Therefore, with respect to α1-6 and X_3, the third tray yielded the results which were significantly different from the first two trays. The presentation and graphical comparison of the results are shown in Figures 5 and 6.

Table 1

Results of the measurement

Distance	Master (mm)	Mean (mm)			Standard deviation (μm)			Difference (μm)			*t*-test *p*			ANOVA *p*
		T_1	T_2	T_3	T_1	T_2	T_3	T_1	T_2	T_3	T_1	T_2	T_3	
X_1	8.507	8.521	8.522	8.52	1.74	2.19	1.94	14	15	13	0.00	0.00	0.00	0.29
X_2	30.002	30.015	30.012	30.012	3.99	5.63	6.66	13	10	10	0.00	0.00	0.00	0.58
X_3	46.015	46.035	46.037	46.051	10.9	13.5	11	20	22	36	0.00	0.00	0.00	0.02
Y_1	18.144	18.150	18.148	18.147	3.4	5	2.89	6	4	3	0.00	0.10	0.07	0.18
Y_2	42.051	42.078	42.078	42.075	7.27	8.36	6.92	27	27	24	0.00	0.00	0.00	0.82
Y_3	24.364	24.380	24.383	24.384	4.28	4.52	4.27	16	19	20	0.00	0.00	0.00	0.32
Y_4	18.319	18.326	18.326	18.323	4.32	4.15	3.65	7	7	4	0.00	0.00	0.01	0.06
Y_5	42.149	42.164	42.161	42.161	7.28	19.4	16	15	12	12	0.00	0.12	0.08	0.93
Y_6	24.277	24.283	24.287	24.282	4.43	9.15	13.5	6	10	5	0.00	0.01	0.27	0.57
Z_1	7.506	7.504	7.502	7.509	9.5	4.9	8.6	-2	-4	3	0.55	0.06	0.33	0.2
Z_2	7.507	7.507	7.506	7.512	8.5	6.2	8.9	0	-1	5	0.82	0.61	0.35	0.5
Z_3	6.923	6.926	6.926	6.924	10.4	7.8	11.7	3	3	1	0.20	0.28	0.71	0.92
Z_4	6.926	6.927	6.919	6.921	7.8	11.7	9.7	1	-7	-5	0.13	0.17	0.24	0.33
Z_5	5.000	4.487	4.485	4.493	8.16	8.66	11.5	-13	-15	-7	0.00	0.00	0.17	0.24
Z_6	4.497	4.491	4.486	4.493	9.41	9.87	13.7	-6	-11	-4	0.09	0.01	0.44	0.46
Angle	Master (°)	Mean (mm)			Standard deviation (mm)			Difference (°)			*t*-test *p*			ANOVA *p*
		T_1	T_2	T_3	T_1	T_2	T_3	T_1	T_2	T_3	T_1	T_2	T_3	
α_1	11.848	11.759	11.733	11.667	0.017	0.031	0.041	-0.09	-0.11	-0.18	0.00	0.00	0.00	0.00
α_2	11.827	11.779	11.721	11.697	0.028	0.023	0.071	-0.05	-0.11	-0.13	0.00	0.00	0.00	0.00
α_3	11.753	11.795	11.743	11.656	0.052	0.055	0.066	0.04	-0.01	-0.09	0.06	0.00	0.00	0.00
α_4	11.795	11.766	11.745	11.669	0.036	0.051	0.043	-0.03	-0.05	-0.13	0.05	0.02	0.00	0.00
α_5	11.912	11.851	11.843	11.747	0.051	0.079	0.087	-0.06	-0.07	-0.16	0.01	0.04	0.00	0.02
α_6	11.793	11.828	11.751	11.647	0.071	0.057	0.136	0.03	-0.04	-0.14	0.19	0.08	0.02	0.00

For abbreviations see Figure 4 .

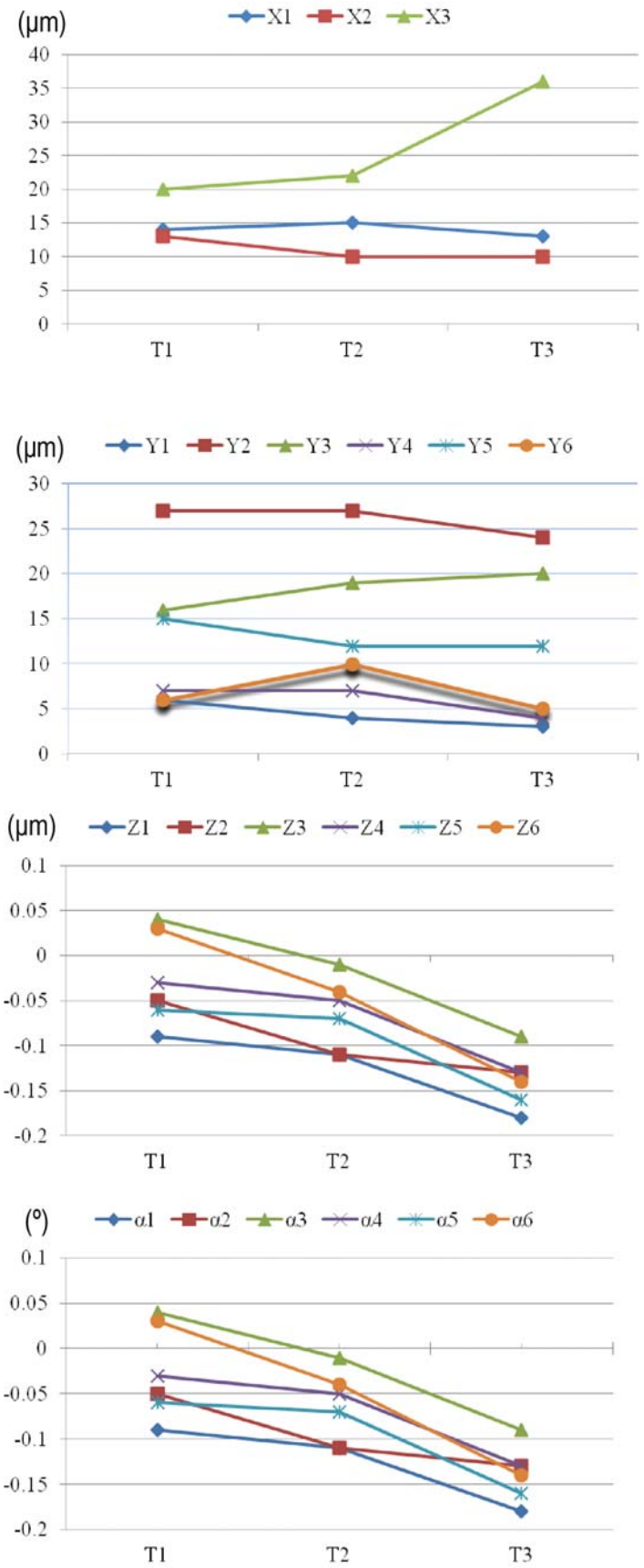

Fig. 5 – Comparison of distance in transversal ($X_{1–3}$), sagittal plane ($Y_{1–6}$), height of the abutments ($Z_{1–6}$) and convergence of axial surface ($\alpha_{1–6}$) between custom impression trays and the master model.

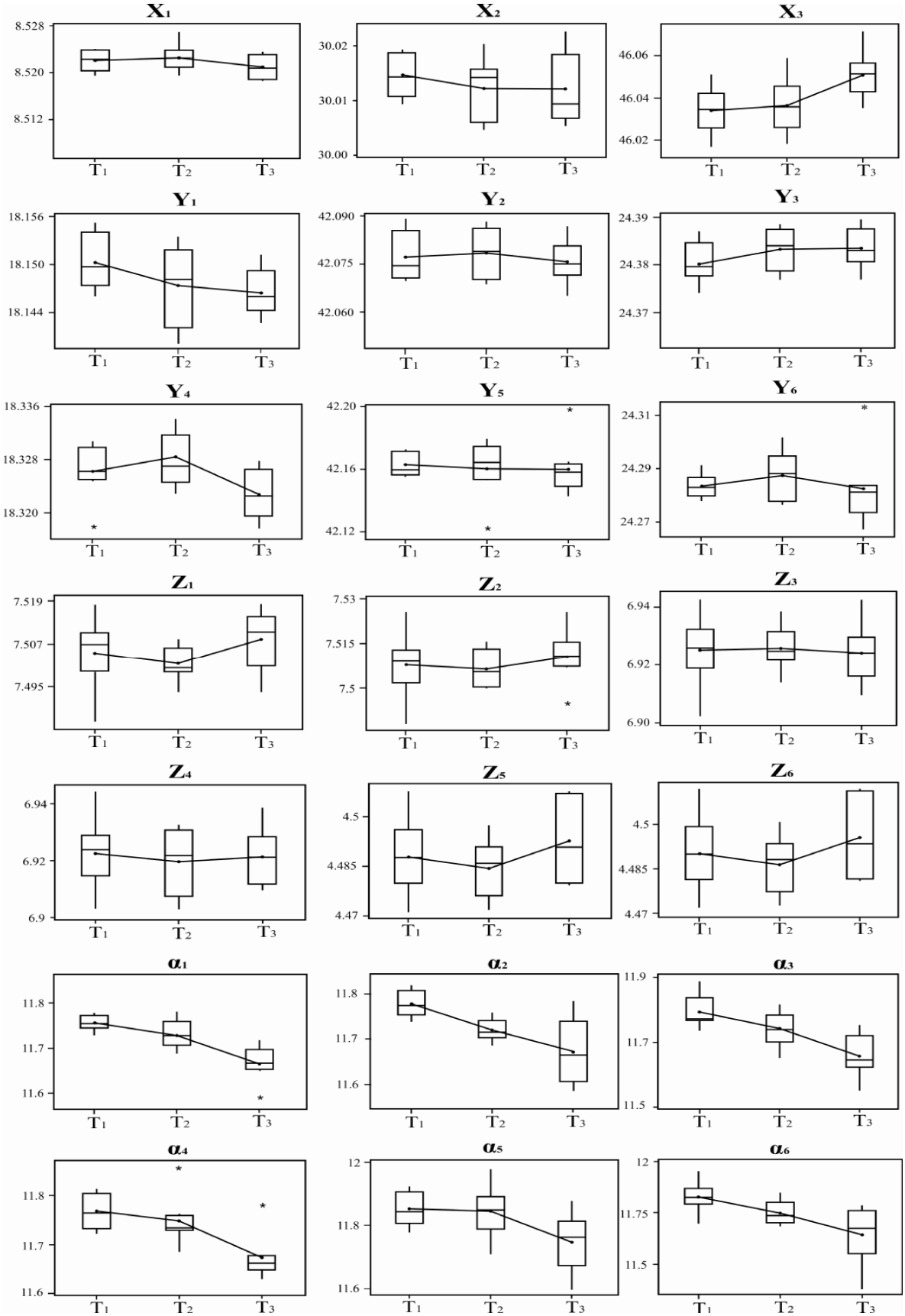

Fig. 6 – Mutual comparison of custom impression trays with 1, 2 and 3 mm spacing (T_1, T_2 and T_3, respectively).

Discussion

The results of this study show that both the experimental master model and the technique used for making the dental impressions have proven to be a reliable method for assessment of the accuracy of dental impression materials. Working casts obtained from all the three custom trays were, in most cases, significantly different from the experimental master model. Most evident deviations were recorded in the sagittal (Y_2) and transversal plane (X_3) for all of the working casts. These were also the greatest distances among the measured abutments, so the differences were most likely to be observed. The measurement of all of the working casts was done by the principles of 3D measurement. Each surface of the abutments was measured with 50–100 surface points. The coordinates of surface points served as a base for measuring the geometry of the abutments. Due to a large number of data and easier analysis of measurement, the results were presented as a cross-section of the model in one plane, which in this case passed through the base of the abutments. This is why the results are presented as the classical 2D measurement, but the principles of measurement are related to the entire surface of the measured model and additional data can be withdrawn for each additional cross-section [25].

The impression material used in this study had a defined polymerization contraction of ≤ 0.2%, while expansion of gypsum was claimed to be 0.19% by the manufacturer. Expansion of gypsum would thus compensate for polymerization contraction of the impression material, but it should be noted that these values refer to the two sizes that are not equal, *ie* the thickness of the impression material in accordance with the height and width of the gypsum abutments [26]. Thickness of the impression material varied from 1 to 3 mm from each side of the abutment, while the height and width of the abutments varied between 4.5 and 7.5 mm. In relation to this, expansion of gypsum should be a more dominant factor, which was proven by this study. Additionally, the influence of the micromechanical retention of the impression material to a custom tray should also be considered. In this case, the expansion of gypsum will be superimposed with the impression materials contraction towards the tray's walls, so that the combined effect will be presented by even wider abutments. As for the height of the abutments, where the upper surface was smaller than the side surface of the custom tray, in most cases the height of the abutments was smaller than that of the master model ($Z_{1\text{-}6}$). This can also be explained by impression materials contraction towards the walls. As the side surface of the tray had a bigger contact area than the upper one, the impression material was pulled down, which resulted in shorter abutments. These results are in accordance with other studies where tray adhesive was used in their research protocols [27–29].

The previously described process indicates that the silicone addition had a good retention with a custom tray made of epoxy resin by the RP technique. Although the RP technique is not widely used in the field of dentistry, it shows promise for manufacturing of dental devices. Its use in everyday practice, as a rational method for fabrication of custom trays, requires advanced systems for intra- or extraoral scanning, together with an adequate software connected to an RP machine, which is currently a limiting factor. Additive technologies such as RP are mainly being used for the production of dental copings for fixed dental restoration, fabrication of surgical guides in implant dentistry and in reconstructive maxillofacial surgery [30, 31]. As there is a large variety of materials that can be used for the production of custom trays with RP technology in different working regimes, these results are just a starting point and require additional investigation.

The analysis of convergence of axial surfaces showed that the largest deviations were observed in the third custom tray (α_1 = -0.18°), while deviations of the second tray ($\alpha_{1,2}$ = -0.11°) and the first tray (α_1 = -0.09°) were smaller. The angle of convergence was measured by scanning the axial surface and further software processing. Although small, deviations detected were oriented towards the increase of convergence. Convergence of the axial wall of the master model abutments was set to 6° (total of 12° when observing both axial planes), while gypsum abutments were detected to have more of a taper. The increase of the convergence angle reduces the overall surface of the abutment, which can influence the retention of the dental restoration [32]. While the width of the abutments was wider in the base cross-section, the axial surface has proven to have more taper. This could be explained by the increase of thickness of the impression material towards the upper base of the abutment and lower retention to the impression tray. Also, during removal of the impression the material deforms elastically. The resulting effect will be more pronounced in the axial surface, because of the overall increase of contact surface and direction of removal force away from the base of the abutment. This may all together explain the behaviour of the material to contract towards the walls of the tray in the base area, while slightly contracting inwards as the height progresses.

The results of this study show that all three custom trays performed satisfactory, but slightly better results were obtained with custom trays with 1 or 2 mm spacing. This is contrary to the claim that the thickness of the impression material should be at least 3 mm to prevent distortion of the material [33]. Due to a problem of correct positioning of the impression tray when thickness of the material is low, the custom tray with 2 mm spacing should be recommended for use in the clinical practice. This is considered to be beneficial regarding the accuracy of the impression, ease of handling and reduction of the quantity of material. Making a precise impression is especially important when constructing long-span bridges, because the accuracy of fit is harder to achieve as the number of abutment teeth and the distance between them increases. In this case, custom trays should be recommended, because they provide uniform thickness of impression material that can influence the accuracy of working casts. The issue of impression material bulk and its effect on accuracy of working casts has been previously adressed. Plausible results were obtained up to 5 mm of the impression material [14–18, 34, 35].

Difficulty of comparison of our own results with other studies, lies in the complexity of the methodological procedure. Even comparison with studies that used the monophasic impression technique, as it was in this study, should be observed not only by input, but also by output parameters, which are obtained through the use of the measurement instrument. As most of the studies used different measurement procedures, a relevant overview is hard to achieve, so we will restrain from further data comparison.

Limitations of this study are related primarily to the method of measurement. Although CMM is an instrument of great accuracy and precision, it is necessary to know the geometry of the measurement object in order to obtain reliable results [3]. Because the measurement is done by a relatively small amount of surface points (in this study up to 100), the measurement object has to be clearly defined before the measurement takes place. Since the master model of known dimensions and shape was used, measuring in this study is characterized by considerable accuracy and precision.

Based on the obtained data, future studies would be based on determing of accuracy of the rest of the production process of dental restorations, including production of wax patterns and the casting procedure. Detection of flaws in each of the stages of the production procedure, will provide useful guidelines for the dentists and dental technicians for the improvement of the quality of dental restorations.

Conclusion

The results of this study show that the impression material bulk of 1–3 mm could provide accurate working casts when using a monophasic impression technique. The increase of the distance between abutment teeth influences the accuracy of working casts. Custom trays produced by rapid prototyping can be successfully used for dental impressions. The experimental master model, combined with a CMM, is a reliable tool for assessment of dental impression accuracy.

Acknowledgements

The results presented in this paper were obtained in the framework of the project entitled "Research and development of modelling methods and approaches in manufacturing of dental recoveries with the application of modern technologies and computer aided systems" – TR 35020, financed by the Ministry of Education, Science and Technological Development of the Republic of Serbia.

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Received on January 5, 2015.
Revised on March 17, 2015.
Accepted on May 19, 2015.
Online First May, 2016.

ORIGINAL ARTICLE

UDC: 579.85:615.33
DOI: 10.2298/VSP150415134S

Antimicrobial susceptibility and β-lactamase production in *Bacillus cereus* isolates from stool of patients, food and environment samples

Osetljivost na antibiotike i proizvodnja β-laktamaza kod *Bacillus cereus* izolata iz stolice pacijenata, hrane i okoline

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Abstract

Background/Aim. *Bacillus cereus* (*B. cereus*) usually ingested by food can cause two types of diseases: vomiting due to the presence of emetic toxin and diarrheal syndrome, due to the presence of diarrheal toxins. Systemic manifestations can also occur. The severe forms of disease demand antibiotic treatmant. The aim of this study was to determine the differences in antibiotic susceptibility and β-lactamase activity of *B. cereus* isolates from stools of humans, food and environment. **Methods.** Identification of *B. cereus* was performed with selective medium, classical biochemical test and polymerase chain reaction (PCR) with primers specific for *bal* gene. Thirty isolates from each group were analysed for antibiotic susceptibility using the disk-diffusion assay. Production of β-lactamase was determined by cefinase test, and double-disc method. **Results.** All strains identified as *B. cereus* using classical biochemical test, yielded 533 bp fragment with PCR. Isolates from all the three groups were susceptible to imipenem, vancomycin, and erythromycin. All isolates were susceptible to ciprofloxacin but one from the environment. A statistically significant difference between the groups was confirmed to tetracycline and trimethoprim-sulphamethoxazole sensitivity. A total of 28/30 (93.33%) samples from the foods and 25/30 (83.33%) samples from environment were approved sensitive to tetracycline, while 10/30 (33.33%) isolates from stools were sensitive. Opposite to this result, high susceptibility to trimethoprim-sulphamethoxazole was shown in samples from stools (100%), while isolates from foods (63.33%) and from environment (70%) had low susceptibility. All samples produced β-lactamases. **Conclusion.** The strains of *B. cereus* from all the three groups showed high rate of sensitivity to most tested antibiotics, except to tetracycline in samples from human stool and to trimethoprim-sulphamethoxazole in samples from food and environment. The production of β-lactamases was confirmed in all the strains.

Key words:
bacillus cereus; anti-bacterial agents; drug resistance, microbial; beta-lactamases.

Apstrakt

Uvod/Cilj. *Bacillus cereus* (*B. cereus*) koji se u organizam čoveka unosi uglavnom putem hrane, može izazvati dva tipa oboljenja: povraćanje usled prisustva emetičkog toksina i dijarealni sindrom, usled prisustva dijarejnih toksina. Moguće su i sistemske manifestacije. Teže forme bolesti zahtevaju lečenje antibioticima. Cilj ove studije bio je da se ispitata osetljivost na antibiotike i utvrdi proizvodnja β-laktamaza kod sojeva *B. cereus* izolovanih iz stolice ljudi, hrane i okoline. **Metode.** *B.cereus* je identifikovan primenom selektivne podloge, klasičnog biohemijskog testa i metodom lančane reakcije polimeraze (PCR) pomoću prajmera specifičnih za *bal* gen. Iz svake grupe analizirana je osetljivost na antibiotike kod 30 izolata, disk-difuzionom metodom. Proizvodnja β-laktamaza rađena je Cefinaza testom i duplom disk metodom. **Rezultati.** Kod svih sojeva identifikovanih kao *B. cereus* primenom biohemijskog testa, metodom PCR umnožen je fragment od 533 bp. Izolati iz sve tri grupe bili su osetljivi na imipenem, vankomicin i eritromicin. Na ciprofloksacin su bili osetljivi svi sojevi osim jednog iz okoline. Statistički značajna razlika između grupa utvrđena je za osetljivosti na tetraciklin i trimetoprim-sulfametoksazol. 28/30 (93,33%) uzoraka iz hrane i 25/30 (83,33%) uzoraka iz okoline bili su osetljivi na tetraciklin, dok je samo 10/30 (33,33%) uzoraka stolice bilo osetljivo. Nasuprot ovim rezultatima, visoka osetljivost na trimetoprim-sulfametoksazol

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utvrđena je kod uzoraka iz stolice i iznosila je 100%, dok je kod izolata iz hrane i okoline bila niža i iznosila je 63,33% i 70%. Svi izolati proizvodili su β-laktamaze. **Zaključak.** Izolati *B. cereus* iz sve tri grupe pokazali su visoku osetljivost na većinu testiranih antibiotika, osim na tetraciklin iz uzoraka poreklom iz stolice i na trimetoprim/sulfametoksazol iz uzoraka hrane i okoline. Produkcija β-laktamaza potvrđena je za sve izolate.

Ključne reči:
bacillus cereus; antibiotici; lekovi, rezistencija mikroorganizama; beta laktamaze.

Introduction

Bacillus cereus, the Gram-positive, spore-forming opportunistic human pathogen, is found frequently as a saprophyte in the environment: many types of soils, sediment, dust and plants [1]. From all these habitats it is easily transferred to food, and to intestinal tract of invertebrates and mammals. *B. cereus* can be found in different foods and food ingredients (rice, dairy products, spices, dried foods, vegetables) and cross-contamination can distribute spores or vegetative cells to other foods (meat, milk). Spores of *B. cereus* are resistant to harsh environments, heat, dehydration, gastric acid and other physical stresses [2]. Regardless of thermal and other types of food processing, a human can be infected by spores that germinate and grow in the intestinal tract. But, disease can be caused by toxins already present in food performed by bacteria *B. cereus* that has also been isolated from stools of healthy humans [1, 3, 4].

B. cereus causes two distinct types of food poisoning in humans: the diarrhoeal (termolabile toxin) and emetic (termostabile toxin) type. Both types can seriously ruin human health [5], causing severe infections including sepsis, meningitis, endocarditis, endophthalmitis, respiratory and surgical wound infections [6]. Recently *B. cereus* was connected to hospital infection [7]. In some countries, diarrheal disease has been a major public health problem causing high morbidity and mortality among children [8].

Resistance to antibiotics is an increasing problem today. It is known that *B. cereus* has developed innate mechanisms of resistance through production of β-lactamases [9, 10]. In *B. cereus*, the production of β-lactamases can lead to resistance even up to the third generation of cephalosporins [9, 11]. Excessive use of antibiotics has led to increased antimicrobial resistance in various bacterial species [12]. Bearing in mind the circulation of *B. cereus* in nature, from soil to plants and different animals (insects, arthropods, others invertebrate and mammalian) to humans, resistance to antibiotics, under certain conditions, can be linked to transfer of resistance genes [9, 13, 14]. It is known that genes for resistance are transferred between the strains in *Bacillus* groups, and between different species [15–17]. However, wild types of strains isolated in nature and in patients, previously not exposed to effects of antibiotics and disinfectants, usually, are more sensitive to antibiotics.

Therefore, this study was conducted to determine the differences of *B. cereus* isolates from stools of patients, food and environment in antibiotic susceptibility and β-lactamase activity.

Methods

Samples

During 2013, 62 diarrhoeal stool specimens collected from outpatients and inpatients, were obtained at the Center for Microbiology, Institute of Public Health, Niš. At the same period, 40 specimens from different types of food (tea, dietary products, spices, milk powder, and ham) and 146 specimens from the environment (110 from soil, 36 from hospital environment) were collected in routine work at the Department of Sanitary Microbiology, Institute of Hygiene, Military Medical Academy, Belgrade and Department of Microbiology, Genetic Laboratory, Institute of Soil Science, Belgrade.

The samples were classified into three groups: isolates from patients, different types of food and from the environment. *B. cereus* ATCC 11778 was used as positive control.

Identification of B. cereus isolates

For identification of *B. cereus*, the first step was screening for the presence of β-hemolysis on 5% sheep blood agar, following the procedure of Collins et al. [18]. After that, positive isolates were tested on the selective Mannitol egg yolk polymyxin agar (MYP) for *B. cereus* (HiMedia, India). Detection of pink colonies and lecithinase reaction indicated that isolates belonged to *B. cereus.* In Gram-staining preparations it appeared as characteristic Gram positive, spore forming bacterium with spore not wider than the body of bacilli. In addition, *B. cereus* was determined with interactive database by using BBL Crystal GP ID Biochemical profiles.

Polymerase chain reaction and detection of bal gene

Polymerase chain reaction (PCR) assay was used for identification of *B. cereus* group (*balFR* gene), using a specific primer (Invitrogen, Vivogen D.O.O.).

For PCR, DNA samples were prepared from a single colony of each isolate of *B. cereus.*They were incubated in the brain-heart infusion broth at 37ºC for 18–24 h. A pellet of 1 mL of overnight culture was rinsed in saline solutions, resuspended in 500 μL of distilled water, and boiled for 10 min. The prepared DNA was used directly for PCR or stored at -20ºC until use.

A PCR mixture was prepared in a volume of 25 μL, with DreamTaqGreen Master Mix (ThermoScientific, Lithuania), 200 nM final concentration of each primer, and 2.5 μL of prepared DNA template. The primer sequences and PCR conditions were the same as described earlier [19]. PCRs

were performed on thermocycler EppeddorfMasterCycler (Eppendorf, Germany).

The PCR products were separated on 1.5% agarose gel (ICN Biomedicals) using elektrophoresis system (Pharmacia LKB), stained with ethidium bromide, visualized on a UV transilluminator (Shimadzu 160UV-Vis) and photographed by the gel documentation system.

Susceptibility testing for antimicrobial agents

Sensibility of *B. cereus* isolates was tested using the disk-diffusion assay recommended by the Clinical and Laboratory Standards Institute (CLSI, 2006) on Mueller Hinton agar (HiMedia, India) plates. Each isolate grown overnight on MYP agar at 37ºC was taken for this test. Fresh bacterial colonies were inoculated in 0.8% NaCl suspension to a turbidity equivalent to a 0.5 McFarland standard. The culture was applied on the Mueller Hinton agar plate using sterile cotton swab. Discs of ampicillin (10 μg), penicillin G (10 U), tetracycline (30 μg), trimethoprim-sulphamethoxazole (1.25/23.75 μg), erythromycin (15 μg), ciprofloxacin (5 μg), gentamicin (10 μg), vankomycin (30 μg) and imipenem (10 U); (Bionalyse, Ankara, Turkey) were placed on the plate. Plates were incubated at 37ºC for 24 h and the diameter of the inhibition zone was determined according to the CLSI (CLSI, 2013) guidelines for *Staphylococcus* spp. Based on the zone of inhibition, strains were classified as sensitive (S), intermediate (I), resistant (R). The strains with intermediate sensitivity were classified in the group of sensitive ones, to the statistical processing.

The production of β-laktamases – penicillinases was determined by cefinase test (Cef-F, *bioMérieux*, *Marcy l'Etoile*, France), while cephalosporinases were detected using double disc method (ampicillin-clavulonic acid (20 μg /19 μg), ceftazidim (30 μg), cefotaxim (30 μg) [20].

Statistical analysis

For statistical analysis the Fisher and Chi-square tests were used. A *p*-value less than 0.01 was considered statistically significant. All statistical analyses were performed with the SPSS statistical software for Windows version 11.5 (SPSS Inc., Chicago, USA).

Results

Pink colonies on MYP agar plates with positive lecithinase reaction, giving β hemolyisis on sheep agar, were used for identification with BBL Crystal. Thirty *B. cereus* isolates identified in each group were taken for further analysis. Belonging to a *B. cereus* group was confirmed by PCR. All *B. cereus* isolates from stools, food and environment yielded 533 bp amplified fragments with primer pair BalF/BalR specific for *B. cereus* group (Figure 1).

Disk diffusion susceptibility testing revealed that all *B. cereus* isolates from stool, food and environment were susceptible to imipenem and vancomycin (Table 1). Furthermore, all *B. cereus* isolates from stools of patients and from food were susceptible to erythromycin and ciprofloxacin. Similarly, all *B. cereus* isolates from environment were sensitive to erythromycin, with only one strain resistant to ciprofloxsacin (3.33%).

There was a statistically significant difference on susceptibility to tetracycline and trimethoprim-sulphamethoxazole between the samples from stools as compared to the samples from foods and the environment. The samples from different foods 28/30 (93.34%) and those from environment 25/30 (83.33%) were sensitive to tetracycline, while 10/30 (33.33%) isolates from stools of humans were sensitive to this antibiotic ($p < 0.001$, Fisher Exact Probability Test). Opposite to this result, high susceptibility to trimethoprim-sulphamethoxazole

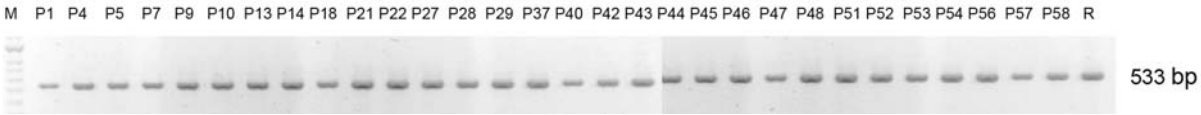

Fig. 1 – *B. cereus* group specific polymerase chain reaction (PCR). Line M: 100 bp DNA ladder; Lines P1-Z58: *B. cereus* isolates; Line R: *B. cereus* reference strain ATTC 11778.

Table 1

Susceptibilities of *B. cereus* strains to the selected antibiotics by disk diffusion susceptibility testing

Antibiotic disc (disk content)	Stool (n = 30)		Food (n = 30)		Environment (n = 30)	
	n (%)		n (%)		n (%)	
	S	R	S	R	S	R
Ampicillin (10 μg)	21 (70)	9 (30)	0	30 (100)	1 (3.33)	29 (96.67)
Penicillin	21 (70)	9 (30)	0	30 (100)	1 (3.33)	29 (96.67)
Imipenem (10 U)	30 (100)	0	30 (100)	0	30 (100)	0
Vankomycin (30 μg)	30 (100)	0	30 (100)	0	30 (100)	0
Ciprofloxacin (5 μg)	30 (100)	0	30 (100)	0	29 (96.67)	1 (3.33)
Erythromycin (15 μg)	30 (100)	0	30 (100)	0	30 (100)	0
Tetracycline (30 μg)	10 (33.33)	20 (66.67)	28 (93.33)	2 (6.67)	25 (83.33)	5 (16.67)
Trimethoprim sulphamethoxazole (1.25/23.75 μg)	30 (100)	0	19 (63.33)	11 (36.67)	21 (70)	9 (30)

S – sensitive; R – resistant

was shown in all samples from stools (100%), while strains from foods and environment in 63.33% (19/30) and 70% (21/30) samples, respectively, had low susceptibility to this antibiotic ($p < 0.01$, Fisher Exact Probability Test) (Figure 2).

All samples were resistant to penicillin and ampicillin. Using the cefinase test in all isolates the production of inducible penicillinases was detected. The presence of cephalosporinases was approved with the double-disc method and the production of these β-lactamase was detected in all *B. cereus* isolates (Figure 3).

well as acting of antibiotics from microorganisms which originated from the soil [15, 17]. Therefore, it was of interest to compare the resistance of *B. cereus* isolates from different environments.

All the tested *B. cereus* isolates were resistant to penicillin and ampicillin. Complete resistance in all strains to these antibiotics and cephalosporins was the consequence of β-lactamases production which was detected by the commercial methods: nitrocefin test and the double-disk method, for detection of penicillinases, and cephalosporinases, respec-

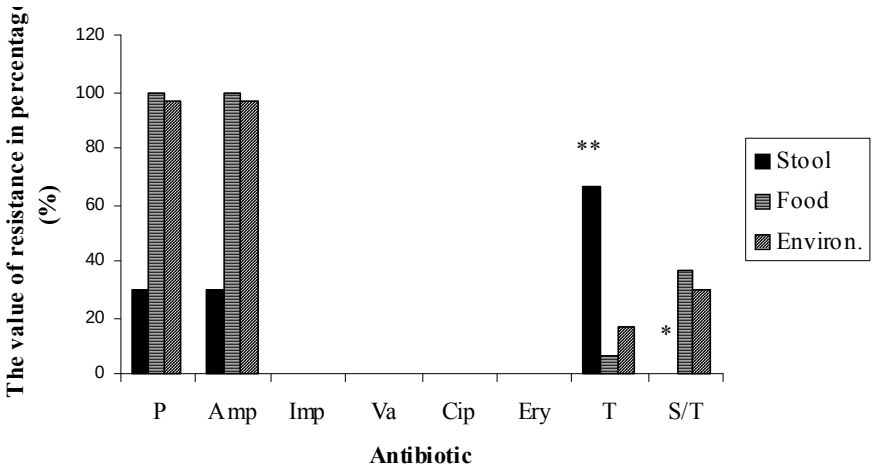

Fig. 2 – Resistance to the investigated antibiotics from different sources.
Amp: Ampicillin; P: Penicillin; Imp: Imipenem; Va: Vankomicin; Cip: Ciprofloxacin: Ery: Erythromycin; T: Tetracyclin; S/T: Trimethoprim sulphamethoxazole; **$p < 0.001$; *$p < 0.01$.
(statistically significant difference in resistance to tetracycline and trimethoprim-sulphamethoxazole was confirmed, by comparing isolates *B. cereus* from stools, food and environment).

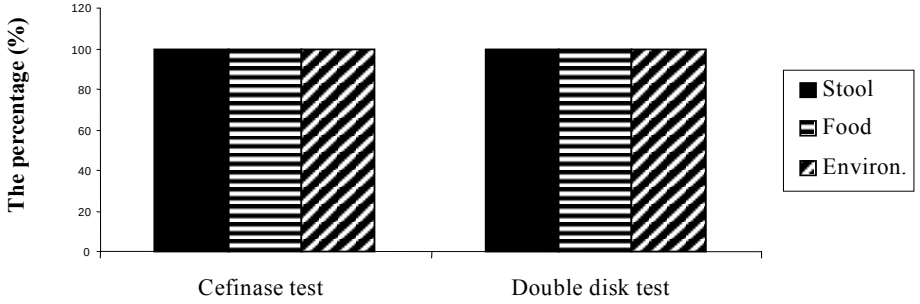

Fig. 3 – The activity of β-lactamases: penicilinases and cephalosporinases.
All *B. cereus* strains from all investigated sources produce β-lactamases.

Discussion

Pathogenic strains *B. cereus* from the environment may directly or indirectly be transmitted through food to man and cause damage to human health. In the transmission cycle, they can be exposed to different effects of environment, as

tively. Many other studies detected the existence of β-lactamases and therefore the resistance to penicillin, ampicillin and cephalosporins in different types of samples [9, 21–24]. Özcelik and Citak [11] assumed that there was the possibility of spreading resistance to β-lactam antimicrobial agents and sporadically resistance to erythromycin and tetracycline, in

strains of *B. cereus* which were isolated from ice-cream. However, there is no explanation whether the resistance of *B. cereus* strains from ice cream is because of transmission of resistant genes from microorganisms in digestive tract, process of conjugation or transduction, or strains already had resistant gene which is circulating in the environment. In addition to the presence of penicillinase and cephalosporinase, Godič-Torkar and Seme [25] confirmed the presence of metallo-β-lactamases in clinical and food samples of *B. cereus*.

All *B. cereus* strains from all the three groups investigated in this study were susceptible to imipenem, vankomycin and erythromycin. Susceptibility to the ciprofloxacin was shown in all the isolates from stools and food, but only one sample from environment was resistant to this antibiotic. Similar to this Banerjee et al. [26] received 100% sensitivity to ciprofloxacin and imipenem in samples from patients, and other authors [11, 14, 27] obtained the same result in testing sensitivity to ciprofloxacin in samples from food. Sensitivity to vancomicin and ciprofloxacin is confirmed by Jensen et al. [28] in *B. cereus* agricultural soil isolates from Denmark. In contrast to our results, Luna et al. [29] confirmed the resistance to karbapenem (meropenem) in 14% isolates from the environment in the USA. Similarly to our results, Özcelik and Citak [11] approved that only 1/34 isolates from ice-cream were resistant to erythromycin, but Oladipo and Adejumobi [14] showed the resistance to this antibiotic in all isolates from street food. In contrast to our results, Al-Khatib et al. [30] and Godič-Torkar and Seme [25] confirmed the resistance to erythromycin in about 40% samples from patient stools. Comparing the resistance to erythromycin between isolates from those of human stool, from meat and ready-to-eat meat products, Tewari et al. [10] determined the difference: 73.91% isolates from human stool were susceptible, while 48.3% and 54.5% from meat and meat products, respectively were resistant. As opposed to this, Aslim [22] and Luna et al. [29] indicated high level of sensitivity to erythromycin of *B. cereus* isolated from environment.

A statistically significant difference in the sensitivity to tetracycline and trimethoprim-sulphamethoxazole was confirmed by comparing isolates from stools, food and environment. Only 33.3% isolates from stools were sensitive to tetracycline, and 100% were sensitive to trimethoprim-sulphamethoxazole. Opposite to this result, a high rate of strains susceptible to tetracycline was shown in samples from the environment (83.33%) and from food (93.34%), but a low rate of susceptibility was detected to trimethoprim-sulphamethoxazole: from foods 63.33% and 70% in isolates from the environment. Similar to our results Özcelik and Citak [11] confirmed resistance to tetracycline in 6/34 isolates of *B. cereus* from ice cream, but Wong et al. [31] showed a high sensitivity to trimethoprim-sulphamethoxazole (78%) and slightly susceptibility to tetracycline (19%) from dairy products. The resistance to tetracycline in the strains from all samples of street vended food was confirmed by Oladipo and Adejumobi [14]. Aslim [22] found sensitivity to tetracycline in 93% samples from the soil and Luna et al. [29] showed 100% sensitivity to this antibiotic in environmental samples. However, the same authors indicate high sensitivity to trimethoprim-sulphamethoxazole (74%) in the tested samples.

We affirmed a difference in sensitivity to tetracycline and trimethoprim-sulphamethoxazole by comparing human stools samples and blood samples in suspected bacteremia [21]. In our study, the sensitivity to trimethoprim-sulphamethoxazole was found in 100% samples and 33.3% to tetracycline, but Weber et al. [21] showed 100% resistance to trimethoprim-sulphamethoxazole and 59% sensitivity to tetracycline. It is known that the resistance to tetracycline occurs through three mechanisms: producing ribosomal protection proteins, actively pumping the antibiotics out of the cell, or enzymatic degradation of antibiotics [32]. However, regardless of the mechanism of resistance, the spread of resistance is quick. Uncontrolled use of antibiotics in agriculture and food industry leads to favoring resistant strains of bacteria in the soil, and with them to transferring of the gene for resistance through food chain.

The question arises: Where does the presence of high resistance to tetracycline in samples from stool of patients and something lower resistance to trimethoprim-sulphamethoxazole in samples from food and environment come from? On the one hand, perhaps the resistance can be related to uncontrolled use of antibiotics, especially tetracycline, in agriculture and veterinary medicine. On the other hand, the resistance can be related to uncontrolled use of antibiotics by patients. In both cases it is the presence of horizontal transfer of antibiotic resistance genes from intestinal bacteria in manure to the soil bacterial population and from the soil to the animal and human population.

Conclusion

Since *B. cereus* can be associated with serious infections, it is of great importance to register the resistance to a particular antibiotic. In our study, the strains of *B. cereus* from all the three investigated groups showed a high rate sensitivity to most tested antibiotics, except to tetracycline in samples from stool of patients and to trimethoprim-sulphamethoxazole in samples tested from food and environment.

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Received on April 15, 2015.
Accepted on June 3, 2015.
Online First November, 2015.

ORIGINAL ARTICLE

UDC: 616.36-02
DOI: 10.2298/VSP150514093P

Obesity and metabolic syndrome as risk factors for the development of non-alcoholic fatty liver disease as diagnosed by ultrasound

Gojaznost i metabolički sindrom kao faktori rizika od razvoja nealkoholne masne bolesti jetre dijagnostikovane ultrazvukom

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Abstract

Background/Aim. Non-alcoholic fatty liver disease (NAFLD) is a chronic liver disease of a broad histological spectrum, characterized by the accumulation of triglycerides in more than 5% of hepatocytes in the absence of consuming alcohol in quantities harmful to the liver. The aim of our study was to determine the importance of anthropometric and laboratory parameters as well as metabolic syndrome for the diagnosis of NAFLD and to estimate their influence on the degree of liver steatosis as evaluated by ultrasound. **Methods.** The study included 86 participants, 55 of whom had fatty liver diagnosed by ultrasound and they comprised the study group. The control group consisted of 31 subjects with no liver diseases. During the course of hospitalization at the Clinic of Gastroenterology and Hepatology, Clinical Centre Niš, the patients had their anamnesis taken, and anthropometric measurements as well as biochemical blood analyses and abdominal ultrasound were performed. **Results.** The patients with NAFLD had statistically higher values of body mass index (BMI), waist circumference (WC), systolic (SBP) and diastolic blood pressure (DBP), levels of alanine and aspartate aminotransferase (ALT, AST), gamma-glutamyl transpeptidase (GGT) ($p < 0.001$), low-density lipoprotein cholesterol (LDL), total bilirubin (TBIL) ($p < 0.05$), total cholesterol ($p < 0.01$), triglycerides (TGL), urates, C-reactive protein (CRP), ferritin, fibrinogenes, fasting blood glucose (FBG), insulin and Homeostasis Model Assessment (HOMA-IR) ($p < 0.001$) compared to the control group, whereas the levels of high-density lipoprotein cholesterol (HDL) were higher in the control than in the study group ($p < 0.05$). In the NAFLD group, there were statistically significantly more patients with hypertension (72.73% *vs* 12.90%, $p < 0.001$) and type 2 diabetes mellitus (DM) (47.27%). Metabolic syndrome was established in 48 (87.27%) patients of the study group. The equal number of patients, *ie* 16 (29.09%), had 3, 4 and 5 components of MS. In the NAFLD group there were 17 (30.91%) overweight (BMI from 25 kg/m^2 to 29.9 kg/m^2) and 38 (69.09%) obese patients (BMI ≥ 30.0 kg/m^2). The largest number of patients in the obesity group, 22 (40.00%) of them, had the first degree obesity (BMI from 30 kg/m^2 to 34.99 kg/m^2). The largest number of the NAFLD group patients, 23 (41.82%), had an ultrasound finding of grade 3 fatty liver, 20 (36.36%) patients had grade 2 and 12 (21.82%) grade 1 fatty liver. Kruskal-Wallis test and ANOVA analysis showed statistically significant differences between groups with different US grade for insulin, LDL-cholesterol, WC, BMI ($p < 0.05$), as well as HOMA-IR and body weight (BW) ($p < 0.01$). Metabolic syndrome was statistically more present in patients with US finding grades 2 and 3 ($p < 0.01$) in relation to US finding grade 1, as well as obesity, hypertension and DM type 2 ($p < 0.05$). **Conclusion.** The results of our study confirm that a high percentage of patients with high risk factors (DM, MS, dyslipidemia, hypertension) have NAFLD.

Key words:
obesity; metabolic syndrome x; ultrasonography; diagnosis; non-alcoholic fatty liver disease; risk factors.

Apstrakt

Uvod/Cilj. Bolest nealkoholne masne jetre (NAFLD) je hronično oboljenje jetre, širokog histološkog spektra, koje karakteriše akumulacija triglicerida u više od 5% hepatocita u odsustvu konzumiranja alkohola u količinama štetnim za jetru. Cilj našeg istraživanja bio je da se utvdi značaj antropometrijskih, laboratorijskih parametara i metaboličkog sindroma u dijagnozi NAFLD i da se proceni njihov uticaj na stepen steatoze jetre procenjene ultrazvukom. **Metode.** Istraživanjem je bilo obuhvaćeno 86 ispitanika, od kojih je 55 imalo masnu jetru, dokazanu ultrazvukom, i oni su činili studijsku grupu. Kontrolnu grupu činio je 31 ispitanik bez bilo kakve bolesti jetre. Prilikom hospitalizacije u Klinici za gastroenterologiju i hepatologiju Kliničkog centra Niš, ispitanicima je uzeta anamneza, izvršena su antropometrijska

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merenja, biohemijske analize krvi i ultrazvučni pregled abdomena. **Rezultati.** Bolesnici sa NAFLD imali su statistički značajno povećan *body mass index* (BMI), obim struka (OS), sistolni i dijastolni krvni pritisak, vrednosti aspartat i alanin aminotransferaze (AST, ALT), gama-glutamil transpeptidaze (GGT) ($p < 0,001$), lipoproteina niske gustine (LDL), ukupnog bilirubina ($p < 0,05$), ukupnog holesterola ($p < 0,01$), triglicerida, urata, C reaktivnog proteina (CRP), feritina, fibrinogena, glikemije našte, insulina i *Homeostasis Model Assessment* (HOMA-IR) ($p < 0,001$), dok je vrednost lipoproteina visoke gustine (HDL) bila veća u kontrolnoj grupi ($p < 0,05$). U NAFLD grupi bilo je statistički značajno više bolesnika sa hipertenzijom (72,73% *vs* 12,90%, $p < 0,001$) i sa diabetesom melitusom (DM) tipa 2 (47,27%). Metabolički sindrom utvrđen je kod 48 (87,27%) bolesnika studijske grupe. Podjednak broj bolesnika, 16 (29,09%), imao je 3, 4 i 5 komponenti MS. U NAFLD grupi bilo je 17 (30,91%) bolesnika sa predgojaznošću (BMI od 25 kg/m^2 do 29,9 kg/m^2) i 38 (69,09%) gojaznih (BMI ≥ 30.0 kg/m^2) bolesnika. Najveći broj bolesnika u grupi gojaznih, 22 (40,00%), bio je sa prvim stepenom gojaznosti (BMI od 30 kg/m^2 do 34,99 kg/m^2). Najveći broj bolesnika NAFLD grupe, 23 (41,82%), imao je ultrazvučni nalaz masne jetre gradusa 3, 20 (36,36%) gradusa 2 i 12 (21,82%) gradusa 1. Kruskal-Wallis test i ANOVA analiza pokazali su da postoje statistički značajne razlike između grupa sa različitim ultrazvučnim gradusom za insulin, LDL holesterol, OS, BMI ($p < 0,05$), kao i HOMA-IR i telesnu masu (TM) ($p < 0,01$). Metabolički sindrom bio je statistički zastupljeniji kod ispitanika sa UZ gradusom 2 i 3 ($p < 0,01$) u odnosu na UZ gradus 1, kao i gojaznost, hipertenzija i DM tip 2 ($p < 0,05$). **Zaključak.** Rezultati naše studije potvrđuju da veliki procenat bolesnika sa faktorima visokog rizika (DM, MS, dislipidemija, hipertenzija) ima NAFLD.

Ključne reči:
gojaznost; metabolički sindrom x; ultrasonografija; dijagnoza; jetra, masna infiltracija, nealkoholna; faktori rizika.

Introduction

Non-alcoholic fatty liver disease (NAFLD) has become the most common chronic liver disease in western countries in the past twenty years, with the prevalence of 20–30% in adults [1]. The prevalence of NAFLD increases parallelly with the epidemics of obesity and type 2 diabetes mellitus (DM), which are the risk factors for developing primary NAFLD [2, 3]. The disease was first described as an entity in 1980 by Ludwig et al.[4]. It is considered that primary NAFLD represents hepatic manifestation of metabolic syndrome (MS), and insulin resistance (IR) is the key pathophysiological mechanism [5, 6]. Non-alcoholic fatty liver disease is histologically categorized into non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH) [7]. Non-alcoholic fatty liver is a benign, non-progressive liver disease, which is histologically characterized by the presence of macrovesicular steatosis in more than 5% of hepatocytes in the absence of consuming alcohol in quantities harmful to the liver, whereas NASH means the presence of hepatic steatosis, lobular inflammation (acute and/or chronic) with hepatocyte damage (ballooning degeneration), with or without accompanying fibrosis [1, 6].

Within NAFLD spectrum, only patients with NASH develop progressive liver damage. NASH progresses to cirrhosis in 10–20% of cases, or more rarely to hepatocellular carcinoma (HCC) [8–10]. NAFLD is usually asymptomatic, clinically silent disease. It is mostly diagnosed incidentally, during routine laboratory blood tests, when higher transaminase values are detected, particularly alanine aminotransferase (ALT) or when ultrasound (US) examination shows fatty liver [3, 6, 11]. More than 80% of patients with NAFLD have normal transaminase values which can remain unchanged even during disease progression [12–14]. Therefore, NAFLD should be suspected in patients with determined risk factors. In establishing the diagnosis of primary NAFLD, four criteria should be met: confirmation of liver steatosis by imaging methods or pathohistologically; absence of consuming alcohol in significant quantities (less than 21 alcohol units for men and 14 units for women on a weekly basis); exclusion of other causes of liver steatosis, i.e. "secondary" NAFLD and exclusion of other etiological factors of chronic liver disease [7].

The primary and mostly used diagnostic method for screening asymptomatic patients with higher aminotransferase values and suspicion of NAFLD is US [15].

Ultrasound is a non-invasive, cheap, available method, with the sensitivity of 60–94%, and specificity of 66–97% [16]. Ultrasound changes in patients with NAFLD are characterized by hepatomegaly, hyperechogenicity of liver parenchyma ("bright" liver), hepatorenal contrast, attenuation of ultrasound waves in subcapsular regions, difficult visualization of the portal vein, gallbladder wall, liver capsule and blood vessels [17, 18]. However, US has certain disadvantages. The sensitivity and specificity of US in diagnosis of NAFLD is significantly lower in obese patients and if steatosis is less than 30% [19, 20]. It is not possible to differentiate steatosis from steatohepatitis and fibrosis by US [15, 21]. Therefore, liver biopsy is still the gold standard in diagnosing NAFLD.

The aim of our research was to determine the importance of laboratory and anthropometric parameters as well as MS in diagnosing NAFLD and to estimate their influence on the degree of liver steatosis as evaluated by US.

Methods

Prospective study was carried out in the period from January 2012 to October 2014, at the Clinic of Gastroenterology and Hepatology, Clinical Center Niš. It included 86 participants, 55 of whom had fatty liver infiltration diagnosed by US. They comprised the study group. The control group consisted of 31 subjects, where the diagnosis of non-alcoholic fatty liver disease and other liver diseases were ruled out on the basis of anamnestic data, biochemical blood analyses and US examination. Inclusion criteria in the study were patients with higher transaminase values and echoso-

nographic finding of fatty liver. Exclusion criteria from research were alcohol consumption (more than 30 g/day for men, and 20 g/day for women), use of hepatotoxic drugs, the presence of metabolic or genetic liver diseases (Wilson's disease, hemochromatosis, α 1-antitripsin deficiency), acute and chronic virus hepatitis (hepatitis B and hepatitis C), autoimmune liver disease (primary biliary cirrhosis, primary sclerosing cholangitis, autoimmune hepatitis). The participants signed the informed consent and thus confirmed that their anamnestic, laboratory and histopathological findings could be used for the purpose of this study.

During hospitalization demographic data and a detailed anamnesis were taken from the patients regarding presence of hypertension, diabetes, existing liver diseases, use of hepatotoxic drugs and alcohol consumption.

Physical examination, anthropometric measurements, biochemical blood analyses and abdominal US were performed. Anthropometric measurements included measurement of body weight (BW), body height (BH) and waist circumference (WC). Body weight (kg) of patients was measured in light clothes, without shoes. Body height (cm) was measured using the standard measuring equipment and the scale.

Waist circumference (cm) and body mass index (BMI) were estimated in each patient according to criteria of the World Health Organization [22]. Waist circumference was taken by flexible meter in standing position, midway between the lower edge of the rib cage and *crista iliacus* horizontally. On the basis of the given values of BW and BH we calculated the value of BMI, as the ratio of BW in kilograms and body height in m^2 (kg/m^2). Following these values of BMI, the patients were divided in several groups: BMI from 25 to 29.9 kg/m^2 – overweight or preobesity, BMI from 30 to 34.99 kg/m^2, class I or mild obesity, BMI from 35 to 39.99 kg/m^2 class II or moderate obesity, BMI higher than 40 kg/m^2, class III or severe obesity.

Arterial pressure was measured by sphygmomanometer in a seating position, after resting the patient for 10 minutes. Values of systolic (SBP) and diastolic blood pressure (DBP) were taken (mmHg). All examinees included in the study had their complete blood count (CBC) and biochemical blood analysis taken in the Central Laboratory, Clinical Centre Niš (Beckman Coulter, AU680). Biochemical blood analyses included C-reactive protein (CRP), activity of ALT and aspartate aminotransferase (AST), alkaline phosphatase (ALP), gamma-glutamyl transpeptidase (GGT), values of total bilirubin (TBIL) and direct bilirubin (DBIL), urates, ferritin, transferrin saturation, ceruloplasmin, iron, fasting blood glucose (FBG), insulin, values of total cholesterol, high-density lipoprotein cholesterol (HDL), low-density lipoprotein cholesterol (LDL), triglycerides (TGL), total proteins and albumins, prothrombin time, international normalized ratio (INR) and fibrinogen. Ratio AST/ALT was determined. Insulin resistance was estimated by model formula Homeostasis Model Assessment [23] (HOMA):

$$\text{HOMA-IR} = \frac{\text{glycaemia (mmol/L)} \times \text{insulin (mU/L)}}{22.5}$$

After detailed examination of the available medical documentation of patients, in certain cases (incomplete medical documentation or diagnostic dilemma) additional serological analyses were performed including: serological examination of viral hepatitis B and C (HBs Ag, anti HCV At), immune complexes, immunoglobulins (IgG, IgA, IgM) and pathological antibodies (antimitochondrial, anti-smooth muscle, antinuclear antibodies) and determining the value of α 1-antitripsin.

The presence of MS and its components were analyzed in each patient using the American National Cholesterol Program definition (The National Cholesterol Educational Program Adult Treatment Panel – NCEP– ATPIII) [24]. Metabolic syndrome is present if the patient shows at least three out of the following five components: central abdominal obesity (WC > 102 cm in men, and > 88 cm in women, respectively), increased triglyceride level: ≥ 1.7 mmol/L, lower HDL-cholesterol level: < 1.03 mmol/L in men, and < 1.29 mmol/L in women respectively, higher blood pressure: systolic ≥130 mmHg and/or diastolic ≥ 85 mmHg, or already treated hypertension; increased FBG: ≥ 5.6 mmol/L, or existing type 2 DM.

Liver US was performed in the morning with previous regime of abstaining from food the night before the examination (instrument ACUSION Siemens model X 300 and ultrasound probe of 3.5 MHz).

We evaluated the size and structure of the liver parenchyma, echo contrast between the liver and the right kidney, degree of parenchyma echogenicity, degree of blood vessels visualization, the diaphragm, as well as the posterior segment of the right lobe, or the degree of attenuation of US waves.

The degree of liver parenchyma fatty infiltration or steatosis evaluated by US can be divided into three stages depending on the severity of US changes: grade 1 US finding, mild steatosis which is shown on US as moderately hyperechogenic parenchyma, with the visible portal vein and the diaphragm (Figure 1A); grade 2 US finding, mild steatosis when the parenchyma is more prominently hyperechogenic, so that intrahepatic blood vessels and diaphragm are less visible (Figure 1B); grade 3 US finding – the liver is highly hyperechogenic, without possibility of good visualization of the portal vein, diaphragm, posterior segment of the right lobe, that is, attenuation of US waves is present [25] (Figure 1 C).

This study was approved by the Ethical Committee of the Faculty of Medicine, University of Niš.

Statistical analysis

Continuous parameters were shown by the mean values, standard deviations and medians. Attributive parameters were presented in frequencies and percentages. Normality of distribution of continuous variables was examined by Shapiro-Wilk or Kolmogorov-Smirnov test depending on the size of examinee groups. Values of continuous parameters of two independent samples were compared by Mann-Whitney and Student *t*-test of independent samples, while comparison of 3 independent samples was performed by Kruskal-Wallis test or ANOVA (depending on the normality of distribution of

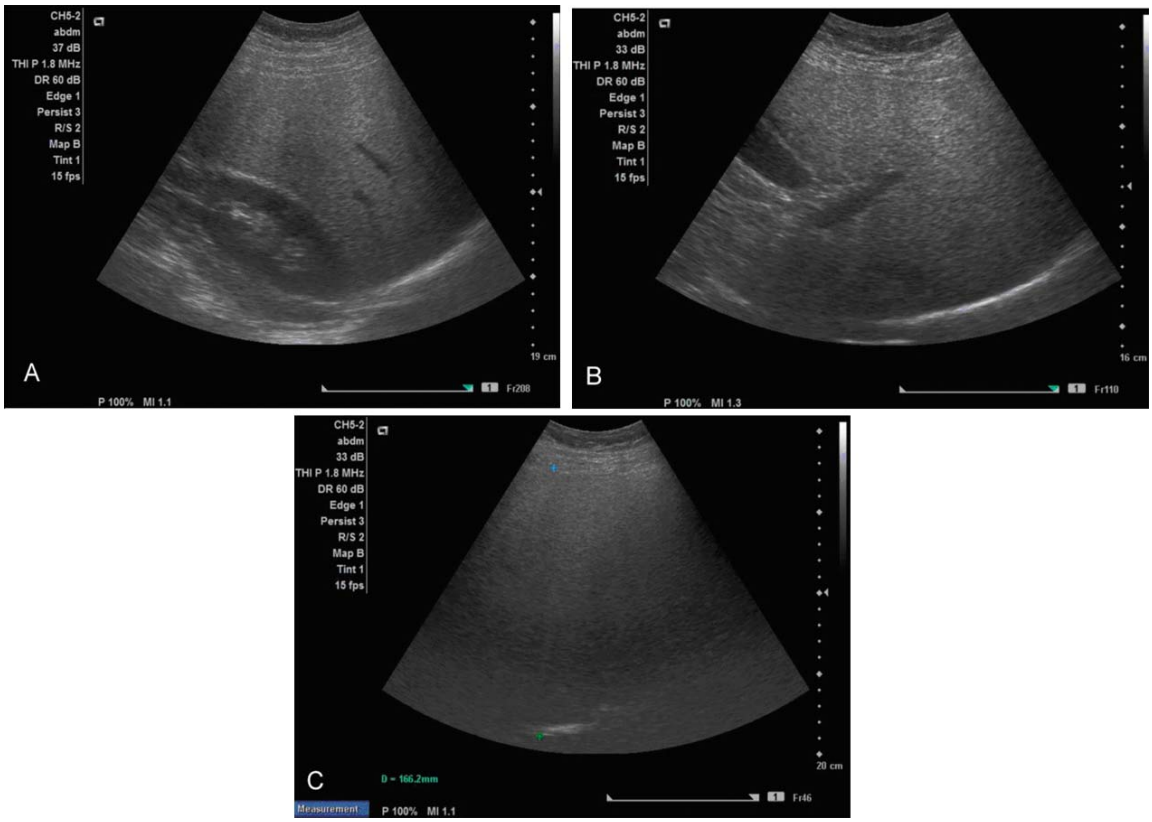

Fig. 1 – Ultrasound findings of liver steatosis: A) grade 1; B) grade 2, and C) grade 3.

variables compared), Pearson χ^2 test, and, if necessary, in case of contingency 2 × 2 table, Mantel-Haenzel or Fisher's exact probability test for comparing frequency and distribution of attributive parameter modalities.

Results

Eighty-six participants were included in the study. The NAFLD (study) group was comprised of 55 (63.95%) patients, whereas the control group included 31 (36.05%) subjects. The mean age of patients [x ± SD (median)] in the NAFLD group was 49.29 ± 12.95 (52.00) and in the control group 47.84 ± 10.08 (49.00) years (Table 1). In the NAFLD group, there were 23 (41.82%) male and 32 (58.18%) female patients, and in the control group 10 (32.26%) male, and 21 (67.74%) female subjects. A statistically significant difference in age and sex of participants in the NFLD and control groups was not found.

The BMI value was statistically significantly higher in the NAFLD group in reference to the control group (32.83 ± 4.20 *vs* 22.52 ± 2.08 kg/m^2), as well as WC (106.36 ± 8.44 cm *vs* 78.87 ± 7.18 cm) ($p < 0.001$) (Table 1).

In the NAFLD patients, statistically significantly higher values of LDL-cholesterol were found, as well as those of TBIL ($p < 0.05$), total cholesterol ($p < 0.01$), TGL, urates, CRP, ferritin, fibrinogene, FBG, insulin and HOMA-IR ($p < 0.001$), while the value of HDL-cholesterol was higher in the control group ($p < 0.05$). Values of INR and platelets did not statistically differ in the examined groups. In the NAFLD group, the values of AST, ALT, GGT ($p < 0.001$) and ALP ($p < 0.01$) were significantly higher (Table 1).

In the NAFLD group, there were statistically significantly more patients with hypertension (72.73% *vs* 12.90%, $p < 0.001$) (Table 2), with values of systolic and diastolic blood pressure significantly higher when compared to the control group ($p < 0.001$) (Table 1). Type 2 DM was present in 26 (47.27%) patients of the NAFLD group, while there were no diabetes patients in the control group ($p < 0.001$) (Table 2).

Most patients in the NAFLD group, 23 (41.82%) of them, had an US finding of fatty liver grade 3, and there were 20 (36.36%) patients with grade 2 finding and 12 (21.82%) with grade 1 finding.

Kruskal-Wallis test and ANOVA analysis showed statistically significant differences between the groups with different US grade for insulin, LDL-cholesterol, WC, BMI ($p < 0.05$), as well as HOMA-IR and BW ($p < 0.01$) (Table 3).

Based on Student's *t*-test and Mann-Whitney test, by comparing the groups with different US grades separately, the patients from the NAFLD group with grade 2 US finding had BMI values significantly higher in reference to those from the group with grade 1 US finding, 33.26 ± 4.16 (33.29) *vs* 29.85 ± 3.11 (33.26) kg/m^2 ($p < 0.05$). Also, the patients with grade 3 US finding, in comparison with grade 1, had statistically significantly higher mean values of WC (109.26 ± 7.76 *vs* 100.75 ± 6.15 cm) and BMI (34.01 ± 4.12 *vs* 29.85 ± 3.11 kg/m^2) ($p < 0.01$). Body weight mean value was the highest in the patients with grade 3 US finding, statistically significantly higher than in the patients with grade 2 ($p < 0.05$) and 1 ($p < 0.001$) (Table 3) findings.

Table 1

Demographic, anthropometric and biochemical parameters in the patients with non-alcoholic fatty liver disease (NAFLD) and control group subjects

Parameter	NAFLD group (n = 55)		Control group (n = 319)	
	$\bar{x}$ ± SD	Md	$\bar{x}$ ± SD	Md
Sex, n (%)				
male	23 (41.82)		10 (32.26)	
female	32 (58.18)		21 (67.74)	
Age (yrs)	49.29 ± 12.95	(52.00)	47.84 ± 10.08	(49.00)
WC (cm)	106.36 ± 8.44‡	(105.00)	78.87 ±7.18	(79.00)
BW (kg)	92.22 ± 14.83‡	(92.00)	65.00 ± 8.90	(62.00)
BH (m)	1.68 ± 0.12	(1.65)	1.70 ± 0.09	(1.68)
BMI (kg/m^2)	32.83 ± 4.20‡	(32.87)	22.52 ± 2.08	(22.77)
SBP (mmHg)	137.18 ± 18.60‡	(140.00)	116.45 ± 13.86	(120.00)
DBP (mmHg)	84.73 ± 10.69‡	(90.00)	72.90 ± 9.98	(70.00)
Urates (μmol/L)	373.01 ± 94.55‡	(375.10)	239.98 ± 56.57	(237.40)
TBIL (μ/L)	12.78 ± 5.66*	(12.20)	10.17 ± 2.91	(9.90)
DBIL (μ/L)	2.23 ± 1.08	(2.00)	1.81 ± 0.54	(1.60)
Albumins (g/L)	45.10 ± 3.09	(45.20)	44.05 ± 3.84	(44.90)
Total cholesterol (mmol/L)	5.86 ± 1.06†	(5.82)	5.16 ± 1.08	(5.12)
HDL (mmol/L)	1.15 ± 0.25	(1.13)	1.28 ± 0.28*	(1.34)
LDL (mmol/L)	3.76 ± 0.92*	(3.80)	3.26 ± 0.87	(3.30)
TGL (mmol/L)	2.28 ± 1.12‡	(1.91)	1.20 ± 0.37	(1.08)
CRP (mg/L)	6.20 ± 10.38‡	(3.40)	1.69 ± 1.63	(1.10)
Ferritin (μ/L)	145.38 ± 113.45‡	(113.90)	45.65 ± 28.17	(39.30)
INR	1.07 ± 0.11	(1.06)	1.08 ± 0.08	(1.06)
Fibrinogen (g/L)	4.47 ± 1.02‡	(4.27)	3.48 ± 0.86	(3.55)
Platelets (x10^9/L)	242.69 ± 66.60	(235.00)	253.90 ± 50.48	(261.00)
AST (U/L)	40.17 ± 21.82‡	(34.80)	20.84 ± 4.25	(20.10)
ALT (U/L)	59.56 ± 43.94‡	(52.50)	16.85 ± 6.23	(16.10)
ALP (U/L)	71.85 ± 28.67†	(63.10)	55.50 ± 16.56	(49.80)
GGT(U/L)	61.62 ± 67.10‡	(38.40)	19.45 ± 14.97	(14.70)
FBG (mmol/L)	6.56 ± 2.38‡	(6.00)	5.00 ± 0.74	(5.00)
Insulin (mu/L)	39.16 ± 28.88‡	(4.27)	12.41 ± 4.37	(13.00)
HOMA-IR	13.67 ± 18.88‡	(7.47)	2.77 ± 1.07	(2.88)

***p < 0.05. †p < 0.01. ‡p < 0.001; $\bar{x}$ – mean value; SD – standard deviation; Md – median; WC – waist circumference; BMI – body mass index; BH – body height; BW – body weight; SBP – systolic blood pressure; DBP – diastolic blood pressure; TBIL – total bilirubin; DBIL – direct bilirubin; LDL – low density lipoprotein cholesterol; HDL – high density lipoprotein cholesterol, TGL – triglycerides, CRP – C reactive protein; INR – international normalized ratio; AST – aspartate aminotransferase; ALT – alanine aminotransferase, ALP – alkaline phosphatase; GGT – gamma-glutamyl transpeptidase; FBG – fasting blood glucose; HOMA-IR – homeostasis model assessment.**

Table 2

Prevalence of diabetes mellitus (DM) and hypertension in the non-alcoholic fatty liver disease (NAFLD) and control group subjects

Parameter	NAFLD group (n = 55) n (%)	Control group (n = 31) n (%)
Hypertension	40 (72.73) ‡	4 (12.90)
DM type 2	26 (47.27) ‡	0 (0.00)

‡p < 0.001.

Table 3

Demographic, anthropometric and biochemical parameters of non-alcoholic falty liver disease (NAFLD) patients in relation to ultrasound (US) grades

Parameter	NAFLD US grade 1 (n = 12)		NAFLD US grade 2 (n = 12)		NAFLD US grade 3 (n = 12)	
	x̄ ± SD	Md	x̄ ± SD	Md	x̄ ± SD	Md
Age (years)	48.00 ± 15.18	(53.00)	50.80 ± 12.74	(54.00)	48.65 ± 12.36	(52.00)
FBG (mmol/L)	5.49 ± 0.93	(5.35)	7.10 ± 2.97 [a*]	(6.00)	6.64 ± 2.24 [a*]	(6.10)
Insulin (mu/L)[*]	22.92 ± 8.16	(22.00)	48.03 ± 33.13 [a†]	(35.40)	39.92 ± 29.15 [a*]	(30.00)
HOMA-IR[†]	5.61 ± 2.12	(5.16)	18.11 ± 21.99 [a†]	(8.50)	14.01 ± 20.03 [a*]	(7.92)
Total cholesterol (mmol/L)	6.27 ± 0.94	(5.83)	5.99 ± 0.98	(6.17)	5.54 ± 1.13	(5.28)
HDL (mmol/L)	1.25 ± 0.24	(1.18)	1.12 ± 0.22	(1.08)	1.12 ± 0.27	(1.12)
LDL (mmol/L)[*]	4.15 ± 0.83 [c*]	(4.05)	3.92 ± 0.90	(3.95)	3.41 ± 0.9	(3.50)
TGL (mmol/L)	2.24 ± 1.25	(1.83)	2.32 ± 0.94	(1.87)	2.27 ± 1.23	(1.96)
AST (U/L)	38.51 ± 23.19	(33.20)	43.32 ± 26.89	(35.00)	38.30 ± 16.14	(34.80)
ALT (U/L)	50.04 ± 21.91	(46.45)	67.36 ± 61.87	(54.55)	57.74 ± 32.93	(55.30)
ALP (U/L)	72.26 ± 26.44	(65.55)	71.88 ± 28.34	(61.80)	71.61 ± 31.21	(65.90)
GGT (U/L)	84.10 ± 113.11	(39.70)	50.68 ± 50.56	(35.10)	59.41 ± 45.37	(41.70)
CRP (mg/L)	5.69 ± 4.90	(4.15)	7.11 ± 14.95	(3.65)	5.68 ± 7.65	(3.20)
WC (cm)[*]	100.75 ± 6.15	(101.00)	106.40 ± 9.00	(103.50)	109.26 ± 7.76 [a†]	(107.00)
BW (kg)[†]	82.10 ± 9.22	(79.10)	89.77 ± 13.59	(91.50)	99.63 ± 14.8 [a‡b*]	(100.00)
BMI (kg/m^2)[*]	29.85 ± 3.11	(29.27)	33.26 ± 4.16 [a*]	(33.29)	34.01 ± 4.12 [a†]	(34.22)
SBP (mmHg)	132.92 ± 13.22	(140.00)	132.50 ± 15.60	(130.00)	143.48 ± 21.92	(140.00)
DBP (mmHg)	82.08 ± 10.33	(80.00)	83.00 ± 9.51	(80.00)	87.61 ± 11.57	(90.00)
AST/ALT	0.78 ± 0.26	(0.69)	0.80 ± 0.34	(0.74)	0.78 ± 0.39	(0.63)

***p < 0.05, †p < 0.01, ‡p < 0.001; [a] *vs* US gr 1, [b] *vs* US gr 2, [c] *vs* US gr 3; x̄ – mean value; SD – standard deviation; Md – median; WC – waist circumference; BMI – body mass index; BW – body weight; SBP – systolic blood pressure; DBP – diastolic blood pressure; LDL – low density lipoprotein cholesterol, HDL – high density lipoprotein cholesterol, TGL – triglyceride; CRP-C – reactive protein; AST – aspartate aminotransferase; ALT – alanine aminotransferase, ALP – alkaline phosphatase; GGT – gamma- glutamyl transpeptidase; FBG – fasting blood glucose; HOMA-IR – homeostasis model assessment.**

In patients with grade 1 US finding, insulin and HOMA-IR values were significantly higher than in the patients with grade 2 ($p < 0.01$), and grade 3 ($p < 0.05$) US findings. The values of FBG were statistically higher in patients with grades 2 and 3 in comparison to those with grade 1 US finding ($p < 0.05$).

We did not find statistically significant differences in transeminase, ALP, GGT, total cholesterol, TGL, CRP, fibrinogen and ferritin values in examinees with different US grades.

On the basis of contingency table 3 × 2, the presence of hypertension and type 2 DM in the NAFLD group was statistically significantly different in reference to US grade ($p < 0.05$).

By separate comparison, hypertension occurred more frequently in US findings grades 2 and 3 in comparison with US finding grade 1 ($p < 0.05$). Also, type 2 DM which was mostly represented in the group with US findings grade 3, which was statistically more frequent in reference to grade 1 US findings ($p < 0.05$) (Table 4).

In the NAFLD group, there were 17 (30.91%) overweight (BMI from 25 to 29.9 kg/m^2) and 38 (69.09%) obese patients (BMI ≥ 30.0 kg/m^2). The largest number of patients in the obesity group, 22 (40.00%) of them, had first degree obesity (BMI from 30 to 34.99 kg/m^2). It was confirmed that obesity as a category of nutritional status (BMI ≥ 30.0 kg/m^2) was statistically more common in patients with grades 2 and 3 US finding in reference to grade 1 US finding ($p < 0.05$), and the same significance was achieved on the basis of contingency table 3 × 2. Mild, moderate and severe obesity as a unique category of obesity are statistically significantly more frequent in patients with grades 2 and 3 US finding in reference to grade 1 US finding ($p < 0.05$) (Table 5).

There were 48 (87.27%) patients in the study group

Table 4

Prevalence of diabetes mellitus (DM) and hypertension in patients with non/alcoholic fatty liver disease (NAFLD) in reference to ultrasound (US) grades

Parameter	NAFLD US grade 1, n = 12		NAFLD US grade 2, n = 20		NAFLD US grade 3, n = 23	
Hypertension[*]	5	41.56%	16	80.00%[a*]	19	82.61%[a*]
DM type 2[*]	2	16.67%	10	50.00%	14	60.87%[*a]

***p < 0.05; a *vs* ultrasound grade 1.**

Table 5

Correlation between body mass index (BMI) and ultrasound (US) grade of fatty liver

Parameter	NAFLD US grade 1 (n = 12)	NAFLD US grade 2 (n = 20)	NAFLD US grade 3 (n = 23)
BMI, n (%)			
< 18.5kg/m^2	0 (0.00)	0 (0.00)	0 (0.00)
from 18.5 to 24.9 kg/m^2	0 (0.00)	0 (0.00)	0 (0.00)
from 25 to 29.9 kg/m^2	8 (66.67)	4 (20.00)	5 (21.74)
≥ 30.0 kg/m^2 *	4 (33.33)	16 (80% a[a*])	18 (78.26 [a*])
Obesity, n (%)			
No obesity	8 (66.67)	4 (20.00)	5 (21.74)
BMI from 30 to 34.99 kg/m^2	3 (25.00)	10 (50.00% [ax*])	9 (39.13 [ax*])
BMI from 35 to 39.99 kg/m^2	1 (8.33)	5 (25.00)	7 (30.43)
BMI higher than 40 kg/m^2	0 (0.00)	1 (5.00)	2 (8.70)

*** $p < 0.05$; [a] – *vs* ultrasound grade I, [x] – patients with BMI 30 kg/m^2 compared as unique category.**

with MS, therefore with 3 or more components of MS. The equal number of the NAFLD group patients, *ie* 16 (29.09%) of them, had 3, 4 and 5 MS components, respectively.

The most frequent MS component in the NAFLD group was hypertension or increased SBP or DBP, which was present in 50 (90.91%) patients. Other components of metabolic syndrome were represented as follows: central obesity (WC) – 87.27%, higher FBG or already existing type 2 DM - 69.9%, higher triglyceride level – 65.45% and lower HDL-cholesterol – 60.00%. The presence of hypertension, as a metabolic component of NAFLD, was statistically more frequent than the presence of metabolic components of higher triglyceride levels, the presence of DM or higher FBG ($p < 0.01$), as well as lower levels of HDL-cholesterol ($p < 0.001$) (Table 6).

Metabolic syndrome was statistically more frequent in the patients with grades 2 and 3 US findings ($p < 0.01$) in relation to grade 1 US finding (Table 7).

Table 6

Frequency of metabolic syndrome (MS) components in the non-alcoholic fatty liver disease (NAFLD) group

MS component	n (%)
Waist circumference (cm)	48 (87.27)
Lower HDL (mmol/L)	33 (60.00)
Higher TGL (mmol/L)	36 (65.45)
Hypertension/ SBP and/or DBP (mmHg)	50 (90.91[bc†,a‡])
FBG ≥ 5.6 mmol/L / type 2 DM	38 (69.09)

[†]$p < 0.01$, [‡]$p < 0.001$; a *vs* HDL, b *vs* TGL, c *vs* FBG ≥ 5.6 mmol/L/ type 2 DM; FBG – fasting blood glucose; HDL – high density lipoprotein cholesterol, TGL – triglyceride; SBP – systolic blood pressure; DBP – diastolic blood pressure; DM – diabetes mellitus.

Table 7

Frequency of metabolic syndrome (MS) components in the non-alcoholic fatty liver disease (NAFLD) group in relation to ultrasound (US) grade

Parameter	NAFLD (US grade 1, n = 12)	NAFLD (US grade 2, n = 20)	NAFLD (US grade 3, n = 23)
MS, n (%)	6 (50.00)	20 (100.00 [a†])	22 (95.65 [a†])
Waist circumference, n (%)	9 (75.00)	18 (90.00)	21 (91.30)
Lower HDL, n (%)	3 (25.00)	15 (75.00 [a†])	15 (65.22[a*])
Higher TGL, n (%))	7 (58.33)	13v65.00)	16 (69.57)
Hypertension, n (%)	10 (83.33)	18 (90.00)	22 (95.65)
FBG ≥ 5.6 mmol/L/ type 2 DM, n (%)	5 (41.67)	15 (75.00)	18 (78.26[a*])
MS component, n (%)			
0	0 (0.00)	0 (0.00)	0 (0.00)
1	1 (8.33)	0 (0.00)	0 (0.00)
2	5 (41.67)	0 (0.00)	1 (4.35)
3	3 (25.00)	6 (30.00)	7 (30.43)
4	1 (8.33)	9 (45.00 [ax])*	6 (26.09 [ax*])
5	2 (16.67)	5 (25.00)	9 (39.13)

***$p < 0.05$; [†]$p < 0.01$ a *vs* US grade 1, x – patients with 4 and 5 MS components compared as unique categories; FBG – fasting blood glucose; HDL – high density lipoprotein cholesterol; TGL – triglyceride; SBP-systolic blood pressure; DBP – diastolic blood pressure; DM – diabetes mellitus.**

The frequency of lower values of HDL-cholesterol as metabolic component in relation to the patients with grade 1 US finding was statistically significantly higher in the patients with grade 2 US finding ($p < 0.01$), as well as grade 3 ($p < 0.05$). Already existing type 2 DM or higher FBG as metabolic component was most frequent in the patients with grade 3 US finding, and as such statistically more frequent than grade 1 US finding ($p < 0.05$) (Table 7).

The number of patients with 4 or 5 metabolic components was statistically significantly higher in the patients with grades 2 and 3 US findings in relation to the patients with grade 1 ($p < 0.05$) (Table 7).

Discussion

Non-alcoholic fatty liver disease is the most common chronic liver disease nowadays and it is the most common reason of high aminotransferase levels in hepatology wards [26].

The presence of multiple metabolic disorders such as DM, obesity, dyslipidemia and hypertension carries a high risk of disease progression and development of non-alcoholic steatohepatitis and fibrosis in NAFLD patients [27, 28]. It is important to recognize the patients with NAFLD so as to enable timely action on joined risk factors and prevent development of more severe diseases [29]. The prevalence of NAFLD is on the increase, which is the consequence of obesity pandemic. Liver biopsy is a gold standard for diagnosing the disease, but it is not widely used due to ethical reasons, since we are dealing with patients mainly without clinical symptoms, with frequently normal transaminase values. Considering a good correlation between fatty liver ultrasound finding and the pathohistological one, the ultrasound is recommended to be the first diagnostic method. In this study we adhered to the criteria of Needleman et al. [25] who, after comparing pathohistological and ultrasound findings of fatty liver, confirmed the precision of ultrasound findings at 88% in diagnosing and studying non-alcoholic fatty liver.

In this study, we compared BMI, the presence of MS, certain components of MS and laboratory parameters of the control and study groups with the aim to estimate the impact of these parameters on fatty liver development, as well as the association between these parameters and the degree of steatosis estimated by ultrasound. The patients in the study group had statistically higher values of BMI, WC, BW, FBG, insulin, HOMA-IR, AST, ALT, ALP, GGT, total cholesterol, LDL-cholesterol, TGL, CRP, ferritin, urates and fibrinogen in reference to the control group. The majority of patients in the study group had the ultrasound finding of grade 3 fatty liver. By comparing the examined parameters of certain grades of ultrasound findings, ANOVA analysis showed that BMI, WC, higher fasting blood glucose, insulin and HOMA-IR were statistically significantly related to the degree of ultrasound grade.

In our study, the majority of NAFLD patients (69,09%) were obese, with BMI higher than 30 kg/m^2 and the mean values of BMI and WC statistically were significantly higher in relation to the control group.

Earlier studies estimate that liver steatosis develops in 57–74% of obese people, in 90% of people with third degree obesity, and that more than a third of asymptomatic people with severe obesity have histological characteristics of NASH [2, 30]. Abdominal obesity correlates with the prevalence of NAFLD, or NASH respectively, and is closely related to insulin resistance, a major pathogenetic factor for developing NASH [31].

In the study of Leite et al. [32] central obesity is an independent risk factor for NAFLD development. Most examinees in NAFLD group in the study of Williams at al. [33] were obese with mean values of BMI statistically higher in relation to the control group (not-NAFLD). Similar results can be found in the study of Kirovski et al. [34] that confirms statistically higher mean values of BMI and WC in examinees from the NAFLD group in relation to the control group.

In the study by Cheah et al. [35], the prevalence of central obesity, DM, hypertension, higher fasting blood glucose and triglycerides was statistically much higher in the NAFLD group. This study showed that NAFLD develops 1.2 times more often in patients with larger waist circumference [35].

In the study by Abangah et al. [36] most examinees had an ultrasound finding of grade 2 fatty liver, while BMI and TG statistically significantly correlated with the degree of steatosis. Our study also confirms statistically significant correlations between BMI and higher ultrasound grade.

The presence of type 2 DM considerably increases the risk of developing NAFLD and progression into more serious forms of the disease, non-alcoholic steatohepatitis and different degrees of fibrosis [37, 38]. The prevalence of NAFLD in patients with diabetes goes up to 79% [32, 33, 39, 40]. Having observing a group of patients with type 2 DM, Dvorak et al. [40] confirmed the NAFLD prevalence of 79%. Patients with NAFLD have a higher body weight, waist circumference, BMI, ALT and triglycerides in relation to non-NAFLD examinees.

The prevalence of NAFLD increases with higher fasting blood glucose, from 27% in patients with normal values, 43% in patients with higher fasting blood glucose, to 62% in patients with type 2 DM [41].

In prospective studies type 2 DM is an independent risk factor of NAFLD progression, fibrosis development, HCC and mortality [42].

The prevalence of diabetes in our NAFLD examining group reached 47,27%, which is similar to results of previous studies. Mean values of fasting blood glucose were statistically higher in the NAFLD group than in the control one. The prevalence of hypertension in our study group was also statistically higher than in the control group (72.73% *vs* 12.90%, $p < 0.001$), which was similarly to the results of other studies [34, 35]. At the same time, hypertension and diabetes were statistically more frequent in the examinees with grades 2 and 3 US findings in relation to grade 1.

Chitturi et al. [43] determined that 87% patients with NAFLD had characteristics of MS (94% central obesity, 82% dyslipidemia and 50% glucose intolerance), with practically 98% of patients having IR, which was more frequent and serious in patients with NASH than in patients with chronic hepatitis C. In the study of Caballería et al. [44],

MS and IR are independent risk factors of NAFLD development.

A larger number of MS characteristics in a person combines with multiply higher risk of developing NAFLD, whereas the presence of only one characteristic carries 3.6 times higher risk [45]. The presence of MS is a predisposing factor for disease progression and development of more acute forms of NAFLD [46, 47].

In our study, the prevalence of MS in NAFLD group was 87.27%. All the examinees showed one or more metabolic risk factors, whereas 29.09% showed at least three metabolic risk factors which is the minimum for diagnosing MS. In earlier studies, more than 90% of patients with NAFLD had one or more components of metabolic syndrome, whereas 33% of them had the complete diagnosis [48, 49].

Examinees with NAFLD had statistically significantly higher prevalence of hypertension, central obesity, higher fasting blood glucose, which is similar to other studies [34, 50]. The study demonstrated that there was the association between the MS components and the US degree of fatty liver infiltration. Among the patients with US finding of fatty liver grades 2 and 3, there were statistically more patients with four or five MS components, similar to studies where the results imply that the presence of a larger number of metabolic disorders carries a higher risk of developing more severe forms of the disease [27, 28, 51].

Insulin resistance is closely connected with NAFLD, both with development of steatosis and progression of the disease to steatohepatitis, cirrhosis and liver carcinoma [52]. Mean values of insulin and insulin resistance expressed by HOMA-IR were statistically much higher in the study group than in the control group, which was similar to the results obtained by de Salgado et al. [53]. The patients in the study group with US findings grades 2 and 3 had statistically higher values of insulin and HOMA-IR than the patients with grade 1 US finding, which was expected since the patients with US findings grades 2 and 3 had higher prevalence of DM and fasting blood glucose, thus confirming the influence of insulin, insulin resistance and the presence of type 2 DM on the degree of the disease severity.

In a study by Ghamar-Chehreh et al. [54], univariate analysis showed a statistically significant association between insulin, HOMA-IR, higher fasting blood glucose, transaminases, triglycerides, body weight and the grade of fatty liver.

It is known that a large number of NAFLD patients have normal transaminase values, so their sensitivity in diagnosing the disease is low.

Higher values of AST and GGT correlate with the degree of liver steatosis, but are less sensitive in relation to ALT [55]. In a study by Leite et al. [32] the examinees from the NAFLD group had higher serum levels of ALT than those from the control group. In a multivariate analysis, a high serum triglycerides level and a high-normal ALT level were independently associated with hepatic steatosis, together with either the presence of obesity or increased waist circumference. Razavizade et al. [56] determined a correlation between the serum levels of ALT and the degree of steatosis estimated by US.

In our study, the mean values of AST, ALT, GGT and ALP were statistically significantly higher in the study group in relation to the control group, however, no statistical significance was found with respect to the degree of steatosis estimated by ultrasound, which indirectly implies that we cannot estimate the degree of disease progression on the basis of aminotransferase levels.

Dyslipidemia is a risk factor for developing NAFLD and progression of the disease [32, 35, 36, 40, 57]. Our study results were similar, *ie* the patients with NAFLD had statistically much higher values of total cholesterol, LDL cholesterol and TGL in relation to the control group.

The values of HDL cholesterol were statistically significantly lower in the study group, which is similar to the results of Kirovski et al. [34]. No statistically significant correlations were found between the degree of liver steatosis estimated by US and dyslipidemia.

However, there are several limitations of our study to be considered. Firstly, patients did not have liver biopsy done, which is a gold standard for establishing NAFLD diagnosis. Secondly, ultrasound has certain disadvantages. The sensitivity and specificity of US in NAFLD diagnosis rapidly decrease in obese patients. In our study, the largest number of patients had BMI $\geq$ 30 kg/m^2, and we should consider the low sensitivity of ultrasound for steatosis less than 30%. It is not possible to differentiate steatosis from steatohepatitis and fibrosis by ultrasound, and the majority of our examinees had multiple risk factors for disease progression, such as DM, MS, hypertension, dyslipidemia, obesity. On the other hand, liver biopsy is an invasive method, frequently demanding patient's hospitalization and sedation, certain costs, and is followed by possible complications. Biopsy sample represents 50 000 part of the liver parenchyma tissue, which during the progression of the disease is not equally affected by pathological changes, which influences the variability of the sample itself, and consequently the validity of pathohistological findings, as well [58].

All this poses a challenge both for the clinician to estimate which patients with US finding of fatty liver should be considered candidates to undergo liver biopsy and for patients themselves to easily undergo this intervention, as they commonly do not present with significant disease symptoms and often have normal transaminase values.

The results of a recent meta analysis show that ultrasound is a precise, reliable method for diagnosing more than 20–30% fatty infiltrated liver parenchyma, in comparison to histology, with sensitivity of 84.8% and specificity of 93.6% [59].

Conclusion

The results of our study show that a large percentage of patients with high risk factors (DM, MS, dyslipidemia, hypertension) have NAFLD. A strong association between certain elements of metabolic syndrome and the presence of NAFLD was demonstrated, particularly between obesity and hypertension.

We also confirmed the importance of insulin resistance estimated by HOMA-IR in the development of the disease,

as well as its influence on development of more severe grades of the disease estimated by ultrasound. Despite statistically significantly higher values of liver enzymes in NAFLD group, a correlation with ultrasound has not been established. A high prevalence of obesity and metabolic syndrome in higher US grades was found, *ie* the association with progressive forms of the disease was confirmed.

Ultrasound is cheap and available, compared with other diagnostic methods, which makes it a technique of choice for screening patients on NAFLD presence, particularly in conditions of obesity pandemic. The severity of hepatic steatosis estimated by ultrasound in the presence of metabolic syndrome is a better non-invasive method of disease monitoring in relation to liver enzymes.

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Received on May 14, 2015.
Accepted on June 24, 2015.
Online First May, 2016.

ORIGINAL ARTICLE

UDC: 616.12-073.7:616.24-005.6/.7
DOI: 10.2298/VSP150512011K

Association of different electrocardiographic patterns with shock index, right ventricle systolic pressure and diameter, and embolic burden score in pulmonary embolism

Povezanost različitih elektrokardiografskih znakova sa šok indeksom, veličinom i sistolnim pritiskom desne komore i skorom embolijskog opterećenja kod akutne plućne tromboembolije

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Abstract

Background/Aim. Some electrocardiographic (ECG) patterns are characteristic for pulmonary embolism but exact meaning of the different ECG signs are not well known. The aim of this study was to determine the association between four common ECG signs in pulmonary embolism [complete or incomplete right bundle branch block (RBBB), S-waves in the aVL lead, $S_1Q_3T_3$ sign and negative T-waves in the precordial leads] with shock index (SI), right ventricle diastolic diameter (RVDD) and peak systolic pressure (RVSP) and embolic burden score (EBS). **Methods.** The presence of complete or incomplete RBBB, S waves in aVL lead, $S_1Q_3T_3$ sign and negative T-waves in the precordial leads were determined at admission ECG in 130 consecutive patients admitted to the intensive care unit of a single tertiary medical center in a 5-year period. Echocardiography examination with measurement of RVDD and RVSP, multidetector computed tomography pulmonary angiography (MDCT-PA) with the calculation of EBS and SI was determined during the admission process. Multivariable regression models were calculated with ECG parameters as independent variables and the mentioned ultrasound, MDCT-PA parameters and SI as dependent variables. **Results.** The presence of S-waves in the aVL was the only independent predictor of RVDD ($F = 39.430$, $p < 0.001$; adjusted $R^2 = 0.231$) and systolic peak right ventricle pressure ($F = 29.903$, $p < 0.001$; adjusted $R^2 = 0.185$). Negative T-waves in precordial leads were the only independent predictor for EBS ($F = 24.177$, $p < 0.001$; $R^2 = 0.160$). Complete or incomplete RBBB was the independent predictor of SI ($F = 20.980$, $p < 0.001$; adjusted $R^2 = 0.134$). **Conclusion.** In patients with pulmonary embolism different ECG patterns at admission correlate with different clinical, ultrasound and MDCT-PA parameters. RBBB is associated with shock, S-wave in the aVL is associated with right ventricle pressure and negative T-waves with the thrombus burden in the pulmonary tree.

Key words:
pulmonary embolism; electrocardiography; diagnosis, differential; tomography; angiography; ventricular function, right; sensitivity and specificity.

Apstrakt

Uvod/Cilj. Pojava pojedinih elektrokardiografskih (EKG) znakova karakteristična je za akutnu plućnu tromboemboliju (APTE). U ovu grupu znakova spadaju kompletan ili nekompletan blok desne grane (BDG), prisustvo S-zupca u aVL odvodu, $S_1Q_3T_3$ znak i prisustvo negativnih T-talasa u prekordijalnim odvodima. Tačno značenje pojave ovih znakova i njihova povezanost sa kliničkim stanjem, ehokardiografskim i angiografskim karakteristikama još uvek nisu utvrđeni. Cilj ove studije bio je da se utvrdi povezanost karakterističnih EKG obrazaca na prijemu kod bolesnika sa APTE sa šok indeksom (ŠI), srednjim pritiskom i prečnikom desne komore (SPDK i DDK) i skorom embolijskog opterećenja (*embolic burden score*, EBS). **Metode.** Prisustvo BDG, S-zupca u aVL odvodu, $S_1Q_3T_3$ znaka i negativnih T-talasa u prekordijalnim odvodima zabeleženi su kod 130 bolesnika na prijemu u jedinicu intenzivne nege jedne tercijarne zdravstvene ustanove tokom pet godina. Ehokardiografsko ispitivanje sa merenjem SPDK i DDK, multidetektorska kompjuterizovana tomografska plućna angiografija (MDKT-PA) sa izračunavanjem EBS i utvrđivanje ŠI vršeni su tokom pri-

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jemne obrade bolesnika. Multivarijabilni regresioni modeli utvrđeni su na osnovu pomenutih EKG znakova kao nezavisnih promenljivih i ŠI, SPDK, DDK i EBS kao zavisnih promenljivih varijabli. **Rezultati.** Prisustvo S-zupca u aVL odvodu jedini je nezavisni prediktor visine SPDK ($F = 29{,}903$, $p < 0{,}001$; usklađen $R^2 = 0{,}185$) i veličine DDK ($F = 39{,}430$, $p < 0{,}001$; usklađen $R^2 = 0.231$). Negativni T-talasi u prekordijalnim odvodima jedini su nezavisni prediktori veličine EBS ($F = 24{,}177$, $p < 0{,}001$; usklađeni $R^2 = 0{,}160$). Jedini nezavisan prediktor veličine ŠI je BDG ($F = 20{,}980$, $p < 0{,}001$; usklađeni $R^2 = 0{,}134$). **Zaključak.** Kod bolesnika sa APTE karakterističnI EKG obrasci povezani su sa kliničkim, ehokardiografskim i angiografskim statusom. Pojava BDG ukazuje na veći ŠI, a shodno tome na težu kliničku sliku. Prisutan S-zubac u aVL odvodu u vezi je sa visinom SPDK i veličinom DDK, pa se njegova pojava može shvatiti kao preteća disfunkcija desne komore. Prisustvo negativnih T-talasa u prekordijalnim odvodima ukazuje na veći EBS, samim tim, na zahvaćenost velikih krvnih sudova plućnog vaskularnog korita trombnim masama.

Ključne reči:
pluća, embolija; elektrokardiografija; dijagnoza, diferencijalna; tomografija; angiografija; srce, funkcija desne komore; osetljivost i specifičnost.

Introduction

Acute pulmonary thromboembolism (APE) is a common and potentially fatal disease caused by the migration of thrombi from the veins to the pulmonary arteries. Thrombi may be small and asymptomatic, may cause pulmonary infarction with secondary pneumonia and large thrombi may overload the weak right ventricle with the shock state and circulatory and respiratory failure [1]. The diagnosis of APE is remarkably improved after introduction of multidetector computed tomography pulmonary angiography (MDCT-PA) which is widely available and after clinical assessment of and D-dimer level determination it becomes the cornerstone for the APE diagnosis [2, 3]. Echocardiography is important for the assessment of right ventricle function which is important for the risk stratification of patients at admission. Urgent estimation of risk for death is extremely important in APE because the treatment modality is based on that [2]. Electrocardiography (ECG) is simple, inexpensive and repeatable diagnostic tool which is part of routine procedures in every acutely ill patient. In APE ECG changes are typical, but have low sensitivity and specificity for the diagnosis [4]. However, ECG changes in APE are extremely dynamic and may follow-up closely hemodynamic deterioration or successful reperfusion and be very useful for the direction of therapeutic measures. Several parameters, like shock index (SI), right ventricle diastolic diameter (RVDD) and systolic pressure (RVSP) measured by echocardiography and embolic burden score (EBS) on MDCT-PA are well-known markers of prognosis for APE.

The aim of this investigation was to examine the association of the most common ECG signs in APE at admission with hemodynamic status (presented by SI), function of the right ventricle (presented by echocardiographic measured RVSP and RVDD) and thrombus burden in the pulmonary arterial tree (presented by EBS).

Methods

This study included 130 consecutive patients with confirmed APE hospitalized at the Clinic of Emergency Internal Medicine in the Military Medical Academy, Belgrade, during a 5-year period, from January 2010 to December 2014. All patients were submitted to clinical, biochemical, electrocardiographic, echocardiographic, and radiological investigations at admission. The diagnosis of APE was confirmed radiologically with MDCT-PA which visualized a thromb in the pulmonary vascular tree.

The basic clinical assesment included the measurement of heart rate and arterial pressure at admission with calculation of shock index according the formula SI = heart rate/systolic blood pressure [5]. Electrocardiographic (ECG) recording was done in all the patients at admission by conventional 12-leads. Five classical ECG characteristics which are commonly used for the estimation of acute PTE were analysed: heart rate, $S_1Q_3T_3$ sign, S-wave in the aVL lead, negative T-waves in the precordial leads and the presence of incomplete or complete right bundle branch block (RBBB). The right ventricle (RV) dysfunction was measured by transthoracic echocardiography examination at admission. The RVD was measured in diastole 1 cm beyond the tricuspid anulus in apical 4-chamber view. Right ventricle systolic blood pressure was measured through the regurgitation blood velocity and adding 10 mmHg for the estimated right atrium pressure. Thrombus burden was measured by the admission MDCT-PA using EBS [6].

All the patients were scheduled for the follow-up visit at 1, 3 and 6 months after disharge. If a patient was not present at the scheduled visit he was contacted by phone.

Statistical analysis

SPSS software (Statistical Package for the Social Sciences, version 20.0, SSPS Inc, Chicago, IL, USA) was used for statistical analyses. Categorical variables were expressed as numbers and percentages, and continuous variables as means and standard deviations. The characteristics of study population (gender, age, risk factors, DDK, SPDK, EBS, heart rate, ECG signs, clinical parameters) were calculated by descriptive methods. Significant differences in shock index, echocardiographic and MDCT-PA parameters between the groups of patients with and without the presence of some ECG parameters were calculated by the Mann-Whitney U-test. Multivariable regression models were calculated with ECG parameters as independent variables and the mentioned RVDD, RVSP, EBS and SI as dependent variables. A p value less than 0.05 was considered statistically significant.

Results

Clinical characteristics

The study enrolled 130 patients (65 men and 65 women; mean age 60 ± 17 years) with APE. The basic patient characteristics are shown in Table 1. The history of the previous surgery in a few last months was present in 33 (25.4%) and active smoking in 25 (19.8%) of the patients. Malignancy was found in 12 (9.2%) and clinical signs of deep vein thrombosis (DVT) in 72 (55.4%) of the patients. Hypotension (systolic arterial blood pressure less than 90 mmHg) at admission was detected in 24 (18.5%) and RV dysfunction in 86 (68.3%) of the patients. The risk for APE was calculated in all the patients and 65 (50.0%) of them had intermediate risk. Pulmanary embolism severity index (PESI) score 0 was present in 41 (31.5%) of the patients. The mean values of SI, RVSP, RVDD and EBS are presented in Table 1.

ECG characteristics

The mean heart rate in the study group was 103 ± 22 (beats/min). Atrial fibrillation was present only in 15 (11.5%) patients. Frequencies of the basic ECG patterns at initial ECG recording are shown in Table 2.

Association of the most common ECG patterns with hemodynamic status, right ventricle function and thrombus burden in pulmonary tree

A multiple stepwise regression analysis with ECG parameters as independent variables and hemodynamic status, right ventricle function parameters and pulmonary thrombus burden score as dependent variables is shown in Table 3. RBBB at presentation was the independent predictor of SI (F = 20.980, $p < 0.001$; adjusted $R^2 = 0.134$). The patients

Table 1

Characteristics of 130 patients with pulmonary thromboembolism

Parameters	Values
Age (years), mean ± SD	60 ± 17
Male, n (%)	64 (49.2)
Female, n (%)	66 (50.8)
Spontaneous APE, n (%)	65 (50.0)
Provoked APE, n (%)	65 (50.0)
Active smoking, n (%)	25 (19.8)
Surgery in last few months, n (%)	33 (25.4)
Malignancy, n (%)	12 (9.2)
Clinical signs of DVT, n (%)	72 (55.4)
DVT or APE cases in family, n (%)	14 (10.8)
Hypotension (SP < 90 mmHg), n (%)	24 (18.5)
RV disfunction (RVMP > 40 mmHg), n (%)	86 (68,3)
Risk, n (%)	
high	23 (17.7)
intermediate	65 (50.0)
low	42 (32.3)
Wells score (Inter Quartal Range)	4.5 (IQR = 4)
PESI score, n (%)	
0	41 (31.5)
1	33 (25.4)
2	29 (22.3)
≥3	27 (20.8)
Shock index, $\bar{x}$ ± SD	0.95 ± 0.41
Right ventricle SP, $\bar{x}$ ± SD	50.70 ± 19.13
RV diameter at four chamber view, $\bar{x}$ ± SD	3.80 ± 0.80
Embolic burden score at MDCT-PA, $\bar{x}$ ± SD	11.76 ± 5.37

APE − pulmonary thromboembolism; DVT − deep vein thrombosis; RV − right ventricle; SP – systolic pressure; RVMP – right ventricle medial pressure; PESI – pulmonary embolism severity index; MDCT-PA – computed tomography pulmonary angiography.

Table 2

The electrocardiographic (ECG) parameters at admission

The ECG characteristics	Values
Heart rate (beat/min), mean ± SD	103 ± 22
Atrial fibrillation, n (%)	15 (11.5)
$S_1Q_3T_3$ sign, n (%)	37 (28.5)
RBBB or incomplete RBBB, n (%)	34 (26.2)
S wave in aVL, n (%)	62 (47.7)
Negative T waves in precordial leads, n (%)	60 (46.2)

RBBB – right bundle branch block.

Table 3

Independent electrocardiographic variables in regression models for the association with shock index (SI), right ventricle diastolic diameter (RVDD), right ventricle systolic pressure (RVSP) and embolic burden score (EBS)

Dependent variable	Indpendent variables	R square	Adjusted R square	Unstandardized coefficient	Standardized coefficient beta	*p*	95.0% CI for B	
							Lower bound	Upper bound
SI	RBBB	0.375	0.141	0.351	0.375	< 0.001	0.200	0.503
RVDD	S wave in aVL	0.237	0.231	0.779	0.487	< 0.001	0.534	1.025
RVSP	S Wave in aVL	0.192	0.185	16.700	0.438	< 0.001	10.657	22.744
EBS	Negative T waves in precordial leads	0.167	0.160	4.382	0.408	< 0.001	2.617	6.146

RBBB – right bundle branch block; CI – confidence interval.

with RBBB had significantly higher SI than those without it [0.77 (0.61−1.02) *vs* 1.10 (0.70−1.54) respectively; $p = 0.002$] (Figure 1). S-wave in the aVL lead at admission was an independent predictor of RVSP (F = 29.903, $p < 0.001$; adjusted $R^2 = 0.185$) and RVDD (F = 39.430, $p < 0.001$; adjusted $R^2 = 0.231$). The patients with S-waves in the aVL lead had a significantly higher RVSP [41.00 mmHg (27.25−58.50 mmHg) *vs* 59.00 mmHg (45.00−68.50 mmHg), respectively; $p < 0.001$] and a larger RVDD [3.20 cm (3.00−4.00 cm) *vs* 4.2 cm (3.57−4.95 cm), respectively; $p < 0.001$] (Figures 2 and 3). Presentation of negative precordial T-waves was independent predictor of EBS (F = 24.177, $p < 0.001$; $R^2 = 0.160$). The patients with negative T waves in precordial leads had significantly higher EBS than the patients without them [9.00 (5.00−13.00) *vs* 14.50 (12.00−18.00), respectively; $p < 0.001$] (Figure 4).

Discussion

The intention of this investigation was to find individual ECG patterns whose occurrence could predict unstable hemodynamic status, massive thrombus burden and

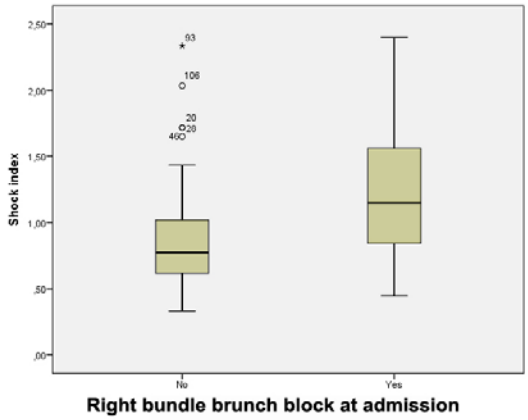

Fig. 1 − Shock index according to the presence of right bundle branch block at admission electrocardiography.

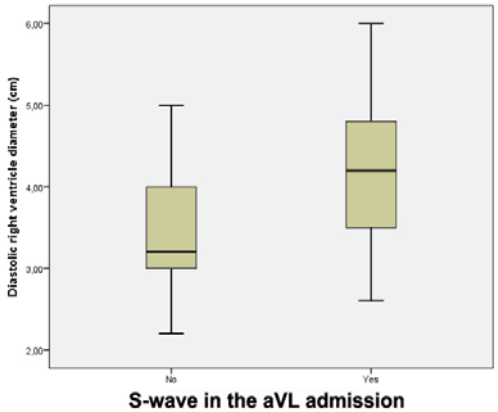

Fig. 2 − Diastolic right ventricle diameter according to the presence of S-waves at admission electrocardiography.

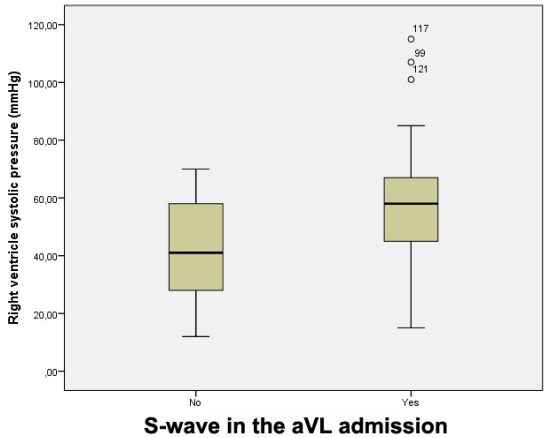

Fig. 3 − Right ventricle systolic pressure according to the presence of S-waves at admission electrocardiography.

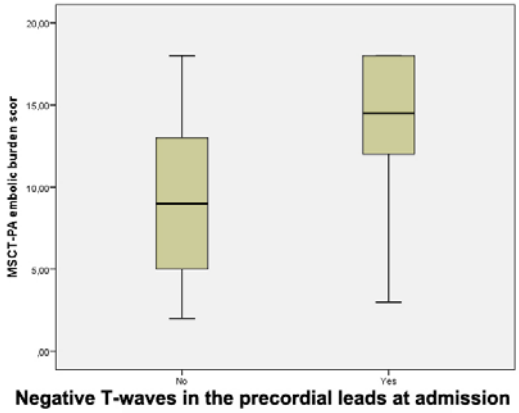

Fig. 4 − Embolic burden score according to the negative T-waves in the precordial leads at admission electrocardiography. MSCT-PA – multislice computed tomography pulmonary angiography.

dysfunction of the right ventricle. Several previous studies have suggested that ECG may be useful for predicting right ventricular dysfunction and the severity of APE.

We concluded that the presence of RBBB on admission, among all other ECG signs, was the only independent predictor of shock index, which was an indication of hemodynamic instability and shock. The presence of RBBB is an indicator of acute right ventricular overload. In our study the RBBB was found in 34 (26.2%) of the patients. Petrov et al. [7] found that patients with autopsy proven trunk pulmonary embolism had the newly emerged RBBB in 80% of cases and in none of the cases with peripheral embolism.The author of this study believes that the appearance of RBBB is a marker of significant obstruction of the main pulmonary artery. However, we did not found a significant correlation between RBBB and EBS which is the more precise method of the measure of central thrombus pulmonary obstruction. Sreeram et al. [8] observed RBBB in 33 (69%) patients. Kukla et al. [9] found RBBB in 20 (22.2%) cases of APE complicated by cardiogenic shock and in 10.2% without shock. They showed the association of low QRS voltage, RBBB, and ST-segment elevation in the lead V1 with cardiogenic shock. According to the current European Society of Cardiology (ESC) guidelines, patients with APE and shock are considered at high risk of death. RBBB is the independent predictor of mortality [10]. All this suggests that RBBB is present when the obstruction is massive, which directly causes saturation decrease, acute right ventricular dysfunction and, consequently, a reduction in left ventricle preload, which leads to shock. RBBB means desinchronization of the ventricles. This situation leads to left ventricle filling reduction and, consequently, to hypotension and higher SI [11].

The presence of S-wave in the aVL lead is associated with the right ventricle diastolic diameter and right ventricle systolic pressure. We found the mentioned pattern in 62 (47.7%) of the patients. Sreeram et al. [8] found this ECG sign in 36 (73%) patients. Presumably, the reason for this discrepancy may have been the presence of high right ventricle peak systolic pressure and the increased right ventricular end-diastolic diameter in all 49 patients included in their study. In the present study the RVSP was 55 ± 13 mmHg (ranged from 33 to 84 mmHg) and the diameter was 40 ± 7 mm (range 28−60 mm). RVSP in our study group ranged from 12 to 115 mmHg (mean 50.7 ± 19.13 mmHg) and RVDD ranged from 2.2 to 6.5 (mean 3.8 ± 0.8) mm. After all, they did not notice the difference in RVSP and RVDD between patients with and without abnormal ECG. Ryu et al. [12] used ECG score proposed by Daniel et al. [13] with tachycardia, T-wave inversion, RBBB $S_1Q_3T_3$. They found that the ECG score was the independent predictor of RVSP. Stein et al. [14] showed sensitivity, positive predictive value, and negative predictive value that were insufficient for the diagnosis or exclusion of RV enlargement in patients without cardiopulmonary disease. Sukhija et al. [15] found similar results. Both groups of authors did not have data about S wave presentation in the aVL lead. Hariharan et al. [16] found the association between right heart strain and tachycardia, T-wave inversion in the leads V1-V3, and S-wave in the lead I. They used that pattern to create TwiST score which can identify patients likely or not likely to have right heart strain with > 80% specificity and sensitivity. In experimental studies, Love et al. [17] show that ECG changes emerge after echocardiography visible right ventricular dilatation that leads to the conclusion that the appearance of ECG changes takes time. Considering that ECG is more accessible and preceding echocardiography in the diagnostic algorithm, based on our results, the S-wave presence in the aVL lead has a great value in identifying the right ventricle enlargement and overload.

Negative T-waves in the precordial leads (electrocardiographic pattern of subepicardial ischemia) are independent predictors of EBS in our study. This means that a patient with APE will have massive thrombus burden if this pattern is present in his/her ECG at admission. We found the mentioned pattern in 60 (46.2%) patients. Sreeram et al. [8] found negative T-waves in the leads V1 to V4 in 13 (27%) patients and in 9 of them symptoms lasted longer than 7 days. Petrov [7] observed T-wave inversion in the V1–V4 in 4 of 20 cases of massive trunk embolism. Geibel et al. [18] found T-wave inversion in the leads V2−V3 in 45%, and in the leads V4−V6 in 35% of patients. Punukollu et al. [19] observed T-wave inversion in the leads V1−V3 in 43% patients. Ferrari et al. [20] reported T-wave inversion in the precordial leads in 68% of patients and concluded that mentioned sign was the independent predictor of severity of APE. Choi and Park [21] observed T-wave inversion in the precordial leads in 35% of patients and showed that this pattern was the independent predictor of right ventricular dysfunction. This conclusion is supported by our results, because right ventricular dysfunction is the result of massive thrombus burden and depends on the sum of occluded pulmonary arteries. According to McIntyre et al. [22] electrocardiographic patterns suggestive of right ventricular overload were present only with angiographic obstruction of $\geq 47\%$. This percentage is equivalent to the value of EBS about 9. EBS in our group ranged from 2 to 18 (mean 11.76 ± 5.37). Kukla et al. [23] concluded that patients having ≥ 5 leads with T-wave inversion in comparison to patients having < 5 leads with the mentioned sign had a higher mortality rate and developed more complications throughout hospitalization period. The group of patients with T-wave inversion in ≥ 5 leads entailed higher rates of thrombolytic therapy and inotrope support. These authors showed that T-wave inversion in the leads V1−V4 is a common pattern in patients with elevated troponin levels [24]. A higher number of leads with T-wave inversion is connected with higher troponin levels, too. On the bases of the mentioned results it is certain that a higher number of occluded arteries (presented by EBS) implies myocardial injury.

Conclusion

In patients with pulmonary embolism different ECG patterns at admission correlate with different clinical, ultrasound and MDCT-PA parameters. Right bundle branch block is associated with shock, S-wave in the aVL is associated with right ventricle pressure and negative T-waves with the thrombus burden in the pulmonary tree.

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Received on May 12, 2015.
Revised on July 6, 2015.
Accepted on July 27, 2015.
Online First January, 2016.

ORIGINAL ARTICLE

UDC: 617.55::616.351-006-089
DOI: 10.2298/VSP150430092M

Local recurrence in patients treated for rectal cancer using total mesorectal excision or transection of mesorectum

Lokalni recidiv kod bolesnika lečenih od karcinoma rektuma metodama totalne mezorektalne ekscizije ili transsekcije mezorektuma

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Abstract

Background/Aim. Rectal cancer is a major health problem throughout the world, despite the great progress in the treatment and control of the disease. The aim of this study was to determine the effect of mesorectal excision type on local recurrence in patients operated on for rectal cancer within a 3-year period. **Methods.** The clinical retrospective study was conducted at the Clinic for General Surgery at the Clinical Center in Niš, Serbia, and included 225 patients with rectal cancer. Postoperatively, the patients were observed 36 months. Total mesorectal excision (TME) method was used in 129 (57.33%) patients, and partial mesorectal excision (PME) in 96 (42.66%). There were 145 (64.44%) man and 80 (35.55%) women, average age 66.8 years. **Results.** In 58 (25.77%) of the patients cancer was localized in the proximal third of the rectum, in 99 (44%) in the medium third, in 68 (30.22%) it was 8 cm of the anocutaneous line. In 167 (74.22%) patients rectal cancer was in T3 stadium. TME was performed in all the patients with cancer in the distal third of the rectum and in 61.61% of the patients with cancer in the medium third of the rectum. PME was performed in all the patients with localized cancer in the proximal third and in 38.38% of the patients with cancer in the medium third of the rectum. Local recurrence occurred in 20 (8.88%) patients, 12 (9.30%) in the TME group and 8 (8.33%) in the PME group, which was not a statistically significant difference. In 75% of the cases, relapse occurred in the patients in T3 stage. Relapse occurred in 55% of the cases in the second year after the surgery. The median survival of all the patients amounted to 35 months. The total mortality of all respondents in a 3-year period amounted to 5.3%. **Conclusion.** There were no statistically significant differences in the incidence of local recurrence and survival among patients who underwent TME and those underwent PME. The type of mesorectal excision does not affect the incidence of local recurrence in node-negative disease stages.

Key words:
rectal neoplasms; neoplasm recurrence, local; digestive system surgical procedures; surgical procedures, operative; neoplasm staging; prognosis.

Apstrakt

Uvod/Cilj. Karcinom rektuma predstavlja veliki medicinski problem širom sveta, uprkos znatnom napretku u lečenju i lokoregionalnoj kontroli bolesti. Cilj rada bio je da se utvrdi uticaj tipa mezorektalne ekscizije na pojavu lokalnog recidiva kod bolesnika operisanih od karcinoma rektuma, u trogodišnjem periodu. **Metode.** Klinička retrospektivna studija sprovedena je na Klinici za opštu hirurgiju Kliničkog centra u Nišu i obuhvatila je 225 bolesnika operisanih od karcinoma rektuma. Bolesnici su postoperativno praćeni 36 meseci. Metodom totalne mezorektalne ekscizije (TME) operisano je 129 (57,33%) bolesnika, a metodom parcijalne ekscizije mezorektuma (PME) 96 (42.66%) bolesnika. Muškaraca je bilo 145 (64,44%), a žena 80 (35,55%); prosečna starost 66,8 godina. **Rezultati.** Kod 58 (25,77%) bolesnika karcinom je bio lokalizovan u proksimalnoj trećini rektuma, kod 99 (44%) u srednjoj trećini, a kod 68 (30,22%) do 8 cm od anokutane linije. Kod 167 (74,22%) bolesnika karcinom rektuma bio je u T3 stadijumu. Metoda TME primenjena je kod svih bolesnika sa karcinomom u distalnoj trećini rektuma i kod 61,61% bolesnika sa karcinomom u srednjoj trećini rektuma. Metoda PME primenjena je kod svih bolesnika sa lokalizacijom karcinoma u proksimalnoj trećini i kod 38,38% bolesnika sa lokalizacijom u srednjoj trećini rektuma. Do pojave lokalnog recidiva došlo je kod 20 (8,88%) bolesnika. U grupi TME bilo je 12 (9,30%), a u grupi PME 8 (8,33%) bolesnika sa lokalnim recidivom, što

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ne predstavlja statistički značajnu razliku. Kod 75% bolesnika recidiv se javio u T3 stadijumu bolesti. Kod 55% bolesnika lokalni recidiv se javio u drugoj godini. Prosečno preživljavanje svih ispitanika iznosilo je 35 meseci. Ukupna smrtnost na trogodišnjem nivou iznosila je 5,3%. **Zaključak.** Nije bilo statistički značajne razlike u incidenciji lokalnog recidiva i dužini preživljavanja između bolesnika kojima je urađena TME i onih kojima je urađena PME. Tip mezorektalne ekscizije ne utiče na incidenciju lokalnog recidiva u *nodus* negativnim stadijumuma bolesti.

Ključne reči:
rektum, neoplazme; neoplazme, lokalni recidiv; hirurgija digestivnog sistema, procedure; hirurgija, operativne procedure; neoplazme, određivanje stadijuma; prognoza.

Introduction

Colorectal cancer (CRC) with the incidence rate of 27 *per* 100,000 people represents the third leading cause of morbidity, right after lung and breast cancer. Annually, about 1.2 milion people are affected by CRC. In Serbia, CRC is the second leading cause of death in men and the third in women. Over the past few years, intensive work on improving the prevention, diagnosis and surgical techniques has been done in order to improve the results of treatment and quality of life of patients with CRC. However, the overall survival percentage remains unsatisfactory, because only 50% of patients live five years after the curative resection [1]. Screening of general population has an important role, because it allows the prevention of the disease, and the early detection of cancer, at a stage when the chances of cure are the largest and most certain. Surgical treatment is the most important link in the treatment of patients with rectal cancer [1]. The decision on the type of surgical intervention, *ie* total mesorectal excision (TME) or transection of mesorectum or partial mesorectal excision (PME), depends on several factors, primarily the tumor location and stage of the disease. For tumors of the distal third of the rectum, surgical method of choice is TME. In tumors of the medium third of the rectum (8–12 cm) it is possible to perform PME in selected cases. For tumors of the rectum laid 12–15 cm above the anocutaneous line, PME is a surgical method of choice [1, 2]. According to the literature [2–4], rectal cancer recurs in 8–50% of the cases. The highest percentage of the disease recurrence is within the first two years of the treatment completion [5, 6]. One of the most important risk factors for the disease recurrence represents the stage of the tumor. In the first stage of the disease local recurrence occurs in 10%, in the second stage about 24%, and in the third in around 41% of the patients who underwent potentially curative procedures [7]. The local recurrence is significantly influenced by poor tumor differentiation and perineureal and vascular space involvement of the tumor [7]. Risk factors for local recurrence are age, general condition of patients, as well as the knowledge and experience of surgeons in this field of surgery [8]. The tendencies of modern treatment of rectal cancer are to decrease the incidence of the local recurrence rate below 10% by constantly improving the surgical techniques and adjuvant therapy [1, 9]. If, in rectal cancer treatment only surgery is applied, local relapse frequency is pretty high, 15–45%. If radiotherapy and chemotherapy are applied together with TME, local relapse percentage is under 10%. Neoadjuvent and adjuvant radiotherapy application improves local disease control in great amount, while with chemotherapy micrometastases can be controlled. In locally progressive rectal cancer in T3 stage, radiotherapy is applied preoperatively, with the aim of tumor resectability enhancing, transferring cancer from the inoperable to operable stage, reducing malignant potential and local relapse percentage.

The aim of this study was to determine the effect of mesorectal excision type on local recurrence in our series of operated patients after the initial treatment for rectal cancer within a 3-year period.

Methods

A retrospective analysis of the initial treatment results in 225 patients with rectal cancer without metastases was conducted at the Clinic for General Surgery at the Clinical Center in Niš, Serbia, in a period 2009–2012. Postoperatively, the patients were observed 36 months. Of the total number of patients, there were 145 males (64.44%) and 80 (35.55%) females, average age 66.8 years. Histopathological examination of biopsy specimens revealed adenocarcinoma in all the cases. The localization and disease stage (TNM) are given in Table 1. The patients underwent potentially curative resection surgery

Table 1

Localization of rectal cancer and TNM stage tumors by the type of mesorectal excision (TME/PME) before the treatment period

Parameter	TME	PME	Total
Localization of rectal cancer, n (%)			
proximal 12–15 cm	-	58 (100)	58 (25.77)
medium 8–12 cm	61 (61.61)	38 (38.38)	99 (44)
distal 2–8 cm	68 (100)	-	68 (30.22)
Stadium of the disease (TNM)			
T1, T2 N0 M0	8	7	15
T1-2 N1-2 M0	19	21	40
T3 N0 M0	74	65	139
T3 N1-2 M0	25	3	28
T4 N1-2 M0	3	-	3
Total, n (%)	129 (57.33)	96 (42.66)	225 (100)

TNM – tumor, nodus, metastasis; TME – total mesorectal exacision; PME – partial mesorectal excision.

(standard resection anterior of the rectum – RAR, in all patients). TME was performed in all the patients with cancer in the distal third of the rectum [129 (57.33%) patients], and in 61.61% of the patients with carcinoma of the medium third of the rectum. PME was performed in all the patients with localized cancer in the proximal third [96 (42.66%)] of the rectum, and in 38.38% of the patients with cancer localized in the medium third of the rectum.

As a part of the resection procedure, preoperative radiotherapy was performed in 63 patients (28.0% of the total number of patients in the series – 225) (all from the group with TME – the sT3 N0 M0, sT3 N1-2 M0, sT4 N1-2- 3 M0), by the protocol 25 gray (5 Gy/fraction each day during the week) and a subsequent operation, as well.

The patients were observed through regular three month check-ups in the first two years, 6 month later on. In some cases when, based on patients' symptoms and physical examination, it was suspicious of local relapse existence, we used tumor markers (CEA and Ca 19-9), computed tomography (CT) of the abdomen and pelvis one *per* year, ultrasonography of the abdomen every six months, later multislice CT (MSCT) and nuclear magnetic resonance (NMR) and chest x-ray after one year.

Statistical analysis

Statistical analysis was performed using SPSS version 18 for Microsoft Windows. Survival analysis was carried by Kaplan-Meier method. Multivariate analysis was performed using the Cox regression model. *P*-values less than 0.05 were considered statistically significant.

Results

Data on localization of rectal cancer, TNM stage of tumors the type of mesorectal excision (TME/PME), before the treatment period are presented in Table 1.

In 167 (74.22%) of the patients rectal cancer was in T3 stadium (TNM).

The patients in stages of the disease N1-2 had 8-14 lymph nodes removed. In 220 (97.78%) operated patients, mechanical anastomoses were performed, in 54 (41.86%) patients from the TME group protective transverse colostomy was performed, and in 75 patients (58.13%) ileostomy was done.

Local recurrence occurred in 20 (8.88%) patients; 12 (9.30%) in the TME group and 8 (8.33%) in the PME group, which was not a statistically significant difference (OR = 0.86; 95% CI = 0291–2496; $p = 0.755$).

During a 3-year follow-up period, 38 (16.88%) of the patients developed distant metastases (liver, peritoneum, bones) and 17 (7.56%) had local recurrence associated with distant metastases.

Characteristics of local recurrence are shown in Table 2.

In 15 (75%) of the cases in T3 stage at the time of the surgery relapse occurred. Relapse occurred, at the earliest, six months after the surgery, and in 55% of the cases in the second year after the surgery.

Three of the patients with potentially curative surgery had positive margin on histology. One of them underwent abdominoperineal resection and the other 2 refused further operation and died of liver metastasis.

In our study 63 patients of the TME group were preoperatively treated with radiation therapy and four relapses were noted in this group of patients, out of total 12 relapses in the TME group.

Treatment of the patients with locoregional disease recurrence and distant metastases (liver) was conducted in accordance with consultative assessment and decision, and included a curative resection, chemotherapy and palliative procedures.

The total mortality of all respondents in a 3-year period amounted to 5.3% (12 respondents died out of 225). Causes of death are shown in Table 3.

Figure 1 shows the Kaplan-Meier survival curves of the patients operated for rectal cancer. The median survival of all

Table 2

Characteristics of local recurrence in the patients operated for rectal cancer

Parameter	TME n = 12	PME n = 8	Total n = 20
Localisation of rectal cancer recurrence			
segment of anastomosis	2	1	3
regional lymph nodes	5	4	9
pelvis, peritoneum, omentum, abdominal wall (implants)	5	3	8
Stadium of the disease (TNM)			
T1-2 N0 M0	-	-	-
T1-2 N1-2 M0	1	-	1
T3 N0 M0	3	4	7
T3 N1-2 M0	5	4	9
T4 N1-2 M0	3	-	3
Time of occurrence (months)			
0–6	1	-	1
7–12	2	2	4
13–24	7	4	11
25–36	2	2	4

TNM – tumor, nodus, metastasis; TME – total mesorectal exacision; PME – partial mesorectal excision.

Table 3
Causes of death of patients operated for rectal cancer by TME or PME methods

Causes of death	Number of patients	
	TME	PME
Anastomotic leakage, peritonitis diffuse, MODS	2	2
Cardiopulmonary insufficiency	2	0
Progressio morbi, n	4	2
Total, n (%)	8 (6.2)	4 (4.2)

TME – total mesorectal excision; PME – partial mesorectal excision; MODS – multiple organ dysfunction syndrome.

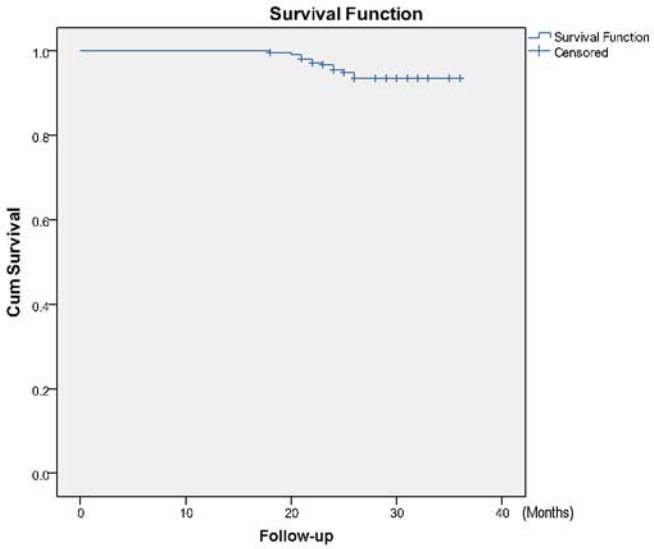

Fig. 1 – Kaplan-Meier survival curves of the patients operated for rectal cancer.

the patients amounted to 35.162 months with a standard error of 0.235 months.

Figure 2 shows the survival of the two groups of patients. The average survival time of the TME patients amounted to 35.078 ± 0.316 months, and the PME patients to 35.287 ± 0.348 months, and no statistically significant difference in the length of survival of analyzed groups was observed (log rank = 0.194; $p = 0.660$). Regarding this, the Cox regression model does not single out the type of surgery as a predictor of the fatal outcome, as well [Hazard Ratio (HR) = 0.764; 95% Confidence Interval (CI) = 0.230–2541; $p = 0.661$].

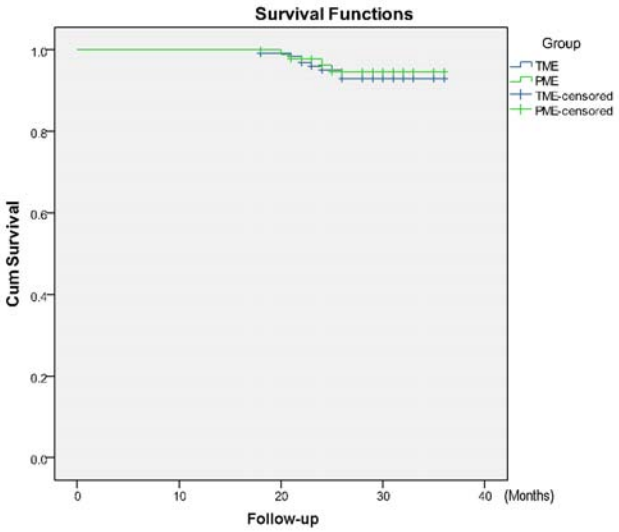

Fig. 2 – Kalpan-Meier survival curve of the patients operated on by total mesorectal excision (TME) and partial mesorectal excision (PME).

Discussion

In the last twenty years, numerous studies on any modalities of therapy for rectal cancer have been carried out and shown that they are subject to the same basic principles [10]. For tumors of the distal and proximal third of the rectum, the attitude on the type of mesorectal excision is clearly defined. However, there are controversies regarding surgical treatment of rectal cancer which is localized in the middle third. In other words, the question is whether to always apply TME in these patients or PME in selective cases [1].

TME was defined as the excision of the rectum with the surrounding mesorectum enclosed by the visceral pelvic fascia at the level of the pelvic flor. Transection of mesorectum at a higher level was considered PME. Local recurrence was defined as the presence of radiologically confirmed or histologically proven tumor in the pelvis within the field of surgery. The time to local recurrence was the duration between the surgical resection and the time of documentation of the recurrence [2]. We analyzed the data from the medical records related to gender, age, tumor location, stage of disease, type of surgical procedures and preoperative radiation.

Most studies indicate that the line of resection to 2 cm below the tumor is enough and oncologically quite safe [1–3, 6, 8, 11]. Also, lateral circumferential propagation is much more predictive of local recurrence compared to the propagation of distal tumors. Incomplete resection of the lateral margin of the tumor is considered the main cause of recurrence. Even with well performed mesorectal excision, a certain number of patients have a positive circumferential resection margin (CRM). Involvement of CRM is a sign of more advanced disease rather than poor surgical techniques. Patients with affected CRM can die of distant metastases before the local recurrence. The bigger the distance of the tumor from CRM the better the prognosis. A margin is positive when the tumor is less than 1 mm of the mesorectal fascia [1].

In the study of Scott et al. [11], the incidence of expansion into mesorectum at a distance of 5 cm, was 20%, which indicated that the excision of mesorectum 5 cm below the tumor is quite sufficient to satisfy the principles of oncology. Postoperative monitoring is essential in the detection of local recurrence. If the local recurrence is detected at an early stage, the chances of recovery are significantly higher. A large number of studies have dealt with the problem of optimal monitoring. The results show that the monitoring program is optimal if 2–3% of patients with recurrent disease are detected at the check-up, if the check-up is done every 2–4 months in the first two years from operation, and then every 6 months [12]. The conclusion is that the monitoring program must be adjusted according to the degree of relapse risk [13]. Local recurrence in 90% of the cases is detected in the first five years postoperatively. Aggravating circumstance is that the patients who relapse are most frequently in bad general condition, with the presence of distant metastases. A small number of patients with local recurrence is in good general condition, with a tumor resectionable at the time of disclosure and without distant metastases [14].

Diagnostic procedures are the most important link in the detection of local recurrence and include: physical examination, tumor markers [CEA and Ca 19-9), endoscopic and radiological methods (CT, NMR, endorectal ultrasound (ERUZ), positron emission tomography (PET) scan]. One of the most important indicators that can induce doubt on the existence of local recurrence are the symptoms of patients. If patients with a suspected local recurrence was not detected by a noninvasive diagnostic procedure, “a second look” laparotomy is indicated. Local recurrence of rectal cancer after TME and PME, mostly depends on the characteristics of the tumor. The highest percentage of relapses occurs in ulcer infiltrative tumor forms (26.3%), while the lowest is in egzofit intraluminal form and in tumors smaller than 3 cm (10.8%) and along with the stage of the disease, the frequency of local recurrence is growing; in the third stage it is 40% [15].

Based on the all above, the decision on the type of mesorectal excision, *ie* TME or PME, shall be adopted for each patient individually, depending on the characteristics and stages of tumors [16, 17].

Our study showed that there was local recurrence in 20 (8.88%) of the patients within a 3-year interval after the initial treatment (Table 2). We found no statistically significant difference in the incidence of local recurrence among the groups, which opens the possibility for the PME to be applied in patients with cancer localized in the middle third of the rectum in the early, favorable cases of so-called "good" group (sT1-2, some early sT3 N0 (sT3a (b) and clear – CRM by magnetic resonance imaging (MRI).

Also, in the present study, local recurrence was reported in 55% of the cases in the second year after surgical procedures, and in 75% of the cases, relapse occurred in the patients in T3 stage of rectal cancer at the time of surgery, confirming the data from numerous studies on this subject, that the local recurrence was mostly caused by the stage of the tumor, rather than the surgical techniques [18–21]. The results of studies on this problem, show no significant difference in the incidence of local recurrence in patients operated by TME and PME methods [1].

Local recurrence is most common in stage C according to Dukes (Heald 7 *vs* 27.4%, Hall 14 *vs* 27.8%, Dickson 9 *vs* 39.9%). The study by Killingback et al. [18] which included 549 patients operated by TME and PME showed that the local recurrence of 7.6% after PME was the approximate percentage of recurrence after TME. In patients with carcinoma of the medium third of the rectum, TME is not commonly peformed, but PME was performed instead in selected cases. The 5-year survival of patients in this study was 72.5% [18].

Lopez-Kostner et al. [19], in their study on the emergence of local recurrence in tumors localized 10–15 cm, in which TME and PME methods were performed, proved that there is no significant difference in the occurrence of local recurrence, as well as in a five-year survival, suggesting performing of PME whenever possible due to smaller functional deficits postoperatively.

A study done by Van Lingen et al. [20], showed the local recurrence in 4.6% of the patients after TME in the follow-up period of 25 months.

In a study of Petronella at al. [21], it was demonstrated that the emergence of local recurrence occurred in 6% of patients after TME.

Krivokapic et al. [22], have also dealt with this problem in a series of 1,000 patients operated for rectal cancer. They accepted TME concept for all tumors up to 8 cm above the anocutaneous line. In cases of rectal carcinoma located above 8 cm they usually performed PME. It was no statistically significant difference in local recurrence rates between TME and PME group. The emergence of local recurrence after TME was 7.6% and in the group of patients who underwent PME it was 5.6% of cases, which is in correlation with the results from our study.

Analyzing our results by Kaplan -Meier test, the survival of two groups was not statistically significantly different in overall survival. The median survival of all the patients was 35 months. The total mortality of all respondents in a 3-year period amounted to 5.3%, which was in correlation with the data available in the literature.

In a study conducted by Law and Chu [17], local recurrence in a 5-year interval was 9.7% and the survival percentage was 74.5% with no statistically significant differences between the two groups.

In a study by van Lingen et al. [20] after a follow-up period of 25 months, mortality was 5.3%.

In a study by Gupta at al. [23], TME was performed in 202 and PME in 96 patients. In the follow-up period of 38.7 months, 32 patients with local recurrence were detected. In a 2-year period after the operation, the local relapse occurred in 7.0% of the cases, and a 5-year monitoring showed the incidence of local recurrence rate of 10.7%. The 5-year overall survival and cancer-specific survival rates were 67.5% and 75.5%, respectively.

Indications for preoperative radiotherapy were patients with preoperatively confirmed T3 and T4 tumor stages (TNM). Local recurrence is the most important measure of the oncologic outcome following rectal cancer surgery [1–3]. The question whether the rate of local recurrence would be reduced in the group of patients with T3 nodule negative and nodule positive stage of the disease, who underwent PME, in accordance with the views and recommendations of other authors [24–27], remains open.

The treatment of rectal cancer is demanding and requires skills and art of the entire multidisciplinary team. Good surgery, good analysis of histological samples, good technique and optimal radiation, chemotherapy, along with a long-term monitoring of morphological and functional results are very important for the quality control [2]. (Non) radicality of the surgical procedure and applied preventive measures against local recurrence by the surgical team, also affect the localization and type of local recurrence [1, 2].

Conclusion

In the series of 225 patients with rectal cancer, after the initial and potentially curable surgical treatment within a 3-year follow-up period, there was a local recurrence in 20 (8.88%) of the patients, while the overall survival was 35 months and the overall mortality 5.3%.

There were no statistically significant differences in the incidence of local recurrence and survival among the patients who underwent TME with low anastomosis and those with PME and high anastomosis.

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Received on April 30, 2015.
Revised on June 6, 2015.
Accepted on July 29, 2015.
Online First May, 2016.

ORIGINAL ARTICLE

UDC: 616.5-006.81-07
DOI: 10.2298/VSP150227042S

Validity of ultrasound-guided aspiration needle biopsy in the diagnosis of micrometastases in sentinel lymph nodes in patients with cutaneous melanoma

Validnost ultrazvučno vođene aspiracije tankom iglom u dijagnostici mikrometastaza u limfnim čvorovima stražarima kod obolelih od melanoma kože

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Abstract

Background/Aim. Cutaneous melanoma is one of the most aggressive solid cancers, that develops local, regional and distant metastases. The presence of metastases in lymph nodes is in correlation with Breslow tumor thickness. According to various researches, in melanoma with more than 4 mm Breslow thickness, lymph node micrometastases can be found in 60–70% of cases. Sentinel lymph nodes biopsy is a diagnostic procedure for lymph node micrometastasis detection, which is necessary for disease staging. In recent studies, ultrasound-guided fine needle aspiration with cytology (US FNAC) of the sentinel lymph node was used as less invasive procedure, but is not accepted as the standard procedure. The goal of this work was to define sensitivity, specification and precision of the ultrasound-guided fine needle aspiration method in comparison with standard sentinel lymph node biopsy. **Methods.** After obtaining the Ethics Committee's permission, from 2012 to 2014 a total of 60 patients with cutaneous melanoma were enrolled, and divided into three groups: group I with thin melanoma, group II with intermediate thickness melanoma and group III with thick melanoma. The presence of micrometastases in sentinel regional lymph nodes was analyzed by US FNAC. The results obtained were compared to sentinel lymph nodes biopsy (SLNB) results. The golden standard for calculating the specific, sensitive and precise characteristics of the method of US FNAC of sentinel lymph nodes was histopathologic lymph node examination of sentinel lymph nodes acquired through biopsy. **Results.** Detection rate of US FNAC was 0% in the group I, 5% in the group II and 30% in the group III. SLNB detection rates were: 10% in the group I, 15% in the group II, and 45% in the group III. In melanoma thicker than 4 mm, 15% of the patients were false negative by US FNAC. The sensitivity of US FNAC for all the patients was 50%: in the group I, 0%; in the group II, 33.3%; and in the group III, 66.6%. The method specificity for all examined patients was 100% and accuracy 88%: group I, 90%; group II, 90%; group III, 85%. The FNAC and SLNB micrometastasis detection rate was significantly higher in melanoma with Breslow thickness > 4 mm (group 3) in comparison to thin and intermediate thickness tumors. **Conclusion.** The method of ultrasound-guided fine needle aspiration of sentinel lymph nodes, according to its sensitivity, has a place in the diagnostics of micrometastasis in regional lymph nodes only in thick melanoma, but not in thin and intermediary thickness melanoma. The results must be confirmed in a larger number of patients. If this observation could be confirmed, it would rationalize treatment of patients with thick melanoma, decrease the number of operations and shorten the time to make the diagnosis.

Key words:
endoscopic ultrasound-guided fine needle aspiration; neoplasm, micrometastasis; sentinel lymph node biopsy; skin; melanoma; diagnosis; sensitivity and specificity.

Apstrakt

Uvod/Cilj. Kožni melanom je jedan od najagresivnijih solidnih malignih tumora, koji se širi lokalno, regionalno i udaljeno metastazira. Prisustvo metastaza u limfnim čvorovima u vezi je sa debljinom tumora prema Breslow-u. Prema različitim istraživanjima, kod melanoma debljih od 4 mm prema Breslow-u, mikrometastaze u limfnim čvorovima mogu se naći kod 60–70% slučajeva. Biopsija

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limfnog čvora stražara je dijagnostička procedura za otkrivanje mikrometastaza u limfnim čvorovima, što je neophodno za određivanje stadijuma bolesti. U poslednjim istraživanjima, ultrazvučno vođena aspiracija tankom iglom (UZ ATI) sa citološkim nalazom limfnog čvora stražara korišćena je kao manje invazivna procedura, ali nije prihvaćena kao standardna peocedura. Cilj ovog rada bio je da se definiše senzitivnost, specifičnost i tačnost metode ultrazvučnog vođenja aspiracije tankom iglom u odnosu na standardnu biopsiju limfnog čvora stražara. **Metode.** Nakon dobijanja dozvole Etičkog komiteta u periodu od 2012. do 2014. godine, 60 bolesnika sa kožnim melanomom grupisano je u tri grupe: grupa I sa tankim melanomom, grupa II sa intermedijarnom debljinom melanoma i grupa III sa debelim melanomom. Prisustvo mikrometastaza u regionalnim limfnim čvorovima stražarima analizirano je UZ ATI sa citološkom analizom. Dobijeni rezultati su poređeni sa rezultatima biopsija limfnog čvora stražara. (BLCS) Zlatni standard za izračunavanje specifičnosti, senzitivnosti i tačnosti metoda UZ ATI limfnog čvora stražara je bio patohistološki nalaz limfnih čvorova dobijenih biopsijom limfnog čvora stražara. **Rezultati.** Dobijeni rezultati UZ ATI bili su 0% u grupi I, 5% u gupi II i 30% u grupi III. Dobijeni rezultati BLČS bili su: 10% u grupi I, 15% u grupi II i 45% u grupi III. Kod melanoma debljih od 4 mm, 15% bolesnika imalo je lažno negativni rezultat UZ ATI. Senzitivnost UZ ATI za sve bolesnike bila je 50%: u grupi I 0%; grupi II 33,3%; u grupi III 66,6%. Specifičnost metode za sve bolesnike bila je 100%, a tačnost 88%: grupa I 90%, grupa II 90%; grupa III 85%. Otkrivanje mikrometastaza UZ ATI i BLČS bilo je značajno veće kod melanoma debljine veće od 4 mm prema Breslow-u (grupa III) u poređenju sa tankim i intermedijarnim tumorima. **Zaključak.** Metod UZ ATI limfnih čvorova stražara prema senzitivnosti ima mesto u dijagnostici mikrometastaza u regionalnim limfnim čvorovima samo kod debelih melanoma, ali ne i kod tankih i intermedijarnih melanoma. Rezultati moraju biti potvrđeni na većem broju bolesnika. Ako se ovo posmatranje potvrdi, može se racionalizovati lečenje bolesnika sa debelim melanomom, smanjujući im broj operacija i skraćujući im vreme dijagnostike.

Ključne reči:
endoskopska, ultrazvukom-vođena, aspiracija tankom iglom; neoplazma, mikrometastaza; limfni čvorovi, stražarski, biopsija; koža; melanom; dijagnoza; senzitivnost i specifičnost.

Introduction

Melanoma, malignant tumor of melanocytes, is one of the most aggressive solid malignant tumors. It develops local, regional and distant metastases. The presence of metastases in lymph nodes is in correlation with Breslow tumor thickness. According to various researches, in melanoma with more than 4 mm Breslow thickness, regional lymph node micrometastases can be found in in 60–70% of cases [1].

Regional lymph node metastases in melanoma are diagnosed by ultrasound examination, biopsy of sentinel lymph nodes in order to detect micrometastases and fine needle aspiration for macrometastases. Ultrasound examination of regional lymph nodes gives data on the presence of macrometastases. The precision of this exam can be enhanced by using color Doppler sonography and power mode [2]. By analysis of ultrasound characteristics of the lymph node, the lymph node length to width ratio, echogenic characteristics of lymph node, arborisation, lymph node vascularization type and resistance index, the sensitivity of the exam of 100% and the precision of 93.3% are obtained [3]. This exam gives secure ultrasound detection of macrometastasis in lymph nodes of 2 mm or bigger. It was also used in recent studies to analyze sentinel lymph node after lymphoscintigrpahy, but with various results [4–7].

Sentinel lymph nodes biopsy (SLNB) is a diagnostic procedure for detection of micrometastases necessary for disease staging. The method of intraoperative marking of lymph nodes and biopsy of sentinel lymph nodes, which were developed by Morton et al. in 1991, represents a more rational and effective way of diagnosing of patients with clinically negative regional lymph nodes [8, 9]. Using this procedure the extent of metastases in regional lymph nodes can be estimated with a high degree of reliability, through biopsy of one, sometimes two or three sentinel lymph nodes [10, 11]. The methodology is based on the theory that the first node among the regional lymph nodes is also the place of the first metastasis. The sentinel lymph node status is predictive of further non-sentinel lymph node metastases. If a sentinel lymph node is negative, it can be expected with 99% accuracy that other regional lymph nodes are also without metastases. The possibility of the presence of skipping metastases (when the nearest lymph node region is skipped and metastases are found in the next one) is 2%. If sentinel lymph node biopsy is positive, the patient is diagnosed as stage III disease according to the American Joint Committy of Cancer (AJCC) classification [12]. In patients with positive sentinel lymph node, complete regional lymph node dissection is indicated [13].

Sentinel lymph node is marked with a radioactive marker [technetium (Tc 99) nano colloid], which is applied around the primary tumor excision scar 24 hours before the procedure. After the placement of the radio marker, gamma camera is used for determination of the lymph node region. During this procedure, the patient is positioned in the same way as he will be positioned during the actual operation: for axilla, arms above head; for inguinal region – shin in flexion, thigh in abduction; for retroauricular salivary gland and neck – head turned to the opposite side from the place of marked sentinel lymph node.

In addition to radioactive tracer, lymph nodes can be marked with 1% methylene blue which is injected 1–2 hours before the procedure (1–2 mL). According to various researches, this double marking of the sentinel lymph node raises the accuracy of the method to 93–99.7% depending on the center where the procedure is done [14, 15]. Possible complications after the sentinel lymph node biopsy are: edema, hematoma, infection, wound dechiscention; lymphocela, and also all complications due to general anesthesia [16].

The significance of this method is both diagnostic and prognostic. This method prolongs disease free survival and in intermediate thickness melanoma, overall survival, too [17–26]. Success of this method depends on a good collaboration of nuclear medicine specialist, surgeon and pathologist.

Ultrasound-guided fine needle aspiration (US FNA) is used as a standard procedure for histopathological confirmation of melanoma macrometastases. This method was also used for evaluation of sentinel lymph node, with various success [27]. The procedure, monitored with ultrasound, is done by moving the needle through the lymphatic node and, simultaneously, aspirating the specimen [27]. According to some authors, in 65% of cases, micrometastases can be found using this method, and hence this matheod could replace the standard SLNB [28–31]. Other authors think that this procedure is not reliable because it is impossible to visualize by ultrasonography sentinel lymph nodes with a diameter less than 4.5 mm and perform the fine needle aspiration. Also, it is possible that the distance between the cortex and medulla of the sentinel lymph node is too small making the ultrasound-guided fine needle biopsy hard to perform [32].

If this method can be proven to be of similar specificity and sensitivity as SLNB, it would be possible to reduce the number of operations and shorten the time for disease staging. The method of sentinel lymph node ultrasound-guided fine needle biopsy would be less aggressive and much cheaper than sentinel lymph node biopsy. Thus, the aim of our work was to determine sensitivity, specificity and accuracy of ultrasound-guided fine needle biopsy method in comparison to standard SLNB.

Methods

After obtaining the Ethics Commity's permission, this prospective study, performed from 2012 to 2014, enrolled 60 patients with histopathologically confirmed cutaneous melanoma with indication for SLNB. They were divided into three groups according to Breslow thickness: group I, 20 patients with melanoma of less than 1 mm Breslow thickness, and the presence of mitoses and/or ulcerations and/or Clark IV and/or regression more than 25%; group II, 20 patients with intermediate thickness melanoma, 1–4 mm; group III, 20 patients with Breslow thickness of more than 4 mm. Analysis included patients with only one positive sentinel lymph node and in only one lymphatic region. Initial staging consisted of palpation, regional lymph node ultrasound, abdominal and pelvic ultrasound chest radiography and laboratory analyses. Sentinel lymph node was determined in all the patients by the method of lymphoscintigraphy using gamma camera, Adac vertex, after subdermal placing of Technetium 99 marked with nanocolloid. Two hours prior to the operation, 1% methylene blue was also injected intradermaly around the excision biopsy scar.

Sentinel lymph nodes were identified by ultrasound examination of the patients from all the three groups, using the ultrasound device type TOSHIBA APLIO X6SA-790A with multi-frequent cannula, frequency rate 6-12 MHz. All lymph nodes were examined in B-mode real time, by pulse color Doppler, in power mode. Then, ultrasound-guided fine needle aspiration of sentinel lymph nodes was done with 27G (0.4 × 20 mm) and 25G (0.5 × 25 mm) needles, dependent of the distance between sentinel lymph node and the surface of the skin (Figure 1). The appearance of hematoma and punctuation in extracted sentinel lymph node was used as evidence of good aspiration (Figure 2). The specimens were stained with May-Grünwald-Giemsa and analysed. After fine needle aspiration, sentinel lymph node biopsy was performed in all the patients. Intraoperative sentinel lymph nodes detection was done by Gamma camera type Europrobe. Histopathological analysis of lymph nodes was done with hematoxylin and eosin staining (HE) and S-100 and HMB 45 imunohistochemistry as per protocol.

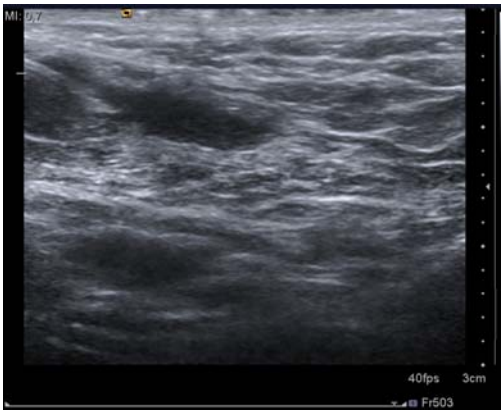

Fig. 1 – Ultrasound image of the needle in sentinel lymph node (LN).

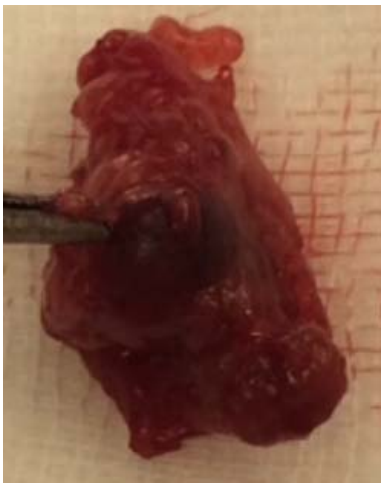

Fig. 2 – Needle mark and hematoma in a removed sentinel lymph node (LN).

Sensitivity was calculated as a quotient between really positive findings and the sum of really positive findings and false negative findings. Specificity was calculated as a quotient between really negative findings and the sum of really negative findings and false positive findings. Accuracy or prediction of positive outcome was calculated as a quotient between total number of positive findings and total number of negative findings and the total number of patients.

Descriptive statistic parameters (mean, standard deviation, spread, median and frequency of appearance of some parameters), and variance of analysis were used in the first direction and finished with Tukey test or non-parameter Kruskal-Wallis test for independent parameters which was finished with Mann-Whitney *U*-test. The presence of statistic significance between frequencies distribution in the particular gro-

up was checked by χ^2 test. Minimal statistic significance was set at the standard level of $p < 0.05$. For statistical analysis commercial statistic software SPSS, version 18 (USA) was used.

Results

Demographical data of all the patients are presented in Table 1. In all the three groups, the average age was almost the same, in the group I 50.25 years; in the group II, 55.45 years and in the group III 52.15 years. There were more male than female patients – in the group I, 11 males; in the group II, 15 males while in the group III the number of male and female patients was equal, 10.

Table 2 shows the distribution of detection rates of cytological analysis made by sentinel lymph node US FNAC method and histopathological analysis made by the sentinel lymph node biopsy method in relation to the tumor thickness.

In the group with melanoma thicker than 4 mm there were 30% of patients with the diagnosis of micrometastasis in SLN by cytological analysis after US FNAC, which was significantly more frequent than in thin and intermediary melanoma ($p < 0.05$). In the group with melanoma thicker than 4 mm there were 45% of patients with the presence of micrometastases in SLN histopathological analysis after SLNB, also significantly more frequent than in thin and intermediate thickness tumors ($p < 0.05$). So, 15% of the patients were falsely negative in US FNAC method.

Table 3 shows that there is a statistically significant difference in sensitivity between SLNB and US FNAC in all the patients and all the patient groups, in favor of SLNB. There was no statistically significant difference in specificity between these two methods. There is a statistically significant difference of $p = 0.017$ in the accuracy in all the patients, while this difference is absent in the patient groups, probably due to the small number of the patients.

Table 4 shows comparative analysis of surfaces of sen-

Table 1

Demographic data of the patients enrolled in the study

Melanoma thickness (mm)	Sex, n (%)			Age (years)		
	male	female	total	x̄	SD	median
< 1	11 (30.6)	9 (37.5)	20 (33.3)	50.05	14.96	52.00
1–4	15 (41.7)	5 (20.8)	20 (33.3)	55.45	15.85	57.00
> 4	10 (27.8)	10 (41.7)	20 (33.3)	52.15	15.64	51.00
Total	36 (100.0)	24 (100.0)	60 (100.0)			
Comparison	Distribution comparison $\chi^2 = 2.91$; $p = 0.23$			Group comparison (ANOVA) F = 0.61; $p = 0.54$		

SD – standard deviation.

Table 2

Detection rates of ultrasound-guided fine needle aspitration cytological analysis (US FNAC) and sentinel limph node biopsy (SLNB) histopathological analysis

Melanoma thickness (mm)	Cytology, n (%)			Histopathology, n (%)		
	negative	positive	total	negative	positive	total
<1	20 (0.0)	0 (0.0)	20 (33.3)	18 (90)	2 (10)	20 (33.3)
1–4	19 (95.0)	1 (5.0)	20 (33.3)	17 (85)	3 (15)	20 (33.3)
> 4	14 (70.0)	6 (30.0)	20 (33.3)	11 (55)	9 (45)	20 (33.3)
Total	53 (88.3)	7 (11.6)	60 (100.0)	46 (76.6)	14 (23.3)	60 (100.0)
Distribution comparison	$\chi^2 = 10.02$; $p = 0.007$			$\chi^2 = 8.01$; $p = 0.018$		

Table 3

Sensistivity, specificity and accuracy of ultrasound-guided fine needle aspitration cytology (US FNAC) in comparison to sentinel limph node biopsy (SLNB) histopatology

Parameters	Procedure	Patient groups regarding melanoma thickness			
		All (n = 60)	< 1 mm (n = 20)	1–4 mm (n = 20)	> 4 mm (n = 20)
Sensitivity	SLNB	1.0	1.0	1.0	1.0
	US FNAC	0.50	-	0.33	0.66
Comparison (*t*-test proportion)		z = 6.11 $p < 0.001$	-	z = 4.15 $p < 0.001$	z = 2.44 $p = 0.015$
Specificity	SLNB	1.0	1.0	1.0	1.0
	US FNAC	1.0	1.0	1.0	1.0
Comparison (*t*-test proportion)		*ns*	*ns*	*ns*	*ns*
Accuracy	SLNB	1.0	1.0	1.0	1.0
	US FNAC	0.88	0.90	0.90	0.85
Comparison (*t*-test proportion)		z = 2.38 $p = 0,017$	z = 0.72 *ns*	z = 0.72 *ns*	z = 1.20 *ns*

Table 4

Surface of sentinel limph node (SLN) and cytological analysis

Surface of SLN (mm^2)	Melanoma thickness (mm)			Cytological analysis	
	< 1	1–4	> 4	positive	negative
mean	73.85	75.75	53.00	254.17	109.06
SD	194.39	224.64	106.95	333.97	101.62
median	109.71	77.92	93.00	179.34	78.00
	Group comparison (Kruskal-Wallis test) $\chi^2 = 8.07$; $p = 0.018$ 1:2 – $p < 0.01$; 1:3 – *ns*; 2:3 – *ns*			Mann-Whitney test z = 1.69; $p = 0.091$	

SD – standard deviation; *ns* – no significance.

tinel lymph node in patients with or without SLN micrometastases according to cytological analysis.

There was no statistical significance between means of surfaces of affected lymph nodes in the patients with positive cytological findings compared to the patients with negative cytological findings ($p = 0.091$).

Discussion

From a surgical point of view, advantage is always given to methods that are more reliable and safer for the patient, with fewer complications during the procedure, and which are simpler to perform and economically more feasible. Due to the above mentioned, one of our goals was to determine the sensitivity, specificity and accuracy of sentinel US FNAC method. The method accuracy was found to be 88%: in the group I, 90%; in the group II, 90%; and in the group III, 85%. The method specificity for all examined patients was 100% in all the groups.

In this part special emphasis should be put on the segment which is the most important in clinical environment – the sensitivity of the method, which gives us data about successfully diagnosed micrometastases in a sentinel lymph node.

We have hypothesized, based on previous studies of that the sensitivity of this method would be similar to the sensitivity of sentinel lymph node biopsy, which was 93–97.3% [15]. However, that was not shown in this study (Table 3). In our study, the method sensitivity for all the patients was 50%, in the group I, 0%; in the group II, 33.3%; and in the group III, 66.6%. The sensitivity of US FNAC was found to be of inferior sensitivity in comparison to SLNB, especially in thin and intermediate thickness melanoma, and thus it cannot be recommended [6, 33] (Table 2). The method shows some validity only in melanoma with Breslow thickness of more than 4 mm, and it could find its use within this group, but this must be confirmed in a larger number of patients. Since the specificity is similar to SLNB, if the results of preoperative FNAC are positive, and micrometastasis is found, regional lymph node dissection could be done immediately. However, if it is negative, sentinel lymph node biopsy must be performed, since 15% of US FNAC cytology was falsely negative in our patient series. Thus, micrometastases found using US FNAC could decrease the number of SLNB, which is a more precise but also a more complex method. Based on this pilot study, 30% of patients with Breslow thickness of more than 4 mm could be diagnosed with micrometastases with US FNAC, skip SLN biopsy and beswitched to regional lymph node dissection. However, these results must be confirmed in a larger number of patients. As expected, and found in previous studies, the positivity rate of both US FNAC detected and SLNB detected micrometastases was higher in tumors with Breslow thickness of > 4 mm in comparison to thin and intermediate thickness melanoma ($p < 0.01$). The possibility that the tumor burden (diameter and location of micrometastatis) within a sentinel lymph node is in correlation with detection rate of the US FNAC must be explored in future studies.

The sensitivity of this method was not dependent of the patient's sex or the surface of the sentinel lymph node (Table 4). The biggest registered affected surface of sentinel lymph nodes was in the group with intermediate thickness melanoma, which was 194.39 mm. Despite that, the number of positive cytology findings and sensitivity of this method was greatest in the group with melanoma thicker than 4 mm (Table 2). In the patients with positive cytological findings of sentinel lymph nodes, the average surface was 254.17 mm compared to 109.6 mm in the patients whose cytological findings were negative. This was not a statistically significant difference. That also showed that sentinel lymph node area did not interfere with the method sensitivity (Table 3).

Voit et al. [28], a group of German dermatologists, are the most cited authors regarding US FNAC method. According to their Berlin ultrasound morphological criteria (loss of central echo of lymph node, balloon shape of lymph node, periphery vascularization of lymph node), four groups of sentinel lymph nodes were formed in their study: benign, probably benign, probably malignant, and malignant. The sensitivity of the US FNAC method in the first two groups was 56%, while in the third and fourth groups it was 82% [34]. Our criteria for analysis of the lymph nodes in the regional lymph basins before US FNAC were stricter than Berlin ultrasound morphologic criteria. We also added the resistance index and arborization of lymph nodes. Our findings can be compared with the total sensitivity of the methods employed

by Voit et al. [35] for the total number of patients, which was 59% [34], compared to our 50%. In the second study of Voit et al. [36] the sensitivity of the US FNAC method in melanoma thicker than 4 mm was 76%. In this study, the sensitivity of US FNAC in melanoma thicker than 4 mm was 66.6%.

Conclusion

The ultrasound-guided fine needle aspiration method with the sensitivity of 66.6% in thick melanomas (Breslow > 4mm), the accuracy of 88% and the specificity of 100% has a place in the diagnosis of micrometastases in regional lymph nodes only in this patient group. However, these findings should be confirmed in a larger sample of patients. If the results from this pilot study could be reproduced in larger number of patients, it could be possible to rationalize the treatment of patients with thick melanoma, decrease the number of operations and shorten the time to diagnosis. In thin and intermediate thickness melanoma, however, this method is of no value, and should not be further explored, since it was 0% in thin melanoma and 33.3%, in intermediate thickness melanoma.

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Received on February 27, 2015.
Revised on June 21, 2015.
Accepted on June 25, 2015.
Online First March, 2016.

SHORT COMMUNICATIONS

UDC: 616.1-089::616.134-007.64-08
DOI: 10.2298/VSP150420091M

Endovascular treatment of the subclavian artery aneurysm in high-risk patients – A single-center experience

Endovaskularno zbrinjavanje aneurizme supklavijalne arterije kod visokorizičnih bolesnika – iskustvo jednog centra

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Abstract

Background/Aim. Subclavian artery aneurysm (SAA) is a rare disease, but with serious complications. Recently, besides open surgical procedure, appearance of the stent-grafts enables endovascular reconstruction. We presented our first experience with endovascular treatment of 6 SAA occurring in five male and one female patient. **Methods.** All the patients, in our studies, according to ASA classification were at high risk of open repair of SAA. The etiology of all aneurysms was atherosclerotic degeneration of the artery. Two aneurysms were at intrathoracic location, and the other ones were extrathoracic. Symptoms related to SAA were present in two of the patients, compression and chest pain in one, and hemorrhage shock in another one. Other patients were asymptomatic. We preferred the Viabhan endoprosthesis for endovascular repair in 5 cases. In one patient with rupture of SAA, who was at high risk of open repair we performed a combined endovascular procedure. First of all, we covered the origin of the left subclavian artery with thoracic stent graft and after that put two coils in a proximal part of the subclavian artery. **Results.** There was no operative mortality, and the early patency rate was 100%. The follow-up period was from 3 months to 3 years. During this period, one patient died of heart failure and another one required endovascular reoperation due to endoleak type I. **Conclusion.** Endovascular treatment is recommended for all patients with SAA whenever it is possible due to anatomical reasons especially in high-risk patients with intrathoracic localization of aneurysm, to prevent potential complications.

Key words:
subclavian artery; aneurysm; aneurysm, ruptured; vascular surgical procedures; stents; transplants; prognosis; mortality.

Apstrakt

Uvod/Cilj. Aneurizma arterije supklavije se retko javlja, ali su komplikacije ozbiljne. U novije vreme, pored otvorenog hirurškog zahvata, pojava stent-graftova omogućava i endovaskularnu rekonstrukciju. Prikazali smo naše prvo iskustvo sa endovaskularnom rekonstrukcijom aneurizme supklavijalne arterije kod šest bolesnika, pet muškaraca i jedne žene. **Metode.** Svi bolesnici u našoj studiji bili su visoko rizični za otvorenu rekonstrukciju prema ASA klasifikaciji. Aterosklerotska degeneracija arterije bila je uzročnik nastanka aneurizme kod svih bolesnika. Dva bolesnika imala su aneurizme supklavijalne arterije intratorakalno, dok su kod ostalih bolesnika aneurizme bile ekstratorakalno. Kod dva bolesnika aneurizma supklavijalne arterije bila je simptomatska, sa simptomima u vidu pritiska i bola u grudima kod jednog i hemoragičnog šoka i bola u grudima kod drugog, dok su kod preostalih bolesnika aneurizme bile asimptomatske. Za endovaskularnu rekonstrukciju koristili smo Viaban stent-graft. Kod jednog bolesnika sa rupturom aneurizme supklavijalne arterije koji je bio visokorizičan za otvorenu rekonstrukciju, primenili smo kombinovani endovaskularni postupak. Prvo smo pokrili ušće supklavijalne arterije torakalnim stent-graftom, a zatim smo postavili dva klema u proksimalni deo supklavijalne arterije. **Rezultati.** Nije bilo operativnog mortaliteta tokom endovaskularne rekonstrukcije, a uspešnost izvođenja procedure bila je 100%. Period praćenja bio je od tri meseca do tri godine. Tokom ovog perioda, jedan bolesnik je umro zbog srčanog popuštanja, a kod jednog bolesnika smo izveli novu endovaskularnu proceduru zbog pojave endolika tipa I. **Zaključak.** Endovaskularno lečenje aneurizme supklavijalne arterije preporučuje se kod bolesnika kod kojih anatomske karakteristike same aneurizme omogućavaju izvođenje iste, a posebno se preporučuju kod visokorizičnih bolesnika sa intratorakalnom lokalizacijom aneurizme radi prevencije komplikacija.

Ključne reči:
a.subclavia; aneurizma; aneurizma, ruptura; hirurgija, vaskularna, procedure; stentovi; graftovi; prognoza; mortalitet.

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Introduction

Subclavian artery aneurysm (SAA) is a rare disease, thus it represents only 0.1% in relation to all other aneurysms of the aorta or peripheral arteries [1, 2]. Possible complications of SAA are rupture, distal embolization, compression and thrombosis, and therefore should be considered for surgical treatment [3]. Atherosclerosis is the most common cause of these aneurysms. Other causes that can lead to SAA are: thoracic outlet syndrome, degenerative connective tissue disorders, infection and trauma. The only way to treat SAA was open, surgical aneurysm reconstruction, until the appearance of stent-grafts and endovascular reconstruction. Open surgical procedure in the treatment of SAA depends on whether aneurysm affects the intrathoracic or extrathoracic segment of the artery [4]. For endovascular reconstruction of SAA localization of aneurysm is not so important but it is very important that aneurysm has adequate anatomical characteristics for the endovascular procedure. That means that SAA has adequate proximal and distal zone for stent-graft fixation.

Methods

We reported our single-center experience with endovascular treatment of 6 SAAs in the period January 2009 – December 2013. Four aneurysms were at extrathoracic location, while two of them were intrathoracic. Most of them were asymptomatic. Symptoms were present in two patients, compression and chest pain in one, and massive hemorrhage and chest pain in another one (Figure 1).

We preferred the Viabahn endoprosthesis (W.L. Gore, Flagstaff, USA) for endovascular repair in 5 cases. In one case, we covered the origin of the left subclavian artery with thoracic stent graft (TAG 3110, W.L. Gore, Flagstaff, USA) and after that we put two coils (Azur 35 Helical Hydrocoil 10 mm, Terumo, Tokyo, Japan) in the proximal part of the subclavian artery because the aneurysm did not have enough proximal neck (Figures 2 a and 2 b).

Under the local anesthesia we combined the transfemoral approach to endovascular treatment with the transbrachial approach to put the diagnostic catheter.

The follow-up period was from 3 months to 3 years. The patients were monitored postoperatively by physical examination, doppler ultrasonography at 3-, 6- and 12-month intervals, and once yearly thereafter. Control computed tomography (CT) angiography was performed in the patients after the first year of operation or more often if there was a need.

Results

Six presented patients were between 72 and 84 years old (five males and one female). All aneurysms were athero-

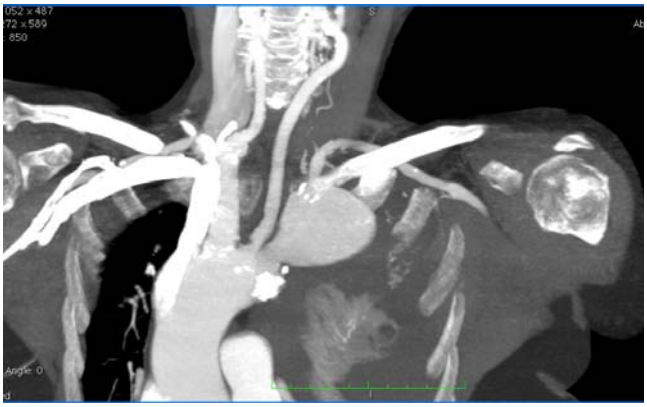

Fig. 1 – Rupture of subclavian artery aneurysm in the intrathoracic part with massive hemothorax.

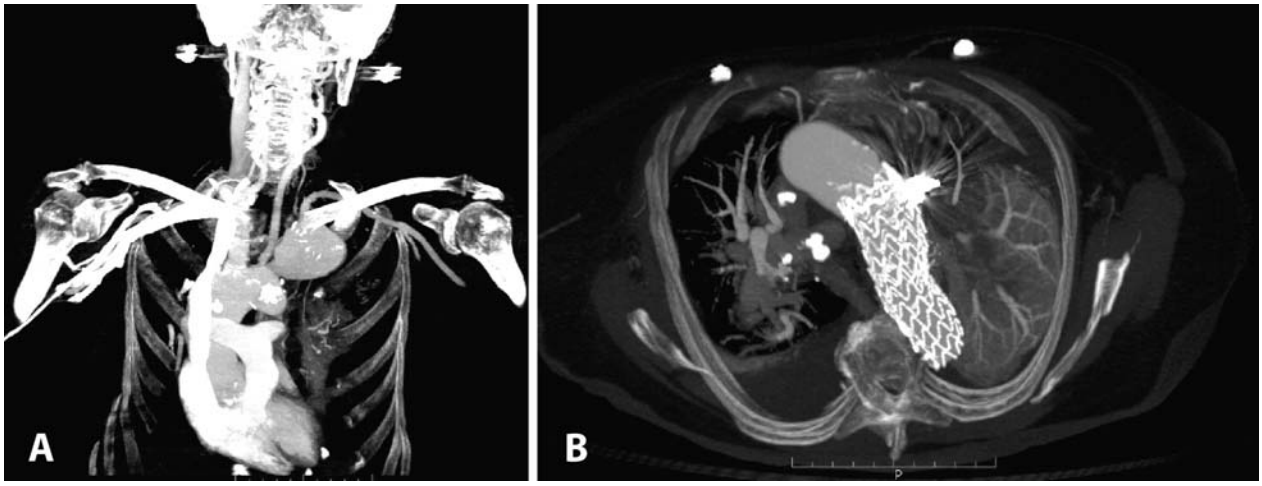

Fig. 2 – A) Short neck of subclavian artery aneurysm; B) Control computed tomography shows good position of the thoracic stent- graft covered origin of the subclavian artery and coils into the proximal part of the subclavian artery.

sclerotic true aneurysm. The diagnosis was established using CT angiography and duplex ultrasonography of the aortic arch and branches. The diameter of SAA ranged from 3.6 cm to 12 cm (mean 5.2 cm).

The most common comorbid conditions in the presented patients were: arterial hypertension, coronary artery disease, cerebrovascular insult, chronic cardiomyopathy, diabetes mellitus, peripheral arterial occlusive disease and chronic obstructive pulmonary disease. All the patients in our group were active smokers. It is interesting that none of the patients in our group had no medical history of aneurysmal aortic disease, nor a peripheral artery aneurysm. Medical history of the patients showed no chest injury, nor other types of trauma in the subclavian artery region (Table 1).

There was no operative mortality, and the early patency rate during the first tree months was 100%. During this follow-up period there was no need for open reconstruction of SAA and there were no complications such as stent graft thrombosis or distal embolization and ishemia.

During follow-up period of 3 years, one patient died of heart failure and another one required endovascular reoperation due to endoleak type I (Figures 3a and 3b).

Discussion

Elective surgical repair is mandatory for subclavian aneurysms, even when asymptomatic, because they tend to increase in size with increased risk of rupture, thrombosis, distal embolization and compression of surrounding structures.

Although aneurysms of SAA are rare, potential risk of rupture and secondary ischemic complications are complications which require surgical treatment. Open repair of SAA, especially of the intrathoracic segment in patients with previous median sternotomy or lateral thoracotomy, is a technical challenge with a lot of postoperative complications. Davidović et al. [3] reported a series of 14 patients with SAA treated with the supraclavicular or trans-sternal approach, depending on aneurysm location. No mortality occurred and the postoperative complication rate was 21%. During the follow-up period one patient required reoperation because he developed aneurysmal degeneration of a saphenous vein graft.

Endovascular techniques offer a minimally invasive option especially in high risk patients. Endovascular repair of SAA have been reported in a small series [5]. MacSweeney et al. [6] appear to be the first to use a stent-graft in endovascular repair of

Table 1

Clinical manifestations and comorbidity of patients with subclavian artery aneurysm (SAA)

Patient	Sex	Age (years)	Loc	Symptoms	Comorbidity	Procedure	Patency
1	M	72	I	Compression and chest pain	CVI, HTA	Viabhan	6 months
2	M	74	E	Asymptomatic	HTA, PAOD	Viabhan	3 years
3	M	78	E	Asymptomatic	CAD, DM	Viabhan	2 years
4	F	84	I	Massive hemorrhage	CAD,COPD,DM, HTA	TAG and coils	3 months*
5	M	76	E	Asymptomatic	CMP,COPD,HTA	Viabhan	1 year
6	M	75	E	Asymptomatic	CAD,HTA	Viabhan	1 year

M – male; F – female; Loc – localization; I – intrathoracic; E – extrathoracic; CVI – cerebrovascular insult; HTA – arterial hypertension; CAD – coronary artery disease; DM – diabetes mellitus; CMO – cardiomyopathy; COPD – chronic obstructive pulmonary disease; PAOD – peripheral artery obstructive disease. *Tree months later, the patient died with patent graft.

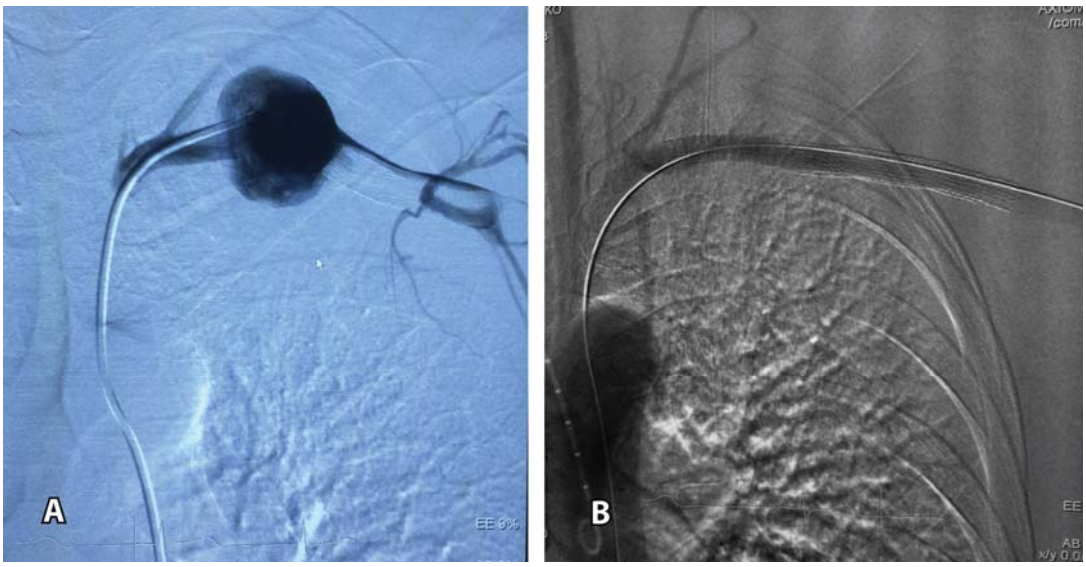

Fig. 3 – A) Aneurysm of the subclavian artery above the previously placed stent-graft with endoleak type I one year after the first reconstruction; B) Endovascular reoperation with one more placed stent-graft.

subclavian artery aneurysm in larger series of patients.

Preoperative duplex ultrasonography and CT angiography are always mandatory for diagnostic and planning the endovascular treatment of SAA [7, 8]. These diagnostic procedures are necessary to determine the proximal and distal neck diameter and proximal and distal landing zone, as well as to determine approprite the length of the stent-graft.

We preferred a Viabahn stent-graft for endovascular repair of SAA. It is a flexible nitinol stent frame covered internally with polytetrafluoroethylene (PTFE) graft. The flexibility of the Viabahn adapted well to the tortuosity of the subclavian artery, with minimal alteration in the native vessel curvature.

In our series, we successfully treated SAA in five patients with Viabhan stent-graft. All the patients were treated with Viabhan stent-graft placed endovasculary as an elective operation, but there are studies that present emergency stent-graft repair of SAA with Viabhan due to rupture [9]. In one patient with ruptured giant intrathoracic SAA who did not have an adequate proximal lending zone of the aneurysmal neck, we placed emergently thoracic stent-graft to cover the origin of subclavian artery, and after that we put two coils in the proximal part of the subclavian artery to prevent endoleak type II. Amiridze et al. [10] have already described the use of coils for treatment of the subclavian artery pseudoaneurysm and arteriovenous fistula. But it seems that endovascular treatment only with coils is reserved for pseudo-aneurysm and small saccular aneurysm.

Conclusion

Endovascular treatment of subclavian artery aneurysm may be a valuable, less invasive alternative to open surgical approach. This treatment is especially good for high risk patients with aneurysm of the intrathoracic part of the subclavian artery. However, long-term results of this technique have not yet been established.

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Received on April 20, 2015.
Accepted on June 25, 2015.
Online First May, 2016.

SHORT COMMUNICATION

UDC: 355/359:61]::616.89-07
DOI: 10.2298/VSP150124090S

Re-evaluating disability assessment in war veterans with posttraumatic stress disorder

Procena tačnosti dijagnoze invaliditeta kod ratnih veterana sa posttraumatskim stresnim poremećajem

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Abstract

Background/Aim. Sametimes war veterans may resort to such strategies as preducing exaggerated symptoms and malingerating in order to obtain material compensation rights. The aim of this study was to assess the accuracy of the diagnosis of posttraumatic stress disorder (PTSD) on the basis of which war veterans were entitled to a financial compensation due to their disability. **Methods.** The diagnoses of 259 war veterans were re-evaluated. Veterans were previously diagnosed by a psychiatrist on local level, while regional state medical commission determined the degree of disability and the right to a financial compensation. A team of experts, consisting of psychiatrists with research experience in the field of traumatic stress and who were trained to use a structured interview for PTSD, conducted the evaluation of medical data from veterans' military records. The diagnostic process was conducted using the standardized diagnostic interview (Clinician-Administered PTSD Scale – CAPS), after which the diagnosis was reaffirmed or reviewed. This influenced disability status and consequential financial compensation. **Results.** There was a remarkable difference between the first diagnostic assessment of PTSD, conducted by the psychiatrists on local level, and the second evaluation conducted by the team of experts. In more than half of 259 veterans (52.1%) diagnosed with PTSD in the first assessment the diagnosis was not confirmed. The diagnosis was confirmed in 31.7% of veterans. Those veterans who were diagnosed with lifetime PTSD (7.3%) should also be treated as accuratelly diagnosed. This means that a total of 39% of the diagnoses were accurate. The rest (8.9%) were diagnosed with other diagnoses, but not PTSD, as was the case in the initial assessment. **Conclusion.** The possibility for war veterans to obtain the right to disability and financial compensation due to a diagnosis of PTSD might interfere with the proper diagnostic assessment and thus the treatment outcome. During the procedures for the obtention of these rights, exaggeration or simulation of symptoms are common. The quality of the diagnostic assessment of PTSD can be improved by applying evidence-based standardized procedures.

Key words:
veterans; stress disorders, post-traumatic; work capacity evaluation; socioeconomic factors.

Apstrakt

Uvod/Cilj. Ratni veterani ponekad mogu pribegavati preuveličavanju simptoma i simuliranju da bi ostvarili pravo na materijalnu kompenzaciju. Cilj rada bio je da se izvrši procena tačnosti dijagnoze posttraumatskog stresnog poremećaja (PTSP) na osnovu koje su ratni veterani ostvarili pravo na invalidsku finansijsku kompenzaciju. **Metode.** Ponovna procena dijagnoza obavljena je kod 259 veterana rata. Kod svih veterana PTSP dijagnostikovao je psihijatar na lokalnom nivou, a zatim su regionalne lekarske komisije određivale stepen invalidnosti i pravo na finansijsku kompenzaciju. Ekspertski tim psihijatara sa istraživačkim iskustvom iz oblasti traumatskog stresa i obrazovanih za korišćenje strukturisanog intervjua za PTSP, vršio je procenu svih medicinskih nalaza i podataka iz vojne evidencije ratnih veterana. Dijagnostička procena vršena je primenom strukturisanog dijagnostičkog intervjua za PTSP [(*Clinician-Administered Post-Traumatic Stres Disorder* – PTSD Scale (CAPS)] nakon čega je potvrđivana ili revidirana dijagnoza, što je uticalo i na promenu statusa invalidnosti i efekata kompenzacije. **Rezultati.** Nađena je značajna razlika između prve dijagnostičke procene PTSP koju je izvršio psihijatar na lokalnom nivou i druge procene, koju je izvršio ekspertski tim psihijatara. Od 259 ispitivanih veterana, dijagnoza PTSP nije potvrđena kod više od polovine (52,1%). Dijagnoza je potvrđena kod 31,7% veterana, a kod 7,3% postavljena je dijagnoza prebolovanog PTSP, što znači da su

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i oni tačno dijagnostikovani tokom prve procene, tako da je dijagnoza potvrđena kod 39% veterana. Kod ostalih veterana (8,9%) dijagnostikovani su drugi mentalni poremećaji a ne PTSP, kao što je bio slučaj kod prve dijagnostičke procene. **Zaključak.** Mogućnost da ratni veterani ostvare finansijsku kompenzaciju i pravo na invaliditet zbog dijagnoze PTSP može da remeti adekvatnu dijagnostičku procenu, a time i ishod lečenja. U toku procesa za ostvarivanje ovog prava često se može uočiti prenaglašavanje ili simulacija simptoma. Iz studije se može zaključiti da se kvalitet dijagnostičke procene PTSP i posledična invalidnost mogu poboljšati primenom standardizovane dijagnostičke procene zasnovane na dokazima.

Ključne reči:
veterani, ratni; stresni poremećaji, posttraumatski; dijagnoza; sposobnost, radna, ocena; socioekonomski faktori.

Introduction

War in former Yugoslavia (1991–1995) is a paradigm of traumatic experience which has led to a severe disruption in mental health not only in war veterans, but also in refugees and the entire population [1, 2]. Research of effects of the air bombing of Federal Republic of Yugoslavia (FRY) in 1999 by NATO forces conducted on 434 civilians has shown that it is in fact personality characteristics that bear a much greater influence on the prediction of traumatic reactions rather than the intensity of experienced stress [3].

Clinical experience with posttraumatic stress disorder (PTSD) diagnosis has shown, however, that there are differences among individuals regarding the capacity to cope with catastrophic stress. Therefore, while most people exposed to traumatic events do not develop PTSD, others develop full symptoms of the disorder. Such observations have prompted the recognition that trauma, like pain, is not an external phenomenon that can be completely objectified. Like pain, the traumatic experience is filtered through cognitive and emotional processes before it can be appraised as an extreme threat. Due to differences among individuals, the thresholds in trauma patients are also different [4]. Research has consistently shown that PTSD is associated with impairments in functioning across a number of psychosocial domains. Such impairments are common among populations at a high risk for PTSD, such as military personnel involved in combat [5].

Many PTSD veterans seek compensation for the traumatic experience they have been exposed to quite different forms of benefits: financial compensation, early retirement or other types of social protection [6]. Receiving the compensation, however, raises doubts that traumatized person's reported levels of distress are motivated by material gain. Therefore, compensation motive is likely to augment symptomatology and relates to the concept of "secondary gain". This is why PTSD is more connected to law than any other disorder. "Non-psychiatric" incentives (desire for material gain or desire to avoid legal responsibility) are present in the legal system and they put in question the validity of PTSD diagnoses [7, 8].

An issue of special importance is delayed PTSD. Researches show that these cases are often connected to symptom exaggeration and malingering in order to obtain material compensation rights [9–11].

The aim of the study was to re-evaluate initial PTSD diagnoses set on local level.

Methods

At the request of the Ministry of Labor and Social Policy which was verifying the validity of disability retirement schemes obtained due to a PTSD diagnosis, team of expert psychiatrists from the Clinic for Psychiatry of the Military Medical Academy (MMA) in Belgrade reevaluated the initial PTSD diagnoses. The obtained results were then compared.

The study included 259 veterans from the entire Serbian territory, who participated in former Yugoslavia wars from 1991 to 1995 and in the NATO bombing in 1999. All of them were diagnosed with PTSD by a psychiatrist on the local level, while a regional medical commission determined the degree of disability on the basis of which veterans obtained the right to a financial compensation and early retirement due to disability. Until the second diagnostic assessment their invalidity lasted on the average [mean ± standard deviation (SD)] 7.8 ± 2.8 years (range, 0–19 years). Re-evaluation of the diagnosis was conducted at the Psychiatric Clinic of the MMA between the 2010 and 2013. A team of experts consisted of military psychiatrists with clinical and research experience in the field of traumatic stress and who were also educated to use Clinican-Administered PTSD Scale (CAPS). Sociodemographic data on marital and family status, education and the social and professional functioning before and after the war was collected through a clinical interview, as well as data on physical and mental health. Veterans' medical records were thoroughly examined, with a special emphasis on data regarding veterans' war participation and above all their traumatic war experiences. The diagnostic assessment of PTSD was done in accordance with the DSM-IV classification of mental disorders because a structured clinical interview CAPS based on this classification was used [12, 13]. The results were presented through descriptive statistics (average and median values), paired sample *t*-test, tabular representations and the use of the appropriate statistical software tools.

Results

The group of 259 war veterans was examined, all male, with the mean age (± SD) of 43.8 ± 8.7 years. Most of them participated as reserve soldiers (91.5%), were married (84.6%) and had secondary level education (83%). As for their employment status, 52.9% were employed, 27.4% unemployed and 11.6% retired. The majority of veterans par-

ticipated in the war during NATO bombing (54.1%), followed by the participants of 1991–1995 wars (21.2%), and only 9 (3.5%) of them participated in both wars. The number of PTSD diagnoses after the first diagnostic assessments conducted by the psychiatrists on the local level is remarkably different from the ones set by the team of experts who conducted re-evaluation. Namely, all 259 subjects were diagnosed was PTSD in the first assessment. The diagnosis was confirmed in 31.7% of veterans. Given that additional 7.3% of them were diagnosed with lifetime PTSD, it means that 39% in total had correct diagnosis. More than half (52.1%) of veterans have not had their diagnoses confirmed (the rest are 39% with the confirmed diagnosis, and 8.9% having other disorders). Mean CAPS intensity score (± SD) was 57.2 ± 12.6 for current PTSD and 45.2 ± 7.7 for lifetime PTSD (Table 1). After looking into complete medical records of 105 study participants, the average rate of attendance of medical appointments was calculated for the period prior to and after establishing their eligibility for disability status. It was found that the average attendance rate had dropped significantly after veterans obtained rights to financial and disability compensation [mean ± SD (after/before) = 1.5 ± 4.4/11.8 ± 10.6; $t_{(104)} = 9.11$, $p < 0.01$].

sing PTSD is more subjective than it is the case with many other disorders that the United States Department of Veteran Affairs benefits for [14].

For compensation purposes, disability is a socially created administrative category. Each disability-compensating scheme is based on the system of rules and the process of assessment. Most systems require medical records documenting physical or mental medical conditions, as well as an administrative rating of the severity of that condition in terms of the loss of ability to work. Compensation is most often proportional to the loss of potential earnings and depends on the level of funding set aside for each specific program.

Many of the issues identified can be addressed by a targeted allocation of time and resources needed for a thorough PTSD clinical examination. This measure will facilitate: more comprehensive and consistent assessment of veterans' reporting exposure to trauma; conduct of standardized psychological testing where appropriate; more accurate assessment of the social and vocational impacts of identified disabilities; evaluation of any suspicious malingering or dissembling using strategies such as standardized tests (where appropriate) and clinical face-to-face assessment; more detailed documentation of claimant's condition to inform rater's decision and an

Table 1

Posttraumatic stress disorder (PTSD) diagnosis reassessment and Clinician-Administered PTSD Scale (CAPS) intensity score in 259 veterans

Diagnosis	Veterans	CAPS intensity score
	n (%)	$\bar{x}$ ± SD
Non-diagnosed	135 (52.1)	
Current PTSD	82 (31.7)	57.2 ± 12.6
Lifetime PTSD	19 (7.3)	45.2 ± 7.7
Other diagnoses	23 (8.9)	
Total	259 (100)	

$\bar{x}$ – mean; SD – standard deviation.

Discussion

The results of this study show a remarkable inconsistency in diagnostic assessment of PTSD conducted by the two separate groups of psychiatric specialists. War veterans, all 259 of them, have been first diagnosed by psychiatrists on the local or regional level, while the second re-evaluated, diagnosis was rendered by the team of experts at the MMA. The aim of this re-evaluation was to establish the presence of the PTSD diagnosis, determine the severity of PTSD symptoms, and establish a logical relationship between the exposure to military stressors and PTSD symptomatology. The PTSD diagnosis was confirmed in 82 (31.7%) of the participants, whereas 19 of them (7.3%) were diagnosed with the lifetime type of PTSD.

Determining the ratings for mental disabilities in general and for PTSD specifically is more difficult than for other disorders because of the inherently subjective nature of reporting the symptoms. In particular, compensation claims for PTSD have attracted attention because of the increasing numbers of claims in recent years and also because diagnoinformed, case-specific determination of whether re-examination is appropriate and, if so, when; evaluation of inter-rater reliability and generate information that can be used to promote the accuracy and validity of ratings [15].

In most part, the inconsistency found in this study is owed to the fact that the second diagnostic procedure involved a standardized, structured interview, unlike the initial diagnosis. The most recent studies suggest that, although many PTSD compensation and pension examiners note the importance of testing and are concerned about exaggeration or outright malingering of PTSD symptoms, the overwhelming majority of them are not using standardized, psychometrically sound assessment instruments to assess PTSD in their examinations for compensation [16]. The second reason is the inadequate or insufficient overview of collateral information obtained from military files, on the basis of which one can confirm or question the severity of traumatic events that preceded the development of the disorder. Many veterans with PTSD diagnosis were not actually exposed to traumatic stressors of war, did not participate in combat, and some did not even witness any traumatic events whatsoever.

Similar study results have been found in Croatian and American war veterans, where it became clear that the diagnostic process involved the use of a structured diagnostic procedure and an insight into the military files [8]. For 32% of war veterans who were treated in hospital conditions, their files showed no record of any participation in combat or exposure to other severe war stressors [17].

Apart from the abovementioned causes regarding structured interviews and military files, it is also possible that the initial group of psychiatrists did not pay enough attention to the effects of secondary gain. Patients' exaggeration of reported symptoms can also influence the psychiatrist into rendering the false positive diagnosis. Thus, exercising the legal right to compensation leads to a doubt regarding the traumatized person's reported level of distress and whether or not the patient was motivated by financial gain [18, 19].

This doubt is augmented by the presence of the so-called "compensational neurosis", a phenomenon firstly recognized in victims of railway accidents, whose ailments never had any organic basis. After the First World War the possibility of early retirement due to "shell shock" was often questioned, as it was apparent that this caused symptoms to be exaggerated. This lead to a proposition that in future war situations this disorder would not be financially compensated, which did happen eventually in Germany after the Second World War. It was claimed that, as soon as trial was over, symptoms of the so-called "compensational neurosis" in patients disappeared. This oversimplified claim was eventually discredited [20].

The augmentation of psychiatric symptomatology is indeed motivated by the possibility of obtaining different forms of compensation. However, the very act of attending trial can aggravate the primary PTSD symptoms and cause a re-traumatization process. The demand that PTSD patients express and relive their trauma history prevents the characteristic efforts to avoid speaking and/or thinking about it. This can in turn lead to the revival of intrusive thoughts and high irritability. The anamnestic process comprises various dilemmas for forensic experts looking into PTSD. However, the well-known tendencies of patients' avoidance of painful past experiences lead to symptoms that are actually present to be unrightfully neglected.

On the other hand, a direct research into the diagnostic criteria of PTSD can motivate the patient to give a series of answers to direct and suggestive questions that would lead to an easy diagnosis [21]. After the diagnostic criteria have become available through medical publications and word of mouth, there is little that can be done to prevent a motivated individual with a compensation goal to understand exactly which symptoms need to be reported in the attempt to be diagnosed with PTSD.

Our study show that there is high probability that veterans who sought psychiatric aid were indeed motivated by financial gain, which was apparent in the number of visits they made to their doctor until they received clearance for financial gain. The second possible cause of discrepancy between the two psychiatric assessments is the time lapse between them. The re-evaluation was done between 2010 and 2013, whereas the initial assessment in most cases was immediately after the wars of 1991–1995 and 1999. The severity and course of PTSD change over time, with some studies proving that as many as 50–60% patients reach full recovery [22, 23]. In this study we have found that only 19 veterans (7.3%) had lifetime PTSD, which confirms the conclusion that the majority of study participants were motivated by financial gain not because their mental health was impaired, but because of the very fact that they had participated in the war.

Conclusion

The inconsistent psychiatric diagnostics of PTSD may be the consequence of differences or inadequacies in the diagnostic process. Objective evaluation of this disorder in war veterans needs to involve, first and foremost, assessment of war stressors they have been exposed to, and analysis of additional information in their military files. For valid assessment of the presence (and severity) of the symptoms, raters need to apply structured standardized interviews and/or assessment scales. They should also be aware of the fact that assessments of functionality and/or disability are often under the threat of false positives led by patients' exaggeration motivated by material benefits. For PTSD to be diagnosed definitively and correctly, it is crucial to merge all the assessment factors into the coherent diagnosis.

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Received on January 24, 2015.
Revised on May 3, 2015.
Accepted on June 24, 2015.
Online First May, 2016.

PRACTICAL ADVICE FOR PHYSICIANS

UDC: 616-08-039.74::616.14-073.432.19
DOI: 10.2298/VSP150609085R

Lung ultrasound for severe acute dyspnea evaluation in critical care patients

Značaj ultrazvuka pluća u proceni etiologije teške akutne dispneje kod bolesnika u jedinicama intenzivne nege

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Key words:
lung diseases; ultrasonography; intensive care units; diagnosis; diagnosis, differential; acute disease; dyspnea.

Ključne reči:
pluća, bolesti; ultrasonografija; intenzivna nega, odeljenja; dijagnoza; dijagnoza, diferencijalna; akutna bolest; dispneja.

Introduction

Acute dyspnea is a very common cause of hospitalisation, especially in intensive care units, and it can be precipitated by heart failure, exacerbation of chronic obstructive pulmonary disease, pulmonary embolism, pleural effusions and many other causes. The differentiation of cardiac from non-cardiac causes of dyspnoea poses a huge clinical challenge, since an accurately established diagnosis is the precondition for an adequate therapy, as well as for disease prognosis [1].

Lung ultrasound exam in intensive care unit patients

Chest radiography (RTG) is used as a regular diagnostic procedure for most patients in intensive care units. It involves the use of a portable X-ray device, which produces limited quality images, especially in recumbent patients. Another option for evaluating lung conditions is computed tomography (CT), which entails the transportation of patients, who are often dependent on mechanical ventilation, and their exposure to strong doses of ionising radiation.

Ultrasound diagnostics is commonly employed for intensive care patients, especially echocardiography, abdominal and vascular ultrasound. Air-filled organs, such as the lung, reflect ultrasound waves badly, which has led to lungs being excluded from the ultrasound diagnostics repertoire. The only structure visible in healthy lungs is the pleura, which is visualised as a hyperechoic horizontal line, moving synchronously with the lung during respiration. Conversely to healthy lungs, in pathological conditions such as pneumonia, heart failure, acute respiratory distress syndrome (ARDS), pulmonary fibrosis and others, the volume of air in the lungs decreases, which leads to the appearance of various images (artefacts), based on which the pathological process is diagnosed [2, 3].

Chest ultrasound enables relatively easy and quick detection and diagnosis of various lung abnormalities, more reliably than radiography. Moreover, the examination is inexpensive, infinitely repeatable and it is conducted at the bedside with a portable ultrasound device. As a result, patients are spared from being exposed to ionising radiation, or to contrast dye potentially damaging to the kidneys, from allergies and transportation to remote hospital wards for CT scans. Despite all of these advantages, ultrasound-based diagnostics of lung diseases is still not sufficiently employed in intensive care units, except for detection of pleural effusions. One of the possible reasons for that could be found in the required training of physicians, which takes several months, since the image quality and, consequently, the diagnosis strongly depend on the experience of a person performing the scan [4, 5].

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Lung ultrasound in decompensated heart failure

In patients with heart failure, fluids are typically accumulated in the lungs and the most common sign of this in an ultrasound scan is the appearance of B-lines (Figure 1). They are long, vertical, hyperechoic lines shaped like sun rays or comet tails. They extend from the pleura towards the inside of the lung and they move synchronously with respiration, i.e. with lung sliding. B-lines erase the physiological A-lines – bright echogenic lines, about 2 cm long, which run parallel to the pleura and indicate normal lung structure [6, 7].

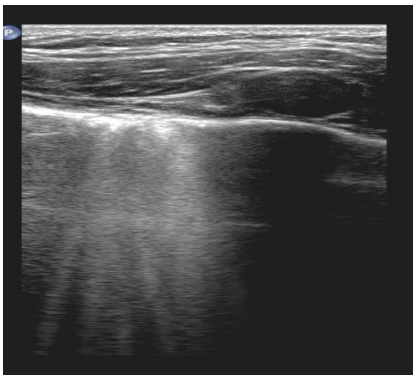

Fig. 1 – Lung ultrasound of a patient with dilated cardiomyopathy and pulmonary oedema. One field of view shows at least 3 B-lines, spreading throughout the lung, while the distance between them is decreased to 3 mm, or they fuse together.

B-lines appear as the consequence of accumulated fluid in the interstitial space, i.e. in interlobular septa. The distance between them is about 7 mm, which is the normal distance between interlobular septa. They correspond to Kerley's B-lines visible in the chest X-ray associated to heart failure, i.e. horizontal lines at the lung periphery, which also indicate interlobular septal thickening (Figure 2).

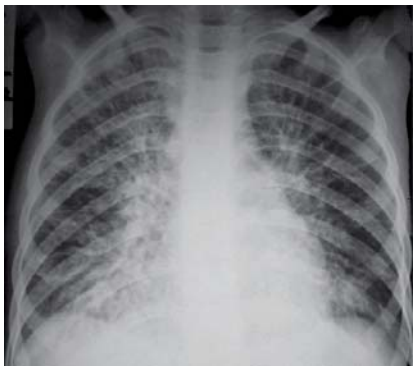

Fig. 2 – Chest radiograph of the same patient, showing Kerley's B-lines – short horizontal lines at the lung periphery, caused by interlobular septal thickening.

The number of B-lines is proportional to the degree of pulmonary congestion. Occasionally, they can also be seen in healthy lungs, two B-lines in one field of view at the most, usually at the bases of the lungs. In pulmonary oedema – the most severe form of heart failure, B-lines are multiple, extending throughout the entire lungs, with the distance between them shrinking from 7 mm to 3 mm, or they fuse together. B-lines are very reliable in diagnosing pulmonary oedema – equally reliable as the brain natriuretic peptide (BNP). In addition, B-lines are a significant indicator of heart therapy failure, since they disappear very shortly after the use of diuretics or after haemodialysis [8, 9].

For establishing a differential diagnosis of pulmonary oedema from pneumonia or ARDS, in which B-lines also appear, the differentiating indicator is pleural sliding, i.e. the sliding of the parietal and visceral pleural layers against each other during respiration, which is not detected in other diseases. In the conditions of heart failure, there is pleural effusion, usually posterolateral, which cannot be detected by radiography in its early stages, i.e. when its volume is smaller than 100 mL, whereas it is fairly easily identifiable by ultrasound [10].

The appearance of B-lines is very important for a differential diagnosis of dyspnoea, i.e. for distinguishing heart failure from chronic obstructive pulmonary disease (COPD), in which there are no B-lines. These artefacts can also be used for prognosis purposes – stress test called "alveolar-capillary stress echo" refers to monitoring the appearance of B-lines during stress, which effectively assesses the left ventricular systolic function. The appearance of B-lines that were absent before the test indicates a serious disorder of the systolic or diastolic function of the myocardium, as well as very substantial heart valve abnormalities, and calls for immediate intervention [11].

Lung ultrasound in chronic obstructive pulmonary disease (COPD)

The second most common cause of acute dyspnoea in intensive care units is COPD. Lung ultrasonography reveals only A-lines, which are part of a normal ultrasound finding, as well as the usual pleural sliding, with no B-lines or other artefacts [12]. Apparently, ultrasonography of COPD patients produces practically normal findings (Figure 3).

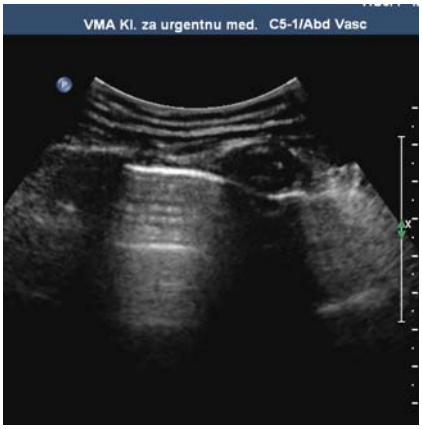

Fig. 3 – Chest ultrasound of a patient with cronic opstructive pulmonary disease (COPD). The scan shows normal A-lines; pleural layers are sliding over each other; there are no B-lines.

Ultrasound evaluation of pneumonia

Ultrasound diagnosing of pneumonia is based on the two types of criteria, parenchymal and pleural.

Parenchymal abnormalities occur due to the inflow of fluid into alveoli as the result of the inflammation process, leading to the decrease of the content of air in them, i.e. to consolidation. As the result, lung tissue resembles the liver and this phenomenon is known as "lung hepatisation" (Figure 4).

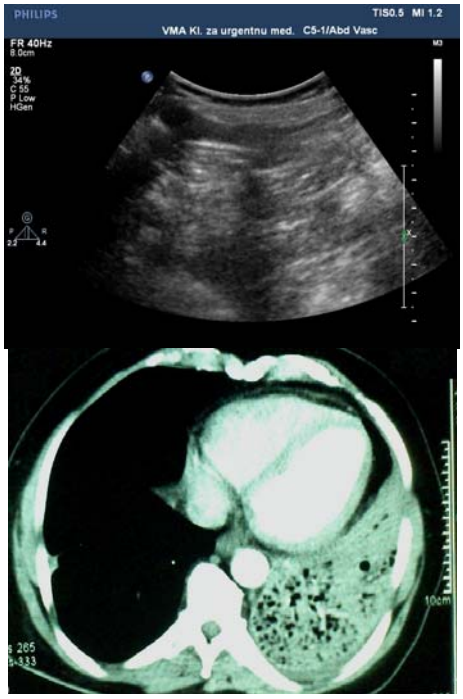

Fig. 4 – Pneumonia – parenchymal abnormalities in chest ultrasound and computed tomography (CT) scan: images reveal consolidation (alveoli are filled with fluid), as well as hepatisation – the lungs look like the liver; B-lines are visible in the inflammation area.

B-lines can be seen in the inflammation area, localised in one part of the lung, as opposed to pulmonary oedema, where B-lines are diffused. Sometimes it is possible to detect the "lung pulse", i.e. the transfer of heart contractions on the lungs due to lung parenchyma consolidation [13, 14]. The scan may also show the "air bronchogram", i.e. the bronchial walls have thick end, so the bronchi are visualised as branching white lines in the longitudinal section, or as lentil-sized hyperechoic circles of few millimetres in diameter in the transverse section (Figure 5).

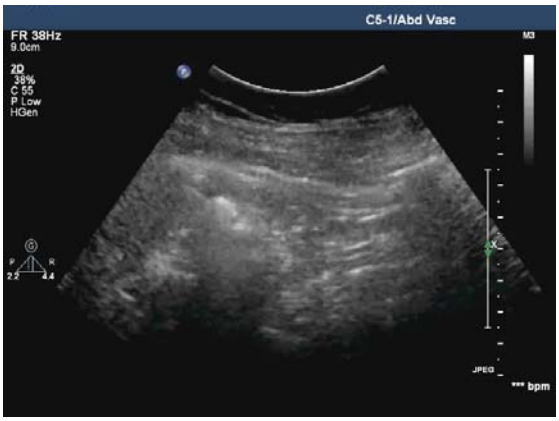

Fig. 5 – "Air bronchogram" – thickened bronchial walls in longitudinal and transverse sections (hyperechoic circles and lines).

Pleural abnormalities include the occurrence of pleural effusions. Sometimes the amount of fluid in the pleural space is so large that one gets the impression that lung tissue is swimming in the effusion. The pleura is inflamed, thickened and missing one of the key features of healthy lungs – the sliding of the parietal over the visceral pleura during respiration [15].

Ultrasound in diagnosing pneumothorax

In intensive care units, pneumothorax is most commonly detected among patients with lung injury or as the complication of subclavian vein puncture for administration of parenteral therapy (Figure 6). Thoracic ultrasound has the same sensitivity as CT in diagnosing pneumothorax (95%), while RTG sensitivity is much smaller (75%). Examination is conducted with a high-frequency linear transducer, positioned in the third or fourth intercostal space bilaterally, along the midclavicular line, since pleural air collects in the non-dependent zones of the lung. Scanning is continued laterally along the anterior or midaxillary lines because of the possibility of partial pneumothorax [16].

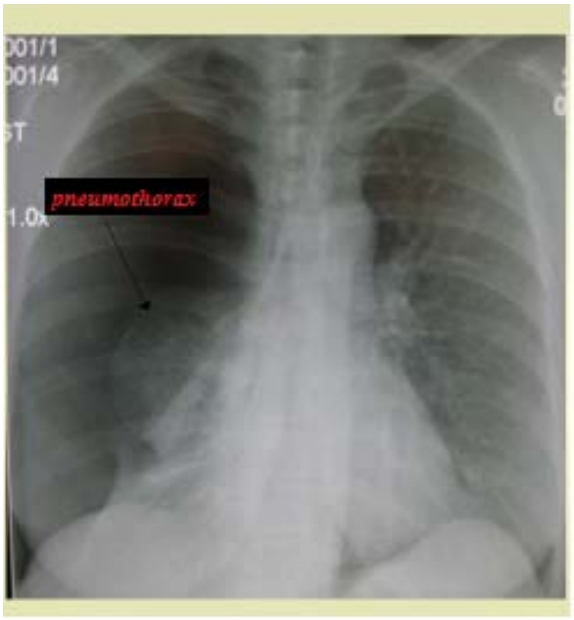

Fig. 6 – Chest and heart RTG – pneumothorax. The right lung has collapsed as a result of pleural air.

It is indicative that there is no lung sliding, i.e. the movement of the visceral and parietal pleura. A-lines are visible just as in the scan of healthy lung tissue. There are no B-lines whatsoever, since the parietal and visceral pleural layers are divided by air, while the presence of a single B-line, as well as of lung sliding, rules out the possibility of pneumothorax.

The M-mode scan produces the "stratosphere sign", i.e. horizontal lines appear both above and below the pleura, resulting from ultrasound reverberation due to the presence of pleural air. Another specific sign of pneumothorax is the "lung point", which refers to the transition point between the normal lung pattern and pneumothorax [17]. At this point, in a two-dimensional image, lung sliding alternates with the absence of lung sliding (Figure 7).

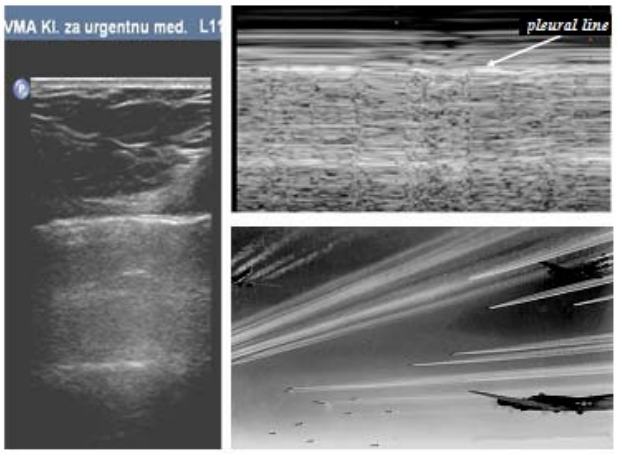

Fig. 7 – Pneumothorax diagnosed by ultrasound the two-dimensional image on the left shows A-lines, with no lung sliding or B-lines. On the right, the M-mode shows the "stratosphere sign" or the "barcode sign" – horizontal lines under the pleura, as a result of the presence of air in the pleural space.

Ultrasound in diagnosing acute respiratory distress syndrome

Acute respiratory distress syndrome is a severe inflammatory lung condition that leads to diffuse damages to alveolar epithelium and vascular endothelium, increasing their permeability, as well as to pulmonary oedema and acute hypoxia. This condition is diagnosed by lung ultrasound with 98% sensitivity and 88% specificity, almost the same as in CT and much higher than in radiography.

Examination findings are quite diverse: zones dominated by B-lines alternate with areas of lung consolidation due to inflammation and atelectasis, and with zones of normal lung parenchyma (Figure 8). B-lines are diffusely distributed in the lung and are typically visible in the posterior chest, influenced by the gravity. They are caused by the interstitial oedema resulting from increased alveolar-capillary membrane permeability [18].

The pleura has thickened to more than 2 mm, it is irregular, and there are zones of subpleural lung consolidation. The occurrence of pleural effusions is very frequent. Lung sliding is missing, as is the lung pulse, which is a very indicative sign.

It is very important to differentiate ARDS from cardiogenic pulmonary oedema. Diffuse B-lines are visible in both of these conditions; in ARDS, however, B-lines alternate with zones of lung consolidation and zones of normal lung tissue. In addition, the pleura in ARDS thickens and the sliding of its layers cannot be perceived, whereas these signs are absent in heart failure. Moreover, in left ventricular insufficiency, effusions are more frequent and usually larger [19].

Lung ultrasound in diagnosing pleural effusions and in guiding thoracentesis

A typical indication for thoracic ultrasonography is pleural effusion. Under the influence of gravity, fluid collects in lower and posterior regions of the lung, so these zones should certainly be scanned during an examination. When a patient is in supine position, a low-frequency (cardiac or abdominal) transducer is placed on the posterior axillary line, above the diaphragm, with the orientation marker pointed cephalad. The scan usually starts from the diaphragm upwards.

Ultrasound allows us to distinguish effusions from elevated diaphragm, tumour, atelectasis or consolidated lung parenchyma, which is sometimes unfeasible through chest X-ray or auscultation. This examination has multiple benefits: pleural effusion diagnosis; effusion volume assessment; provisional identification of the content of effusion; ultrasound can be used as guidance in pleural puncture [20].

Pleural effusion is visualised as a hypoechoic or anechoic space between the two pleural layers, often with a zone of lung consolidation visible underneath it – atelectasis, resulting from compressive effect of effusion and disappearing if effusion is evacuated. If effusion is small, it is the consequence of the pathological process in the lungs (inflammation, tumour) and the

Fig. 8 – Acute respiratory distress syndrome (ARDS) – chest radiograph on the left and ultrasound on the right: consolidation zones alternating with zones of normal lung parenchyma and zones with fluid in the interstitial tissue.

consolidation zone also remains after the pleural puncture. If the pleura and the effusion are scanned in M-mode, the resulting image shows the "sinusoid sign", i.e. the undulating movement of the visceral pleura and the lung during respiration (Figure 9).

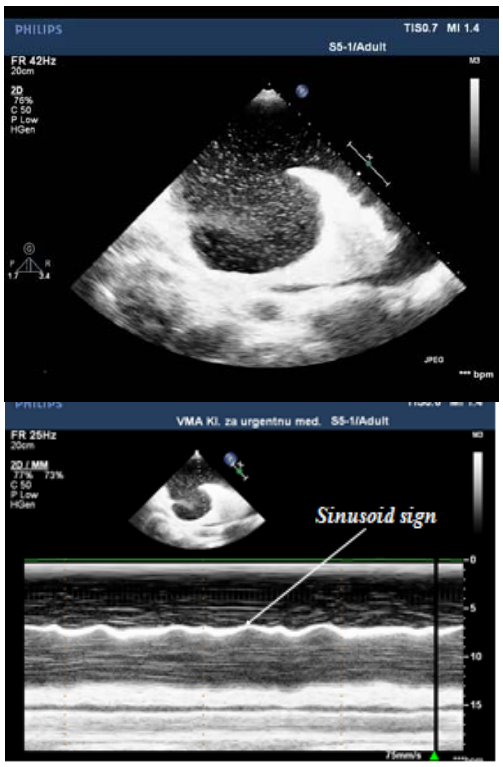

Fig. 9 – Pleural effusion: above is a twodimensional image showing compressive atelectasis of the lung; bellow is an M-mode image showing the "sinusoid sign", i.e. the undulating movement of the lung and visceral pleura.

Ultrasonography enables differentiation between different types of effusions: transudate is visualised as an anechoic, dark, homogeneous space; exudate generates a stronger reflection, it is heterogeneous, fibrinous, often with visible pleural septa creating pockets; hemothorax has a strong reflection, it is heterogeneous and often contains small pieces of tissue, although it may also be anechoic if it is of recent onset.

Ultrasound can be used as a guide in thoracentesis, especially in cases where puncture was previously unsuccessful. It is convenient because it prevents injury of the liver on the right side, or the spleen and kidneys on the left side, while the patient is sitting or in semi-recumbent position. The thickness of the effusion needs to be at least 1 cm for a pleural puncture, and it is made at the point where the effusion is thickets [21,22].

Conclusion

Lung ultrasound is a new domain of ultrasound-based diagnostics, which has gained popularity in the past 15 years. The key aspect in the development of this technique is the detection of abnormalities in lung parenchyma, in addition to its traditional use for diagnosing and evacuating pleural effusions. It is exceptionally useful in intensive care units for differentiating cardiogenic pulmonary oedema from acute lung conditions such as acute repiratory distress syndrome, pneumonia, chronic obstructive pulmonary disease, pneumothorax and pulmonary embolism. Examination is done at the bedside, using a minimum of equipment; it is simple, harmless and inexpensive. In the future, thoracic ultrasonography may also find application in other areas including cardiology, pulmonology, dialysis, or as part of the out-of-hospital emergency medical service.

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Received on June 9, 2015.
Accepted on July 17, 2015.
Online First August, 2015.

CASE REPORTS

UDC: 575::616.13/.14-007]::616.24
DOI: 10.2298/VSP150515094L

Hereditary hemorrhagic telangiectasia with bilateral pulmonary vascular malformations – A case report

Nasledna hemoragijska teleangiektazija sa obostranim plućnim vaskularnim malformacijama

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Abstract

Introduction. Hereditary hemorrhagic telangiectasia (HHT) also known as Osler-Weber-Rendu syndrome is an autosomal dominant disease that occurs due to vascular dysplasia associated with the disorder in the signaling pathway of transforming growth factor β (TGF-β). The clinical consequence is a disorder of blood vessels in multiple organ systems with the existence of telangiectasia which causes dilation of capillaries and veins, are present from birth and are localized on the skin and mucosa of the mouth, respiratory, gastrointestinal and urinary tract. They can make a rupture with consequent serious bleeding that can end up with fatal outcome. Since there is a disruption of blood vessels of more than one organic system, the diagnosis is very complex and requires a multidisciplinary approach. **Case report.** We reported a 40-year-old female patient with a long-time evolution of problems, who was diagnosed and treated at the Clinic for Lung Diseases of the Military Medical Academy in Belgrade, Serbia, because of bilaterally pulmonary arteriovenous malformations associated with HHT. Embolization was performed in two acts, followed with normalization of clinical, radiological and functional findings with the cessation of hemoptysis, effort intolerance with a significant improvement of the quality of life. **Conclusion.** HHT is a rare dominant inherited multisystem disease that requires multidisciplinary approach to diagnosis and treatment. Embolization is the method of choice in the treatment of arteriovenous malformations with minor adverse effects and very satisfying therapeutic effect.

Key words:
telangiectasia, hereditary hemorrhagic; arteriovenous malformations; lung diseases; hemoptysis; diagnosis; embolization, therapeutic; treatment outcome.

Apstrakt

Uvod. Hereditarna hemoragijska teleangiektazija (HHT) ili Osler-Weber-Rendu sindrom je autozomno dominantno oboljenje nastalo usled vaskularne displazije povezane sa poremećajem u signalnom putu transformišućeg faktora rasta β (TGF β). Klinička posledica jeste poremećaj krvnih sudova u više organa, sa postojanjem teleangiektazija koje uzrokuju dilataciju kapilara i vena. Promene su prisutne od samog rođenja i lokalizovane su po koži i mukozi usne duplje, respiratornog, gastrointestinalnog i urinarnog trakta; mogu napraviti rupture sa posledičnim ozbiljnim krvarenjem koje se može završiti i smrtnim ishodom. Kako postoji poremećaj na krvnim sudovima više organskih sistema, postavljanje dijagnoze je veoma kompleksno i zahteva multidisciplinarni pristup. **Prikaz bolesnika.** Prikazali smo 40-godišnju bolesnicu sa dugogodišnjom evolucijom tegoba, dijagnostikovanu i lečenu u Klinici za pulmologiju Vojnomedicinske akademije u Beogradu, zbog bilateralnih plućnih arteriovenskih malformacija udruženih sa HHT. Urađena je embolizacija u dva akta, nakon čega je došlo do normalizacije kliničkog, radiološkog i funkcijskog nalaza, uz prestanak hemoptizija, intolerancije na napor i uz značajno poboljšanje kvaliteta života. **Zaključak.** HHT je retka, dominantno nasledna multisistemska bolest, koja zahteva multidisciplinarni pristup u dijagnostici i lečenju. Embolizacija je metoda izbora u lečenju arteriovenskih malformacija u plućima, sa neznatnim neželjenim efektima i veoma zadovoljavajućim terapijskim ishodom.

Ključne reči:
teleangiektazija, nasledna, hemoragijska; arteriovenske malformacije; pluća, bolesti; hemoptizije; dijagnoza; embolizacija, terapijska; lečenje, ishod.

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Introduction

Hereditary hemorrhagic telangiectasia (HHT), also known as Osler-Weber-Rendu syndrome, is an autosomal dominant disease with the prevalence of 1/5,000–10,000 in general population. More common are cases in isolated populations such as the island Curacao in the Netherlands Antilles (1/1,331) with African-Caribbean population, or in isolated regions of the French Alps. The disease is rare with the population of African-Americans [1, 2].

It is basically a vascular dysplasia associated with disorders in the signaling pathway of transforming growth factor (TGF) β. TGF-β superfamily of proteins are: TGF β, bone morphogenic protein (BMP) 9, BMP10 and growth differentiation factor (GDF) 2. In order to transmit the signal, it is necessary to achieve binding to the type II receptor that activates – phosphorylates the type I receptor, which further activates the complex of small mothers against decapentaplegic (SMAD) proteins (predominantly SMAD1, SMAD5 and SMAD8). This complex binds to SMAD4 and migrates to the nucleus where it works as a transcription factor for genes that play a role in the development, repairing, angiogenesis and migration of leukocytes.

Five genetic mutations that are responsible for the occurrence of HHT are described. The most common is mutation in the endoglin (ENG) gene (9q34) encoding endoglin (ENG), a glycoprotein predominantly in the TGF β1 receptors on endothelial cells, which results in altered extracellular part of the proteins – receptors [1–5]. Activin A receptor type IL (ACVRL) gene (12q11-14) encodes Alk-1 protein (activin receptor-like kinase 1) also TGF β1 receptor. In about 80% of patients with HHT, one of the two mutations is present and the level of ALK-1 and ENG on the endothelial membrane is reduced. A higher incidence of pulmonary arteriovenous malformations (AVM) was noted with patients with ENG mutations, while AVMs of liver are more present in ACVRL1 subtype1.

For the last mentioned above, recent studies suggest the association of mutations in ACVRL1 with pulmonary arterial hypertension [6]. Mutation of the malate dehydrogenase 4 (MADH4) gene encoding SMAD 4, intracellular signaling protein of the superfamily of TGF receptors occurs in juvenile polyposis and HHT [7]. With this same gene it is described that the acquired and *de novo* mutations also cause disease. A correlation between the disease and mutations in the gene loci 5q31 and 7p14 also were proven, but not fully understood [1, 2, 8].

The clinical consequence is a disorder in blood vessels in multiple organ systems whose clinical presentation suggests the diagnosis of the disease. It is characterized by the existence of telangiectasia caused by dilation of capillaries and veins, they are present from birth, localized on the skin and mucosa of the mouth, respiratory, and gastrointestinal and urinary tract. Any of these innumerable lesions can make a rupture, which rarely causes serious bleeding of upper and lower respiratory, gastrointestinal and urinary tract [1–3].

Histologically, the most common are cellular infiltrates with the appearance of acute neutrophilic inflammation and vascular capillary dilatation and proliferation [3].

According to the Curaçao Criteria established in 2000, the definite diagnosis should be based on at least 3 out of 4 of the following criteria: nose bleeding – spontaneous and recurrent; mucocutaneus telangiectasia, including the lips, oral cavity, fingers, and nose; the presence of internal lesions – telangiectasia AVMs, gastric-intestinal AVMs, pulmonary AVMs, cerebral AVMs, spinal AVMs; family history of the phenomena mentioned above.

The diagnosis is considered possible if 2 criteria are present, and it is unlikely in the presence of just one criterion [1, 2]. It was found that the same criteria cannot be applied to children because they generally do not have all the manifestations of the disease demonstrated yet.

Nose bleeding and telangiectasis of skin, face and hands are first signs of the disease and they are present in about 95% of patients with HHT. Pulmonary AVMs are found in about 15–50% of these patients, in about 30–70% they are found in the liver, in 10% in the brain and in about 1% in the spinal canal. Of all the causes of pulmonary AVMs, HHT is responsible for 70–80% [1, 2, 8].

Due to the existence of changes in the lips and facial skin, the differential diagnosis most often suspects Kaposi sarcoma.

In therapeutic terms there is no specific, causal treatment. When it is necessary, antibiotic therapy is prescribed, compensations of iron and blood transfusion are made, epistaxis can be treated with laser coagulation, a septoplasty is applied, as well as intranasal spray with an inhibitor of vascular growth factor (VGF). Anticoagulant/antiplatelet therapy is generally avoided due to the potential risk of bleeding, but it is considered that there are no absolute contraindications for the same [1]. Surgical treatment of excision and ligature are significant in preventing the sequelae of AVM [1, 2].

Embolization proved to be efficient method with only minor side effects and rare complications during many years of follow-up.

The results are evident immediately after the intervention: malformation exclusion from circulation, improving perfusion in the remaining lung tissue and the improvement of oxygenation. Over the longer follow-up after treatment, reperfusion occurs in around 10% of patients and the increase in new small pulmonary AVMs occurs in 15% of cases, other complications are rare [2, 8–10]. Regarding complications, the choice of embolization materials is very important because of the possibility that the material breaks off and causes thrombosis. A relatively new method, which is still being evaluated, is the use of arteriovenous (AV) occluders for closure of pulmonary AVM.

Case report

A 40-year-old female patient with the long history of arterial hypertension, recurrent nose bleeding and occasional hemoptysis (the last 10 years) was examined in local primary health care center because of pain in the projection of arches of the ribs and symptoms of respiratory infection. Chest auscultation showed a continuous vascular murmur, paravertebrally on the level of thoracic base. Then the patient was sent to abdominal ultrasound and to abdominal multislice computer tomography

(MSCT), which showed two ovoid formations with the diameter of 23 mm in the right lower pulmonary lobe, localized posteriorly next to the pleura with intensive postcontrast activity of 160 Hounsfield Units (HU). In the left lower lobe, an identical formation next to the pleura and posterior thoracic wall, with diameter of 16 mm was found.

The patient was sent to our clinic for further diagnosis. On hospital admission we found that she had the long history of hypertension, with difficulties to tolerate physical stress in the last 2–3 years. She had frequent epistaxis since childhood and one episode of massive hemoptisis, which were undiagnosed because she did not accept further diagnostic procedures. The family history was positive for nose bleeding and telangiectasis of tongue and mouth with the patient's mother and a few other relatives on the mother's side. There was one case of death due to pulmonary hemorrhage. It was the patient's relative, age 16, and because of this the patient showed concern for her own health and the health of her children. Further treatment included the assistance of a psychologist.

On physical examination, discrete mucocutaneous telangiectasia of lips, oral mucosa, tongue, lips, and few on lower limbs were found (Figure 1).

Auscultatory paravertebrally over posterior thoracic base we found the presence of tunnel-like continual vascular murmur. Mild hipoxemia and decreased oxygen saturation were found.

Laboratory findings revealed microcytic hypochromatic anemia 4.6 × 10^{12}/L red blood cell (RBC) [normal value (NV) 4.5–6.5 × 10^{12}/L]; mean cell volume (MCV) 75.2 fL (NV 76–96 fL); mean corpuscular hemoglobin (MCH) 25.9 pg (NV 27–32 pg); red blood cell distribution width (RDW) 16.6% (NV 11.5–14.5%); Fe 5 mmol/L (NV 6.6–26 mmol/L).

MSCT contrast pulmonary angiography showed hyperdense peripheral zones in posterobasal segments of both lungs with the diameter of 20 × 18 mm for the right and 28 × 14 mm for the left one. After *iv* contrast, feeding and drainage blood vessels were clearly presented (Figure 2).

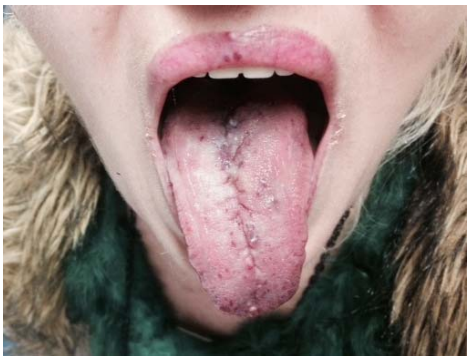

Fig. 1 – Mucocutaneous telangiectasia of the tongue and the lips.

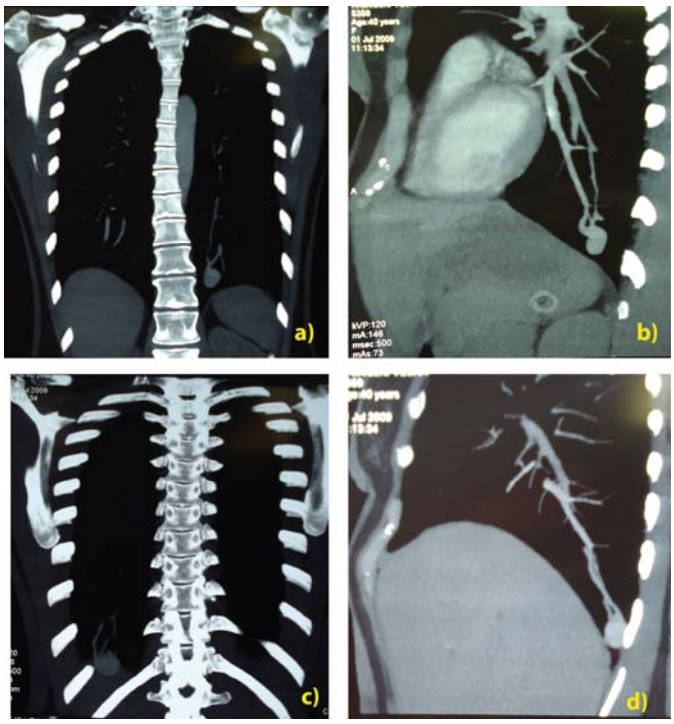

Fig. 2 – Multislice computed tomography (MSCT) contrast pulmonary angiography: a) left lower lobe arteriovenous malformation (coronal section); b) left lower lobe arteriovenous malformation (sagital section); c) right lower lobe arteriovenous malformations (coronal section); d) right lower lobe arteriovenous malformation (sagital section).

Screening for other manifestations of the disease was performed: MSCT angiography of endocranium did not show brain AVMs and stool testing for occult blood was negative. Echocardiography findings and morphological findings were normal, without signs of right heart load and indirect pulmonary hypertension. Based on the clinical course and performed analysis, the diagnosis was HHT with bilateral pulmonary vascular malformations.

Because of the proven bilateral AVMs in the lung parenchyma, it was decided to carry out the treatment of embolization. Active therapeutic approach was selected, pneumoangiography with embolisation of pathological vascular malformations was done in two acts. In the first act, AVM in the left lover lobe and feeding artery was successfully embolized with embolisation coils with dacron tails. This fistula was completely out of circulation.

Control angiography did not show drainage vein, and there was significantly better perfusion of blood vessels for the left lower lobe (Figures 3–5). After a few days, the embolisation of fistula was performed on the right side of the lungs, but with partial success. Control angiography showed the presence of drainage vein. After the patient's recovery at home, AV malformation in the right lower lobe was reembolised successfully (Figure 6). After this intervention, normalization of clinical findings (the vascular murmur disappeared) and arterial blood oxygen saturation were achieved. Control hospitalization after three months, with iron supplementation therapy, showed a significant improvement in the quality of the patient's life: the patient was not bothered with physical exertion, epistaxis and hemoptysis did not repeat. The patient was without problems and returned to normal life.

Discussion

After ten years of testing epistaxis, hypertension and hemoptysis, the patient was diagnosed with HHT or Osler-

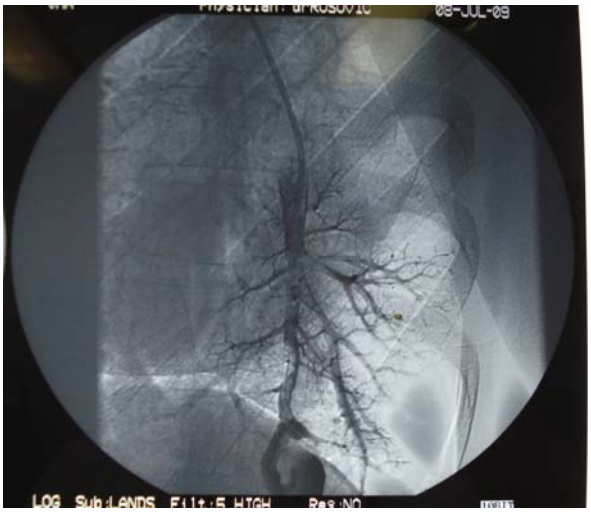

Fig. 3 – Left lower lobe arteriovenous malformation catheterization.

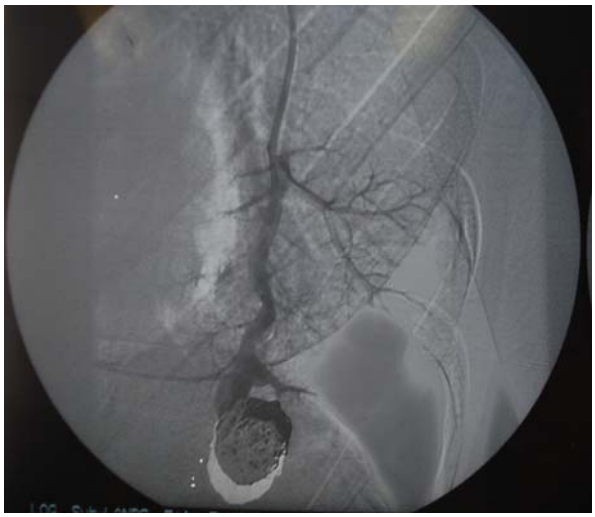

Fig. 4 – Left lower lobe arteriovenous malformation partially embolized.

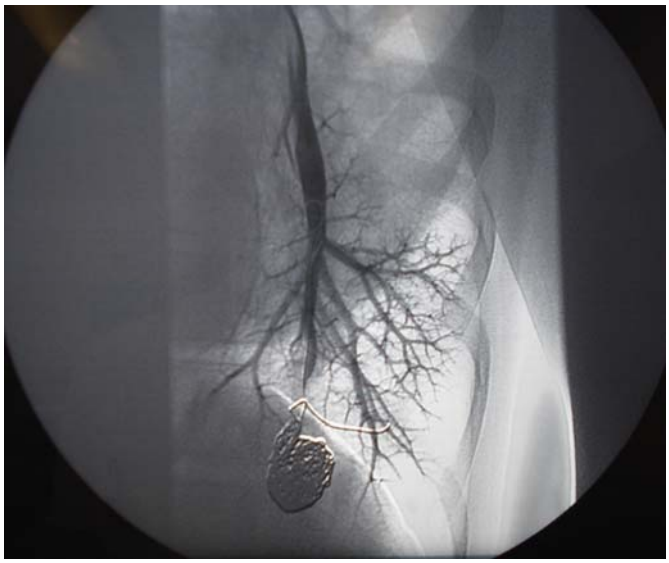

Fig. 5 – Left lower lobe arteriovenous malformation and feeding vessel embolized, complete out of circulation.

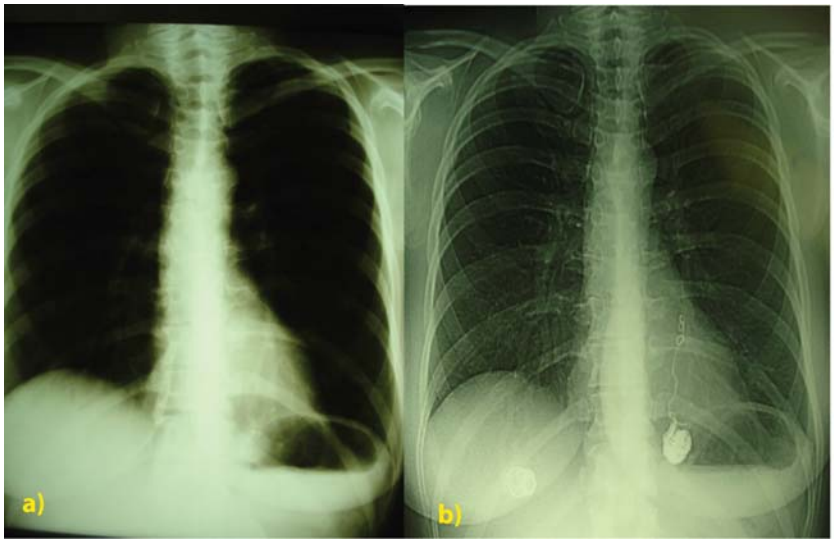

Fig. 6 – Chest x-ray findings: a) before, and b) after embolization.

Weber-Rendu syndrome. MSCT pulmoangiography showed the existence of AVMs on both sides of the lungs, which was the cause of hemoptysis. After the disease diagnosing, due to the extensive changes on both sides of the lungs, the embolization was performed in two acts, which excluded malformations from the circulation and thus perfusion was improved, with subsequent normalization of gases of arterial blood. The patient, who had spent ten years with medical problems, was diagnosed, went through embolization, followed by normalization of clinical, functional and radiographic results, and achieved termination of problems and a significant improvement in the quality of life, and most importantly, the possibility of massive hemoptysis often resulting in death was excluded.

Embolization is the method of choice in the management of AVMs with minor complications.

Conclusion

Hereditary hemorrhagic telangiectasia is diagnostically undervalued, doctors as well as patients and their families are not aware of the potential for screening and treatment of this disease. The consequences can be severe hemorrhage, brain infarction or death. In the present report, medical history, examination and insisting on additional diagnostics have led to the diagnosis of the disease and favorable treatment outcomes.

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Received on May 15, 2015.
Accepted on July 1, 2015.
Online First May, 2016.

CASE REPORT

UDC: 575:61]:[616.432:616-007.6
DOI: 10.2298/VSP150620014D

Coexisting diseases modifying each other's presentation - lack of growth failure in Turner syndrome due to the associated pituitary gigantism

Istovremeno postojanje Tarnerovog sindroma i gigantizma: atipična klinička manifestacija bez zastoja u linearnom rastu

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Abstract

Introduction. Turner syndrome presents with one of the most frequent chromosomal aberrations in female, typically presented with growth retardation, ovarian insufficiency, facial dysmorphism, and numerous other somatic stigmata. Gigantism is an extremely rare condition resulting from an excessive growth hormone (GH) secretion that occurs during childhood before the fusion of epiphyseal growth plates. The major clinical feature of gigantism is growth acceleration, although these patients also suffer from hypogonadism and soft tissue hypertrophy. **Case report.** We presented a girl with mosaic Turner syndrome, delayed puberty and normal linear growth for the sex and age, due to the simultaneous GH hypersecretion by pituitary tumor. In the presented case all the typical phenotypic stigmata related to Turner syndrome were missing. Due to excessive pituitary GH secretion during the period while the epiphyseal growth plates of the long bones are still open, characteristic stagnation in longitudinal growth has not been demonstrated. The patient presented with delayed puberty and primary amenorrhea along with a sudden appearance of clinical signs of hypersomatotropinism, which were the reasons for seeking medical help at the age of 16. **Conclusion.** Physical examination of children presenting with delayed puberty but without growth arrest must include an overall hormonal and genetic testing even in the cases when typical clinical presentations of genetic disorder are absent. To the best of our knowledge, this is the first reported case of simultaneous presence of Turner syndrome and gigantism in the literature.

Key words:
turner syndrome; gigantism; pituitary neoplasms; adolescent; women; puberty; growth hormone; insulin-like growth factor I.

Apstrakt

Uvod. Tarnerov sindrom je jedna od najčešćih hromozomskih aberacija kod osoba ženskog pola, koja se tipično manifestuje zaostajanjem u rastu, insuficijencijom jajnika, karakterističnim crtama lica i drugim različitim somatskim poremećajima. Gigantizam je izrazito retko oboljenje koje nastaje kao posledica pojačane sekrecije hormona rasta (HR) tokom detinjstva, a pre srastanja epifiznih zona rasta dugih kostiju. Osnovna klinička karakteristika obolelih od gigantizma je visok rast, mada ove osobe tipično imaju i hipertrofiju mekih tkiva i hipogonadizam. **Prikaz bolesnika.** Prikazali smo bolesnicu sa Tarnerovim sindromom, zakasnelim pubertetom i normalnim linearnim rastom, nastalim zbog istovremenog postojanja hipersekrecije HR iz pituitarnog tumora. U tom slučaju, izostajale su sve fenotipske karakteristike tipične za Tarnerov sindrom. Usled pojačane sekrecije HR koji je delovao na otvorene epifizne ploče dugih kostiju, izostao je karakterističan zastoj u longitudinalnom rastu. Bolesnica je razvila sliku zakasnelog puberteta i primarne amenoreje, uz nagli razvoj kliničkih pokazatelja hipersomatotropinizma, što je i bio razlog za obraćanje lekaru u 16. godini života. **Zaključak.** Svi slučajevi zakasnelog puberteta koji se manifestuju bez zastoja u rastu, zahtevaju detaljno hormonsko i genetsko ispitivanje, čak i kada ne postoji tipična klinička manifestacija genetskog obolenja. Prema nama dostupnim podacima, ovo je prvi opisani slučaj istovremenog postojanja Tarnerovog sindroma i gigantizma u literaturi.

Ključne reči:
tarnerov sindrom; gigantizam; hipofiza, neoplazme; adolescenti; žene; pubertet; somatotropin; iGF1.

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Introduction

Turner syndrome presents with one of the most frequent chromosomal aberrations in female that occurs in about 1 *per* 2,500 newborn girls. For the diagnosis of this syndrome the presence of characteristic somatic stigmata in phenotypic females, is coupled with complete or partial absence of the second sex chromosome, with or without cell line mosaicism [1]. Typical clinical features are growth retardation, gonadal dysgenesis and numerous congenital somatic stigmata. In those cases, in which the diagnosis was not made at birth, it was determined during childhood or puberty, due to the growth retardation or primary amenorrhea [2].

Gigantism is an extremely rare condition with approximately 100 reported cases altogether. Gigantism results from an excessive secretion of growth hormone (GH) that occurs during childhood, while the epiphyseal growth plates of the long bones are still open. Cases of GH hypersecretion could originate from a primary pituitary source like somatotroph adenomas, or could be caused by disturbed regulation or an increase of growth hormone-releasing hormone (GHRH) secretion followed by pituitary hyperplasia. The major clinical feature of gigantism is growth acceleration. Those patients often suffer from hypogonadism or delayed puberty, macrocephaly and soft tissue hypertrophy, hyperhidrosis, headache or weakness [3].

Coexistence of Turner syndrome with pituitary tumors is extremely rare. As an example of coexisting diseases modifying each other's presentation, we presented a girl with delayed puberty due to the Turner syndrome but with normal linear growth, for sex and age, due to the simultaneous GH hypersecretion by pituitary tumor. According to a standard procedure, written informed consent for diagnostic procedures, as well as for publishing a case was obtained from the patient.

Case report

A 16-year-old female with a 2-year history of rapid linear growth, soft tissue hypertrophy, hyperhidrosis, weakness and primary amenorrhea was referred to our Clinic. She was full term born, with weight of 2.500 g and length of 51 cm. Her growth velocity was practically normal until 14 years of age, when marked growth acceleration (15 cm/year) was noticed with increasing size of hands and feet. Menarche was absent. Mental development was quite normal. There was no family history of tall stature.

Physical examination revealed enlarged hands and feet, coarse facial features, discrete frontal bossing, mild prognathism and macroglossia. Her height was 171 cm [(height score + 1.75 standard deviation (SD); 90th for-age percentiles)], while her weight was 74 kg, with body mass index (BMI) of 25.4 kg/m^2. She was normotensive, with heart rate of 80 beats per minute, without signs of organomegaly. She was in Tanner stage III of puberty with the normal appearance of external genitalia.

Laboratory data listed in Table 1 revealed increased GH and insulin-like growth factor-I (IGF-1) levels, according to the age-adjusted reference range, and prepubertal serum concentrations of sex steroids and gonadotropins. The patient was eucortisolemic and euthyroid with negative thyroid peroxidase antibody titer. There was no other functional abnormality of the anterior pituitary. The increased serum phosphate level of 2.25 mmol/L (the age-adjusted reference range being 0.97–1.81 mmol/L) was observed. Genetic analysis pointed at a mosaic karyotype 46,X,der(X)/45,XO (45/55%) suggestive of Turner syndrome.

Magnetic resonance imaging (MRI) pituitary scans revealed a hypodense microadenoma of the pituitary gland, on the right side within the sellar region, with dimensions of 7 × 6.5 × 8.5 mm (Figure 1). Pelvic ultrasonography revealed normal dimensions of the uterus and ovaries, with thin endometrium and absent ovarian follicles. Ulnar epiphyseal growth plates appeared open on MRI. Echocardiography showed normokinetic left ventricle with mild mitral valve prolapse (MVP). Her bone mineral density was normal. She was short-sighted. No other endocrine, cardiovascular, renal, intestinal, hearing or mental disorder could be detected.

The patient underwent transsphenoidal surgery but with pituitary tumor only partly removed. Postoperative MRI showed reduced dimensions of previously described pituitary microadenoma with the rest tissue of 3 mm in diameter. The-

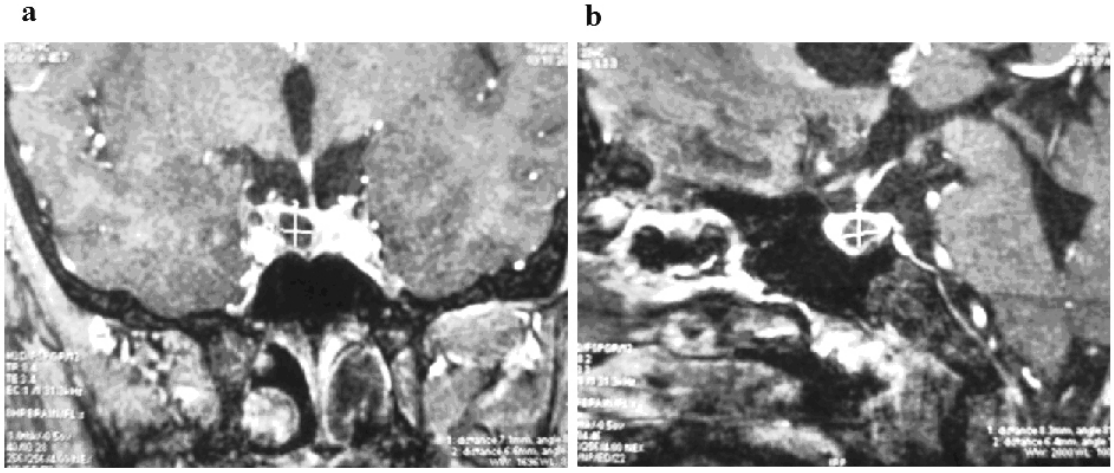

Fig. 1 – Magnetic resonance imaging (MRI) pituitary scans with a hypodense intrasellar tumor on the right side of the fossa sugesting pituitary adenoma (a – coronal section; b –sagittal section)

re was also a newly visualized hypodense mass, on the left side of the pituitary fossa, with dimensions of 3 × 7 mm, suggesting possible pituitary hyperplasia or multiple adenomas (Figures 2). Unfortunately, this speculation could not be histologically proven due to the insufficient amount of tissue material provided at surgery.

Two months after the surgery the patient's condition subjectively improved, but with the clinical evidence of moderate hypersomatotropism still present. Circulating levels of GH and IGF-1 were reduced, but remained above normal and without suppressibility in oral glucose tolerance test (OGTT) (Table 1).

Further treatment of Turner syndrome included sex hormone replacement therapy which was followed with the occurrence of regular menstrual bleeding. The patient was also treated with the long-acting release somatostatin analogue, intramuscularly at monthly intervals over an 18 months period with the ensuing normalization of biochemical parameters of GH secretion and clinical remission. However, as there were no reductions of the size of intrasellar masses the patient was preparing for stereotactic radiosurgery.

Discussion

Observation that coexisting disorders could influence each other's clinical presentations is not unusual. However, it rarely concerns major clinical signs and symptoms.

Cases of Turner syndrome accompanied by pituitary adenomas are very rare. The search of the MEDLINE database retrieved only two cases of Turner syndrome accompanied by acromegaly [4, 5]. The association of Turner syndrome with pituitary hyperplasia verified by histological examination, unfortunately at autopsy, was published in one case [6], with nonfunctioning pituitary microadenoma in two cases [7, 8], while in two more casesthe presence of prolactinoma was demonstrated [9, 10]. To the best of our knowledge no case of Turner syndrome associated with gigantism was reported until now.

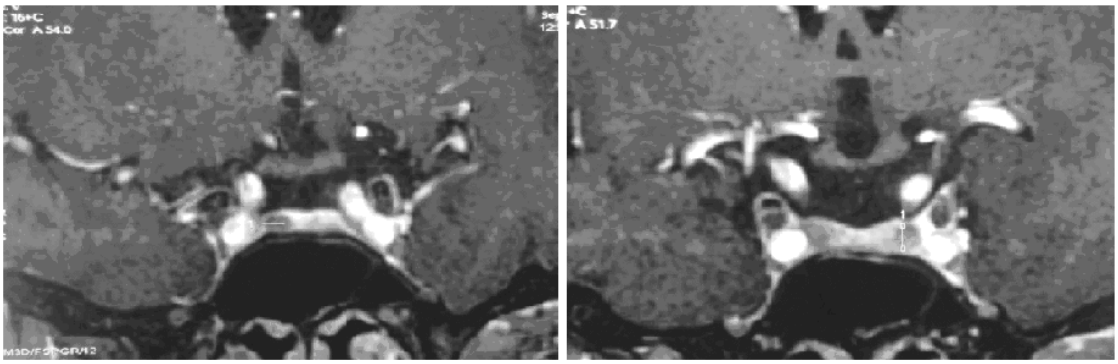

Fig. 2 – Postoperative control. Magnetic resonance imaging (MRI) of the pituitary showing reduced intrasellar tumor on the right side and hypodense mass on the left side of the fossa suggesting possible pituitary hyperplasia or multiple adenomas.

Table 1

Endocrine data in patient before and 2 months after pituitary tumor surgery

Parameter	Values		
	preoperative	postoperative	normal range
GH (ng/mL)	34.3	12.6	< 11.4
IGF-1 (mU/L)	1388.0	1141.0	193–731
LH (U/L)	20.9	12.2	5 – 20
FSH (U/L)	59.84	44.0	5 – 20
Estradiol (pmol-L)	68.4	70.6	95.5–704.8
Testosteron (nmol/L)	0.66	0.7	< 0.922
FT4 (pmol/L)	14.65	20.41	11.5–22.7
TSH (mU/L)	5.29	2.875	035–5.5
TPO-Ab (U/mL)	51.7	50	o-eo
Prolactin (mU/L)	195	137	< 380
Cortisol (nm.ol/L)	498.0	515.5	853–459.6
0800 h			
1600 h	292.7	323.2	64–327.2
ACTH (pmol/L), 0800 h	10.7	5.2	1.6–13.9
GH (ng/mL), nadir during OGTT	-	6.9	0–1

GH – growth hormone; iGF-1 – insulin-like growth factor; LH – luteinizing hormone; FSH – follicle-stimulating hormone; FT4 – free thyroxine; TSH – thyroid stimulating hormone; TPO-Ab – thyroid peroxidase autoantibodies; ACTH –adrenocorticotropic hormone; OGTT – oral glucose tolerance test.

Gigantism results from GH excess that occurs during childhood when open epiphyseal growth plates allow excessive linear growth. Hypothalamic GHRH excess or dysregulation has been considered to be the most common cause of GH hypersecretion affecting the pediatric population [3].

Due to the small number of affected patients, there are no clearly defined signs and symptoms typical for gigantism. Nevertheless, the major clinical feature of gigantism is growth acceleration; these patients can also have coarse facial features, soft tissue enlargement and disproportionately large hands and feet. Those people also suffer from delayed puberty or hypogonadism, menstrual irregularity, headache, weakness, peripheral neuropathy or joint pain [3, 11].

The genetic background for Turner syndrome is highly variable and includes numerous anomalies of the sex chromosomes. Most of the female patients with Turner syndrome are the carriers of the "typical" karyotype of 45X; in 10% of all cases there is a karyotype with isochromosome X [i(Xq) or i(Xp)], while the rest of the patients are individuals with mosaic karyotype of 45,X/46,XX [12]. The most common clinical features are short stature, gonadal dysgenesis and insufficiency. Facial dysmorphism, webbed neck, cardiovascular and kidney malformations as well as lymphedema could also be present. Some patients suffer from cognitive disorders and behavior issues. The absence of puberty with primary amenorrhea represents one of the most frequent symptoms of Turner syndrome, although those patients exhibit spontaneous puberty in 30% and spontaneous pregnancy in 2–5% of cases. However, the major clinical sign of the patients suffering from Turner syndrome remains short stature [1, 12].

In the presented case all the typical phenotypic stigmata related to Turner syndrome were missing. Beside the discrete MVP, we were not able to discover any other systemic malformations or anomalies. Also, due to excessive pituitary GH secretion during the period while the epiphyseal growth plates of the long bones are still open, characteristic stagnation in longitudinal growth has not been demonstrated. The patient presented with delayed puberty and primary amenorrhea along with a sudden appearance of clinical signs of hypersomatotropinism, which were the reasons for seeking medical help at the age of 16. Low serum estrogen levels, increased gonadotropins and karyotype analysis confirmed the existence of Turner syndrome. Biohumoral markers of hypersomatotropinism, in the presence of pituitary microadenoma and still open epiphyseal growth plates, revealed by MRI, confirmed the diagnosis of associated gigantism. Simultaneous occurrence of Turner syndrome and gigantism abolished major clinical sign from both conditions – short stature from Turner syndrome and pronounced growth acceleration from gigantism. This could be the reason for relative delay in diagnosis of these coexisting diseases in our case.

Girls born with Turner syndrome have intrauterine growth retardation and exhibit growth failure during early childhood. Slowdown of growth becomes more pronounced during puberty due to the absence of characteristic peak in pulsatile secretion of GH and IGF-1. Previously, it was considered that short stature represents the consequence of reduced spontaneous GH secretion [12, 13]. Thereafter, it was concluded that estradiol is necessary for the neuroendocrine regulation of pulsatile GH secretion during normal puberty, and became evident that low daily concentrations of GH in patients with Turner syndrome was the result of estradiol deficiency [5, 14]. However, although estrogen replacement therapy applied in girls with Turner syndrome during puberty normalizes daily level of GH in serum, the growth deficit remains. Therefore, it is concluded that growth defect in the majority of patients with Turner syndrome is not solely the result of classical GH deficit [12].

Pathogenesis of growth failure in patients with Turner syndrome is not still completely understood. Most of the authors consider longitudinal growth retardation as the consequence of GH/IGF-1 resistance, particularly on the epiphyseal growth-plate level [2, 15].

Firstly, GH/IGF-1 insensitivity in Turner syndrome was demonstrated on molecular level. Monocyte-macrophage cells in peripheral blood of patients with Turner syndrome expressed lower values of LDL degradation, compared to healthy controls, alongside with the reduced monocyte-stimulated T-lymphocyte proliferation and IL-2 secretion. This indirectly showed lower GH/IGF-1 sensitivity of these cells [16]. Similarly, skin fibroblasts from girls with Turner syndrome release significantly lower amount of IGF-1 and IGF-2 compared to normal fibroblasts [17]. However, female patients with Turner syndrome treated with GH alone or in combination with estradiol, have supraphysiological IGF-1 levels, suggesting the presence of the mechanism that overcomes IGF-1 resistance [18].

On the other hand, GH/IGF-1 resistance may contribute to the explanation of pituitary tumorigenesis in Turner syndrome.

Pituitary function is under tight hypothalamic control, mediated by the effects of releasing and inhibiting hormones. It has been proposed that pituitary tumors are derived from an intrinsic pituitary cell defect leading to monoclonal expansion of a single transformed cell [19]. Hypothalamic hormones may have an important role in promoting the growth of already transformed cell clones and expansion of small adenomas into large tumors. However, it is important that chronic GH excess may be capable to overcome the functional GH resistance causing increase in circulating IGF-1. Induction of GH-secreting pituitary adenomas could be the result of GHRH hypersecretion or decreased somatostatin control [20].

IGF-1 is peripheral hormone helping GH to produce numerous physiological effects on peripheral tissues, including longitudinal growth. Circulating IGF-1 acts as a negative feedback regulator of the GH-gene expression. It inhibits hypothalamic GHRH secretion and acts directly on somatotrophs abolishing stimulatory action of GHRH [21]. Increased need of peripheral tissues for larger amount of IGF-1 could link GH/IGF-1 resistance and GHRH hypersecretion. Similar mechanism could explain the occurrence of acromegaly in patient with anorexia nervosa, a psychosomatic disorder characterized by functional GH resistance [19].

Experimental data on transgenic mice demonstrated that GHRH hyperstimulation resulted primary in somatotroph hyperplasia and, after 8–10 months, in multifocal somatotroph

adenomas. Clinical confirmation of this phenomenon occurs in the development of acromegaly or gigantism in patients with ectopic secretion of GHRH, such as neuroendocrine tumors [22].

It is well-known that target gland hormones (adrenal, thyroid and sex hormones) have strong negative feedback on transcription of genes that encode synthesis of trophic hormones and their secretion. Failure of target gland is accompanied with the loss of negative feedback inhibition and consequent compensatory hyperplasia of respective pituitary trophic hormone cells. Consequently, longstanding primary target gland failure may be associated with pituitary enlargement, as frequently seen on MRI [20].

Although gonadotroph adenomas could also be expected in patients with Turner syndrome, literature data revealed no association among these two conditions. Histochemical and immunochemical studies, conducted on pituitary tissue of four autopsies of cases with Turner syndrome, showed neither gonadotroph hyperplasia nor adenoma. In these cases only corticotroph hyperplasia and adrenocorticotropic hormone (ACTH) immunoreactive adenoma have been perceived [23]. Also, it has to be mentioned that gonadotropin-releasing hormone (GnRH) receptor expression has been documented in the different types of pituitary adenomas [22, 23]. Therefore, the authors speculated that corticotroph cells have expressed GnRH receptors during the time they were exposed to chronic follicle-stimulating hormone (FSH) or luteinizing hormone (LH) hyperstimulation [23].

Thus, we can assume that the GH/IGF resistance, potentially already existing in the presented patient due to the Turner syndrome, has caused hypersecretion of GHRH with ensuing somatotroph hyperplasia and consecutive GH hypersecretion. We could also speculate that somatotroph cells exposed to GnRH and GHRH hyperstimulation, accompanying primary ovarian insufficiency characteristic for Turner syndrome, would express GnRH receptor during the time course. The drawback of our case presentation was the absence of conclusive histological verification of pituitary adenomas or hyperplasia visualized by MRI before and after transsphenoidal surgery. So, definite histological and immunohistochemical confirmation for the existence of acidophilic adenoma, hyperplasia and/or possibly accompanying gonadotroph adenoma was lacking due to the insufficient amount of tissue material provided at surgery. Obviously, autonomic hypersecretion of GH during puberty was sufficient to overcome potential GH/IGF resistance at the level of epiphyseal growth plates and provide accelerated linear growth in our patient. Ensuing lack of growth failure has caused the late detection and postponed treatment of gonadal dysgenesis.

Conclusion

Physical examination of children presenting with delayed puberty but without growth arrest must include an overall hormonal and genetic testing even in the cases when typical clinical presentations of genetic disorder are absent. Authors also believe that this unique case of gigantism coexisting with Turner syndrome threw some more light on still not completely elucidated mechanisms of tumorigenesis in Turner syndrome and possible role of GH/IGF-1 axis in this process.

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Received on June 20, 2015.
Revised on July 23, 2015.
Accepted on July 24, 2015.
Online First February, 2016.

CASE REPORT

UDC: 616.527/.529.1
DOI: 10.2298/VSP150617044S

Pemphigus herpetiformis – A case report of a rare form of pemphigus and review of the literature

Pemphigus herpetiformis – prikaz bolesnika sa retkom formom pemfigusa i pregled literature

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Abstract

Introduction. *Pemphigus herpetiformis* is the rare variant of pemphigus with characteristic clinical features, histopathological findings different from the convectional pemphigus, and immunological findings consistent with pemphigus. **Case report.** We presented a 65-year-old woman with initial pruritus followed by pruritic urticarial papules and plaques, some with annular rings of tense vesicles on the periphery, on the trunk and extremities, with no mucous lesions. Histopathological examination demonstrated spongiosis and intraepidermal vesicles in the mid or subcorneal epidermis in some biopsy specimen, with neutrophil and eosinophil infiltrate. Direct immunoflorescent microscopy revealed intercellular IgG deposition, most prominent in the upper layers of epidermis. Indirect immunoflorescent microscopy showed intercellular binding of IgG autoantibodies in the patient's sera. Initially the patient was threated with systemic corticosteroids and azathioprine, but dapson provided complete clinical remission. **Conclusion.** This entity was established 40 years ago, and around 100 patients have been reported worldwide. It is important to be aware of this particular form of pemphigus because clinical presentation, course of the disease and therapeutic approach are different from conventional forms of pemphigus.

Key words:
pemphigus; rare diseases; diagnosis; drug therapy; treatment outcome.

Apstrakt

Uvod. *Pemphigus herpetiformis* predstavlja retku varijantu pemfigusa, sa karakterističnom kliničkom prezentacijom i patohistološkim nalazom koji se razlikuju od klasičnih formi pemfigusa, i imunološkim karakteristikama koje odgovaraju pemfigusu. **Prikaz bolesnika.** U radu je prikazana bolesnica stara 65 godina sa početnim pruritusom, a potom pojavom pruritičnih papula i plakova, sa mestimično anularno raspoređenim vezikulama na periferiji pojedinih lezija, na trupu i ekstremitetima. Na mukozama nije bilo patoloških promena. Patohistološkim pregledom utvrđena je spongioza i intraepidermalne vezikule u srednjim slojevima epiderma i supkornealno, uz ćelijski infiltrat sačinjen od neutrofila i eozinofila. Direktnom imunoflorescentnom mikroskopijom uočeni su intercelularni depoziti IgG autoantitela, izraženije u gornjim slojevima epiderma. Indirektnom imunoflorescentnom mikroskopijom u serumu bolesnika dokazana su autoantitela IgG klase. Bolesnica je inicijalno lečena opštom kortikosteroidnom terapijom i azatioprinom, ali je do kompletne kliničke remisije dovela terapija dapsonom. **Zaključak.** Od kada je ovaj entitet prvi put opisan pre 40 godina, u literaturi je prikazano oko 100 bolesnika. Pemfigus *herpetiformis* je važno prepoznati s obzirom na drugačiju kliničku prezentaciju, tok bolesti i terapijski pristup u odnosu na konvencionalne forme pemfigusa.

Ključne reči:
pemfigus; retke bolesti; dijagnoza; lečenje lekovima; lečenje, ishod.

Introduction

Pemphigus represents a group of potentially life-threatening autoimmune blistering diseases affecting the skin and mucous membranes [1]. It is characterized by intraepdermal blisters due to achantholyslis, separation of the epidermal cells from each other caused by the antibody-induced disruption of the structural components of keratinocytes, cell-cell anchoring complex, desmosomes [1]. Pathophysiologically, the underling intraepithelial blister formation is caused by immunoglobulin G (IgG) antibodies against desmosomal adhesion proteins desmoglein 3 (Dsg3) and/or desmoglein 1 (Dsg 1)

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on the epidermal keratinocyte cell surface [1]. They can be detected in tissue by direct immunofluorescent microcopy (DIF) of the perilesional skin, in circulation by indirect immunofluorescent microscopy (IIF), as serological detection of antibodies against epidermal components, or specific target antigen, by enzyme-linked immunosorbent assay (ELISA) or immunobloting [1–3].

Pemphigus can be divided into three major forms: pemphigus *vulgaris* (with its localized form pemphigus *vegetans*), pemphigus *foliaceus* (with its localized form pemphigus erythematous and endemic form *fogo selvagem*) and paraneoplastic pemphigus [4]. Pemphigus *vulgaris* (PV) and pemphigus *foliaceus* (PF) are originally characterized as classic or main types of pemphigus [1, 3–5]. In addition, rare forms are included: pemphigus *herpetiformis* (PH), IgA pemphigus [3–6], drug-induced pemphigus, [4, 6] neonatal pemphigus [6] and IgA/IgG pemphigus [5].

In general, pemphigus is uncommon disease. The epidemiology is dependent on the area of the world that is studied as well as the ethnic population in that area. In Europe, the incidence has been reported as 0.5–1.0 [1] up to 2.0 [2] new cases *per* one million inhabitants *per* year.

Pemphigus *herpetiformis* is one of the rare subtypes of pemphigus. It was first introduced by Jablonska et al. [7] in 1975. With the clinical presentation atypical for the most common types of pemphigus, but the immunologic characteristics of pemphigus, this entity presents challenges in the diagnosis. Therefore, a delay in the diagnosis is common. Also, the treatment may be puzzling.

Case report

A 65-year-old Caucasian female was admitted to the Clinic for pruritic urticarial eruption of 3 months duration. Her initial symptom was pruritus, started few weeks before skin changes that initially emerged on the trunk. Physical examination revealed pruritic urticarial papules and plaques, some with annular rings of small or abortive vesicles frequently in herpetiform pattern (Figure 1). The lesions were scattered on the trunk (Figure 2a) and, more prominent, on the extremities (Figures 2b and 2c). Mucous lesions were not present. The patient complained of mild pruritus during the course of skin changes. Histopathological examination of the lesonal skin demonstrated eosinophilic spongiosis with

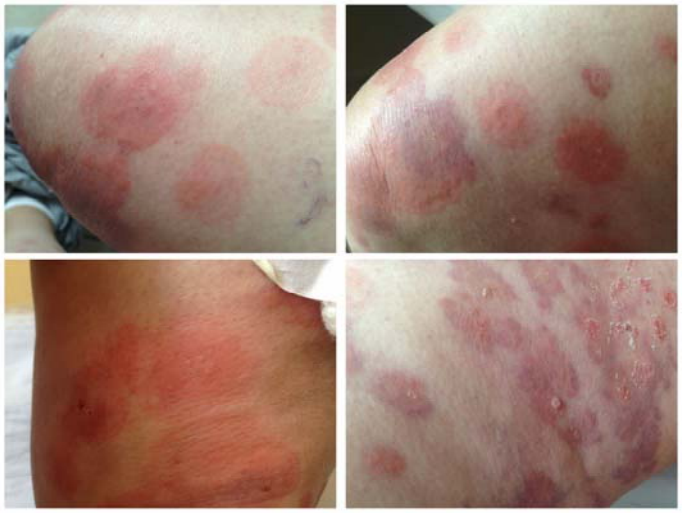

Fig. 1 − Pemphigus *herpetiformis* − groups of small and abortive vesicles, in herpetiform pattern, on erythematous plaques.

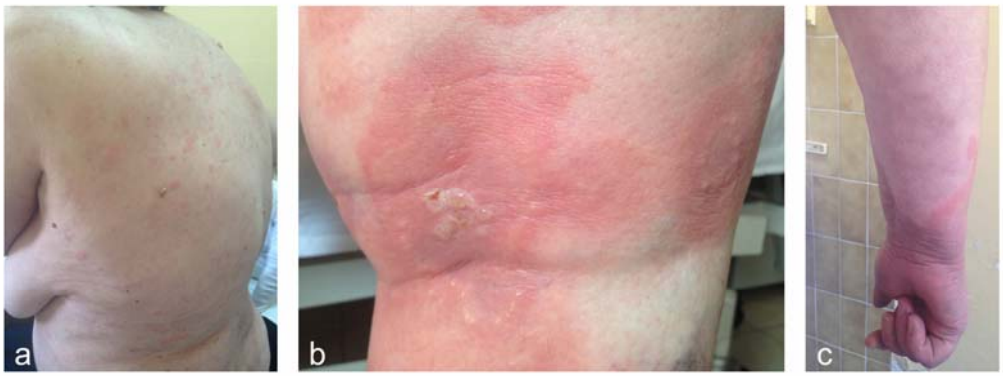

Fig. 2 − Pemphigus *herpetiformis*: a) Erythematous, urticarial plaques on the trunk; b) Tense, vesicles on erythematous base on the leg; c) Annular erythematous, edematous plaques on the distal arm.

formation of intraepidermal vesicles in the mid or subcorneal epidermis and perivascular and interstitial infiltration of eosinophils and lymphocytes in the dermis. (Figures 3a, 3b and 3c) A perilesional skin biopsy for DIF revealed intercellular IgG deposition, most prominent in the upper layers of epidermis (Figure 3d); IgA, IgM and C3 were negative. IIF, using the monkey esophagus as the supstrate, shows intercellular binding of IgG (Figure 3e). Laboratory examination showed slightly elevated levels of urea (9.8 U/L), creatinine (101 U/L) proteins (51 U/L) and gamma-glutamyl transferase (GGT − 49 U/L), while other serum parameters, complete blood count, and tumor markers were within normal limits; urinalysis was normal. The patient had been taking antihypertensive medications for years. Based on clinicopathological and immunological features the diagnosis of pemphigus *herpetiformis* was made. Chest X-ray, computed tomography (CT) scan of the thorax and abdominal ultrasonography were normal, as well as gynecological examination. Oral prednisone in a dosage of 0.59 mg/kg daily was started, as well as topical corticosteroids (fluocinonide 0.05%, clobetasole-propionate 0.05%). In addition, azathioprine 100 mg daily has been administered achieving significant improvement. In further course the prednisone dosage was slowly reduced to 20 mg daily. After two months of treatment mild flare appeared. Azathioprine was excluded and the dosage of prednisone increased. After serum glucose-6-phosphate delydrogenase (G6PD) activity check, dapson, up to 100 mg daily was initiated, and complete clinical remission was achieved in 15 days. Three months later the patient was still free of lesions.

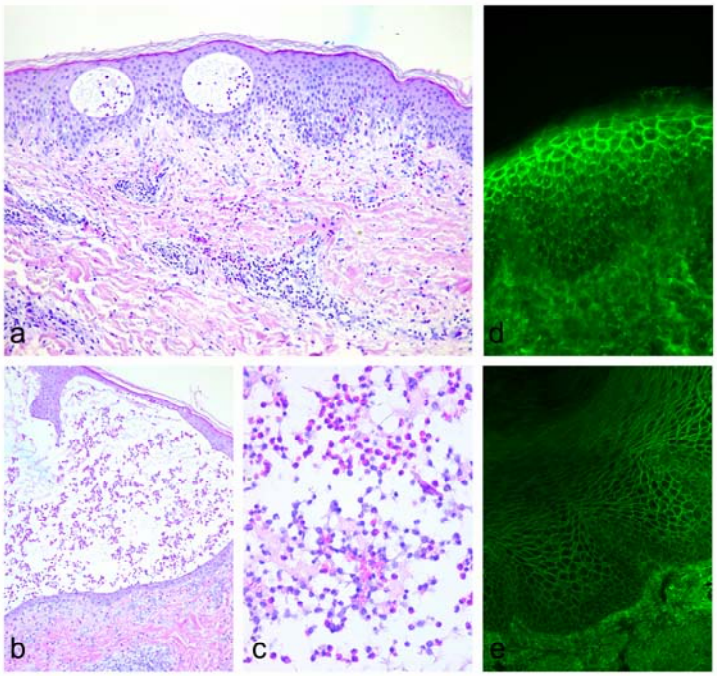

Fig. 3 − Pemphigus *herpetiformis*: a) Spongiosis and intraepidermal vesicles with eosinophils (HE, ×40); b) Spongiotic vesicle containing numerous eosinophils and neutrophils (HE, ×200); c) Numerous eosinophils within vesicle (HE, ×400); d) Direct immunofluorescence of perilesional skin showing intercellular deposits of IgG, more prominent in the upper layers of the epidermis; e) Indirect immunofluorescence on monkey esophagus demonstrating intercellular distribution of anti-IgG antibodies in the patient's sera (1 : 320).

Discussion

Pemphigus *herpetiformis* is a uncommon and sporadic variant of pemphigus with the incidence estimated at 6% [5, 8–10] up to 7.3% [5, 10] of all cases of pemphigus. So far, around 100 patients have been reported [10]. There is no ethnic or gender predilection [5, 10]. Although PH was reported in patients from 5 to 92 years of age, most of the patients were adults [10]. So far, only 4 pediatric patients have been reported [8, 9, 11, 12]. PH is considered to be a distinct entity due to its specific clinical characteristics and distinctive benign course, different from the classical forms of pemphigus. It is characterized by clinical features that resemble dermatitis *herpetiformis* (DH), but immunological findings are consistent with pemphigus [3, 5–7, 9–11]. Although Jablonska et al. [7] established the name of this entity in 1975, similar clinical presentations were described by Floden and Gentele [13] in early 1955, named dermatitis *herpetiformis* with achantolysis. Skin lesions of PH are usually atypical comparing to PV and PF. Erythematous, edematous, vesicular, bullous or papular lesions may be presented [5, 7, 10]. Resulting from centrifugal spread of inflammatory process, the lesions tend to form annular shape [7, 10]. Usually, the groups of small or abortive vesicles, sometimes even pustules, often in herpetiform pattern, are shown on erythematous

base and/or plaques [4–6, 10]. Occasionally, the dominant lesions might be just urticarial erythematous papules and plaques [4, 14]. The lesions frequently affect the trunk and proximal extremities, but they can be shown on other sites as well [7, 10]. Mucous membranes are speared in the majority of the cases [5–7, 10]. Pruritus often accompanies skin lesions, sometimes it might be severe [5, 7, 10], even the initial clinical symptom [14]. Eosinophilia can be found in peripheral blood samples [5], reported in 37.5% cases by Laws et al. [14]. PH may occasionally evolve into PV and PF [4, 7, 15–17], in one case even *fogo selvagem*. Also, the opposite has been reported, PH initially misdiagnosed as other classic variants of pemphigus [4, 5, 7]. Due to the diversity of the clinical presentation, differential diagnosis includes DH, PF, IgA pemphigus, bullous pemphigoid and IgA linear dermatosis [5, 7, 10]. Biopsy findings may also be variable and nonspecific [3, 5, 10]. The eosinophilic spongiosis is the most typical [3–5], but neutrophilic spongiosis or even mixed neutrophilic-eosinophilic spongiosis may be presented, also found in early, urticarial lesions [3, 5]. The assumption is that autoantibody-amplified signaling pathways lead to the secretion of cytokines, chemokines (especially IL8 as potent granulocyte chemoattractant), which cause stimulation and recruitment of eosinophils and neutrophils, resulting in intercellular edema and spongiosis [18], or developed antibodies, despite their minimum acantholytic activity, could activate eosinophils and neutrophils through the Fc portion of IgG [15]. Another characteristic of PH is the presence of intraepidermal bullae [3, 5, 10] or pustules [4–5, 10] variable in composition, in most cases in the subcorneal epidermis, occasionally suprabasally or in the spinous layer [3, 5, 10]. Dermal papillary neutrophilic microabscesses may also be seen [3]. Acatholysis is often absent [3–5, 10]. If present it appears later in the disease process [7, 19]. In practical terms, multiple biopsies are required because of the variable histopathology among patients, even in one patient [3, 5, 7], and the correlation with immunopathology is crucial for final diagnosis. Furthermore, performing direct immunofluorescence (as the gold standard in the diagnosis) when histology reveals neutrophilic and/or eosinophilic spongiosis is recommended. On DIF, intercellular IgG and C3 deposits are most often seen in the superficial layers of the epidermis, less frequently in the lower layers, mainly when circulating anti-Dsg3 antibodies are present [7, 19]. IIF with the monkey/guinea pig esophagus, rat bladder or healthy human skin as substrate can reveal intercellular binding of IgG antibodies [20]. So far, there has not been a clear explanation why autoantibodies produce unusual lesions in PH, different from classic types of pemphigus. The assumption is that the pathogenic blister-inducing activity of the IgG autoantibodies might be weaker [4]. Moreover, the suggested hypothesis is that there is different antibody profiles and broader epitope distribution in patients with PH compared with classic pemphigus [21]. Although ELISA or immunoblotting can show circulating antibodies against epidermal components, usually Dsg1 [4, 5], less commonly Dsg-3 [4, 5], Dsg1 and 3, the same target antigens of the classic pemphigus [5], in PH antibody binding is probably different or target functionally different epitopes of Dsg-1 or 3, therefore do not lead directly to achantolysis, and causing clinicopathological diversity [5, 21]. Furthermore, an epitope spreading phenomenon can be crucial in the pathogenesis, since inflammatory event releases and exposes new antigens inducing autoimmunity to other antigens [22]. Some patients with PH have shown immunoreactivity to 150- and 230-kd antigens [23], 178-kd antigen [24], Dsc3 [25, 26], Dsc1 [20, 27], BP 180 C-terminus and laminin 332 γ2 subunit [20]. PH has been described coexisting with malignances and other diseases. Cases related to malignances are sporadic; to date, five patients with PH and coexisting of lung cancer have been reported [23, 24, 28–30], one esophageal cancer [31], prostate cancer [32] and cutaneous angiosarcoma [33]. In regards to other comorbidities, PH has also been reported in association with another autoimmune diseases, like autoimmune hemolytic anaemia [34], psoriasis [35, 36] and systemic lupus erythematodes [37]. In addition, some cases have been reported with HIV infection [38], drug intake (penicillamine, thiopronine) [39–41] and ultraviolet light exposure [36]. PH generally has an indolent course, good prognosis, [5, 10] and responds well to treatment [5]. It is less life threatening than other types of pemphigus [10]. In this sence, even low doses of systemic corticosteroids can be enough to achieve complete remission [5]. The drug of first choice is dapson (100−300 mg daily), as monotherapy or in combination with systemic steroids [5, 10]. Other therapeutic options are methylprednisolon as puls therapy (1 mg/day for 3 days) together with azathioprine 150 mg/day [5], or azathioprine as monotherapy [19], cyclophosphamide [42, 43], sulfapyridine [7, 44], mycophenol mofetil [45], mycophenolate sodium [46], methotrexate [8], high dose intravenous immunoglobulin [26] and plasmapheresis [26, 42] for more severe cases or cases evolving to classical forms of pemphigus. Recently, the treatment with minocycline and nicotinamide has been published [47].

Conclusion

PH is an uncommon variant of pemphigus with unusual clinical and immunopathological findings, and still unclear underlining pathogenesis. The rarity of this disease and its specificity makes the diagnosis a challenge, so the delay in distinction of this form of pemphigus is often. Therefore, establishing the early diagnosis is important because of the specific course that necessitates a different approach in treatment than for the conventional forms of pemphigus.

R E F E R E N C E S

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Received on June 17, 2015.
Accepted on July 17, 2015.
Online First March, 2016.

CASE REPORT

UDC: 617.7-089-06
DOI: 10.2298/VSP141109088J

Urrets-Zavalia syndrome after deep anterior lamellar keratoplasty

Sindrom Urets-Zavalia nastao posle duboke prednje lamelarne keratoplastike

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Abstract

Introduction. Urrets-Zavalia syndrome is an uncommon complication of the deep anterior lamellar keratoplasty in keratoconus. The manifestations of this syndrome are an irreversible mydriasis, iris atrophy and secondary glaucoma. **Case report.** Deep anterior lamellar keratoplasty was done for keratoconus with a presumably healed corneal hydrops in a 21-year-old Caucasian man. The graft remained clear, but the surgery was complicated by a fixed, dilated pupil, patches of iris atrophy, ectropium of the iris pigment layer and glaukomflecken in the lens. **Conclusion.** Although safer than penetrating keratoplasty, the deep anterior lamellar corneal transplantation is not devoid of complications. Urrets-Zavalia syndrome can be avoided by not trying to secure an unhealed Descemet's membrane with air. Instead, a new Descemet's membrane transplanted within a penetrating graft is a safer choice.

Key words:
corneal transplantation; keratoconus; postoperative complications; syndrome.

Apstrakt

Uvod. Sindrom Urets-Zavalije je neuobičajena komplikacija posle operacije keratokonusa metodom duboke prednje lamelarne keratoplastike. Manifestacije ovog sindroma su trajna midrijaza, atrofija dužice i sekundarni glaukom. **Prikaz bolesnika.** Kod bolesnika starog 21 godinu, urađena je duboka prednja lamelarna keratoplastika sa keratokonusom i naizgled zaceljenim hidropsom rožnjače. Kalem je ostao providan, ali se operacija komplikovala trajnom midrijazom, poljima atrofije dužice, izvrnutim rubom pigmentnog sloja dužice i mrljastim zamućenjima ispod prednje kapsule sočiva (*glaukomflecken*). **Zaključak.** Mada bezbednija od perforativne keratoplastike, duboka prednja lamelarna transplantacija rožnjače nije bez komplikacija. Sindrom Urets-Zavalije može se izbeći ako se umesto zaptivanja otvora u Descemetovoj membrani pomoću vazduha, presadi nova Descemetova membrana zajedno sa kalemom, tehnikom perforativne keratoplastike.

Ključne reči:
transplantacija rožnjače; keratokonus; postoperativne komplikacije; sindrom.

Introduction

Deep anterior lamellar keratoplasty (DALK) has become an aleternative to penetrating keratoplasty (PK) in the treatment of keratoconus (KC) [1]. It permits preservation of the healthy endothelium of a young recipient, which makes late endothelial failure less probable and excludes endothelial rejection. Urrets-Zavalia syndrome (UZS) is a rare complication of DALK. Since the description of fixed, dilated pupil, iris atrophy and secondary glaucoma by Urrets-Zavalia in 1963 [2], this syndrome, bearing his name, limited mainly to penetrating keratoplasty for keratoconus, has been the subject of many theories of its etiology. In the 1980s, it appeared to be extinct, probably due to less surgical trauma [3]. However, new surgical techniques, barosurgery [4] and phakic lens implantation have brought the syndrome into the focus, singling out pupillary block as its cause. Yet, the review of the literature has revealed only five papers describing 17 patients with UZS after DALK [5–9]. To our knowledge, cases of this syndrome have not been presented in our literature. Thus, it seems worthwhile to address this issue again. We reported a case with UZS after DALK for keratoconus with a presumably healed Descemet's membrane after hydrops.

Case report

A 21-year-old Caucasian man was submitted to DALK in his left eye, which had developed hydrops six months earlier, leaving it with the best corrected visual acuity (BCVA) of counting fingers at 1 m. An initial 7.5 mm/300 μm trephination was used to remove the superficial slice of the cornea. While removing the deeper corneal layers by manual dissec-

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tion, a small scar was noted at the site of a presumably healed Descemet's membrane (Figure 1) and a drop of aqueous protruded through a microperforation (Figure 2). An air-bubble injected into the anterior chamber enabled the removal of the remaining stroma and suturing an 8.0 mm graft with 16 interrupted 10–0 nylon sutures. Intravenous infusion of 100 mL 10% mannitol was started. Two hours later, approximately half of the bubble was aspirated, and the balanced salt solution was added. Dexamethasone and gentamycin were injected subconjunctivally at the end of surgery. Carbonic anhydrase inhibitor (acetozolamide, 500 mg) was given *per os* two hours after surgery. Intraocular pressure was checked digitally twice during the afternoon, and estimated to be normal. Another infusion of 100 mL 10% mannitol was given intravenously two hours before bedtime in order to prevent acute glaucoma during the night.

On the first postoperative day, the graft was clear, Descemet's membrane was attached, intraocular pressure was 19 mmHg, and the pupil was round, about 4 mm in diameter. It reacted to light, but less promptly and completely as did the pupil in the intact eye. There was still a small air bubble in the anterior chamber. On the second postoperative day, the pupil was fixed and dilated, with uveal ectropium from 10–2 o'clock, and *glaukomflecken* in the lens (Figure 3). The therapy with 1% pilocarpin did not constrict the pupil. During the following two weeks, patches of iris atrophy became visible, but the intraocular pressure was never higher than 19 mmHg. At three months follow-up, intraocular pressure was 16 mmHg, correction for astigmatism 2.25 × 120, and BCVA was 20/50. These findings remained stationary during the following three months.

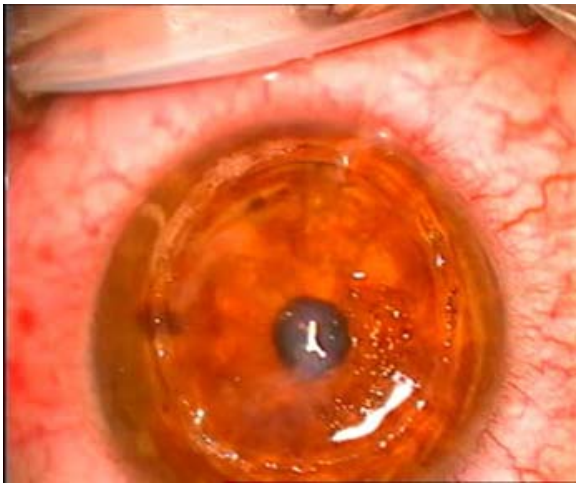

Fig. 1 – A scar at the site of presumably healed Descemet's membrane.

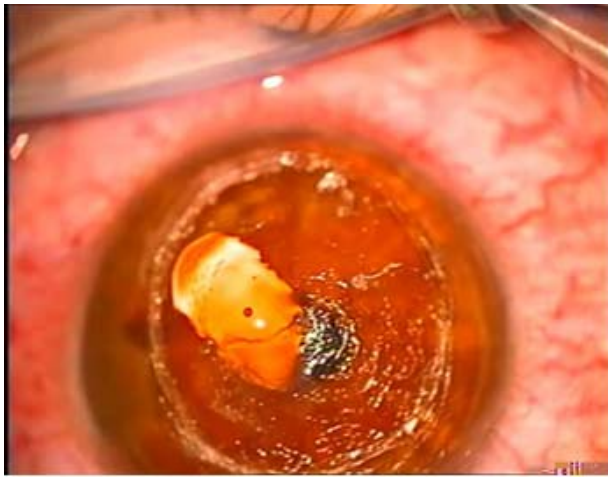

Fig. 2 – A drop of aqueous protruding through the unhealed microperforation in the Descemet's membrane.

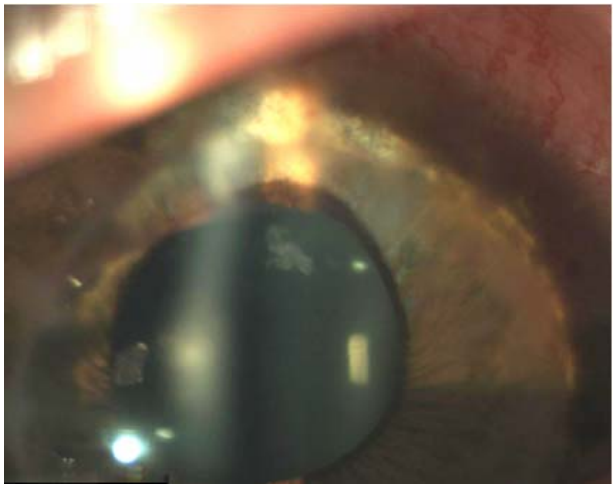

Fig. 3 – Fixed, dilated pupil, ectropium of the iris pigment layer, pathches of iris atrophy and *glaukomflecken* in the lens.

Discussion

The presented patient developed all classic signs of UZS, except for the late secondary glaucoma from angle closure by peripheral synechiae. Yet, the patient might have got it after the follow-up period. The influence of mydriatics and cycloplegics on the irreversible mydriasis in this case can be excluded, as the patient did not receive any of these drugs either pre- or postoperatively. Also, we have no data to sup-

port other etiologies of UZS, proposed in the older literature: abnormal reaction of the dilator musculature of the iris [10], or a paralysis of parasympathetic nerves [11].

The trigger for the pathophysiological mechanism of UZS in this case seems to be an acute raise of intraocular pressure due to the pupillary block by air bubble during the first postoperative night, in spite of the therapy with mannitol and carbonic anhydrase inhibitor. *Glaukomflecken* in his lens are "the smoking gun" of acute glaucoma. The location of the patches of iris atrophy seems to correspond to the position where the air bubble exerted its pressure on the iris longer than elsewhere. This fits into the fluorescein and indocyanine green anterior segment angiography findings of ischemia of the iris [12]. Although there are still cases of inexplicable etiology of UZS, like its development in only one eye after a bilateral trabeculectomy [13], and a postoperative IOT of 19 mmHg, a recent retrospective study points to a rise of postoperative IOT as a serious indicator of an imminent UZS [14]. Even stronger confirmation of the significance of the pupillary block comes from a sudden rise of UZS after the use of air, gas or phakic intraocular lenses [9, 15–17]. Finally, two recent papers report more cases of UZS after DALK for various indications than the complete previous literature [8, 9]. A constant event in these cases is a prolonged presence of an air bubble in the anterior chamber in order to seal a microperforation of Descemet membrane. Antiglaucoma medications and/or iridectomy, do not seem to offer an absolute protection of UZS [13]. Even if the pupillary block is not the sole explanation of the pathophysiology of UZS, it seems clear that its frequency is higher after a prolonged pressure of air on the iris [18].

Conclusion

Irreversible mydriasis, iris atrophy and secondary glaucoma (Urrets-Zavalia syndrome) are infrequent complications after DALK. Yet, the doom of the lasting effects of this syndrome should govern surgeons to lower the incidence of UZS by converting to penetrating keratoplasty when corneal microperforation is evident, or even suspected.

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Received on November 9, 2014.
Accepted on June 30, 2015.
Online First May, 2016.

HISTORY OF MEDICINE

UDC: 61-05::[796:061(091)
DOI: 10.2298/VSP150401095V

Contribution of Dr. Laza Popović to the development of Serbian and Yugoslav Sokol movement

Doprinos dr Laze Popovića razvoju srpskog i jugoslovenskog sokolskog pokreta

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Key words:
history, 20th century; physicians; famous persons; sports; serbia.

Ključne reči:
istorija medicine, xx vek; lekari; slavne ličnosti; sport; srbija.

Introduction

Doctor Laza Popović (Figure 1) was born in Sremski Karlovci in July 1877. He finished primary school and Serbian Orthodox Gymnasium *("Velika gimnazija Karlovačka")* in Sremski Karlovci. Then he studied and graduated medicine in Vienna, where he acquired the title of the Doctor of General Medicine. While studying he made friends and spent time with South-Slav students: the Czechs, Poles, Slovenians, Croats and others. During these meetings he was especially thrilled by the ideas, organisation and programme activities of the "Soko" society he was presented by the Czech colleagues.

Fig. 1 – Dr. Laza Popović, founder of the Sokol gymnastics association.

In university students that could act only through cultural and literary associations and through their youth assemblies, new hopes were awaken in their efforts to better address issues of vital interests of the Serbian people in the territories under the administration of Vienna and Budapest. Thus, in the city Karlovci, the youth initiated the idea of establishing a kind of an association in which they could gather. In the beginning, these were ideas about the formation of fencing, football, or gymnastics association, originating because of the previous efforts of famous physicians Laza Kostić, Jovan Jovanović Zmaj and Milan Jovanović Batut to spread the idea of gymnastic exercising in the aim of "improvement of public health of the nation" [1].

Laza Popović – A founder of Serbian Sokol Associations under the Austro-Hungarian rule

Attracted by the romantic enthusiasm of the youth of Karlovac, young physician Laza Popović, after returning to Sremski Karlovci from his studies, professional, but also handsome, approachable, with a reputation in his native town and the surrounding area, found himself right in the midst of the storm. He quickly gained great trust and popularity, especially among the progressive nationally oriented youth that accepted his idea to establish the Sokol gymnastics association, *ie* "Belgrade Soko Gymnastics Association" (1892), which existed in the Kingdom of Serbia. He was suggested this idea by his patient, Joseph

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Kraus, the Czech, who also made it possible for the Czech members of the Sokol to send him instructions.

This was how "The Serbian Soko" was founded in Sremski Karlovci on January 19, 1904, the work of which was soon approved by the authorities. This was the beginning of wide acceptance of Popović's exceptionally devoted engagement to "spread the Sokol idea throughout the Serbs, for national gathering and awakening of freedom of the Serbs under Austro-Hungarian authorities". This was what he himself wrote about later: "I believe in the Serbian national thought ... this thought will emerge as a storm and carry us all with its sudden irresistible force. This thought of the Serbian folk, the content of which are unification, liberation and progress of the whole Serbian nation, will become the natural leader of all our thoughts, and will lead us to victory" [2].

The beginning of the "Serbian Sokol" of Karlovci was not easy at all because of the resistance in various circles. However, Popović's dedication to the development of the Sokol gymnastic association, and, first of all, awakening of the Sokol spirit of the youth of Karlovci, would make the young Sokol association of Karlovci become the centre and the source of the Serbian Sokol movement in the Austro-Hungarian Monarchy in just a year, and to found the "Serbian Sokol parish of Fruška Gora" in Sremski Karlovci (1905). Doctor Laza Popović made connection with the Czech, Croatian, and Slovenian Sokol associations, and since he was a gifted speaker and writer, he began publishing his first Sokol articles in the papers "*Branik*" (Defender), "*Srbobran*" and "*Omladinski glasnik*" (Youth Gazette). The outcomes of his written words spread as rapidly as a flame throughout Srem, Bačka, Banat, Slavonia, and western Croatia. He promoted the idea that "exercise, mass meetings and public appearances of the Sokol are the most important elements of the Sokol movement to achieve health – both physical and spiritual, strength and patience, a strong will to withstand the strain and tension in life, when being faced with sacrifice and pain" [1]. Besides exercising, he stressed the importance of fostering sociability of members, advocated for freedom, unity and brotherhood, but also for the suppression of political and party influence, chauvinism and bigotry [3].

Since the foundation of "The Serbian Sokol", Popović was always voted the head of the association, and he managed to increase the number of members of all categories. He made friends with many of them who were to become his close associates, especially Nikola Maksimović and Milan Teodorović. He chose excellent gymnasts among the young members who were enthusiastic about the Sokol idea, seeing the future leaders of the Sokol in them. In a short period of time, according to "The Serbian Soko", with his associates, Popović helped founding of 30 new associations in Šid, Novi Sad, Zemun, Vukovar, Ruma, Sremska Mitrovica, Korenica, Pakrac, Stara Pazova, Vinkovci, Inđija, and Zagreb.

The contribution of Laza Popović to education and spreading physical exercise among Serbian youth

This enthusiastic physician initiated work everywhere, founded the Sokol reading room with a library and an archive in Karlovci and Sokol libraries in villages in order to make rural population literate, introduced the Sokol lectures on playgrounds, during events and parties, and also initiated the establishment of "The Peasant Sokol", sent the best members to leadership courses in Prague. A great interest in the work of Sokol could be felt among students and theological youth that respected him infinitely. Female divisions were also established. Many theologians from Karlovac joined the Sokol then. He organized associations for joint appearances at *slet* (a mass gymnastics festival). At the First Croatian all-Sokol jamboree (*slet*) (1906) around 200 Sokol members took place, and since then all Serbian Sokol members acted as a national entity. At the Second Croatian all-school *slet* in Zagreb (1911), there were 762 exercisers, while the *slet* in Prague (1907) and the First Slavonic *slet* in Prague (1912) involved over 1,000 members and 585 exercisers in a separate Serbian exercise [2].

Dr. Popović was open for cooperation, and therefore accepted the idea of Tihomir Ostojić, a high school teacher from Novi Sad, known for promoting gymnastics practicing to include the Sokol *slet* in the programme on the *Vidovdan* (June 28) gathering of people in the monastery of Ravanica on Fruška gora. Since 1905, several thousands of people from Srem and Bačka and the Sokol members had gathered in Ravanica. On *Vidovdan* gatherings, in addition to *slets* exercises, there were heroic and traditional folk games, gymnastic competitions in running, jumping, throwing stones from shoulder. Besides, there were speeches about *Vidovdan*, one of the most important Serbian holidays; poetry was recited to remind the gathered of the Serbian Kosovo heroes, Prince Lazar and Miloš Obilić. The famous "*Vidovdan slet* exercise" was often seen, performed during the *slet* in Prague in 1912 [2]. This national symbolism – the connection of *Vidovdan* and the Sokol movement was not broken until the start of the World War II.

Dr. Popović, having acquired the highest reputation among prominent Czech officials of the Sokol, took advantage of a great friendship with Dr. Joseph Shiner, the head of the Czech Sokol community in Prague, after several unsuccessful attempts to mediate in reconciling the two rival Belgrade gymnastic currents in the Kingdom of Serbia. Thus, the Sokol association "Sokols" and civil gymnastics association "Dušan the Mighty" – "Dušanovci", with its associations, united into a single "Union of Sokol associations "Dušan the Mighty" (1910), to which King Peter I Karađorđević of Yugoslavia and the patron of both associations, Prince Đorđe Karađorđević both contributed. That prevented further disputes and led to better relations and the development of the Serbian Sokol movement [4].

The influence of Laza Popović on the unity of all the Serbian Sokol Associations

Popović worked tirelessly for the unity of all Serbian Sokol associations. He initiated the meeting of representatives of all Serbian Sokol associations in Zagreb in 1911, where "The Union of Serbian Sokol associations" was formed [5]. This union, with its president StevanTodorović, did

not recognize national borders of that time. Soon, the mentioned union, thanks to Popović who was a member of the Presidency of the Union, joined in the All-Slavic Sokol Association (1910). The Association comprised 1,300 Sokol associations with 140,000 members – Czechs, Slovenians, Croats, Poles, Bulgarians and Serbs [6]. Popović was not satisfied with that, but continued writing and collecting new members; he proposed Belgrade as the centre of the Serbian Sokol movement. "Karlovac can never be Serbian Prague", he used to write, as well as: "Conditioned by large fragmentation of the national entity into several parts, our national position became unnatural, so that it obstructs and suffocates any general work of the people." These words were later cited at court – the "high-treason" process in Zagreb, where he was charged and convicted [7].

At the end of 1911 and the beginning of 1912, the center of the Serbian Sokol movement moved from Sremski Karlovci to Belgrade, because the Sokol organization had already strengthened significantly in the free and independent Kingdom of Serbia, with a large number of Sokol associations and experts and with the support of the state. The proposal of Dr. Popović and the Sokol members from Karlovac was brought out of love and faith in the future of Serbia.

This Popović's gesture was generous and visionary, as if he had known that soon, when the First Balkan War began, the Austro-Hungarian authorities would prohibit the operation of "The Serbian Sokol". The position of the Serbian Sokol movement became even more difficult because some of the members went to Serbia to fight for the Serbian cause as volunteers. Just before the beginning of the First World War, the establishment of the Yugoslav association in Zagreb, the members of which would be Serbian, Croatian and Slovenian associations, was initiated and negotiated. And Popović spoke to the youth of Sokol with enthusiasm: "This is the beginning of that majestic poetry, which will follow the national rebirth, liberation and union" [2]. But the union did not occur because of the fateful *Vidovdan* shot at Archduke Franz Ferdinand in Sarajevo. The long-time aspirations of the Sokol members for union were to be sealed with blood on the Salonika Front.

The assassination in Sarajevo happened in the moment when the Sokol members of Karlovac and numerous guests were on the *Vidovdan slet* in the Monastery of Ravanica in Fruška Gora. The gendarmerie immediately drove them away; somewere arrested, and several frontmen were executed. The main leader of the "Serbian Sokol", Dr. Laza Popović, together with another 50 members, was arrested and charged with treason, which was not the first time for him because he had been already charged in 1910. During a short trial in Zagreb, Popović, eloquent and dignified, successfully defended not only himself, but the idea and the Serbian Sokol movement, so the prosecutor dropped the charges, and Laza was sentenced to 14 months of severe imprisonment for disruption of public peace (1915) [1], due to which his physician diploma was taken away from him by the Vienna University.

In trials of both Banja Luka and Sarajevo, 56 members of the Serbian Sokol of the Bosnia-Herzegovinian province were charged because of their work in the Sokol association and their attempted secession of Bosnia from the Austro-Hungarian Monarchy (in September 1915). A total of 97 of the accused were found guilty of high treason – 16 were sentenced to death and the others to severe imprisonment (April 1916). The indictment alleged that some mentioned collaboration with Dr. Popović [8].

After World War I, on *Vidovdan*, during the First Sokol Assembly in Novi Sad (June 28, 1919), in the presence of the highest state representatives and delegates of the "tribal "Sokol members, the united "Sokol Association of the Kingdom of Serbs, Croats and Slovenes" was declared [9] and the fundamental principle of "*One country, one nation, one Sokol movement*" was proclaimed.

Popović later wrote about this: "My people are on the first place, and everything is for my people; all the rest is on the second place. In this great first and true love, the whole Sokol movement will burn to the end. When I felt, learned and saw after the war that my people have two names, but are the one, the embodiment of my thoughts on the national unity of the Serbs and Croats is my Yugoslav idea, and my Yugoslav Sokol movement as I first cried it out and in one swing forged it from January to June 1919 [10]."

In the following years, Popović participated and contributed to resolving numerous issues of the new Sokol organization: the changes of the organization's name ("The Yugoslav Sokol Association" in 1920; "The Union of Sokols of the Kingdom of Yugoslavia" in 1929), solving organizational problems in reconstruction of activities of the united association, promoting ideological, aesthetic, human and educational values of the Sokol movement, as well as developing patriotic, dynastic and state-building qualities of each individual, especially the Sokol children and youth, as well as overcoming misunderstandings and problems because a part of the Croatian Sokol movement tried to leave the union. His views on the pernicious influence of politics on the Sokol movement were very open and unambiguous. Here are a few of his messages to the members of Sokol, which are beyond time:

"The Sokol movement and everyday politics neither have, nor can have any connection. First and foremost, we have to determine the fact that one of the main and fundamental principles of the Sokol idea is: full and absolute freedom"; "The Sokol division is a pure, imminent, passive consequence of our political division and political orientation, our political struggle..."; "Many Yugoslav politicians believe that the Yugoslav Sokol movement is an ordinary circus...monkey business"; "First and foremost, cleansing the Sokol membership from all who are not dedicated to the Sokol idea and from politicians!...; "The future culture and Sokol historians will not believe, when they come across and read, what was left after the "Sokol battle" to lie and rot in our country"; "Let the members of Sokol keep their flag clean and unspoilt by anything dirty!"; "In the idea of Sokol, there is not and there must not be any religious, tribal, or class differences [11]!"

Publicist and editor of Sokol journals

Journalistic and editorial opus of Dr. Laza Popović is also extremely rich. In addition to very impressive speeches

at public meetings, that inspired others with his enthusiasm in the Sokol idea, he left a voluminous writer's mark not only in Sokol, but also in other papers, inviting people to join the Sokol movement, spreading the idea of the unity and strengthening of the Serbian people. But his patriotism was not strictly national. He worked closely with the Czech, Slovenian and Croatian Sokols. Thanks to romantic, but strong and vital ideological and propaganda style, he touched feelings of a great number of the Sokol members. In 1906 he founded the "The newspaper for the matters of the Sokol" – Serbian Sokol, which he edited and published in Karlovac. Later, that newspaper was united with the Belgrade newspaper "Serbian Sokol Herald" (1911). Laza edited and prepared the paper in Sremski Karlovci (up to 1914), while it was published in Belgrade; at that time he also wrote in "The Serbian Knight" (1904–1914). After the First World War, he wrote and edited "The Sokol Herald" (1919–1929). His articles were published in the Sokol papers "Soko of "Dušan the Mighty" (1925–1929), "Sokol Gazette" (1930–1941), "Eagle Eye" (1936–1941), as well as the Sokol yearbooks, calendars, memorials and jubilee almanac. It is interesting that he published his first works, stories and short stories while he was a student in the Mostar magazine "*Zora*" (The Dawn) in "*Bosanska Vila*" (Bosnian Fairy) and "Literary South" (up to 1918). He also wrote about thirty articles and literary criticism in "*Brankovo kolo*" (1913–1914) and in "New Europe"(1921–1926), of which he was an editor for a while. He was also the editor of "Serbian Folk Poems" in Zagreb.

The scientist and the Professor of the University

After the war, since 1918, Laza Popović had his permanent residence in Zagreb. That same year, in the Sokol of Prague, he was promoted once again by returning him the degree of general practitioner. At the end of 1918, he became a consultant physician and the head of the Roentgen Laboratory of the Charity Hospital (*Zakladna bolnica*) in Zagreb. At the Faculty of Medicine, the University of Zagreb, he was elected an associate professor in 1921 and full professor in radiology in 1931. In 1935 and 1936 he was the Dean of the Faculty of Medicine. He was the first president of the Association of Roentgenology founded in Zagreb in 1927. He dedicated the last ten years of his life, between 1931 and 1941, to science and teaching career at the University of Zagreb. As an outstanding Serb, a Yugoslav and the member of Sokol, he had to flee from Zagreb to Belgrade in 1941. He died in May 1945 at the age of 67. He was buried at the Military Cemetery in Belgrade, but his wife Sofija (née Jeftić) transferred his remains to Zagreb in 1947.

Conclusion

Because of its ideology, the Sokol movement was an important factor in achieving national unity and liberation of the enslaved South Slavic peoples. Members of the Sokol movement had an important place in these goals. A Serbian physician, writer, social and the Sokol movement, Dr. Laza Popović, found himself on this path. Numerous data related to his organizational operations and patriotic behaviour have confirmed that the majority of authors who have written about the idea of the Sokol movement, justifiably considered him the spiritual leader of the Serbian, as well as the prominent constructor (ideologist or a visionary) of the Yugoslav Sokol movement. In the beginning, his charismatic nature attracted, gathered and popularized this gymnastic organization, in the role of a long-year Sokol leader, he tirelessly spread the idea of the need for national gathering, strengthening and cultural revival of the Serbian people under the Austro-Hungarian rule. He later promoted the unity of Yugoslav peoples, fought against the political influence in the Sokol movement and damaging national phenomena. He also influenced the development the Sokol idea as the founder, editor and writer of articles in Sokol journals. Numerous difficulties he was exposed to because of his social and sports work, national feelings and patriotism in the turbulent times in which he lived, did not diminish his enormous contribution to the Sokol movement. Looking at his versatile work in the Sokol movement, from this distance in time, we can conclude that the name of Laza Popović is inseparably linked to the history of the Sokol movement of Vojvodina, Slavonia, Bosnia, Dalmatia, Southern Serbia, as well as the Kingdom of Serbia, the Kingdom of Serbs, Croats and Slovenes and the Kingdom of Yugoslavia.

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Received on April 1, 2015.
Accepted on July 3, 2015.
Online First May, 2016.

LETTER TO THE EDITOR

UDC: 579.62::616.993
DOI: 10.2298/VSP160809222B

Neglected zoonosis – The Prevalence of *Salmonella* spp. in pet reptiles in Serbia

Zanemarene zoonoze – prevalencija *Salmonella* spp. kod reptila, kućnih ljubimaca, u Srbiji

To the Editor:

Non-typhoidal *Salmonella* represents an important human and animal pathogen worldwide. Most human salmonellosis cases are foodborne, but each year infections are also acquired through direct or indirect animal contact [1]. Contact with reptiles represents an important source of this zoonotic agent, and recent increases in the popularity of exotic pets have resulted in an increase in the number of cases of reptile-associated salmonellosis and rapidly emerging public health problems. Reptile associated *Salmonella* infections in humans tend to be more likely associated with systemic disease than with foodborne infections. Especially among children, the elderly or pregnant women, septicemia, meningitis, arthritis, soft-tissue abscesses, osteomyelitis, pericarditis, myocarditis, peritonitis and urinary tract infections have been repeatedly described, leading to severe disease and comparably high mortality rates. Most reports of reptile-associated salmonellosis concern babies (under one year of age) and young children (up to six-year-olds) [2].

Reptiles have become popular pets and, for example, approximately 3% of households in the USA own one or more reptiles as pets, resulting in a total of approximately 7.3 million reptiles. The number of pet turtles has doubled in recent years, and approximately 2 million turtles are now kept in over 1 million households, and more than 400,000 USA households keep snakes and in excess of 700, 000 households own lizards [3]. In the European Union, exotic reptiles have been enjoying increasing popularity as pets during the last few years. In 2007, more than 500,000 reptiles were imported to Germany only *via* the Frankfurt/Main Airport. This increase in popularity has led to an increase in the number of reptile-associated salmonella infections which occur every year [4].

In the 1970s, it was estimated that each year 280,000 out of 2,000,000 registered cases of human salmonellosis in the United States were associated with turtles, annually contributing an estimated 14–23% of salmonellosis cases among children [5]. One study, conducted in 2004, estimated that in the USA reptile exposure contributes to approximately 74,000 human cases each year [6]. This represents 6% of all sporadic human cases, and reptile-associated cases are estimated to contribute 11% of sporadic human cases in the population < 21 years of age. In the European Union, apparent prevalence estimates vary considerably among member states and over time, ranging from 1% in the UK and the Netherlands up to 5% in Sweden [7].

To the best of our knowledge, the occurrence and prevalence of species, subspecies and serovars of *Salmonella* in reptiles kept as pets has not been investigated in the Republic of Serbia.

From September 2015 to May 2016 cloacal swabs and faeces were taken from snakes, lizards and turtles kept as pets. Isolation and identification of *Salmonella* spp. was performed according to EN ISO 6579: 2008, Annex D - Horizontal method for the detection of *Salmonella* spp., using prescribed bacterial culture media. Identification of suspected *Salmonella* isolates was confirmed by commercial biochemical test set BBL™ Crystal™ Enteric/Nonfermenter ID and then reconfirmed by matrix assisted laser desorption ionisation time-of-flight mass spectrometry (MALDI-TOF MS, Vitek MS, bioMérieux) according to the manufacturer's instructions. All *Salmonella* isolates were typed to the serovar level in the National Reference Laboratory for *Salmonella*, *Shigella*, *Vibrio cholerae* and *Yersinia enterocolitica*. Serotyping was performed by slide agglutination and the detection of the presence of *Salmonella* O- and H- antigens with the Institute of Public Health of Serbia and the Statens Serum Institut from Copenhagen antisera in accordance with the Kauffman White Le Minor scheme.

In the period observed, the presence of *Salmonella* spp. was examined in 34 snakes, 47 lizards and 7 turtles, representing 10 reptile species as shown in Table 1. In total, *Salmonella* was isolated from 19 out of 88 reptiles (21.59%). All isolates belonged to species *Salmonella enterica,* and two different subspecies of *S. enterica* subsp. *enterica* and *S. enterica* subsp. *diarizonae*. The presence of *Salmonella* spp. was detected in 6 out of 10 reptilian species: royal python – *Python regius*, milk snake – *Lampropeltis triangulum,* bearded dragon – *Pogona vittice,* leopard gecko – *Eublepharis*

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Table 1

List of all tested reptiles and isolated *Salmonella enterica* serovars from reptiles

Name (Latin, English)	Number of animals tested	*Salmonella enterica* subsp. *enterica* (*Salmonella enterica* subsp. *diarizonae*)	Number of isolates
Snakes			
Python regius (Royal python)	19	Serovar *S.* Apapa 45 : m,t : -	1
		Serovar *S.* Benin 9,46 : y : 1,7	2
Lampropeltis triangulum (Milk snake)	5	Serovar *S.* Benin 9,46 : y : 1,7	2
Pantherophis guttatus (Corn snake)	4	/	/
Heterodon nasicus (Western hognose snake)	3	/	/
Boa constrictor (Red-tailed boa)	2	/	/
Euprepiophis mandarinus (Mandarin rat snake)	1	/	/
Lizards			
Eublepharis macularius (Leopard gecko)	30	Serovar *S.* Ago 30 : z38: -	3
		Serovar *S.* Apapa 45 : m,t : -	2
		Serovar *S.* Hadar 6,8 : z10 : e,n,x	1
		(Serovar *S.* IIIb 50 : r : z)	1
Pogona vittice (Bearded dragon)	10	Serovar *S.* Ago 30 : z38: -	2
Hemitheconyx caudicinctus (African fat-tailed gecko)	7	(Serovar *S.* IIIb 47 : - : z35)	1
Turtles			
Trachemys scripta elegans (Red-eared slider)	7	Serovar *S.* Thompson 6,7 : k : 1,5	3
		Serovar *S.* Umbilo 28 : z10 : e,n,x	1
Total		All subspecies	19
		Salmonella enterica subsp. *enterica*	17
		Salmonella enterica subsp. *diarizonae*	2

macularius, african fat-tailed gecko – *Hemitheconyx caudicinctus,* and red-eared slider *Trachemys scripta elegans. Salmonella* was isolated from 5 out of 34 snakes (14.71%), from 10 out of 47 lizards (21.27%) and from 4 out of 7 turtles (56.14%). In total, 8 different serovars of *Salmonella enterica* were isolated from reptiles as shown in Table 1. In this study, five out of eight *Salmonella* serovars were found for the first time in Serbia: *S.* Ago, *S.* Apapa, *S.* Benin, *S* IIIb 47 : - : z35 i *S* IIIb IIIb 50 : r : z.

Salmonella is frequently isolated from reptiles kept in private homes, a study from Italy showed that 24% of reptiles carried *Salmonella* [8], and in Austria and Germany *Salmonella* was isolated from 54.1% of reptiles sampled in their home environment [9]. *Salmonella* infections are highly prevalent in captive lizards in Belgium, where different serovars were isolated from 75.8% of cloacal and 59.5% of faecal samples from captive lizards respectively [10]. In our study, *Salmonella* was isolated from 21.59% of the reptiles, and the prevalence rate was lower than in many other studies and surveys.

The presence of two serovars S. Thompson and S. Hadar was detected in examined turtles, whereas the said serovars have been occasionally isolated from human clinical material in Serbia. The largest outbreak of salmonellosis ever recorded in the Netherlands, with 1,149 confirmed cases, was caused by *S.* Thompson [11]. Cases of human salmonellosis, due to S. Thompson among other serovars, have been reported in Japan following consumption of raw blood, viscera and raw meat as well as cooked meat of turtle *Trionyx sinensis japonicas* [12]. In Europe, S. Hadar is the 4th most common serovar isolated from humans.

Serovar S. Apapa, which belongs to Reptile Exotic Pet Associated Salmonellosis (REPAS), was isolated from lizards and snakes. Zoonotic potencial of *S.* Apapa associated with reptiles have been confirmed in Germany and the United States [13].

Findings of this study confirm that the prevalence of *Salmonella* spp. is considerable in pet reptiles in Serbia, which as potential source of this zoonotic pathogen poses a threat to human health. Although the Republic of Serbia defined national legislation governing import, export and trade of these animals, including the mandatory registration of pet shops and pet kennels, there are no available data on the number of reptiles and their health status.

In order to improve animal health and welfare, as well as to prevent the occurrence and spread of infectious animal diseases, including zoonoses, that can be transmitted from animals to humans, it is necessary to establish an adequate veterinary control of keeping, breeding

and trade of exotic animals. This, together with the training programme of pet reptile owners and public awareness campaign, would contribute to the reduction of risk of contagious diseases that could compromise animal or human health.

Acknowledgements

This study was supported by the Ministry of Education, Science and Technological Development of the Republic of Serbia (Grant No. 31087).

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

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