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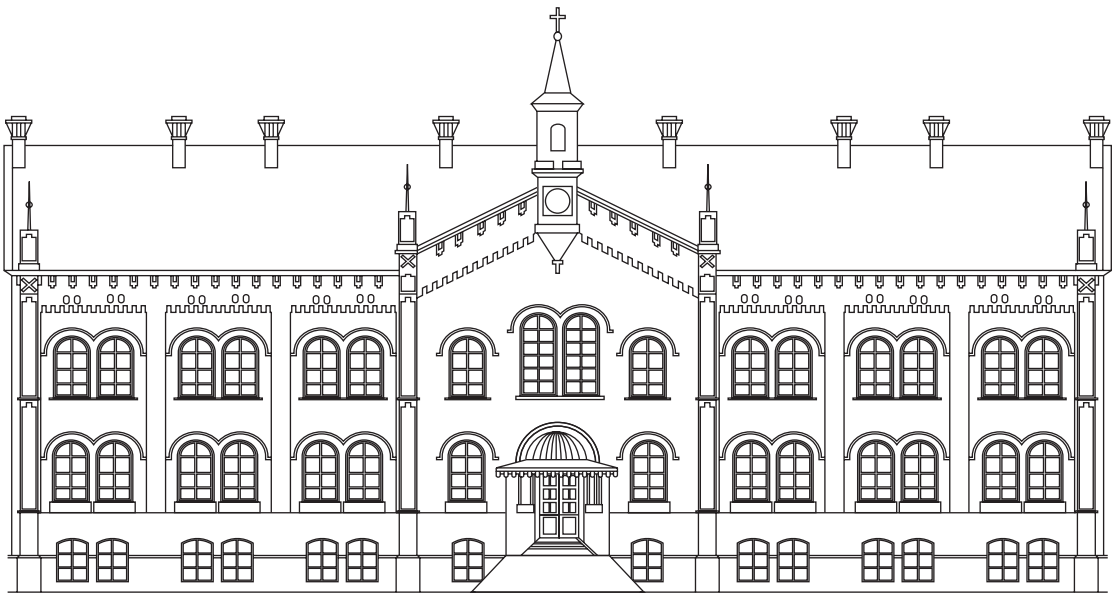

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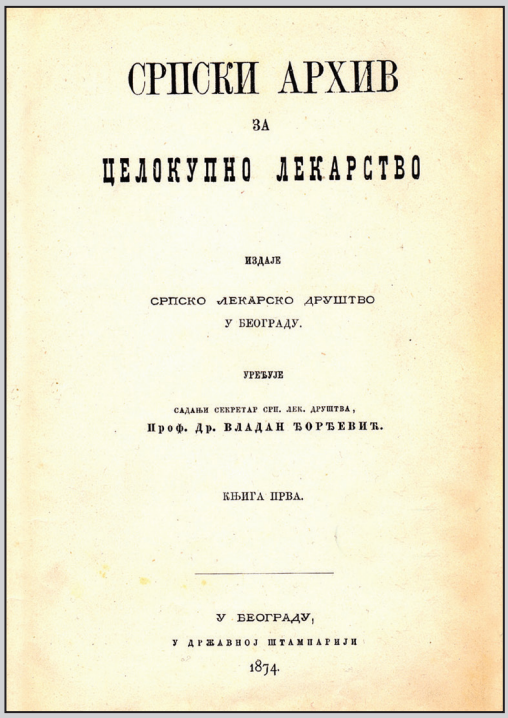

СРПСКИ АРХИВ

ЗА

ЦЕЛОКУПНО ЛЕКАРСТВО

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СРПСКО ЛЕКАРСКО ДРУШТВО

У БЕОГРАДУ.

УРЕЂУЈЕ

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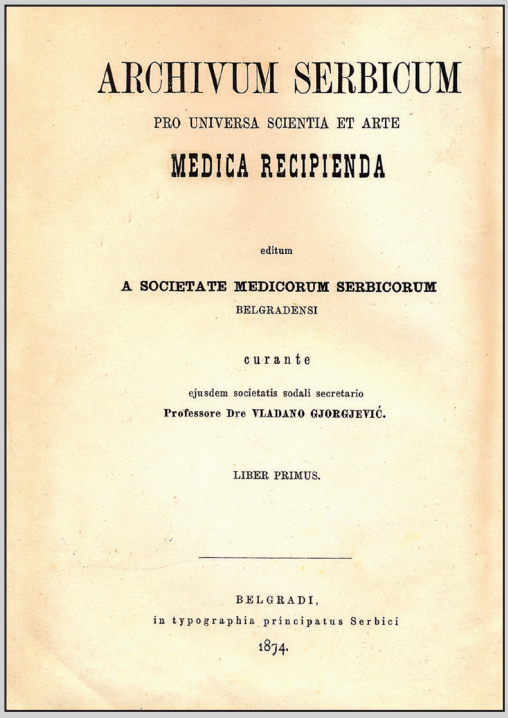
У БЕОГРАДУ,

У ДРЖАВНОЈ ШТАМПАРИЈИ

1874.

Прва страна првог броја часописа на српском језику



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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Effects of sodium hypochlorite on corrosion of the rotary nickel-titanium endodontic instruments – SEM analysis

Milica Jovanović-Medojević[1], Jelena Nešković[1], Marijana Popović-Bajić[1], Đorđe Stratimirović[2], Slavoljub Živković[1]

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SUMMARY

Introduction/**Objective** of this study is to use scanning electron microscopy (SEM) analysis to examine surfaces of new and same sets of Ni-Ti instruments after canal preparations, to check their suspectability to corrosion.

Methods In this study, we used five different endodontic Ni-Ti instruments: K3, Mtwo, ProTaper Universal, HyFlex and BioRaCe. Instruments were analyzed before and after preparation of canals of different curvature, using SEM (150–2000 ×).

Results Corrosion of the working part was observed in 5.5% of new Ni-Ti instruments of the K3 system (apical and middle segment), in 5.5% of Mtwo instruments (apical third), and in 11.1% of ProTaper Universal systems (apical and middle third). Corrosion was not observed on the new instruments of the HyFlex and BioRaCe kits. After instrumentation, disinfection, and sterilization, corrosion was observed in all sets of K3 and ProTaper Universal systems and in all HyFlex instruments of the first group. Corrosion was observed in the HyFlex system in the second group in 16.7% of instruments (apical and middle third) and in the third 83.3% in the apical and 66.7% in the middle segment. In the Mtwo set, corrosion was observed in 16.7% of instruments in the first (apical and middle third), in the second group in 33.3% of instruments in the apical part and 50% in the middle third, while in the third group, corrosion was observed in 16.7% of instruments in the middle third of instruments.

Conclusion Rotary Ni-Ti instruments K3 and ProTaper Universal are susceptible to corrosion in a very high percentage. Ni-Ti systems with post-heat treatment of the working part (HyFlex) are somewhat more resistant to corrosion, while in Ni-Ti systems with electropolished surface (BioRaCe), corrosion is not observed.

Keywords: corrosion; Ni-Ti file; scanning electron microscopy (SEM)

INTRODUCTION

During the last decades, machine instrumentation of root canals with the use of rotary Ni-Ti instruments has become a standard clinical procedure and this Ni-Ti instruments, have enabled easier and faster instrumentation with a predictable outcome [1, 2]. The fact is that various factors during mechanical instrumentation of the canal can affect the occurrence of deformations and unexpected fractures of Ni-Ti instruments (anatomomorphological characteristics of the canal, inadequate choice of endodontic instruments and preparation techniques, means and techniques of irrigation, knowledge, expertise and experience), which significantly frustrates many practitioners and limits the use of these instruments in certain clinical indications [3]. One of the factors that can affect the efficiency and safety of their application is the occurrence of corrosion on the working part of Ni-Ti rotary instruments [4].

Analyzing the surface of new, unused Ni-Ti instruments, many studies have shown that on the surface of their working part there are numerous defects (pitting, fretting, metal strips...) [5, 6, 7]. The reason for this is the significantly more complex process of making rotating Ni-Ti instruments than the making of steel instruments, which often leads to irregularities on the surface of new Ni-Ti instruments [6, 7]. These changes represent centers with dislocation of the crystal structure that can compromise the cutting efficiency of the instruments and become sites for potential corrosion. Also, these points represent the sites of initiation of defects and may contribute to the degradation of mechanical properties and the appearance of micro or complete fractures during the clinical use of Ni-Ti instruments [8]. The presence of numerous defects on the working part of the instruments as a consequence of the production process often leads to corrosive effects on the used instruments.

Corrosion, as an oxidative reaction, leads to the release of electrons from metals, their movement towards the surface and the formation of molecular hydrogen [4]. It has been confirmed that the occurrence of corrosion can affect the reduced blade efficiency of rotary

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Ni-Ti instruments, as well as the possible propagation of other defects [2].

Although it has been proven that there are several types of corrosion (uniform, galvanic, cracked, pitted, intergranular, selective corrosion, corrosion in the form of erosion and grooves, corrosion caused by material stress), pitting corrosion is the type of corrosion most common on Ni-Ti alloy surface [4]. The big problem with this form of corrosion is that the defects are difficult to detect, until the moment of exposure to aggressive ions (mainly chlorides) which further propagate this defect. In the mechanism of this type of corrosion the main role is played by the microstructure of the material and environmental conditions, such as Ph chloride concentration and temperature [4].

The appearance of corrosion on Ni-Ti instruments can be caused by the use of different irrigants during instrumentation, specific conditions of the oral environment (body temperature, saliva with salts and electrolytes, blood) as well as multiple cycles of chemical disinfection and sterilization [9]. The most commonly used irrigant, sodium hypochlorite (NaOCl), is highly corrosive to Ni-Ti alloy, ranging in concentrations 1.2–5.25% [2, 10]. Namely, NaOCl selectively removes nickel from the surface of the instrument, and thus leads to the formation of microcracks, which negatively affect the physical and mechanical properties of rotary Ni-Ti instruments [2].

Although many studies have been conducted in the last two decades regarding the effect of NaOCl on Ni-Ti instruments, using microscopy and electrochemical analysis, quantitative data on corrosion of Ni-Ti rotary instruments are still scarce [2].

The aim of this research was to analyze the surfaces of new (unused) and the same sets of rotary Ni-Ti instruments after the preparation of the canals, ie to check their susceptibility to corrosion.

METHODS

The study included five sets of new rotary Ni-Ti instruments of different design: K3 (SybronEndo Co, Orange, CA, USA), Mtwo (VDW, Munich, Germany), ProTaperUniversal (Dentsply Maillefer, Ballaigues, Switzerland), HyFlex (Coltene Whaledent group, Altstätten, Switzerland) and BioRaCe (FKG DENTAIRE Swiss Dental Products, Le Crêt-du-Locle, Switzerland) (Table 1).

The research was performed *in vitro* conditions, on permanent, multi-rooted teeth, extracted for various reasons after obtaining the consent of the Ethics Committee of the School of Dentistry, University of Belgrade number 36/6 from January 21, 2013. After extraction, the teeth were stored for two hours in 4% sodium hypochlorite solution, and until the beginning of the preparation, they were stored in physiological solution with 0.2% thymol. With the help of a high-speed elbow with water spray and a tungsten-carbide cylindrical drill with a rounded tip E0153/012 (Dentsply/Maillefer), the existing fillings and cariously changed tissues were removed, and a round drill with an extended handle E0123/014 (Dentsply) has done trepanation of the coronary chamber. With the diamond disk, the crown of the tooth was shortened to the level of 2 mm coronally from the enamel-cement border.

The final treatment of the walls of the access cavity and the coronal chamber was completed with a carbide conical drill with a passive tip Endo Z (Dentsply/Maillefer). Probing and assessment of the initial patency of the channel was determined by K-files of ISO 15 size (MicroMega, Besancon, France) and the working length was determined to be 1 mm shorter than the length obtained by the appearance of the instrument at the top. An X-ray was performed on each canal and then the degree of bending for each canal was determined using an online protractor (*https://www.ginifab.com/feeds/angle_measurement/)*.

The degree of canal curvature was determined by Schneider's radiographic technique [10] and based on that, the teeth were divided into three categories:

a) 50 straight canals – less than 10°
b) 50 slightly bent canals – from 10° to 25°
c) 50 strongly bent canals – over 25°.

To mimic *in vivo* conditions, the apex was sealed with pink wax to simulate apical counter-pressure and prevent irrigation from leaking during instrumentation.

In order to achieve uniform experimental conditions, each instrument was used in ten canals or until the moment of its fracture (one set from all five examined threads of the system was applied for processing of 10 canals in

Table 1. Basic characteristics of tested sets of rotary Ni-Ti instruments

Instrument manufacturer	Design specifics	Diameter	Taper	The process production
K3 SybronEndo	triple blades with, positive angle and asymmetric radial surfaces	25	0.12–0.02	micromilling conventional Ni-Ti
Mtwo VDW	S-shape with two active cutting surface angle	10–35	0.04 0.05 0.06	micromilling conventional Ni-Ti
ProTaperUniversal Dentsplay-Sirona	convex triangle variable progressive taper along the instrument	17–30	regressive taper	micromilling conventional Ni-Ti
HyFlex CM Coltene	double Hedstrom design with positive rake angle	20–40	0.04 0.06 0.08	micromilling CM-wire
BioRaCe FKG	triangular with alterations of the cutting edges along the instrument	15–40	0.04 0.05 0.06 0.08	micromilling conventional Ni-Ti electropolished surface

Table 2. Presence of the signs of corrosion on new rotary Ni-Ti instruments

K3		Mtwo		ProTaper Universal		HyFlex		BioRaCe	
Apical third	Middle third	Apical third	Middle third	Apical third	Middle third	Apical third	Middle third	Apical third	Middle third
(5.5%)	(5.5%)	(5.5%)	0	(11.1%)	(11.1%)	0	0	0	0

each experimental group). The instrumentation of the canal was realized in accordance with the manufacturer's instructions, crown-down technique and application of X-Smart Endodontic Rotary Motor (Dentsply, Sirona, Maillefer, Ballaigues Salzburg, Austria).

As irrigants, after each instrument, in the amount of 5 cm^3, 2% NaOCl solution (Chloraxid 2%, Cerkamed, Stalowa Wola, Poland) and then distilled water (Iva, Serbia) were used. Irrigants were applied using a plastic syringe and an endodontic irrigation needle with a closed tip and side openings (Side-vented needle, SmearClear, SybronEndo). Ethylenediamine tetra-acetic acid gel (Glyde-Dentsply, Maillefer, Switzerland) was used as a lubricant during the preparation, applied to the working part of the instruments. During the processing of the canal, each used instrument was carefully inspected with a magnifying glass, in order to detect any change (possible cracks, fractures, unscrewing of twist or other deformations).

The experimental protocol included:

1. Scanning electron microscopy (SEM) analysis, new unused instruments directly from the factory packaging, the apical and middle thirds of the instrument from two different directions were analyzed and three SE images were made for each surface of the instrument
2. preoperative preparation, cleaning in an ultrasonic bath using a mild disinfectant Orocid Multisept plus (Oro Clean Chemie AG, Fehraltorf, Switzerland) for 15 minutes
3. Instrumentation according to the proposed protocol with abundant irrigation (2% NaOCl solution, Distilled water, and EDTA)
4. Cleaning in an ultrasonic bath after use with a mild disinfectant (Orocid Multisept plus) for 15 minutes
5. Sterilization of used instruments performed in an autoclave (MELAG Medizintechnik GmbH & Co. KG – Vacuklav 23B +, Berlin, Germany) at 134°C for five minutes.
6. SEM analysis of used instruments

For defect analysis, the following was inspected:

- 540 recordings of new instruments and 700 recordings of instruments after canal preparation
- Reconciliation of the results of the two researchers was performed by Cohen's kappa analysis

Statistical analysis

Statistical analysis the obtained data was performed using the Fisher test.

RESULTS

The results of the SEM analysis are shown in Tables 2 and 3 and Figures 1–6.

Corrosion of the working part of new Ni-Ti instruments was not observed in HyFlex and BioRaCe instruments, while in other Ni-Ti systems it was observed in a small percentage (Table 2).

In new Ni-Ti instruments, corrosion was observed in 5.5% of K3 system instruments (apical and middle segment), in 5.5% of Mtwo instruments (apical third) (Figure 1) and in 11.1% of ProTaper Universal system instruments (apical and middle third). Corrosion was not observed on the new sets of HyFlex and BioRaCe instruments (Table 2).

SEM analysis of Ni-Ti instruments after their use, cleaning and sterilization indicated the occurrence of corrosion in 62.8% of the analyzed instruments. After chemo-mechanical instrumentation, disinfection and sterilization, the presence of corrosion was not registered on the Ni-Ti instruments of the BioRaCe system. The presence of corrosion was observed on all analyzed Ni-Ti instruments

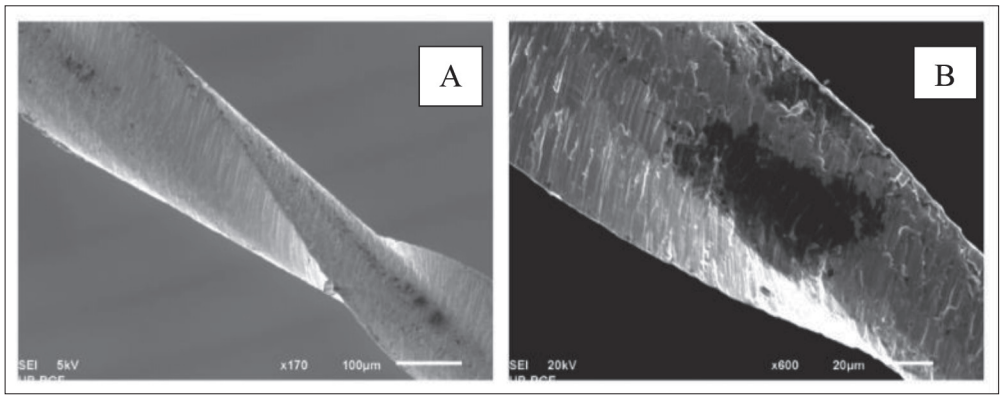

Figure 1. Spin echo image of the new Mtwo instrument (10/0.4): A – surface of the middle third on which the presence of corrosion is observed (170 ×); B – detail from the previous image at higher magnification (600 ×)

Table 3. The presence of corrosion on the sets Ni-Ti instruments after instrumentation, disinfection and sterilization

Ni-Ti instruments	I group		II group		III group	
K3	100%	100%	100%	100%	100%	100%
Mtwo	16.7%	16.7%	33.3%	50%	0	16.7%
ProTaper Universal	100%	100%	100%	100%	100%	100%
HyFlex	100%	100%	6.7%	16.7%	83.3%	66.7%
BioRaCe	0	0	0	0	0	0

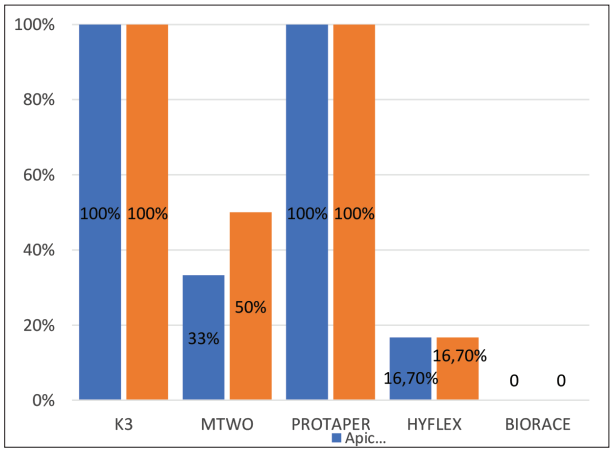

Figure 2. Prevalence of corrosion on Ni-Ti instruments after instrumentation of disinfection and sterilization in the canals of the first group; letters (a or b) indicate no statistically differences ($p > 0.05$)

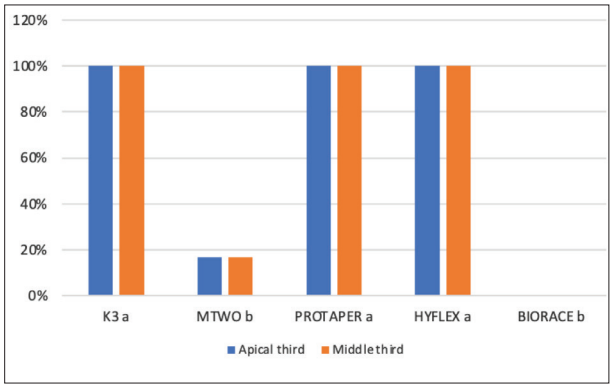

Figure 3. Corrosion prevalence on Ni-Ti instruments after instrumentation, disinfection and sterilization in the channels of the second group; letters (a or b) indicate no statistically differences ($p > 0.05$)

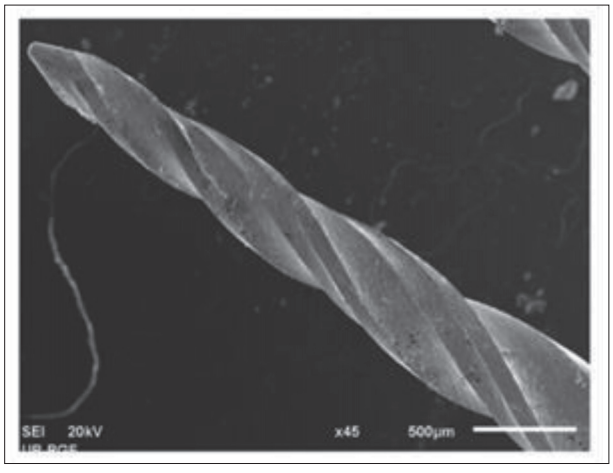

Figure 4. Spin echo image K3 instrument (25–0.08), second experimental group (corrosion on the apical third) (45 ×)

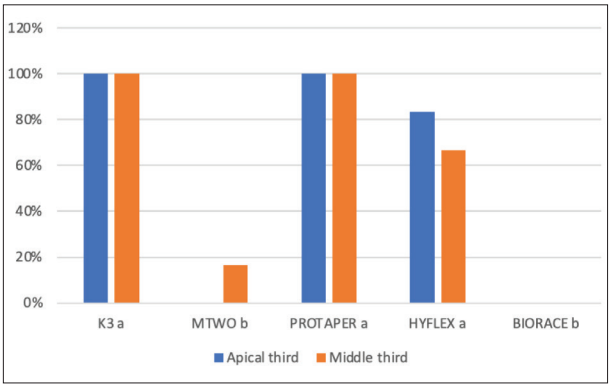

Figure 5. Prevalence of corrosion by Ni-Ti instruments after instrumentation, disinfection and sterilization of third group; letters (a or b) indicate no statistically differences ($p > 0.05$)

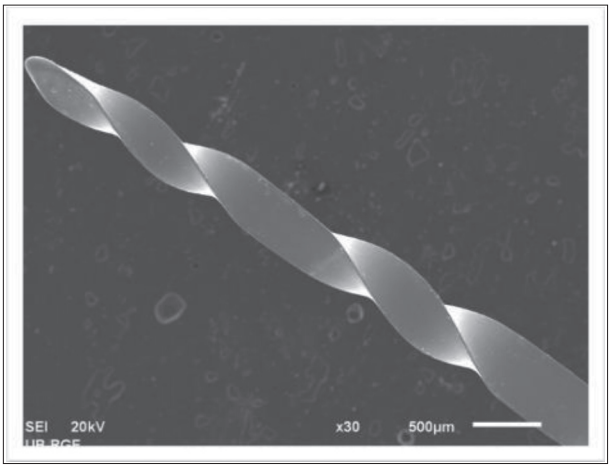

Figure 6. Spin echo image BioRaCe instrument no. 4 without incorrection and corrosion (30 ×)

(100%) K3 and ProTaper Universal system (apical and middle third), in all three experimental groups (Table 3).

In the first group, corrosion was observed on all instruments K3, ProTaper Universal and HyFlex systems (apical and middle third), and in Mtwo systems on 16.7% of instruments (apical and middle third). Corrosion was not observed in BioRaCe instruments. A statistically significant difference was observed between the K3, ProTaper Universal and HyFlex systems compared to the Mtwo and BioRaCe systems of the first experimental group ($p < 0.05$) (Table 3, Figure 2).

In the second experimental group, corrosion was observed on all analyzed Ni-Ti instruments K3 (Figure 3) and ProTaper Universal system (apical and middle third), in HyFlex set on 16.7% of instruments (apical and middle third), and in Mtwo set instruments, to 33.3% of instruments in the apical segment and 50% in the middle third. No corrosion was observed on BioRaCe instruments. A statistically significant difference was observed between K3 and ProTaper Universal systems compared to Mtwo, HyFlex and BioRaCe system ($p < 0.05$) (Table 3, Figure 4).

In the third experimental group, corrosion was observed on all analyzed Ni-Ti instruments K3 and ProTaper Universal system (apical and middle third), in 83.3% of HyFlex set instruments in the apical and 66.7% in the

middle segment (Figure 5). In the Mtwo set, corrosion was observed only in the middle third in 16.7% of the instruments. A statistically significant difference in the occurrence of corrosion was observed between K3, ProTaper Universal and HyFlex systems in relation to Mtwo and BioRaCe system ($p < 0.05$) (Table 3, Figure 6).

Comparing the results of SEM analysis, it was found that corrosion is the only defect that shows a statistically significant difference between new and used instruments. This difference was found between new and used K3 and ProTaper Universal instruments in all groups, and between new and used HyFlex instruments in the first and third groups ($p < 0.05$).

DISCUSSION

The results of this study indicate a low prevalence of corrosion on K3, ProTaper Universal and Mtwo sets, as well as the absence of corrosion on HyFlex and BioRaCe system instruments. This result is in accordance with the statements indicating the biocompatibility and good corrosion resistance of Ni-Ti alloy [2, 11, 12].

Corrosion resistance of Ni-Ti alloy is based on the presence of a passive oxide film of titanium oxide on the surface which prevents the development of uniform corrosion. However, beneath this thin layer of titanium oxide (which also contains small amounts of nickel) is a nickel-rich sublayer that is responsible for corrosion [11]. It is believed that this surface oxide film of titanium oxide increases the stability of the surface layers of the alloy, protects against corrosion and ensures the stability of the material itself [13]. According to the results of research by Stokes and associates who examined the corrosive effect of new, unused Ni-Ti instruments from five different manufacturers, it was confirmed that the occurrence of corrosion on new instruments is influenced by the instrument production process, ie the quality of control of the manufacturing process [14].

The finding of a significantly higher percentage of corrosion of Ni-Ti rotating instruments after instrumentation, disinfection and sterilization is in line with the results of studies confirming that corrosion processes and mechanisms can be activated during chemo-mechanical treatment of channels and application of various organolytic and mineralolytic substances (CHX, citric acid, etc.) which with their chemical and electrochemical potential can act on the surface structure of instruments [15]. It was found that changes in the surface of the working part of Ni-Ti instruments can occur as a result of chemo-mechanical treatment of instruments after instrumentation (cleaning, chemical disinfection or sterilization) [2, 9, 11].

The current results of studies on the impact of sterilization on the occurrence of corrosion are contradictory and there is no clear position on this issue. Multiple sterilization cycles can cause corrosive changes on the surface of Ni-Ti files due to changes in the surface layer of titanium oxide [16, 17]. However, it has also been observed that after sterilization, resistance to cyclic fatigue and torsional stress increases in certain types of Ni-Ti instruments because the sterilization process acts as a form of heat treatment [18]. Manufacturers recommend the mandatory use of gel lubricants during machine instrumentation, either directly on the active part of the Ni-Ti instrument or by application to the pulp chamber [9]. The most commonly used chelating, mineralolytic agent is ethylenediaminetetraacetic acid (EDTA, 15–17%), which according to the literature has no effect on the surface structure of Ni-Ti instruments [19, 20]. In the study of Fajad and Mahran, after immersing the instruments in a 17% solution of EDTA, there was no change in their surface structure [19]. According to research by Reinhard et al. [20], EDTA protects and passivates the surface of Ni-Ti instruments by forming complexes with metal ions (at pH values less than four), thus creating an inhibitory barrier to oxidation and corrosion.

Experimental evidence does not support the use of these gels, as they not only do not reduce the friction between the instrument and the dentinal canal, but in some cases even increase it [13]. Aqueous solutions (or even distilled water) are much more useful, as they wash the dentinal detritus from the grooves of the instruments more efficiently [9, 13]. The use of NaOCl solution during instrumentation is standard because in addition to acting on bacteria and dissolving tissue debris, it is also very effective as a lubricant. NaOCl was confirmed to be highly corrosive to Ni-Ti alloy (in the concentration range 1.2% to 5.25%). A lower NaOCl concentration (1%) does not lead to corrosion and does not affect torsional and cyclic resistance after a cumulative exposure of 2.5 hours, while a longer exposure of 18 hours indicates clear signs of corrosion [21, 22].

The problem that arises during the disinfection process and the long-term complete immersion of the instruments in the sodium hypochlorite solution, arises due to the metallurgical characteristics of the instruments. The handle of the instrument is usually made of different metal in relation to the working part, so the presence of two metals in the solution can affect the release of ions and the creation of galvanic reactions that can accelerate corrosion [21]. It was confirmed that Ni-Ti alloy corrodes in NaOCl solution with high pH values (pH 12.3) due to galvanization (due to gilding on the handle). It is believed that the corrosion resistance of Ni-Ti alloy can be increased by lowering the pH value of the solution to about 10, because then passive oxides, TiO_2 and NiO_2 are formed [23].

The occurrence of corrosion on Ni-Ti instruments in this study could be explained by the fact that the application of various chemical procedures before or during instrumentation (disinfection, sterilization and irrigation) can cause corrosion or deepening of existing corrosive defects [22, 23]. Although the surface of rotating Ni-Ti instruments is usually covered with a protective film of titanium oxide, this layer can be easily disturbed and damaged during instrumentation and contact of the instrument with the root canal wall [23, 24, 25].

O'Hoy et al. [22] showed strong corrosion after immersion of instruments in NaOCl solution and Yokoyama et al. [26] pointed to corrosion as the main reason for the occurrence of Ni-Ti instrument fracture due to stress due to cyclic fatigue. Berutti et al. [27] pointed to a significant

effect of 5% NaOCl solution (within five minutes) on the appearance of pits and cracks on the surface of Ni-Ti instruments and Peters et al. [28] proved a decrease in resistance to cyclic fatigue of Race and ProFile instruments after their exposure to NaOCl. The reason for such contradictory results is probably a consequence of different methodological procedures (different immersion times of instruments, different concentration of solution, different exposure during irrigation, cleaning and disinfection) [21–28].

The results of the study by Darabara et al. [29] indicated that continuous irrigation with 2.5% NaOCl solution does not lead to corrosion of stainless-steel instruments and Ni-Ti instruments, but that mechanical rather than corrosive factors are responsible for the fracture.

The results of studies by Shahi et al. [30] indicated the resistance of Mtwo instruments to NaOCl solution. Immersion of Ni-Ti instruments in 2.5% NaOCl solution for 12–48 hours did not indicate significant changes in the working part of the instruments, which is in accordance with the findings of this study [30].

The BioRaCe system of Ni-Ti rotating instruments is subjected to an electropolishing procedure during final processing, which reduces the possibility of surface irregularities and increases resistance to corrosion and cyclic fatigue, which is also in accordance with the results of this study [2, 9]. Shahi et al. [30] hypothesized that corrosion occurs due to manufacturing defects on the surface of instruments that amplify the effect of cyclic fatigue and change the fracture mechanism due to cyclic fatigue into a corrosive fracture [30].

Improving corrosion resistance, manufacturers are trying to achieve by additional surface treatment of the working part by electropolishing, heat treatment, implementation, physical deposition or coating of various elements [2, 9].

CONCLUSION

The results of this study showed that the rotary Ni-Ti instruments of the K3 and Pro aper Universal systems are subject to corrosion in a very high percentage. Ni-Ti heat-treated systems are somewhat more resistant (HyFlex), while corrosion is not observed in instruments with electropolished surface (BioRaCe).

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Утицај натријум-хипохлорита на појаву корозије ротирајућих *Ni-Ti* инструмената – СЕМ анализа

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САЖЕТАК

Увод/Циљ Циљ истраживања је био да се применом скенирајуће електронске микроскопије (СЕМ) анализирају површине некоришћених и истих сетова никл-титанијмских (*Ni-Ti*) ротирајућих инструмената након препарације канала, односно да се провери њихова подложност за појаву корозије.

Метод У истраживање је укључено пет различитих сетова машинских ендодонтских *Ni-Ti* инструмената: *K3*, *Mtwo*, *ProTaper Universal*, *HyFlex* и *BioRaCe*. Инструменти су анализирани пре употребе и након препарације канала различите повијености, помоћу СЕМ-а (150–2000 ×). Сваки сет инструмената је коришћен за обраду 10 канала у три експерименталне групе (прави, благо и изразито повијени канали).

Резултати Корозија радног дела уочена је код 5,5% нових *Ni-Ti* инструмената система *K3* (апикални и средњи сегмент), код 5,5% *Mtwo* инструмента (апикална трећина) и код 11,1% система *ProTaper Universal* (апикална и средња трећина). Корозија није уочена на новим инструментима *HyFlex* и *BioRaCe* сетова. Након инструментације, дезинфекције и стерилизације корозија је примећена код свих сетова система *K3* и *ProTaper Universal* и код свих *HyFlex* инструмената прве групе. Корозија је уочена код *HyFlex* система у другој групи код 16,7% инструмената (апикална и средња трећина), а у трећој групи код 83,3% инструмената у апикалном и 66,7% у средњем сегменту. Код *Mtwo* сета корозија је уочена код 16,7% инструмената у првој групи (апикална и средња трећина), у другој групи код 33,3% инструмената у апикалном делу и 50% у средњој трећини, док је у трећој групи корозија примећена код 16,7% инструмената у средњој трећини инструмената.

Закључак Ротирајући *Ni-Ti* инструменти *K3* и *ProTaper Universal* подложни су корозији у веома високом проценту. *Ni-Ti* системи са накнадном термичком обрадом радног дела (*HyFlex*) нешто су отпорнији на корозију, док код *Ni-Ti* система са електрополираном површином (*BioRaCe*) коризија није уочена.

Кључне речи: корозија; ротирајући *Ni-Ti* инструменти; скенирајућа електронска микроскопија (СЕМ)

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Soft tissue profile changes during treatment of patients with class II malocclusion

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SUMMARY

Introduction/Objective The class II malocclusion results in disbalanced facial harmony, primarily noticeable in the profile and the lower facial third. Aside from skeletal evaluation, orthodontic diagnosis and treatment planning should include facial soft tissue analysis. The aim of the study was to identify the soft tissue profile outcomes of orthodontic treatment of class II, division 1 malocclusion patients and to determine if these changes are related to different treatment protocols.

Methods The first group was the non-extraction group (25 patients) initially treated with the Herbst appliance, and the second group was four premolars extraction group (25 patients) treated with a multibracket appliance. The patients' cephalograms and pre- and post-treatment profile photographs were used.

Results The improvement in the non-extraction group was evident in the decrease of the nasomental angle, the angle representing the projection of the upper lip to the chin, as well as the upper lip angle. In the extraction group, the nasolabial angle showed a significant increase. Soft tissue variables showed significant differences between the groups: the total facial angle or facial convexity including the nose and the angle presenting the projection of the upper lip to the chin.

Conclusion The patients treated without extractions showed a significant improvement of the convex profile and favorable soft tissue changes in the lower third of the face.

Keywords: facial esthetics; class II malocclusion; facial convexity; profile changes; soft tissue profile

INTRODUCTION

The improvement of facial features is the patient's main aspiration when starting an orthodontic treatment, and thus of primary importance for clinicians. An attractive facial appearance affects social acceptance and psychological well-being, which has a profound effect on a person's self-esteem and social adjustment ability [1]. Soft tissue of the face, together with the underlying dentoskeletal tissues, determines the facial features of a person [2]. Orthodontists, maxillofacial, and plastic surgeons are expected to achieve not only functional, but also esthetic goals for their patients, both equally important [3].

Patients with class II, division 1 malocclusion have undesirable facial esthetics caused by increased overjet and convex profile. Previous studies showed that the convex profile is one of the least desirable features of the face [4]. Patients with class II, division 1 malocclusion are unsatisfied with their smile and facial look, especially in their teenage years, since they are often being perceived by peers as unattractive [5]. As self-esteem is strongly influenced by facial appearance, solving this problem is of primary importance in achieving esthetic treatment goals. Therefore, improvement of facial appearance in teenage patients could improve their quality of life through their most vulnerable years [4, 5].

Orthodontists should comprehensively understand the importance of developing an individualized treatment plan, adjusted to the patient's specific dental and skeletal problems, needs, and desires. Class II, division 1 malocclusion can be treated with functional or fixed functional appliances combined with the multibracket appliance, with or without extractions. Small skeletal discrepancies may only need multibracket appliance treatment for the correction of existing malocclusion and teeth alignment [6]. On the other hand, more severe skeletal discrepancies may require an orthognathic surgical treatment to modify the position and length of skeletal structures, to obtain better esthetic results [7]. Despite the numerous studies conducted on the consequences of extractions, it is still a question of debate among orthodontists. Some investigators reported flattening of the soft tissue profile after extraction treatment, while others claim no such effect [8–11].

Although cephalometric analysis is one of the most common parts of diagnosis and treatment planning among orthodontists, the validity of cephalometric measurements has been questioned [3]. Several authors proposed lateral photographs for the esthetic facial profile evaluation [12, 13, 14].

This study, therefore, aimed to identify the soft tissue profile outcomes of the orthodontic treatment of class II, division 1 malocclusion. A further aim was to determine if soft tissue

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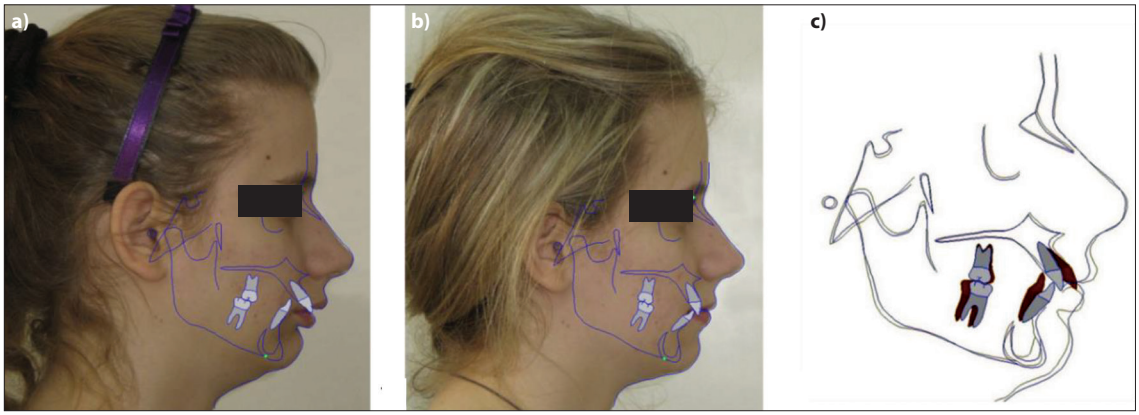

Figure 1. Non-extraction case; superimposition of the cephalometric drawing to the patient's profile: a) before treatment, b) after treatment, c) superimposition of the cephalometric drawings before (brown) and after (gray) treatment with visible changes of the soft tissue profile

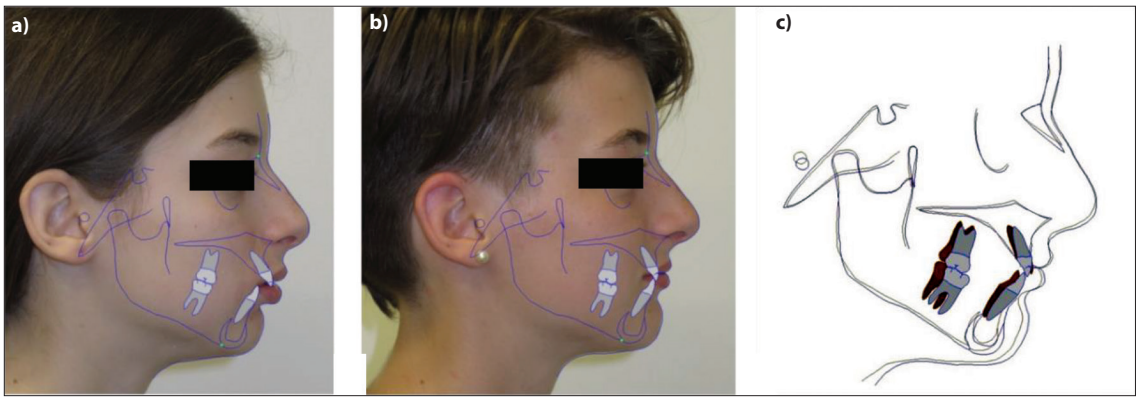

Figure 2. Extraction case; superimposition of the cephalometric drawings to the patient's profile: a) before treatment, b) after treatment, c) superimposition of the cephalometric drawings before (brown) and after (gray) treatment with visible changes of the soft tissue profile

profile changes are connected with different treatment protocols. The hypothesis underlying this investigation is that orthodontic treatment of class II, division 1 malocclusion changes the soft tissue profile, and moreover, that those changes depend on different treatment protocols.

METHODS

The sample for this study consisted of 50 Caucasian patients (22 males; 28 females), with a mean age of 15.8 ± 1.4 years, treated at the Clinic for Orthodontics between 2014 and 2018. This retrospective study was approved by the Ethics Committee of the University (Protocol number 46/15) and informed consent was obtained from the patients' parents/guardians. All subjects were selected according to the following inclusion criteria (pretreatment): full permanent dentition (excluded third molars), class II molar occlusion, division 1 (with characteristic convex profile, deep mentolabial sulcus, retruded chin, and reverted lower lip), overjet of more than 7 mm, moderate irregularity of anterior crowding according to the Little's Irregularity Index [15], and post-pubertal stage of skeletal maturity (CS6) [16]. Exclusion criteria encompassed patients with a systemic disease, craniofacial anomalies, patients with vertical growth pattern, impacted teeth, and poorly visible cephalograms. After successful orthodontic treatment, all the patients achieved class I occlusion, and received a vacuum-formed retainer on the same day the appliance was removed.

The subjects were divided into two study groups.

1. The first group consisted of 25 patients treated with the combined two-phase therapy. The first phase included the cast splint Herbst appliance type I for an average period of seven months. Afterwards, each patient underwent a standardized non-extractive treatment protocol. The treatment duration was on average 20 months. The skeletal and dentoalveolar changes in this group of patients are visible with superimposition in Figure 1.
2. The second group consisted of 25 patients treated with four premolars extractive treatment protocol, followed by class II intermaxillary elastic. The treatment duration was on average 19 months. The skeletal and dentoalveolar changes in this group of patients are visible with superimposition in Figure 2.

The patients' pre-treatment and post-treatment profile photographs were used [4]. The right-side profile photographs were taken in the standing position, in central occlusion. The subjects' Frankfort horizontal plane was kept as parallel to the floor as possible during the taking of the photographs. Before every recording, the operator ensured

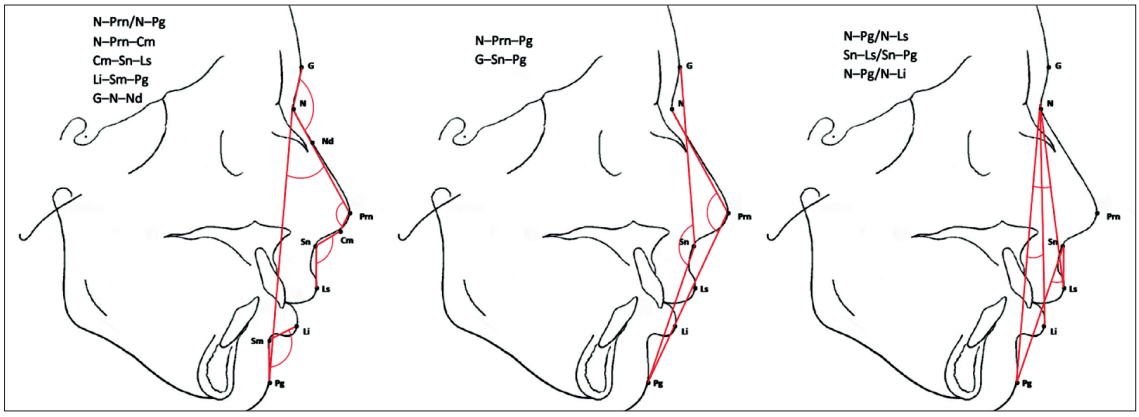

Figure 3. Soft tissue profile landmarks and angular parameters

that the subject's forehead, neck, and ear were clearly visible [6]. The photographs were then printed, and the soft tissue landmarks were identified. The landmarks used in this investigation were the following: glabella (G), nasion (N), nasal dorsum (Nd), pronasale (Prn), columella (Cm), subnasale (Sn), labiale superior (Ls), labiale inferior (Li), supramentale (Sm), pogonion (Pg) [10]. Afterwards, the angular parameters were determined on each photo and used in evaluating soft tissue profile changes. The photogrammetric analysis was based on comparing changes in parameter values before and after the treatment, regardless of the average values for these parameters. These measurements are illustrated in Figure 3. Table 1 provides the definition of angular measurements used in the study. The whole sample was measured by one researcher (JM), repeated once again after two months. Also, all measurements were performed by the second researcher (NN). This was done to evaluate intra- and inter-observer reliability. Radiographic analyses rely on skeletal and dental measurement, whereas soft tissue facial measurements are less emphasized. Therefore, for providing a complete overview of changes during and after orthodontic treatment, photogrammetric analysis has been used.

Table 1. Definitions of angular measurements

Angular measurement	Definition
N–Prn–Pg (°)	Nasomental angle
N–Prn–Cm (°)	Nose tip angle
Cm–Sn–Ls (°)	Nasolabial angle
Li–Sm–Pg (°)	Mentolabial angle
G–N–Nd (°)	Nasofrontal angle
N–Prn–Pg (°)	Total facial angle or facial convexity including the nose
G–Sn–Pg (°)	Facial angle or angle of facial convexity excluding the nose
N–Pg–Ls (°)	Projection of the upper lip to chin
Sn–Ls–Pg (°)	Upper lip angle
N–Pg–Li (°)	Projection of the lower lip to chin

Statistical analysis

The collected data were subjected to statistical analysis using SPSS Statistics for Windows, Version 17.0 (SPSS Inc., Chicago, IL, USA). The Kolmogorov–Smirnov test was applied to test whether the data distribution fits the probability density function, also known as Gaussian function or the bell curve. Subsequently, had the test not rejected the assumed normal distribution, the parametric tests would have been used. Paired-sample t-test was used for intragroup comparisons. For testing the differences in all parameter values between groups, the two-sample t-test was used. In all analyses, the significance level was set at 0.05. The Kolmogorov–Smirnov test showed normality of distribution of the obtained data in both groups. In order to evaluate intra- and inter-observer reliability, intra-class correlation coefficient (ICC) was calculated.

RESULTS

Intra- and inter-observer agreement was found to be excellent (ICC = 0.983 for intra-observer, ICC = 0.974 inter-observer agreement). Angular measurements in the first group treated with the Herbst appliance and without extractions are demonstrated in Table 2. Several statistically significant profile changes could be observed. The nasomental angle (N–Prn–Pg) decreased significantly ($\bar{x}$ = -1 ± 1.0; p = 0.02); furthermore, the angle representing projection of the upper lip to the chin (N–Pg–Ls)

Table 2. Descriptive statistics of the soft tissue profile variables in Herbst/non-extraction group

Herbst/non-extraction treatment protocol				
Variable	Before	After	Difference	p-value change over time
	Mean ± SD	Mean ± SD	Mean ± SD	
N–Prn–Pg	35.93 ± 2.69	34.93 ± 2.81	-1 ± 1.01	0.02*
N–Prn–Cm	80.37 ± 5.61	78.93 ± 6.1	-1.44 ± 0.19	0.41
Cm–Sn–Ls	107 ± 6.64	108.33 ± 9.88	1.33 ± 2.81	0.01*
Li–Sm–Pg	107.06 ± 15.65	119.74 ± 20.16	12.68 ± 12.57	0.02*
G–N–Nd	141.54 ± 7.38	140.43 ± 6.84	-1.11 ± 0.19	0.08
N–Prn–Pg	121.8 ± 3.91	124.17 ± 7.3	2.37 ± 0.95	0.18
G–Sn–Pg	159.56 ± 5.55	163.41 ± 7.07	3.85 ± 4.43	0.05
N–Pg–Ls	10.46 ± 1.46	8.35 ± 2.54	-2.11 ± 2.04	0.01*
Sn–Ls–Pg	21.33 ± 5.17	16.39 ± 5.77	-4.94 ± 10.1	0.01*
N–Pg–Li	4.15 ± 2.33	6.59 ± 10.75	2.44 ± 1.3	0.29

*Statistically significant differences at p < 0.05

Table 3. Descriptive statistics of the soft tissue profile variables in the extraction group

Extraction treatment protocol				
Variable	Before	After	Difference	p-value change over time
	Mean ± SD	Mean ± SD	Mean ± SD	
N–Prn–Pg	35.68 ± 3.01	36.3 ± 2.94	0.62 ± 3.73	0.21
N–Prn–Cm	78.62 ± 6.5	79.92 ± 7.97	1.3 ± 3.44	0.56
Cm–Sn–Ls	103.26 ± 7.39	107.22 ± 10.16	3.96 ± 4.43	0.03*
Li–Sm–Pg	112.18 ± 24.18	119.92 ± 15.86	7.74 ± 2.89	0.33
G–N–Nd	138.04 ± 6.79	136.28 ± 9.18	-1.76 ± 2.04	0.29
N–Prn–Pg	123.96 ± 6.2	124.76 ± 7.58	0.8 ± 2.1	0.29
G–Sn–Pg	162.88 ± 6.45	163.3 ± 3.92	0.42 ± 1.61	0.52
N–Pg–Ls	9.94 ± 3.32	8.96 ± 1.88	-0.98 ± 1.01	0.07
Sn–Ls–Pg	20.24 ± 4.65	18.38 ± 3.46	-1.86 ± 1.72	0.06
N–Pg–Li	4.62 ± 1.71	5.36 ± 2.07	0.74 ± 1.47	0.08

*Statistically significant differences at $p < 0.05$

showed a significant decrease ($\bar{x}$ = -2.11 ± 2.04; p = 0.01). The upper lip angle showed a significantly large decrease ($\bar{x}$ = -4.94 ± 10.1; p = 0.01) over time in the non-extraction group of patients. On the other hand, the nasolabial angle (Cm–Sn–Ls) increased significantly in this group of patients ($\bar{x}$ = +1.33 ± 2.81; p = 0.01). Moreover, the mentolabial angle (Li–Sm–Pg) showed a significantly large increase ($\bar{x}$ = +12.68 ± 12.57; p = 0.02).

Changes in soft tissue profile variables in the extraction group of patients are presented in Table 3. This group showed a greater significant increase in nasolabial angle (Cm–Sn–Ls) ($\bar{x}$ = +3.96 ± 4.43; p = 0.03). However, no significant differences were detected in other soft tissue variables.

Table 4 describes intergroup comparisons of the soft tissue variables. Only two soft tissue variables showed significant differences between two groups: total facial angle or facial convexity including the nose (N–Prn–Pg) increased significantly ($\bar{x}$ = -2.09 ± 1.1; p = 0.04). As for the angle presenting projection of the upper lip to chin (N–Pg–Ls), its value showed a significant decrease ($\bar{x}$ = +0.65 ± 3.73; p = 0.01).

Table 4. Descriptive statistics of the soft tissue profile variables comparing both treatment groups

Herbst/non-extraction versus extraction treatment protocol				
Variable	Before	After	Difference	p-value change over time
	ΔMean ± SD	ΔMean ± SD	Mean ± SD	
N–Prn–Pg	-1.04 ± 3.08	1.04 ± 2.25	2.08 ± 2.92	0.33
N–Prn–Cm	-1.08 ± 5.89	1.23 ± 4.3	2.31 ± 2.81	0.71
Cm–Sn–Ls	-3.52 ± 7.04	-1.45 ± 8.26	2.07 ± 3.71	0.34
Li–Sm–Pg	-8.39 ± 17.35	-11.95 ± 21.54	-3.56 ± 7.32	0.61
G–N–Nd	0.96 ± 6.69	1.34 ± 4.45	0.38 ± 1.46	0.51
N–Prn–Pg	-0.52 ± 5.18	-2.61 ± 5.84	-2.09 ± 1.1	0.04*
G–Sn–Pg	-0.78 ± 5.69	-3.89 ± 4.61	-3.11 ± 2.29	0.05
N–Pg–Ls	1.46 ± 2.2	2.11 ± 2.25	0.65 ± 3.73	0.01*
Sn–Ls–Pg	1.78 ± 4.77	5.09 ± 5	3.31 ± 0.24	0.11
N–Pg–Li	-0.72 ± 1.14	-2.66 ± 10.39	-1.94 ± 2.04	0.15

*Statistically significant differences at $p < 0.05$

DISCUSSION

The success of orthodontic treatment is closely related to facial appearance improvement. A balanced soft tissue profile is an important factor to achieve during orthodontic treatment [2]. This type of malocclusion is frequently reported as the irregularity that alters facial proportions, symmetry, and balance. Thus, correction of facial features will lead not only to facial profile correction, but also to long-term psychosocial well-being of patients [5]. Orthodontic treatment modifies the position, length, and relation between skeletal and dentoalveolar structures, and subsequently, facial expressions and esthetics are modified and enhanced (these effects are shown in Figures 1 and 2). Facial harmony can often be described as dependent on morphological relations, and proportions between three facial structures – nose, lips, and chin [17]. The facial profile consists of five facial prominences – the forehead, nose, lips, chin, and submental-cervical region.

The nasomental angle (N–Prn–Pg), or nasal prominence angle, is in the 20–30° range in class I patients [18], whereas the value is increased in class II patients. In this study, the nasomental angle showed a statistically significant decrease in the non-extraction group of patients, although it was not clinically relevant (-1°). This favorable outcome could have occurred as a result of anterior movement of the soft tissue point pogonion (Pg). This movement promoted positive changes on the soft tissue profile and was reported also by do Rego et al. [19].

Significant improvements in facial profile were recorded in the first group of patients (treated with the Herbst appliance without extractions). In particular, the nasolabial (Cm–Sn–Ls) and mentolabial (Li–Sm–Pg) angles showed significant increase after the treatment. The nasolabial angle (Cm–Sn–Ls) can be changed with both orthodontic and surgical treatment. It plays an important role in a facial profile appearance, and in some cases, it can be used as a guideline for the extraction decision. According to a study by Bergman [20], regardless of the type of treatment needed for the patients (whether it is surgical or orthodontic correction), this angle should be 102 ± 8°. After orthodontic treatment, this angle increased significantly, since the upper lip moved backwards and downwards, and its prominence has been decreased, mostly due to retrusion of the upper incisors. The nasolabial angle also showed a significant increase in the second group of patients, treated with premolar extractions. The increase of this angle was also reported by Iared et al. [21], who confirmed that a backward movement of the upper lip occurred because of orthodontic treatment with extraction of premolars.

The mentolabial angle (Li–Sm–Pg) also showed great variability. A more pronounced mentolabial angle can be seen in class II and vertical maxillary deficiency cases. In both groups of patients, this angle has been increased after the treatment, as a result of achieving a balanced dentoalveolar relation, due to upper incisors retrusion [22].

Significant improvements in facial profile concerning the chin and the upper lip balance were recorded in the

first group of patients. In particular, the angle determining the projection of the upper lip to the chin (N–Pg–Ls), as well as the upper lip angle (Sn–Ls–Pg), showed a significant reduction. This result is related to a less pronounced upper lip. The value of these angles showed a statistical significance in the non-extraction group, given the fact that point Pg moved forward, while point Ls moved backward, which is an expected result of treatment with the Herbst appliance [23]. Moreover, this is also a result of decreasing of the upper lip prominence, as a consequence of upper incisors retrusion, in a ratio of 1:3. Many authors confirmed the relation between the upper lip position and the upper incisors retrusion, in the ratio of 1:3 [24, 25]. Furthermore, esthetical modification depends on the upper and lower incisors position, as well as on the change of the position and development of the lower jaw [26].

The angle N–Pg–Ls showed a statistically significant difference comparing the two groups of patients. The lower lip is the adjacent esthetic subunit to the chin, and its features play an important role in determining facial esthetics in the lower third of the face [27]. As such, the prominence of the lower lip may influence the perception of chin prominence and thus the overall management plan in terms of camouflage *vs.* orthognathic surgery and extraction *vs.* non-extraction decisions [21, 28, 29].

Therefore, a change in the lower lip position and consequent change in the lip/chin relation influences facial esthetics, as these entities determine the profile type. As mentioned, the facial profile in patients with this type of malocclusion is altered and considered unattractive before treatment. As a result of improvement of these proportions and of the profile, the esthetic perception is changed from unattractive to attractive, which is one of the main reasons why patients seek orthodontic treatment [30].

The profile angles are used to assess convexity or concavity of the facial profile. The angle of facial convexity excluding the nose or facial angle (G–Sn–Pg) is supposed to be in the range of 165–175° [20]. This angle is decreased in class II and increased in class III. In our sample, all patients had a decreased value of this angle before treatment. After treatment, the facial angle was increased in both groups of patients – however, not significantly. The favorable outcome, not statistically significant, yet esthetically relevant, was the profile strengthening caused by reduction of facial convexity, which had been one of the main reasons of the patients' dissatisfaction.

CONCLUSION

Photogrammetric analysis is a simple and valid method to assess orthodontic treatment effects on the soft tissue profile. This study confirms previous reports on the improvement of the convex profile, and favorable soft tissue changes at the lower third of the face, after the orthodontic treatment of class II, division 1 malocclusions. Patients treated with the Herbst appliance without extractions presented better results in facial profile parameters than the group of patients treated with premolar extractions. This result is important for orthodontists treating patients with this type of malocclusion, as facial esthetics improvement is a key factor for determining the treatment protocol and achieving patients' satisfaction.

ACKNOWLEDGEMENTS

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Conflict of interest: None declared.

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Мекоткивне промене профила током терапије пацијената са II класом малоклузије

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САЖЕТАК

Увод/Циљ Малоклузије II класе доводе до нарушавања хармоније лица и погоршаног изгледа профила и доње трећине лица. Ортодонтска дијагноза и план терапије треба да укључе и анализу мекоткивног профила.

Циљ рада је био да се одреде промене меких ткива профила пацијената са малоклузијом II класе, 1. одељења, као и да се утврди да ли ове промене зависе од начина лечења.

Методе Прва група од 25 болесника лечена је Хербстовим апаратом без вађења зуба. Друга група од 25 болесника лечена је фиксним апаратом са вађењем четири премолара. Мерења су вршена на профилним снимцима главе и фотографијама пре и после терапије.

Резултати Побољшан је изглед меких ткива профила прве групе болесника у виду смањења назоменталног угла, као и углова који говоре о положају горње усне. У другој групи пацијената значајно је повећан назолабијални угао. Статистички значајна разлика поређењем обе групе болесника нађена је за угао конвекситета лица укључујући нос и угао горње усне.

Закључак Разлика у изгледу мекоткивног профила постојала је у групи пацијената лечених без вађења зуба у виду смањења конвекситета профила и промена у доњој трећини лица.

Кључне речи: естетика лица; малоклузија II класе; конвексно лице; мекоткивни профил

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Comparison of bromazepam and ibuprofen influence on tooth pulp-evoked potentials in humans

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SUMMARY
Introduction/Objective Somatosensory evoked potentials are a neurophysiological tool for testing the effects of drugs in humans and animals.
The aim of this study was to estimate the way that bromazepam and ibuprofen had on tooth pulp-evoked potentials (TPEPs) after non-painful stimuli, as well as to detect possible differences in this activity.
Methods Sixty young healthy subjects were included in the study. They were arranged into three groups: ibuprofen, bromazepam, and placebo. To record TPEPs response, dental pulp were electrically stimulated through intact enamel with non-painful stimuli. For stimulation and registration we used Xltek Protektor 32 system, software EPWorks, version 5.0 (Natus Medical Incorporated, Oakville, ON, Canada). The experiment consisted of two testing sessions. Five recordings were performed in each session. The first test session was before, and the second was 45 minutes after administration of a single dose of the ibuprofen (400 mg), bromazepam (1.5 mg) or placebo.
Results The results of the present study exhibit that both ibuprofen and bromazepam significantly increased all the latencies; ibuprofen decreased amplitudes of all the waves except the first one ($p < 0.05$), and bromazepam decreased amplitudes of all the waves except the first one ($p < 0.05$); placebo did not modified TPEPs waves ($p > 0.05$). Additionally, there were no significant differences in influence on TPEPs between bromazepam and ibuprofen ($p > 0.05$).
Conclusion Our study showed that both bromazepam and ibuprofen had the same influence on TPEPs after non-painful stimuli. That indicates that anxiolytic dose of bromazepam affects neurotransmission in the same manner as non-opioid analgesics ibuprofen.
Keywords: somatosensory evoked potentials; non-painful stimulus; analgesic; anxiolytic

INTRODUCTION

Somatosensory evoked potentials (SEPs) represent electrical activity changes of the nervous system caused by a somatosensory stimulus. Their waves reflect neural activations along somatosensory pathway with different sensory information processing at subcortical and cortical levels. Contrary to spontaneous electrical activity, evoked response occurs at a specific time after stimulation in a particular cortical region. Although electroencephalography equipment is used to record evoked potentials, only signals from electrodes placed above the region of interest are observed [1]. Therefore, the region of interest for tooth pulp-evoked potentials (TPEPs) is vertex because TPEPs show a bilateral symmetrical scalp distribution with a maximum at the vertex [2].

Since the middle of the previous century, SEPs have been the standard assessment tool for nociception, as well for testing and quantifying the effects of analgesics in humans and animals [3–6]. Various studies have shown specific effects on SEPs characteristics in an experimental pain model after analgesic application [2, 3, 6, 7]. Furthermore, it has also been observed that SEPs were useful neurophysiological tool for assessing the emotional aspects of pain. Examining the effect of sedatives on pain-related SEP components, it was revealed that they also change SEPs characteristics by modifying emotional responses to pain [8–11].

It is widely accepted that ibuprofen, a non-steroidal anti-inflammatory drug, in contrast to opioid analgesics, does not show sedative non-specific side effects [12, 13, 14], as well as that bromazepam, acting via gamma aminobutyric acid (GABA) type A receptors, reduces anxiety and consequently reduces the emotional response to pain, but provide no analgesia [15, 16, 17]. However, recent studies suggest that GABA agonists show anti-nociceptive effects, too [13, 18, 19, 20].

So far known to us, no studies have compared the effect of both anxiolytic and analgesic drugs on TPEPs in humans. Therefore, the aim of this study was to analyze the influence of bromazepam and ibuprofen on TPEPs in healthy subjects. Since SEPs are objective method for assessing neurotransmission, we also included a placebo in the study, assuming it would not cause change of TPEPs. Considering that emotional and cognitive aspect of pain could affect perception and consequently SEPs [10, 21], we decided to use non-painful stimulus.

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METHODS

Ethical approvals

The study was conducted at the Clinic for Oral and Maxillofacial Surgery at the Institute of Faculty of Stomatology, Pančevo, between October 2018 and March 2019. The study was approved by the Ethics Committee of the Institute (1240/1-20-2015) and was in accordance with the Principle of Good Clinical Practice and the Declaration of Helsinki [22]. All subjects gave their written informed consent after a full explanation of the study, focusing on the purpose of the study and the precise procedures.

Subjects

Sixty young healthy male and female participants were included in the study. They were randomly arranged into three equal groups of 20 subjects each. The first group received ibuprofen, the second group received bromazepam, and the third group received placebo.

Regardless of using any drug, exclusion criteria were avital central incisors of the upper jaw, as well as fillings and prosthetics on the same teeth. In addition, exclusion criteria were oral mucosal changes, and fractures, trauma or surgery in the maxillofacial region. All subjects were examined under the same conditions, between 8 a.m. and 2 p.m.

Drugs

Ibuprofen (Brufen®, Galenika AD, Belgrade, Serbia), film coated tablet 400 mg, was used as an analgesic. Bromazepam (Bromazepam HF®, Hemofarm AD, Vršac, Serbia), tablet 1.5 mg, was used as an anxiolytic. As placebo was used Betavitevit Folna 400 (folic acid, 400 μg, and vitamin B12, 3 μg, Esensa d.o.o., Belgrade, Serbia), tablet. All tablets were in same bottles. Subjects were told they were receiving one of the investigated tablets.

Evoked potentials registration and analysis

Before starting the TPEPs registration, stimulus intensity for dental pulp stimulation was determined for each subject based on two criteria: subjective experience of the stimulus intensity and sufficient intensity to evoke characteristic SEPs curve. The stimulus intensity was rated by a 5-level ordinal category scale (1 – no sensation, 2 – barely perceptible, 3 – tingling, 4 – mild pain, 5 – moderate pain). The stimulation of central maxillary incisor began with an intensity of 0.2 mA and increased by 0.2 mA until the subject reported a tingling sensation, level 3 on the scale. The average pulse intensity for dental pulp stimulation was 1 mA.

The cortical somatosensory-evoked responses were recorded from vertex, with reference to inion, after pulp of central maxillary incisor were electrically stimulated through intact enamel (for more information of stimulation parameters and the recording technique see our previous study [23]).

The experiment consisted of two testing sessions; five recordings were performed in each session. The first was before, and the second test session was 45 minutes after the single dose of the drug administered.

Obtained average recordings were numerically, graphically and statistically processed. The peak latency and the peak amplitude of all components were measured. Values of latencies and amplitudes after drug administration were compared with the same values before drugs, as well as with previously standardized values of latencies and amplitudes. Finally, SEP records after administration of ibuprofen, bromazepam and placebo were compared with each other.

Statistical analysis

Data were statistically analyzed with SAS (The SAS System for Windows, release 9.3. Cary, NC, USA) [24]. To determine statistical significance, we used the Wilcoxon signed rank sum test and the Kruskal–Wallis test. Values of $p < 0.05$ were considered significant. Results are expressed as mean ± the standard error of the mean.

RESULTS

TPEPs were successfully recorded in 56 subjects (31 male and 25 female participants mean age 22.5 ± 0.7). Four subjects from the bromazepam group were rejected because the records after drug administration were illegible.

At the beginning of the research, in a pilot study, we have standardized values of latencies (LN1 55 ms, LP1 100 ms, LN2 145 ms, LP2 195 ms) and amplitudes (AN1 7.5 μV, AP1 8.0 μV, AN2 9.5 μV, AP2 8.5 μV), which represented the control group. In this pilot study, no significant differences in TPEPs between the sexes were found (data not shown).

The effect of ibuprofen on tooth pulp-evoked potentials

The results obtained 45 minutes after ibuprofen administration showed significantly longer all latencies compared to the same group pre-drug and to the control group ($p < 0.05$). Furthermore, the amplitudes of the first three waves were significantly decreased post-drug versus pre-drug and control group ($p < 0.05$). The detailed data are shown in Figure 1 and Table 1.

The effect of bromazepam on tooth pulp-evoked potentials

All latencies 45 minutes after bromazepam administration were significantly longer compared to the same group pre-drug and to the control group ($p < 0.05$). Additionally, the amplitudes of the last three waves were significantly decreased post-drug versus pre-drug and control group ($p < 0.05$). The detailed data are shown in Figure 2 and Table 2.

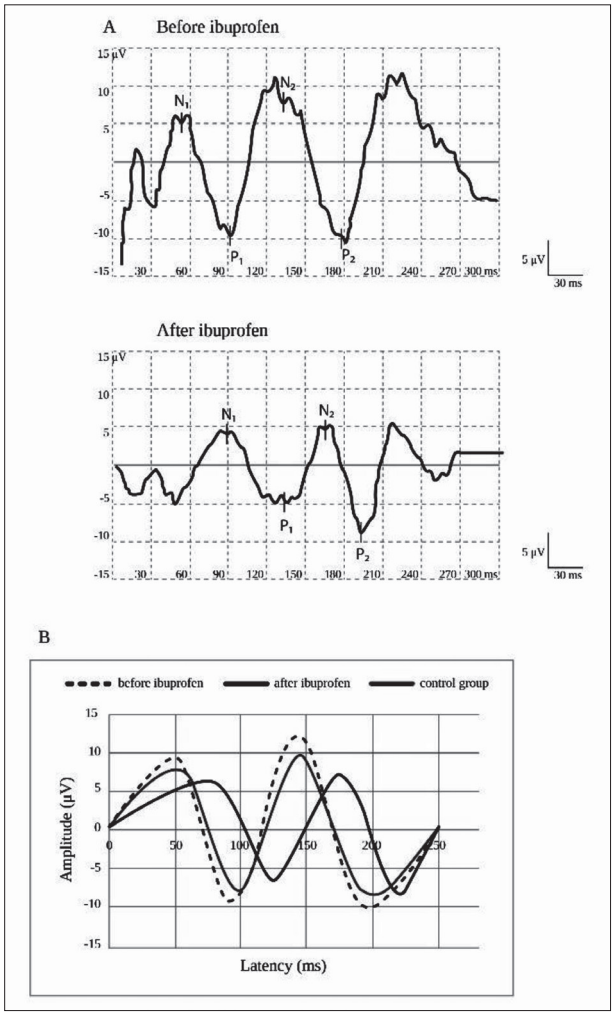

Figure 1. Influence of ibuprofen on tooth pulp-evoked potentials

A) Original waveforms recording from vertex after toot pulp stimulaton before and after ibuprofen administration; B) the pattern of the mean values of evoked potentials before and after ibuprofen administration and control group; all latences were significantly longer ($p < 0.05$) after ibuprofen compared to the same group pre-drug and control group; the amplitudes of the first three waves significantly decreased ($p < 0.05$) after ibuprofen compared to the same group pre-drug and control group

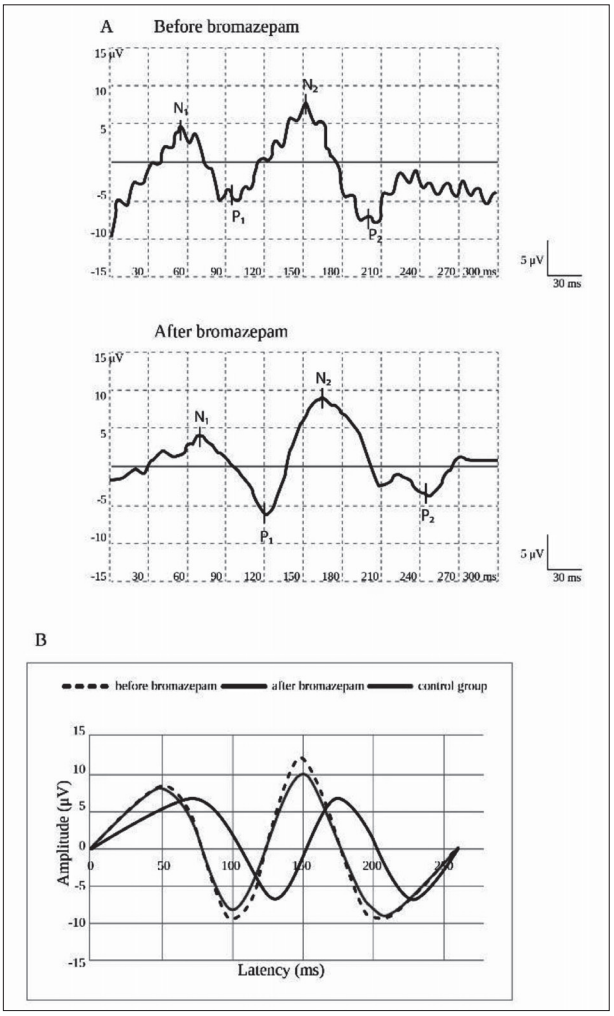

Figure 2. Influence of bromazepam on tooth pulp-evoked potentials

A) Original waveforms recording from vertex after toot pulp stimulaton before and after bromazepam administration; B) the pattern of the mean values of evoked potentials before and after ibuprofen administration and control group; all latences were significantly longer ($p < 0.05$) after bromazepam compared to the same group pre-drug and control group. The amplitudes of the last three waves significantly decreased ($p < 0.05$) after bromazepam compared to the same group pre-drug and control group

Table 1. Comparison of tooth pulp- tooth pulp-evoked potentials parameters before and after ibuprofen administration and control group

Evoked potentials parameters	Pre-drug	Post-drug	Pre-drug *vs.* post-drug p*	Post-drug *vs.* controls p**
Latency (ms)				
N1	52.9 ± 2.2	80.6 ± 4.6	**< 0.0001**	**< 0.0001**
P1	94.5 ± 3.1	127.1 ± 4.5	**< 0.0001**	**< 0.0001**
N2	142.8 ± 3.5	175.7 ± 4.8	**< 0.0001**	**< 0.0001**
P2	191.8 ± 5.9	218.7 ± 6.5	**0.0037**	**< 0.0001**
Amplitude (μV)				
N1	8.9 ± 2.8	5.9 ± 0.8	**0.0153**	**0.0021**
P1	10.4 ± 3.5	6.7 ± 0.2	**0.0056**	**0.0078**
N2	12.2 ± 3.8	7.1 ± 0.7	**< 0.0001**	**0.0078**
P2	9.9 ± 5.3	8.2 ± 0.8	0.0826	0.1502

Pre- and post-drug values are expressed as mean ± standard error;
*Wilcoxon signed rank sum test;
**Wilcoxon–Mann–Whitney test

Table 2. Comparison of tooth pulp-evoked potentials parameters before and after bromazepam administration and control group

Evoked potentials parameters	Pre-drug	Post-drug	Pre-drug *vs.* post-drug p*	Post-drug *vs.* controls p**
Latency (ms)				
N1	57.9 ± 1.1	78.5 ± 1.7	**< 0.0001**	**< 0.0001**
P1	100.6 ± 1.9	125.8 ± 1.3	**< 0.0001**	**< 0.0001**
N2	144.1 ± 2.7	171.1 ± 2.1	**< 0.0001**	**< 0.0001**
P2	190.5 ± 3.1	216.8 ± 2.8	**< 0.0001**	**< 0.0001**
Amplitude (μV)				
N1	7.9 ± 0.7	6.3 ± 0.9	0.4615	0.4839
P1	9.5 ± 0.5	6.5 ± 0.6	**0.0087**	**0.0057**
N2	12.0 ± 1.5	6.8 ± 0.4	**0.0087**	**0.0059**
P2	8.9 ± 0.3	6.7 ± 0.3	**0.0087**	**0.0112**

Pre- and post-drug values are expressed as mean ± standard error;
*Wilcoxon signed rank sum test;
**Wilcoxon–Mann–Whitney test

The effect of placebo on tooth pulp-evoked potentials

After placebo administration, there were no significant differences in the TPEPs components either within the same group pre-drug, or in relation to the control group ($p > 0.05$). The detailed data are shown in Figure 3 and Table 3.

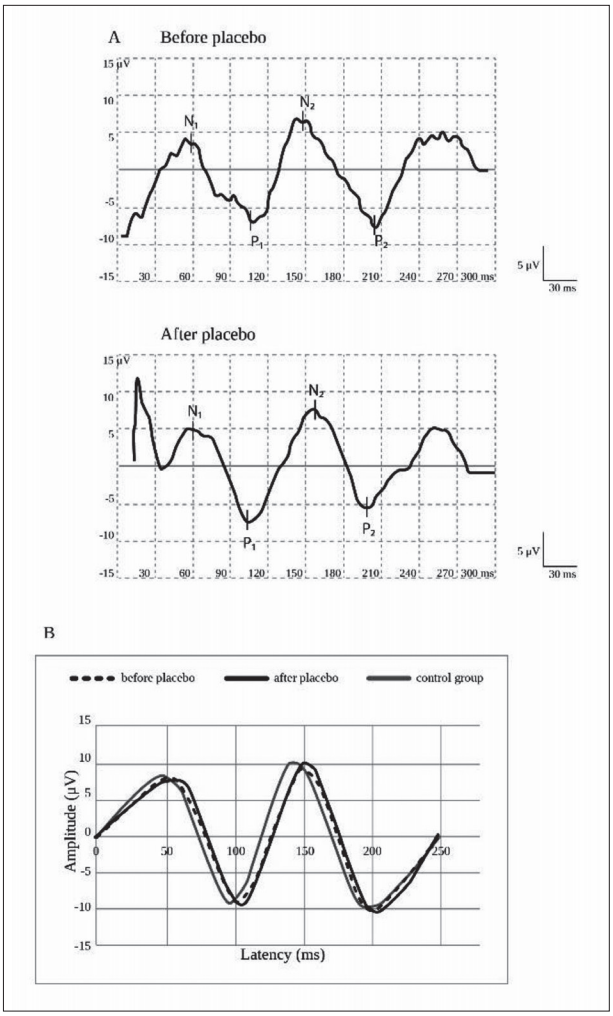

Figure 3. Influence of placebo on tooth pulp-evoked potentials

A) Original waveforms recording from vertex after toot pulp stimulaton before and after placebo administration; B) the pattern of the mean values of evoked potentials before and after placebo administration and control group; there were no significant differences ($p > 0.05$) in the all latencies and amplitudes either within the same group pre-drug or in relation to the control group

Comparison between influence of ibuprofen, bromazepam and placebo on tooth pulp-evoked potentials

Comparing the obtained mean values of wave latencies and amplitudes after ibuprofen administration and the mean values of same parameters after bromazepam administration, no statistically significant differences were found ($p > 0.05$). Contrary, all latencies of both, ibuprofen and bromazepam, were significantly longer than latencies after placebo, while the first three values of amplitudes after ibuprofen, and the last three values of amplitudes after bromazepam were significantly decreased comparing to the same parameters after placebo. The detailed data are shown in Figure 4 and Table 4.

Having in mind that all groups consisted of different subjects, we compared TPEP components between controls

Table 3. Comparison of tooth pulp-evoked potentials parameters before and after placebo administration and control group

Evoked potentials parameters	Pre-drug	Post-drug	Pre-drug *vs.* post-drug p*	Post-drug *vs.* controls p**
Latency (ms)				
N1	58.5 ± 2.1	61.9 ± 1.9	0.1272	0.8858
P1	105.4 ± 2.8	107.1 ± 2.6	0.5879	0.2017
N2	152.9 ± 3.8	154.2 ± 3.6	0.7869	0.0545
P2	199.7 ± 4.7	201.2 ± 4.5	0.7737	0.1078
Amplitude (μV)				
N1	6.8 ± 0.4	7.1 ± 0.5	0.6355	0.3469
P1	7.9 ± 0.5	8.2 ± 0.7	1.0000	0.9700
N2	8.5 ± 0.6	9.5 ± 0.8	0.2439	0.9400
P2	8.9 ± 0.5	9.1 ± 0.6	0.2163	0.1879

Pre- and post-drug values are expressed as mean ± standard error;
*Wilcoxon signed rank sum test;
**Wilcoxon–Mann–Whitney test

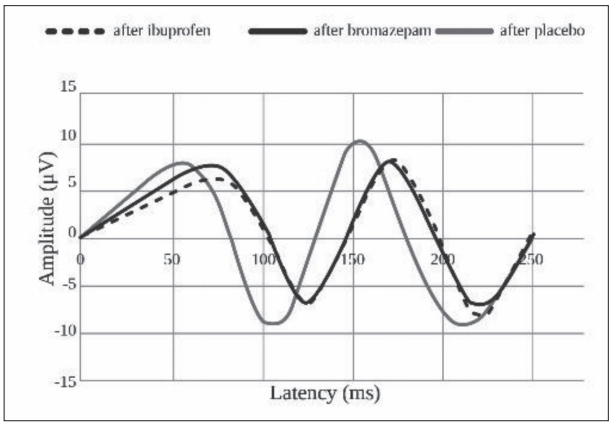

Figure 4. The pattern of the mean values of evoked potentials after ibuprofen, bromazepam and placebo

There were no significant differences ($p > 0.05$) in the all latencies and amplitudes between groups after ibuprofen and after bromazepam; the amplitudes were significantly less comparing to amplitudes after placebo ($p < 0.05$)

Table 4. Comparison of tooth pulp-evoked potentials parameters after drug administration between ibuprofen, bromazepam and placebo groups

Evoked potentials parameters	ibuprofen *vs.* bromazepam p	ibuprofen *vs.* placebo p	bromazepam *vs.* placebo p
Latency (ms)			
N1	0.6327	**< 0.0001**	**< 0.0001**
P1	0.8986	**0.0002**	**< 0.0001**
N2	0.3897	**0.0005**	**0.0006**
P2	0.5664	**0.0128**	**0.0075**
Amplitude (μV)			
N1	0.2141	**0.0024**	0.2141
P1	0.1810	**0.0081**	**0.0018**
N2	0.3724	**0.0072**	**0.0024**
P2	0.5664	0.5664	**0.0014**

Pre- and post-drug values are expressed as mean ± standard error;
Wilcoxon–Mann–Whitney test

and each group before drug administration, as well as between all groups before drug administration. Analysis showed no significant differences in all comparisons ($p > 0.05$) (data not shown). Therefore, post-drug results could be compared between groups.

DISCUSSION

In this study TPEPs modulation by analgesic and anxiolytic was studied. TPEPs are the most appropriate method for assessing orofacial pain, because any supra-threshold stimulus that affects the tooth-pulp is perceived as pain [2, 9, 25]. Each of the four waves is characterized by two components: latency and amplitude. An upward deflection of the TPEPs waveform was defined as N (negative) and downward deflection as P (positive). The latency reflects rate of neurotransmission, and the amplitude stimulus intensity [7, 26]. Amplitudes with peak occurring at a mean latency less than 100 ms (exogenous SEP components) were proportional to stimulus intensity, while amplitudes with peak occurring at a mean latency greater than 100 ms (endogenous SEP components) were proportional to the intensity of perception [26]. Therefore, early waveform components manifest the energy transmission at the first-order synapses in the pons and along trigeminal lemniscus, and the late components reflect the brain processes during stimuli perception at thalamus-cortical and thalamus-limbic levels [7, 8].

The results of the present study, that ibuprofen at a dose of 400 mg significantly increases all latencies and decreases amplitudes of first three waves, are in accordance with the previous studies which examined the influence of different doses of analgesics on SEPs [2, 3, 6, 7]. Moreover, our findings indicate that ibuprofen, as a cyclooxygenase inhibitor that affects transmission at the first-order synapses in the pain pathway [14, 20], slows down neurotransmission along the entire pain pathway and reduces the stimulus intensity perception at the level of the pons and trigeminal lemniscus, despite non-painful stimuli.

The dose-dependent effects of benzodiazepines range from anxiolytic and sedative to loss of consciousness [13, 15]. It is well-known that sedative doses of benzodiazepine, as well as opioid analgesics, affect the emotional aspect of pain, in contrast to non-opioid analgesics which affect the sensory aspect of pain [9]. Gonzalez-Liencres et al. [27] reported that endogenous evoked potentials are associated with attention and stimulus evaluation. Since their components correlate with state of the subject, attention level and meaning of the stimulus [10, 21], they can be affected by centrally acting drugs [10, 20]. Many previous studies showed that sedative drugs modify late SEP waves. In fact, they cause a dose related significant increase in latencies and decrease in amplitudes [8–11]. The same modifications of these SEP components caused by analgesics were actually a consequence of their nonspecific sedative effects [2, 28]. In order to avoid sedative effect of bromazepam, in this study, anxiolytic dose was administered. Furthermore, non-painful stimuli were applied since various studies have shown that intensity of painful stimuli positively correlated with amplitudes and negatively correlated with latencies [2, 3, 7, 11], as well as non-painful stimuli did not affect amplitudes [29]. Moreover, in order to eliminate the influence of fear of pain, the subjects were told that the stimulation of TPEPs would be painless and that the drug they receive is an analgesic. Indeed, our findings exhibit that bromazepam even at a dose of 1.5 mg significantly increased all latencies, and decreased amplitudes of last three waves.

According to other studies, benzodiazepines increase the inhibitory postsynaptic potential via GABA-ergic membrane hyperpolarization, which leads to a decrease in the firing rate of neurons [13, 15, 30]. Our results indicate that anxiolytic dose of benzodiazepines slows down neurotransmission along the entire somatosensory pathway and reduces the stimulus intensity perception from the trigeminal lemniscus, through the thalamus, to the limbic system and cortex, even if non-painful stimuli were applied.

Our results showed that placebo did not modify TPEPs waves, as we assumed. Furthermore, there are significant difference between results of placebo and other drugs, which implies that the drug effects on TPEPs are valid. Cruccu et al. [31] examined whether the late components of TPEPs are a reliable index of pain intensity. They found that changing the experience of expected pain under the influence of placebo reduces the amplitude of TPEP and subjective assessment of pain, while input from the periphery remains unchanged. Because TPEP, instead of being an event specifically related to the nociceptive message, represents the electrical equivalent of an unspecific associative activity which seems to depend more on the novelty and affective correlate of the stimulus than on the stimulus intensity. According to Thürauf et al. [10] and von Mohr et al. [21], emotional and cognitive aspect of pain could affect perception and consequently SEPs. Since we applied non-painful stimulus and our subjects did not expect pain, there was no change in the characteristics of the evoked potential, as we assumed.

Even though we found that bromazepam changed last three TPEPs amplitudes, as well as the ibuprofen changed first three TPEPs amplitudes, there were no significant differences in influence on TPEPs when these two groups are compared. Considering that there are no studies that examined the effect of both anxiolytic and analgesic on TPEPs, and based on the knowledge of all factors that affect the SEPs, which we mentioned earlier, we assume that these findings are outcome of non-painful stimuli application.

It is important to note that this part of our experiment have certain limitation. The second part of our exploration is including the effects on TPEPs after painful stimulation of the dental pulp. Due to the appropriate procedures regarding the selection and consent of patients, it was necessary to include a modified sample of patients in the study. We thought that due to the change in study conditions, participants and sample size, it would be more correct approach to present this part of the study separately after completion, and also to compare these subsequent results with result presented here. Further ongoing research, that involves painful stimulation of the dental pulp, will provide

a more complete insight into the effects on TPEPs of these two drugs with different modes of action.

CONCLUSION

In this study, we showed that both bromazepam and ibuprofen had the same influence on TPEPs after non-painful stimulus. In other words, that indicates that anxiolytic dose of bromazepam affects neurotransmission in the same manner as non-opioid analgesics ibuprofen.

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Conflict of interest: None declared.

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Поређење утицаја бромазепама и ибупрофена на евоциране потенцијале зубне пулпе код људи

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САЖЕТАК

Увод/Циљ Соматосензорни евоцирани потенцијали су неурофизиолошко средство за тестирање ефеката лекова на људе и животиње.

Циљ овог истраживања био је да се процени начин на који бромазепам и ибупрофен утичу на евоциране потенцијале зубне пулпе након примене безболног стимулуса, као и да се открију могуће разлике у овој активности.

Методе У истраживање је укључено 60 младих здравих испитаника, који су сврстани у три групе: ибупрофен, бромазепам и плацебо. У циљу добијања одговора на евоциране потенцијале зубне пулпе, зубна пулпа је стимулисана електричном струјом преко интактне глеђи стимулусима који не изазивају бол. За стимулацију и регистрацију користили смо апарат *Xltek Protektor* 32 систем, софтвер *EPWorks*, верзија 5.0 (*Natus Medical Incorporated*, Оквил, ОН, Канада). На сваком испитанику је урађено два пута по пет снимања евоцираних потенцијала, први пут пре примене лека, а други пут 45 минута након примене појединачне дозе ибупофена (400 *mg*), бромазепама (1,5 *mg*) или плацеба.

Резултати Резултати ове студије су показали следеће: и ибупрофен и бромазепам изазвали су значајно продужење свих латенци; ибупрофен је изазвао снижење амплитуда свих таласа осим првог ($p < 0,05$), а бромазепам је изазвао снижење амплитуда свих таласа осим последњег ($p < 0,05$); плацебо није модификовао таласе евоцираних потенцијала ($p > 0,05$). Такође, нису уочене значајне разлике у променама евоцираних потенцијала под дејством бромазепама у односу на ибупрофен ($p > 0,05$).

Закључак Наша студија је показала да су бромазепам и ибупрофен имали исти утицај на евоциране потенцијале зубне пулпе након примене безболног стимулуса. Добијени резултати указују да бромазепам у малим дозама на исти начин утиче на неуротрансмисију као и ибупрофен, који је неопиоидни аналгетик.

Кључне речи: соматосензорни евоцирани потенцијали; безболни стимулус; аналгетик; анксиолитик

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Morphometric analysis of somatotropic and folliculostellate cells of human anterior pituitary during ageing

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SUMMARY
Introduction/Objective In this study, we have pointed out the immunohistomorphometric characteristics of somatotropic (GH) and folliculostellate (FS) cells of the male pituitary gland during ageing.
Methods On histological sections of the pituitary gland of 14 male cadavers of different ages, the GH and FS cells were immunohistochemically labeled with corresponding antibodies, monoclonal anti-GH antibody, and polyclonal anti-S100 antibody, respectively. Immunopositive GH- and FS-cells were further morphometrically analyzed using ImageJ software.
Results The obtained results of morphometric analysis showed that the surface area of GH cells increased significantly with age. In these cells, the nuclear–cytoplasmic ratio gradually decreased and became significantly higher after the age of 70 years. The volume density of GH cells has not changed during ageing, while in FS cells this parameter significantly increased in the cases older than 70 years. The nuclear–cytoplasmic ratio of GH cells is negatively correlated with the volume density of FS cells.
Conclusion Based on the obtained results, we concluded that hypertrophy of GH and FS cells occurs in men with ageing and that correlation between the morphometric parameters of these two cell types indicates their mutual interaction.
Keywords: ageing; men; GH cells; folliculostellate cells; immunohistomorphometry

INTRODUCTION

It is well known that ageing brings with it various physiological changes within the human organism, which are especially pronounced in the functioning of the somatotropic and reproductive axis. Decreased secretion of the growth hormone (GH) with ageing is referred to as somatopause [1]. The basis of somatopause is a multiple neuroregulatory collapse, such as the lack of secretion of the growth hormone (regulated by growth hormone-releasing hormone from the hypothalamus), ghrelin and insulin-like growth factor 1 (IGF-1), as well as excessive secretion of somatostatin [1, 2].

This ultimately results in a reduced amount of secreted GH per one secretory pulse. Ageing does not have an impact on the frequency of pulsatile secretion of GH, basal secretion of GH, half-life of GH and its elimination kinetics [3]. These changes in GH secretion patterns with ageing are probably in part the consequence of certain structural changes at the hypothalamic level [4], and in part the consequence of changes in anterior pituitary somatotropic cells, which have not been sufficiently studied so far [5]. In contrast to hypogonadism, the negative feedback mediated by GH and/or IGF-1 becomes stronger with advancing age. The consequence of this phenomenon is the biochemical hyposomatotropism with an exponential decline of GH and IGF-1 concentrations starting from early adult age, which clinically manifests in the form of osteopenia, sarcopenia, intraabdominal obesity, insulin resistance, hyperlipidemia, increased risk for atherosclerosis and lower quality of life [1, 6].

The knowledge of the function of hypothalamo-somatotropic axis during ageing has led to therapeutic use of GH and other hormones in the elderly as a certain 'elixirs of youth.' However, the expected results have not been obtained, and the incidence of adverse effects and potential malignancy risks in the elderly have led endocrinologists to decide that there are no valid reasons for clinical use of GH to reverse age-related changes [7]. Naturally, future research of the pathways responsible for GH deficiency during ageing should hopefully resolve the present dilemmas as to the use of GH in the attempts to reverse ageing and to prolong human life.

In their fundamental work, Schwartz et al. [8] reported that the function of the anterior

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lobe of the pituitary gland probably relies on the integration of multiple received input signals, including hypothalamic, peripheral, and intrapituitary, which may have stimulatory or inhibitory effects on the hormone production. Interactions of somatotropic (GH) cells with other hormonal and non-hormonal pituitary cells in healthy elderly appear to be very important [9, 10, 11]. Pituitary folliculostellate cells (FS) can release various products into the intercellular space, and thus can influence the functioning of neighboring hormone-producing cells [12].

The internal hormonal milieu of older men changes with advancing age, and we may well suppose that such changes can stimulate FS cell interactions with somatotropes via paracrine loops [13]. These interactions can consequentially alter the function of somatotropes, which would ultimately result in age-related changes of GH levels in the plasma.

In view of the fact that hyposomatotropism during ageing can occur as the consequence of structural changes at all levels of the somatotropic axis and that age-related histomorphometrical changes at the level of the anterior lobe of the pituitary gland are insufficiently studied, the aim of our study was to detect and quantify the changes in morphology and density of human pituitary gland anterior lobe somatotropic cells, using immunohistochemical and morphometric analysis in cases of different ages. We also tried to discover similar changes in adenohypophyseal folliculostellate cells, as the structures which, via paracrine pathways, could have an impact on the function of anterior pituitary somatotropic cells. Additionally, we evaluated statistically the possible association of the above changes during human ageing.

METHODS

Pituitary tissue sampling

The study material consisted of pituitary tissue samples taken from 14 men corpses, aged 41–87 years, which were divided into three groups. The first (I) group consisted of cases aged 41–49 years; the second (II) group implied cases aged 50–69 years, and the third (III) group included cases older than 70 years.

Tissue samples were taken at a routine autopsy at the Center for Forensic Medicine in Niš, Serbia, with the approval of the Ethics Commission of the University of Niš, Faculty of Medicine (Decision No. 12-2307-2/8 of March 10, 2016), described in detail in our previous work [14].

Pituitary tissue processing and immunohistochemistry

Isolated pituitaries of the male corpses were histologically prepared according to the procedure described in detail earlier, while the morphometric analysis of GH and FS cells was performed according to the previously described procedure [15, 16]. The stained histological sections were then immunohistomorphometrically analyzed using light microscopy under 4 × and 40 × magnifications.

Morphometric analysis

Morphometric analysis was performed on digital images obtained by a 1.3-megapixel digital camera. Thirty visual fields were selected from both dorsal and ventral halves, i.e. 60 visual fields in total per analyzed case. We obtained 10 visual fields each from both lateral anterior pituitary wings and 10 from the middle portion of both dorsal and ventral halves from all analyzed cases (20 visual fields in total from each lateral anterior pituitary wing and 20 from the middle portion in each analyzed case). Image analysis was performed using the ImageJ software (https://imagej.nih.gov/ij/).

Regarding GH immunoreactive cells, our analysis involved measurements of their area (A_{GH}) and the area of their nuclei (A_{NGH}). The nuclear–cytoplasmic ratio (N/C_{GH}) was calculated as the quotient of nuclear area and cytoplasmic area, with the cytoplasmic area obtained as the difference of area of the above cells and the area of their nuclei. We performed the measurement of 60 GH cells and 60 FS cells in the dorsal and ventral anterior pituitary halves in all analyzed cases (in total, 120 cells per case).

The analysis was performed using the multipurpose test system M168 (d = 17.88 μm, a = 15.49 μm^2, AT = 2601.54 μm^2, LT = 1501.92 μm), placed over the analyzed digital image of histological sections. Volume density of GH (V_{VGH}) and FS cells (V_{VFS}) was obtained as the quotient of the number of dots in the test system which hit immunopositive cells (PF) and the total number of dots in the system (PT = 168) per each analyzed field of the dorsal and ventral pituitary halves [17].

The values of area, nuclear area, nuclear–cytoplasmic ratio, and volume density of GH cells and volume density of FS cells per each analyzed case were obtained as average values for all measured visual fields.

Statistical analysis

The statistical analysis was performed using SPSS, Version 16.0 (SPSS Inc., Chicago, IL, USA). Dynamics of the values of morphometric parameters for the studied age groups was analyzed using the One Way ANOVA and Tukey–Kramer post hoc test. Due to a small size of the analyzed sample, the obtained statistically significant differences were additionally verified by the calculation of the corresponding effect sizes.

RESULTS

Qualitative histological analysis

In younger individuals (the first group; age 41–49 years), pituitary somatotropic cells were rare and scattered within the *pars intermedia,* while their presence was markedly greater in anterior pituitary lateral wings. They were predominantly polygonal, with eccentric euchromatic nuclei (with prominent nucleoli in some of the cells). A positive immunohistochemical reaction was observed in the

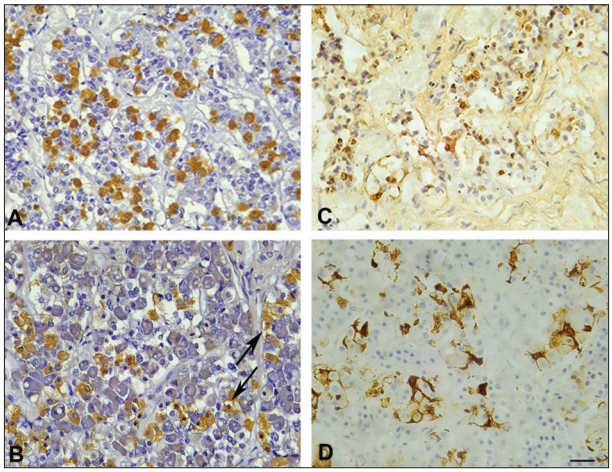

Figure 1. Representative micrograph of a young man's (41 years old) pituitary somatotropic cells A); older man's (87 years old) pituitary somatotropic cells B); S100 immunopositive folliculostellate cells in the 41- (C) and the 87-year-old man (D); Novocastra Peroxidase Detection System; magnification 40 ×, bar = 30 μm

cytoplasm of somatotropes, while in their nuclei the reaction was immunonegative (Figure 1A). Somatotropic cells in older cases (the third group; above 70 years) demonstrated a slightly stronger immunopositive reaction in the *pars intermedia* of the anterior pituitary. In contrast to the above, in these cases there was a significant decline of the number of somatotropes in both *pars intermedia* and anterior pituitary lateral wings. Somatotropes were either single or in groups, located near the capillaries. The cells were larger, with an eccentric, hyperchromatic, immunonegative nuclei and occasional transparent cytoplasmic vacuoles (Figure 1B).

Folliculostellate cells in the anterior pituitary of younger cases (age 41–49 years) were irregular to star-shaped in appearance. Their immunopositive cellular body has thin projections extending between endocrine cells. These cells were rare and irregularly distributed within the lateral wings, as well as in the mucoid wedge of the anterior pituitary (Figure 1C). The bodies of FS cells and their projections were visualized between endocrine cells. In cases over 70 years of age (the third group), we encountered a significantly increased number of FS cells in the middle and in the lateral wings of the anterior pituitary (Figure 1D). Although the irregular shape of their bodies made it difficult to estimate the size of the cells, it could be concluded that in general their bodies became larger and that the immunopositivity was stronger in the more advanced years of life.

Morphometric analysis

The results of morphometric analysis of anterior pituitary immunoreactive GH and FS cells in the studied cases are shown in Table 1.

The correlation analysis of age and morphometric parameters of GH immunoreactive cells showed that their area significantly increased (R = 0.7; p = 0.005; N = 14) (Figure 2A), and nuclear–cytoplasmic index significantly decreased with advancing age (R = -0.69; p = 0.006; N = 14) (Figure 2B). Volume density of FS cells significantly increased during ageing (R = 0.80; p = 0.001; N = 14) (Figure 2C). Nuclear area of GH immunoreactive cells did not change significantly with ageing (p > 0.05), while volume density of these cells increased with ageing, but the increase was not significant (p > 0.05). The results of bivariate linear regression additionally demonstrated that age was a significant predictor of area and nuclear–cytoplasmic ratio of GH immunoreactive cells and volume density of FS cells as well (Table 2). The factor of age was able to explain 49% of area variance, 48% of nuclear–cytoplasmic ratio of GH immunoreactive cells, and 61% of volume density variance of adenohypophyseal FS cells. In all three instances, age represented a large effect size and could be shown using the models presented in Table 2.

Table 1. Morphometric analysis of immunoreactive growth hormone and folliculostellate cells of the anterior pituitary in all 14 analyzed cases

Case	Age	Group	V_{VGH} (%)	V_{VFS} (%)
1	41	I	19.87	1.59
2	45	I	15.2	1.16
3	48	I	19.56	1.88
4	48	I	21.43	1.6
5	57	II	11.71	1.33
6	61	II	19.72	2.94
7	65	II	25.35	3.44
8	65	II	26.35	2.46
9	66	II	19.65	2.26
10	76	III	28.32	2.26
11	76	III	22.69	2.81
12	77	III	19.35	3.13
13	78	III	13.42	3.37
14	87	III	22.74	4.79

V_{VGH} – volume density of growth hormone cells; V_{VFS} – volume density of folliculostellate cells T

Table 2. Regression analysis between the age as predictor and area, nucleocytoplasmic ratio of somatotropes (GH) immunoreactive cells, as well as volume density of folliculostellate cells (FS) cells as outcome variables

A_{GH}					
Variable	B	SEB	β	t	p
Constant	94.00	18.53		5.07	< 0.001
Age	0.97	0.28	0.70	3.41	0.01
$R^2 = 0.49$; $F(1,12) = 11.63$, $p = 0.005$; Model: $A_{GH} = 94.00 + Age \times 0.97$					
$(N/C)_{GH}$					
Variable	B	SEB	β	t	p
Constant	0.35	0.04		8.57	< 0.001
Age	-0.002	0.0006	-0.69	-3.32	0.06
$R^2 = 0.48$; $F(1,12) = 11.05$, $p = 0.006$; Model: $(N/C)_{GH} = 0.352 - Age \times 0.002$					
V_{VFS}					
Variable	B	SEB	β	t	p
Constant	-1.06	0.78		-1.37	0.20
Age	0.06	0.01	0.80	4.62	0.001
$R^2 = 0.61$; $F(1,12) = 21.32$, $p = 0.001$; Model: $V_{VFS} = Age \times 0.06 - 1.06$					

A_{GH} – area of GH cells; $(N/C)_{GH}$ – nuclear–cytoplasmic ratio of GH cells; V_{VFS} – volume density of folliculostellate cells; R^2 – the coefficient of determination; B – unstandardized coefficient; SEB – standard error of B; β – standardized coefficient beta; t – t-test

Table 3. Results of univariate ANOVA, testing between the average values of morphometric parameters of somatotropic cells immunoreactive cells and folliculostellate cells of the anterior pituitary in the analyzed age groups

Parameter	Group	N	Average	SD	SE	95% CI		Tukey post hoc test
						LB	UB	
A_{GH} (μm²)	I	4	141.39	13.94	6.97	119.20	163.57	a
	II	5	150.55	21.18	9.47	124.26	176.85	/
	III	5	172.46	9.20	4.12	161.03	183.89	a
ANOVA	F(2,11) = 4.77, p = 0.03							
A_{NGH} (μm²)	I	4	29.05	2.27	1.13	25.44	32.66	/
	II	5	26.99	5.27	2.36	20.45	33.53	/
	III	5	26.70	5.30	2.37	20.12	33.29	/
ANOVA	F(2,11) = 0.32, p = 0.73							
$(N/C)_{GH}$	I	4	0.26	0.02	0.01	0.23	0.29	a
	II	5	0.22	0.04	0.02	0.17	0.27	/
	III	5	0.18	0.04	0.02	0.14	0.23	a
ANOVA	F(2,11) = 6.38, p = 0.01							
V_{VGH} (%)	I	4	19.02	2.67	1.34	14.76	23.27	/
	II	5	20.55	5.84	2.61	13.31	27.80	/
	III	5	21.30	5.46	2.44	14.53	28.08	/
ANOVA	F(2,11) = 0.24, p = 0.79							
V_{VFS} (%)	I	4	1.56	0.30	0.15	1.08	2.03	a
	II	5	2.41	0.83	0.37	1.37	3.44	/
	III	5	3.21	0.94	0.42	2.04	4.38	a
ANOVA	F(2,11) = 5.08, p = 0.03							

a – I:III, $p < 0.05$;
A_{GH} – area of somatotropic cells; A_{NGH} – area of somatotropic nuclei; $(N/C)_{GH}$ – nuclear–cytoplasmic ratio of somatotropic cells; V_{vGH} – volume density of somatotropic cells; V_{VFS} – volume density of folliculostellate cells; SD – standard deviation; SE – standard error; LB – lower bound; UB – upper bound; CI – confidence interval

A detailed dynamics of age-related changes in average values of morphometric parameters of adenohypophyseal immunoreactive GH and FS cells in the studied age groups was evaluated using the One Way ANOVA test. The results showed that the mean values of area and nuclear–cytoplasmic ratio of GH immunoreactive cells differed significantly between the studied age groups (Table 3). The average area of anterior pituitary GH immunoreactive cells significantly increased during the process of ageing ($F(2,11) = 4.77$, $p = 0.03$) (Figure 2D). The post hoc Tukey–Kramer test showed that this parameter had an increasing tendency during ageing, with the value in age group III being significantly higher compared to group I ($p < 0.05$), although not compared to age group II ($p > 0.05$). The average value of this parameter in age group II was higher than that in group I, but the difference was not statistically significant ($p > 0.05$). The average nuclear–cytoplasmic ratio of anterior pituitary GH immunoreactive cells significantly declined with ageing ($F(2,11) = 6.38$, $p = 0.01$) (Table 3, Figure 2E). The post hoc Tukey–Kramer test showed that this parameter had a declining tendency during ageing, with the value in age group III being significantly lower than that in group I ($p < 0.05$), but not compared to age group II ($p > 0.05$). The average value of this parameter in age group II was lower compared to group I, but the difference was not statistically significant ($p > 0.05$). The average volume density of anterior pituitary FS cells ($F(2,11) = 5.08$, $p = 0.03$) significantly increased during the process of ageing (Table 3, Figure 2F). The post hoc Tukey–Kramer test showed that volume density of FS cells showed an increasing tendency with advancing age, with the values in age group III being significantly higher than those in age group I ($p < 0.05$), but not when compared to age group II ($p > 0.05$). The average value of this parameter in age group II was higher compared to age group I, but the difference was not statistically significant ($p > 0.05$). The remaining two analyzed morphometric parameters did not differ significantly between the studied age groups ($p > 0.05$).

The analysis of correlation between the morphometric parameters of GH immunoreactive and FS cells indicated the presence of a statistically significant negative correlation of average volume density of anterior pituitary FS cells and nuclear–cytoplasmic ratio of GH immunoreactive cells ($R = -0.71$, $p = 0.005$, $N = 14$). The linear regression analysis showed that volume density of FS cells in examined cases represented a statistically significant predictor of nuclear–cytoplasmic ratio of GH immunoreactive cells ($F(1,12) = 12.08$, $p = 0.005$), which could be represented using the following model: $(N/C)_{GH} = 0.287 - V_{VFS} \times 0.031$. In particular, the increased volume density of anterior pituitary FS cells was accompanied by a statistically significant decline of nuclear–cytoplasmic ratio of GH immunoreactive cells (Figure 2G), with the FS cell volume density of the studied cases being able to explain 46% of overall variance of nuclear–cytoplasmic ratio of GH immunoreactive cells ($R^2 = 0.46$), which represented a large size effect.

DISCUSSION

Ageing of the pituitary gland functionally manifests by its declining secretory activity, especially affecting the levels of growth hormone, prolactin, and thyroid-stimulating hormone in the blood. These changes lead to so-called age-related diseases, which predominantly affect the target organs of these hormones [6, 18]. Some previous studies reported a fall in different parameters of somatotropic cells with ageing in detail [1]. In the study conducted by Sano et al. [19], the presence of interstitial, perivascular fibrosis was documented semiquantitatively: the fibrosis progressed with time and involved anterior pituitary parenchyma in 88% of older individuals (predominantly males). The study reported a declining number of somatotropes in the lateral portions of the organ with ageing, but could not establish the dynamics of this decline. According to the same study, the number of other anterior pituitary cells did not change significantly during ageing. In the pituitary glands of individuals aged over 90 years, focal necrosis or scarring tissue, iron or amyloid deposits, basophilic invasions, accumulations of squamous cells, adenomas, and granular cells were occasionally seen.

The results of our present immunohistomorphometric study of anterior pituitary glands, obtained from human male cadavers, indicated a significant increase in size of

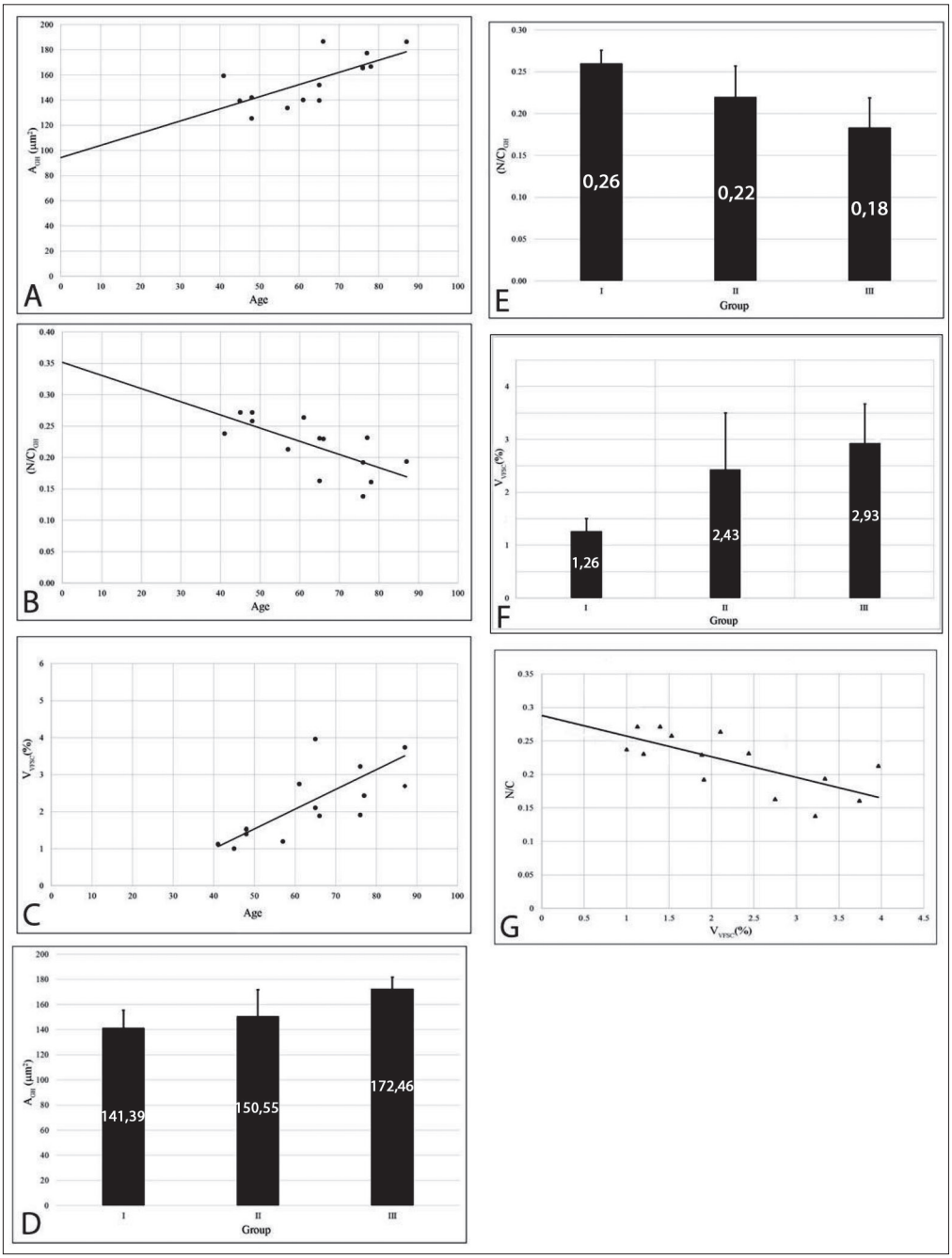

Figure 2. The graphical representation of the anterior pituitary gland parameters in the analyzed cases: correlation between age and area (A), nuclear–cytoplasmic ratio (B), average area (D), and average nuclear–cytoplasmic ratio (E) of somatotropic cells; correlation between age and volume density (C) and average volume density (F) of folliculostellate cells; correlation between volume density of folliculostellate cells and nuclear–cytoplasmic ratio of somatotropic cells (G);

V_{VGH} – volume density of somatotropic cells; V_{VFS} – volume density of folliculostellate cells; A_{GH} – area of somatotropic cells; $(N/C)_{GH}$ – nuclear–cytoplasmic ratio of somatotropic cells

somatotropic cells with ageing, while the size of their nuclei remained unaffected by this physiological process. The increase in size of somatotropic cells was slow and steady, so statistically significant differences exist only between age groups I and III. On the other hand, nuclear–cytoplasmic ratio declined uniformly with years of age, given that statistically significant differences were observed only between age groups I and III. The increase in size of somatotropes during ageing, as shown in this study, is in accordance with the research of Antić et al. [20]. However, in contrast to these authors, we could not confirm any significant decline of volume density of somatotropic cells with advancing age. Our study showed that the appropriate structural changes in somatotropic cells are responsible for the functional decline of the GH/IGF-1 axis during human aging, which is consistent with the earlier work of Antić et al. [20]. The dynamics of these changes at the cellular level was influenced by numerous, insufficiently known, distant and local factors in the cell environment. It is certain that the reported age-related changes of somatotropes can be

at least partly explained by structural changes at the level of hypothalamus occurring with advancing age [4].

Our results showed an increase in the volume density of FS cells in the elderly, compared to younger cases. This fact may indirectly indicate increased FS cell function in the elderly, as shown by Pavlović et al. [21] during examination of this parameter in the case of both sexes, when the significant increase was noticed after the age of 80 years. However, except for the fact that the male cases studied by Pavlović et al. [21] were older and with greater volume density of FS cells than in our study, this significant increase of volume density of FS cells was explained with simultaneous increases in the size and number of these cells in the pituitary mucoid wedge in their oldest group. Our histological analysis could not reveal any regional differences in the dynamics of FS cells during the process of ageing. From our results, we could make an assumption that age-related increased volume density of FS cells is a two-phase process. The first phase, probably occurring in men aged 50–70 years, would predominantly be the consequence of increased size of FS cells or their hypertrophy, while in the second phase, occurring in men aged over 70 years, a further increase in size of FS cells occurs with a simultaneous increase of their number, leading thus to their significantly greater volume density. These facts may indirectly indicate increased function of FS cells in the elderly.

Analysis of the correlation between morphometric parameters of GH and FS cells indicated the presence of a statistically significant negative correlation between the average volume density of anterior pituitary FS cells and the nuclear–cytoplasmic ratio of GH cells, suggesting that homeostasis of the growth hormone production/secretion exists [22].

CONCLUSION

From all the above, we concluded that in men, the size of GH cells increased with age. According to our results, it can be indirectly hypothesized that long-term hypertrophy of GH cells results in their functional decline after the age of 70. Furthermore, density and size of FS cells increased during ageing, which indicated their increased function. The strong correlation between morphometric parameters of FS and GH cells might point to age-related interactions between these two cell groups.

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Морфометријска анализа соматотропних и фоликулостелатних ћелија аденохипофизе човека током старења

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САЖЕТАК

Увод/Циљ У овој студији смо указали на имунохистоморфометријске карактеристике соматотропних (ГХ) и фоликулостелатних (ФС) ћелија хипофизе мушкараца током старења.

Методе На хистолошким пресецима хипофизе 14 мушких кадавера различите старости, ГХ и ФС ћелије су имунохистохемијски обележене одговарајућим антителима (моноклонско анти-ГХ антитело и поликлонско анти-С100 антитело). Имунопозитивне ГХ и ФС ћелије су морфометријски анализиране коришћењем софтвера *ImageJ*.

Резултати Добијени резултати морфометријске анализе су показали да се површина коју заузимају ГХ ћелије значајно повећава са старењем. У овим ћелијама нуклеарно-цитоплазматски однос се постепено смањивао и након 70. године старости постао значајно већи. Волуменска густина ГХ ћелија се није мењала током старења, док је код ФС ћелија овај параметар значајно повећан у случајевима старијим од 70 година. Нуклеарно-цитоплазматски однос ГХ ћелија је у негативној корелацији са волуменском густином ФС ћелија.

Закључак На основу добијених резултата закључили смо да се хипертрофија ГХ и ФС ћелија јавља код мушкараца са старењем и да корелација између морфометријских параметара ова два типа ћелија указује на њихову међусобну интеракцију.

Кључне речи: старење; мушкарци; ГХ ћелије; ФС ћелије; имунохистоморфометрија

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

High risk population screening for Fabry disease in hemodialysis patients in Vojvodina – pilot study

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SUMMARY

Introduction/Objective Fabry disease (FD) is an X-linked lysosomal storage disease that develops as a consequence of mutation in the alpha-galactosidase A (*GLA*) gene. There are more than 1080 known variants in the *GLA* gene. Some of them are pathogenic, but most of them are benign or represent the genetic change that can be classified as a genetic variant of unknown significance or simply be a representation of genetic polymorphism. There are two main features of FD, classic form and late-onset variants of disease. The main target organs in patients with FD are the kidneys, heart, and nervous system. Bearing in mind the fact that FD is a rare disease, the best way for active searching of patients is high-risk population screening, after which family screening for every proband case should be performed.

Methods In this paper, we present results of a multicentric pilot study that represents findings from the screening of hemodialysis patients for FD in six hemodialysis units in Vojvodina.

Results We have found one patient with benign mutation and 16 patients with genetic polymorphisms in *GLA* gene. We have learned that genetic changes in *GLA* gene can be frequent, but very rarely are of clinical significance and lead to manifestations of FD.

Conclusion Results of this screening study will give us important insights into our future work.

Keywords: Fabry disease; hemodialysis; high-risk population screening

INTRODUCTION

Fabry disease (FD) (OMIM 301500) is an X-linked lysosomal storage disorder caused by mutations in the *GLA* gene that result in markedly reduced or absent activity of the enzyme α-galactosidase A (α-Gal A, EC 3.2.1.22). This leads to the intracellular accumulation of substrates like globotriaosylceramide (Gb3) and also its deacylated derivative globotriaosylsphingosine (lyso-Gb3). Lyso-Gb3 is a valuable biomarker and speaks mainly about the burden of FD, since it reflects the severity of disease and also corresponds well with tissue accumulation of Gb3. So, Lyso-Gb3 is of great importance in establishing the diagnosis of FD, but also in assessing the disease severity and therapeutic monitoring [1–4]. Until now, more than 1080 variants in the *GLA* gene have been identified in the Human Gene Mutation Database (HGMD) [5]. Some of them are pathogenic but others are benign or represent genetic change that can be classified as a genetic variant of unknown significance. After the findings of genetic change in *GLA* gene in each patient, we can scroll through the HGMD and seek information about the clinical significance of found mutation [1, 5, 6].

Prevalence of FD is in the range of 1:40,000–1:117,000 live male newborns, but the exact prevalence is very hard to establish due to the very heterogeneous nature of the disease (even within a group of patients with the same genetic variant) and lack of awareness about the disease [7]. In contrast to other X-linked diseases, in which females can only be carriers, in FD females may be as severely affected as male patients [8]. There are two basic phenotypes of FD: a classic form of the disease that is mainly associated with deletions, frameshifts and nonsense variants in the *GLA* gene, and late-onset variants of FD that are associated with missense variants in the *GLA* gene [9]. The classic form of FD starts at a younger age with earlier manifestations on target organs (the heart, kidneys, nervous system) and usually with some other clinical presentations of the disease, like cornea verticillata, angiokeratomas and acroparesthesia (burning sensations on hands and feet). On the other hand, later-onset variants are becoming clinically evident later in life, with cardiomyopathy and/or nephropathy alone [1]. Diagnostic

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criteria for FD require the confirmation of low enzyme activity and presence of genetic variants in the *GLA* gene for male patients or *GLA* gene variants for female patients together with characteristic clinical features of the disease and/or a family member with the diagnosis of FD and/or elevated plasma lyso-Gb3 level [10]. In some cases of suspected FD target organ biopsy should be performed, with electron microscopy analysis of the tissues, so typical cellular inclusions, "zebra bodies," can be found [1, 2].

The current mainstay of treatment for FD patients includes the administration of enzyme replacement therapy. Nowadays, in clinical practice, there are two available forms of the drug: agalsidase alpha and agalsidase beta, with different dosage regimes (0.2 mg/kg intravenously every other week and 1 mg/kg intravenously every other week, respectively) but similar therapeutic efficacy. In some countries, there is also a possibility for oral therapy with migalastat for some types of amenable mutations [11].

There are only six established cases of FD in Serbia, for now. The exact number of FD patients in our country is hard to establish, but should probably be in the range of 50–100 patients. One way of finding of FD patients is high-risk population screening which represents an active search for FD patients in certain patient populations like hemodialysis patients, patients with hypertrophic cardiomyopathy of unknown/uncertain origin or patients with an early stroke (before the age of 55).

FD often remains undiagnosed according to the prevalence calculated in some studies because of the heterogeneous nature of its clinical manifestations. The clinical diagnosis of both phenotypes (classic form and late-onset variants) is challenging, since many of the main symptoms and signs are common in other diseases, and the time between the first symptoms and the settling of diagnosis can take more than ten years [1, 10]. Therefore, the precise establishment of the molecular-genetic diagnosis and the earliest possible treatment is essential to avoid significant disease progression. Molecular analysis of FD is also crucial for segregation studies, enabling early diagnosis of family members with pathogenic variants in the *GLA* gene allowing monitoring before the first symptoms appear, and therefore promoting better disease management.

The aim of this study was to perform molecular-genetic analysis of FD in the group of hemodialysis patients to provide an early application of appropriate therapy as well as the provision of genetic advice to families with a high risk for the birth of a child with FD.

METHODS

After the approval of ethic committees from every institution which participated in the screening program and the signing of full informed consent by every patient who participated in the study, we performed a multicentric pilot study from December 2020 until May 2021. The study was based on a FD screening of patients in six hemodialysis centers from different parts of Vojvodina (Stara Pazova, Pančevo, Kikinda, Subotica, Bačka Palanka and Sremska Mitrovica). At the time of the screening process, these hemodialysis centers had a dialysis population of 529 patients in total (58, 135, 82, 70, 64, and 120, respectively). Patients eligible for screening were male patients under 55 years of age and with an unknown cause of end-stage renal disease (ESRD), as well as female patients of all ages and unknown cause of ESRD. Because of the variable clinical presentations of FD we analyzed the presence of other clinical manifestations, besides ESRD, that can be a part of the clinical spectrum of FD in our patient eligible for screening process (left ventricular hypertrophy (LVH), stroke before the age of 55, existence of white matter lesions, cornea verticillata, angiokeratoma, tinnitus, hearing loss, heat or cold intolerance, postprandial pain, acroparesthesia, hypo-, or hyperhidrosis). The screening protocol for FD was based upon genetic analysis of peripheral blood from 117 selected patients. Genetic analysis was performed at the Institute of Molecular Genetics and Genetic Engineering, University of Belgrade.

In patients with genetic polymorphisms or genetic variants of unknown significance in the *GLA* gene, we performed biomarker (Lyso-Gb3) analysis in Centogene (Centogene Rostock, Germany) from the blood of our participants through dried blood spots (DBS, CentoCard, Centogene) analysis with the liquid chromatography mass spectrometry method (HPLC-MS/MS; Thermo Fisher Scientific, Waltham, MA, USA).

Detection of genetic variants in the GLA gene

Genomic DNA was isolated directly from whole peripheral blood using QIA amp DNA Blood Mini Kit (QIAGEN, Hilden Germany), and quantified with a Qubit® 3.0 Fluorimeter (Thermo Fisher Scientific) and Qubit™ dsDNA HS Assay Kit (Thermo Fisher Scientific). All seven exons and flanking intron regions of the *GLA* gene (GenBank: NM_000169.3) were individually PCR amplified using PCR System (Eppendorf, Master cycler Nexus, Hamburg, Germany). Primers were designed in the way that an average length of fragments was between 400 and 700 bp and that all known single nucleotide polymorphisms were avoided. We used different online available tools: single nucleotide polymorphisms Check [http://www.ngrl.org.uk], OligoAnalyzer [http://www.idtdna.com/pages/tools/oligoanalyze r], In silico PCR [http://genome.ucsc.edu]. A list of all primers is given in Table 1.

Reactions were carried out in a final volume of 25 μl containing 100 ng of genomic DNA, 1 × PCR buffer (with Mg^{2+}), 1 × Q solution, 1.0 mM $MgCl_2$, 0.2 mM dNTP each, 1 U of HotStarTaq DNA® Polymerase (QIAGEN, Hilden, Germany), 10 pmol of each primer and distilled water. Cycling conditions for PCR were as follows: initial denaturation at 95°C for 15 minutes; 30 cycles at 94°C for 30 seconds, 54°C for 30 seconds, and 72°C for one minute, followed by a final extension at 72°C for five minutes.

All PCR fragments were directly sequenced using the Big Dye terminator cycle sequencing kit and the ABI PRISM 310 automated sequencer (Applied Biosystems Life Technologies, USA). Segregation analysis was performed in families when it was possible to determine the carrier status of detected genetic variants.

Table 1. List of all primers used for amplification of coding and flanking regions of alpha-galactosidase A (GLA) gene

Primer name	Sequence 5'-3'	Length (bp)
GLA_ex1_F	5`-AGCGGAACGTCTTACGTGAC-3`	522
GLA_ex1_R	5`-CGGGACAGTTTGCTGGG-3`	
GLA_ex2_F	5`-AGGGCGGGAATATTAACGGG-3`	620
GLA_ex2_R	5`-GTTACAGGCGTTCACCACC-3`	
GLA_ex3_F	5`-CTAGCTCAGCAGAACTGGGG-3`	522
GLA_ex3_R	5`-GAGATGGGAGCTCTGGCAC-3`	
GLA_ex4_F	5`-GGGAAGCTGAGACAGAAGAGTC-3`	477
GLA_ex4_R	5`-CCAGGTGATGGTAGCTTAGGC-3`	
GLA_ex5_F	5`-GGTTTAGACCTCCTTATGGAGACG-3`	420
GLA_ex5_R	5`-GCATCCTGCTCTAAGTACTCTCAC-3`	
GLA_ex6_F	5`-GTGAGAGTACTTAGAGCAGGATGC-3`	555
GLA_ex6_R	5`-AGCAACTAGTGATAAGTGGCCC-3`	
GLA_ex7_F	5`-CAATACCAACTTTGTCTTGGGCC-3`	675
GLA_ex7_R	5`-AGGCGGGTCTCAAAGTCC-3`	

Table 2. Patients involved in Fabry disease screening per hemodialysis (HD) center

HD center	Patients on HD (n)	Tested female patients (n)	Tested male patients (n)	Tested – both genders (n)	Age of patients
Bačka Palanka	64	4	18	22	**55.4 ± 6.4**
Stara Pazova	58	3	2	5	**36.2 ± 7.5**
Kikinda	82	5	3	8	**45.4 ± 7.1**
Subotica	70	5	9	14	**51.2 ± 6.3**
Sremska Mitrovica	120	13	29	42	**55.7 ± 4.4**
Pančevo	135	10	16	26	**47.7 ± 7.5**
In total	529	40	77	117	**48.6 ± 6.3**

RESULTS

Hemodialysis, together with peritoneal dialysis and kidney transplantation, represents methods of renal replacement therapy. There are 16 hemodialysis centers in Vojvodina, with around 1600 patients. From the yearly reports of the Registry of Serbian Society of Nephrology, we have learned that around 8% of patients with ESRD in Serbia have chronic kidney disease of unknown etiology [12].

FD is a rare disease that physicians usually don't think about in their everyday clinical practice. Having in mind that hemodialysis patients belong to a high-risk population group for FD, we decided to perform a pilot study of screening for FD patients in hemodialysis centers in Vojvodina. According to the previously mentioned criteria for screening eligibility, we have screened 117 patients (40 females) from the aforementioned hemodialysis units with a total dialysis population of 529 (22%) (Table 2). The majority of screened patients had LVH (63.2%), but stroke before the age of 55 and white matter lesions were rare (4.3% and 2.6%, respectively). Around one-fifth of screened patients also had tinnitus, hearing loss, heat or cold intolerance, postprandial pain and acroparesthesia. Complete list of comorbidities is given in Table 3.

In this study, we conducted the genetic analysis of 117 patients suspected of FD, and the *GLA* sequencing revealed only one male patient (with a frequency of 0.8%) with a variant in a coding region (p.Asp313Tyr), 16 patients (13.7%) had only non-coding variants, while 100 subjects had no variants in the analyzed regions (85.5%). All detected genetic changes were previously described in the literature. Five different combinations of number of cited variants, described as complex intronic haplotypes (CIHs), in the *GLA* gene were identified in our group of subjects (Table 4 and 5). The most frequent haplotype is formed by the four variants c.-10C>T, c.370-81_370-77delCAGCC, c.640-16A>G, c.1000-22C>T, and was detected in eight of 117 (6.8%) patients. Lyso-Gb3 biomarker levels were within normal range in each tested patient, so all genetic changes we have found could be accounted for nonpathogenic.

DISCUSSION

FD is a rare systemic metabolic disorder that leads to the accumulation of lipid substrates in lysosomes in various tissues and organs. Having in mind a fact that most important target organs in FD patients are kidneys, heart

Table 3. Comorbidities in patients involved in Fabry disease screening

Comorbidities	Bačka Palanka (N = 22)	Stara Pazova (N = 5)	Kikinda (N = 8)	Subotica (N = 14)	Sremska Mitrovica (N = 42)	Pančevo (N = 26)	In total (N = 117)
Left ventricular hypertrophy	12 (54.5%)	2 (40%)	3 (37.5%)	6 (42.8%)	28 (66.7%)	25 (96%)	74 (63.2%)
Cerebrovascular incidents < 55 years	2 (9%)	0	1 (12.5%)	1 (7.2%)	1 (2.4%)	0	5 (4.3%)
White matter lesions	1 (4.5%)	0	1 (12.5%)	0	0	1 (4%)	3 (2.6%)
Cornea verticillata	0	1 (20%)	0	0	1 (2.4%)	1 (4%)	3 (2.6%)
Angiokeratoma	0	0	0	2 (14.4%)	1 (2.4%)	0	3 (2.6%)
Tinnitus	3 (13.5%)	1 (20%)	1 (12.5%)	9 (64.3%)	2 (4.8%)	7 (27%)	23 (19.7%)
Hearing loss	8 (36.4%)	0	1 (12.5%)	2 (14.4%)	6 (14%)	4 (15%)	21 (18%)
Heat/cold intolerance	1 (4.5%)		2 (25%)	8 (57.2%)	1 (2.4%)	8 (31%)	20 (17%)
Acroparesthesia	0	2 (40%)	2 (25%)	2 (14.4%)	6 (14.3%)	13 (50%)	25 (21.4%)
Hypo-/hyperhidrosis	0	1 (20%)	0	3 (21.4%)	7 (16.4%)	0	11 (9.4%)
Post prandial pain	2 (9%)	3 (60%)	1 (12.5%)	2 (14.4%)	3 (7%)	16 (62%)	27 (23%)

Table 4. The summary of complex intron haplotypes found in patients with suspicion of Fabry disease

Genes	Alpha-galactosidase A region	Variant/Haplotype	Patients	
			N = 117	%
Variant 1	5′UTR	c.-10C>T	2	1.7
Complex intronic haplotype 1	5′UTR, intron 2, intron 4, intron 6	c.-10C>T, c.370-77_-81delCAGCC, c.640-16A>G, c.1000-22C>T	8	6.8
Complex intronic haplotype 2	intron 2, intron 4, intron 6	c.370-77_370-81delCAGCC, c.640-16A>G, c.1000-22C>T	4	3.4
Complex intronic haplotype 3	5′UTR, intron4	c.-12G>A, 639+68A>G	1	0.8
Complex intronic haplotype 4	5′UTR, intron 2, intron 4, intron 6	c.-12G>A, c.370-77_-81delCAGCC, c.640-16A>G, c.1000-22C>T	1	0.8

Table 5. Genetic changes in alpha-galactosidase A gene in our patients

Patient	Gender	Age	Genetic change	Importance
1.	♀	44	Incomplete CIH (c.-10C>T (g.1170 C>T))	Polymorphism
2.	♂	51	p.Asp313Tyr (D313Y)	Benign
3.	♀	61	CIH (c.- 10C>T (g.1170 C>T), c.370-77_-81delCAGCC (g.7188-7192 del5), c.640-16A>G (g.10115A>G), c.1000-22 C>T (g.10956 C>T))	Polymorphism
4.	♀	32	CIH (c.- 10C>T (g.1170 C>T), c.370-77_-81delCAGCC (g.7188-7192 del5), c.640-16A>G (g.10115A>G), c.1000-22 C>T (g.10956 C>T))	Polymorphism
5.	♀	52	CIH (c.- 10C>T (g.1170 C>T), c.370-77_-81delCAGCC (g.7188-7192 del5), c.640-16A>G (g.10115A>G), c.1000-22 C>T (g.10956 C>T))	Polymorphism
6.	♀	47	CIH (c.- 10C>T (g.1170 C>T), c.370-77_-81delCAGCC (g.7188-7192 del5), c.640-16A>G (g.10115A>G), c.1000-22 C>T (g.10956 C>T))	Polymorphism
7.	♀	44	CIH (c.- 10C>T (g.1170 C>T), c.370-77_-81delCAGCC (g.7188-7192 del5), c.640-16A>G (g.10115A>G), c.1000-22 C>T (g.10956 C>T))	Polymorphism
8.	♀	53	Incomplete CIH (c.-10C>T (g.1170 C>T))	Polymorphism
9.	♂	51	CIH (c.- 10C>T (g.1170 C>T), c.370-77_-81delCAGCC (g.7188-7192 del5), c.640-16A>G (g.10115A>G), c.1000-22 C>T (g.10956 C>T))	Polymorphism
10.	♂	46	CIH (c.- 10C>T (g.1170 C>T), c.370-77_-81delCAGCC (g.7188-7192 del5), c.640-16A>G (g.10115A>G), c.1000-22 C>T (g.10956 C>T))	Polymorphism
11.	♀	35	Incomplete CIH (c.-12G>A (g.1168 G>A), c.639+68A>G (g.8479>G))	Polymorphism
12.	♀	63	Incomplete CIH (c.370-77_-81delCAGCC (g.7188-7192 del5), c.640-16A>G (g.10115A>G), c.1000-22 C>T (g.10956 C>T))	Polymorphism
13.	♂	44	Incomplete CIH (c.370-77_-81delCAGCC (g.7188-7192 del5), c.640-16A>G (g.10115A>G), c.1000-22 C>T (g.10956 C>T))	Polymorphism
14.	♂	29	Incomplete CIH (c.370-77_-81delCAGCC (g.7188-7192 del5), c.640-16A>G (g.10115A>G), c.1000-22 C>T (g.10956 C>T))	Polymorphism
15.	♂	33	CIH (c.-12 G>A (g.1168 G>A), c.639+68A>G (g.8479>G), c.1000-22 C>T (g.10956 C>T))	Polymorphism
16.	♀	66	Incomplete CIH (c.370-81_-77delCAGCC (g.7188-7192del5), c.1000-22C>T (g.10956C>T))	Polymorphism
17.	♀	53	Incomplete CIH (c.-12G>A (g.1168 G>A), c.370-81_-77delCAGCC (g.7188-7192del5), c.639+68A>G (g.8479>G), c.1000-22 C>T (g.10956 C>T))	Polymorphism

and brain, most of the screening programs worldwide are directed at patient populations on renal replacement therapies, patients with LVH on unknown origin and patients with early cerebrovascular incidents.

In this paper we have summarized the efforts from our pilot study in the screening of hemodialysis patients from six hemodialysis centers in Vojvodina. In different screening studies on patients in hemodialysis units the prevalence of *GLA* mutations was up to 0.87% [13, 14], but if only pathogenic mutations were checked for, then the prevalence of FD dropped to around 0.14–0.3% [15]. Some screening programs are oriented just on male patients which is unacceptable, since female patients can be as severely affected as their male counterparts. Inconsistency of genotype–phenotype correlation in females is due to X-chromosome inactivation.

Detection of genetic variants in the *GLA* gene is essential to confirm the clinical diagnosis of FD [16]. In the present study, we sequenced the entire *GLA* gene including coding regions and flanking intron sequences to find alterations that could explain observed FD-like characteristics in our group of subjects. In 13.7% (16 of 117) of subjects, we identified five different combinations of CIHs in the *GLA* gene. The most frequent (6.8%) was the haplotype consisting of four variants (c.-10C>T, c.370-81_370- 77del-CAGCC, c.640-16A>G, c.1000-22C>T), which coincides with the results of other studies, where the frequency of this haplotype varied 8.9–3.4% in subjects with clinically suspected FD [17, 18].

According to van der Tol et al. [19], the prevalence of pathogenic variants in the *GLA* gene is 0.12%. When variants of uncertain significance (VUS) are included, this number increases to 0.62%. The frequencies reported in our study do not reflect the Van der Tol data since these intronic variants have been reported as polymorphic variants in the general population and, as in most cases, have been observed individually rather than in haplotypes. Furthermore, most studies perform sequencing only

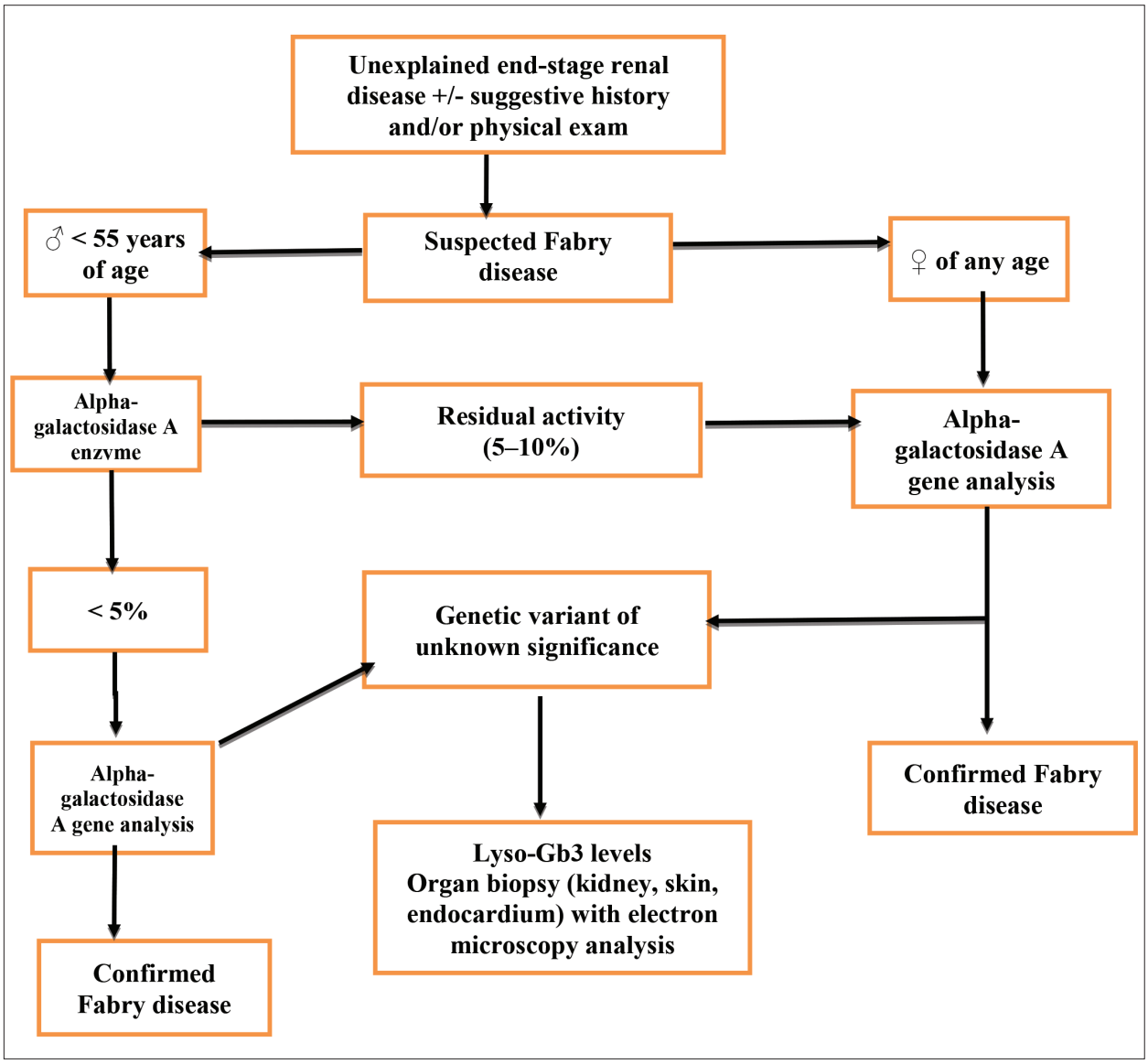

Figure 1. Diagnostic algorithm for Fabry disease patients with end-stage renal disease

through coding regions of the *GLA* gene and thus fail to detect heterozygosity in intron regions [20]. Depending on the sequencing design, CIHs often remain unidentified since these regions are not investigated routinely by gene sequencing; consequently, the prevalence of FD may be underestimated [21]. Previous reports have found that CIHs can be associated with different clinical manifestations reflecting mild renal, neurological, and cardiac disorders [22–25]. However, measuring the level of biomarker Lyso-Gb3 in DBS showed the normal level in all tested patients with detected CIHs in this study. Our results are in agreement with the findings of Ferri et al. [18], in which are detected seven different *GLA* haplotypes in control males, indicating that these CIHs are not involved in the development of FD manifestations. But Gervas-Arruga et al. [21] suggest that in patients with CIHs, environmental factors, as a pro-inflammatory state, in addition to the accumulation of Gb3 may influence the symptoms. Moreover, it has been shown that DBS lyso-Gb3 levels are not solely enough for defining diagnosis of FD and that secondary analysis should be made, due to possible false-positive or false-negative results. The estimated sensitivity of the HPLC-MS/MS method used for measuring DBS lyso-Gb3 levels is 67%, which indicates the limits of accurate/precise screening outcomes [26]. Hence, the lack of abnormality in Lyso-Gb3 levels is not a reliable parameter for excluding FD. Therefore, further analysis, such as gene expression analysis, should be performed to confirm or reject the FD diagnosis in subjects with detected CIHs in the *GLA* gene and doubtful diagnosis of disease.

High-risk population screening program represents the best and the easiest way of finding a new FD patient. A diagnostic algorithm can be proposed for screening of patients with ESRD in hemodialysis centers (Figure 1). It is based on the appropriate usage of enzyme activity testing, genetic testing as well as other types of testing that can helped physicians in determining the significance of findings during screening process. This algorithm can also

be used for patients with milder degrees of chronic kidney disease. In male patients, first step should be enzyme testing, and if needed (when enzyme levels are below normal values), further genetic testing should ensue, while in suspected female FD patients genetic testing should be the first step due to the well-known process of X-chromosome inactivation.

After appropriate high risk population screening is performed, on every index case one could find 3–5 new cases during family screening that should comprise three generations in the family tree.

Having in mind the fact that the prevalence of a pathogenic mutation in the *GLA* gene is very low in hemodialysis population, there is a place for future investigations since the total number of patients on hemodialysis in Vojvodina is around 1600 and in Serbia around 5500–6000 patients [12].

CONCLUSION

The challenges in the establishment of the precise diagnosis of FD and indications for treatment are part of today's clinical practice. In the literature has been described that the enzymatic assay is diagnostic for male patients, while female patients most often need molecular-genetic analysis to get a definite diagnosis. Moreover, the correlation between DBS enzyme activity and *GLA* variants revealed that this screening method is useful for diagnosing previously described mutations. However, when the patient presents CIH, although our study indicates a possible non-pathogenicity, the diagnosis may not be conclusive and other tools may be necessary to confirm or discard the disease. Because the genotype–phenotype correlation often shows inconsistency with FD manifestations and the effects of multiple intronic variants are not yet fully understood and seem to have individual variations, future expressional and functional studies or research focused on the discovery of possible modifier genes are necessary to confirm FD in these patients.

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Скрининг високоризичне популације на Фабријеву болест међу хемодијализним болесницима у Војводини

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САЖЕТАК

Увод/Циљ Фабријева болест (ФБ) *X*-везана је лизозомна болест складиштења која се развија као последица мутације гена алфа-галактозидазе А (ГЛА). Постоји више од 1080 познатих варијанти гена ГЛА. Неки од њих су патогени, али већина их је бенигна или представљају генетску промену која се може класификовати као генетска варијанта непознатог значаја или једноставно представљати генетски полиморфизам. Постоје две главне карактеристике ФБ, класични облик и касније варијанте болести.

Главни циљни органи код болесника са овом болешћу су бубрези, срце и нервни систем. Имајући у виду чињеницу да је ФБ ретка болест, најбољи начин за активну претрагу болесника је скрининг популације високог ризика, након чега би требало извршити породични скрининг за сваки случај пробанда.

Методе У овом раду представљамо резултате мултицентричне пилот студије која представља налазе скрининга болесника на хемодијализи на ФБ у шест хемодијализних центара у Војводини.

Резултати Идентификован је један болесник са бенигном мутацијом и 16 болесника са генетским полиморфизмом гена ГЛА. Утврдили смо да генетске промене на гену ГЛА могу бити честе, али су веома ретко од клиничког значаја и ретко доводе до манифестација ФБ.

Закључак Резултати ове скрининг студије ће нам омогућити увид у преваленцију ФБ у хемодијализној популацији и усмерити наш будући рад.

Кључне речи: Фабријева болест; хемодијализа; скрининг популације високог ризика

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Left breast radiotherapy – the impact of heart and left anterior descending artery doses to cardiovascular diseases developed eight years after treatment

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SUMMARY

Introduction/Objective Left breast cancer patients undergoing radiotherapy are at higher risk of cardiovascular diseases (CVD), as a partial volume of the heart is anatomically close to target volume. This may cause CVD in the years following cancer treatment.

The aim of this work was to develop a scoring system which identifies patients with increased risk of development of CVD, as a consequence of the left breast irradiation.

Methods The patients followed up in this study were treated during 2009. Eight years later, they were invited to participate in a study where they underwent a cardiology evaluation. Their current condition was statistically correlated to the doses received by their heart and left anterior descendant artery (LAD).

Results Out of 114 patients, 31 women were evaluable for cardiology assessment. Out of these 31 subjects, six women were with a history of CVD before cancer treatment. Four women never developed any kind of heart associated disease, while in the other 27, newly onset CVD were diagnosed ranging from hypertension to myocardial infarction, strongly positively correlated to doses to heart and LAD ($p = 0.003$). Severity of developed cardiovascular toxicity was formulated through the correlation of mean heart and mean LAD doses with CVD developed in the form of a scoring system.

Conclusion The doses to critical organs depend on patient anatomy and technique of irradiation. The cardiovascular complications are proven as consequence of radiotherapy. Scoring system based on doses received by heart and LAD is a reliable tool in predicting CVD.

Keywords: cardiotoxicity; computer-assisted radiotherapy planning; left-sided breast cancer; radiotherapy

INTRODUCTION

Breast cancer is a global health care problem worldwide and in the Republic of Serbia: 26% of all new cancer cases in Serbian female population were breast cancer patients, where approximately half of them are left-breast patients [1, 2].

Cardiovascular diseases (CVD) are the first cause of death worldwide according to the World Health Organization. Together, malignant and CVD are the cause of 3/4 of all deaths (both sexes, all ages) in Serbia, where CVD are responsible for 52.1% and cancer for 22.8% of all deaths [3]. Incidences of both diseases are rising.

Radiation therapy of the breast is known to contribute to CVD, and has been reported as a possible cause of cardiac mortality since 1950s [4].

Due to increased reporting on correlation between cancer therapy and CVD, the European Society of Cardiology and International Atomic Energy Agency have published documents on the cardiovascular toxic effect of cancer therapy, including radiotherapy and chemotherapy summarizing evidence [5, 6]. Increase in number of patients receiving chemotherapy and radiotherapy, earlier detection of disease and longer survival, lead to an increase in the number of new patients in cardiology, and may pose a global future problem.

The implementation of modern radiation therapy techniques has significantly lowered the dose to the heart and to the left descending coronary artery (LAD) both often very close to target volume. The usual doses to these two structures are far above 0.5 Gy, stated as limit in International Commission on Radiological Protection (ICRP 118).

The objective was to assess the toxic effect to these structures in our patient set, from radiotherapy aspect. The constraints given in the literature were very limited, so we conducted a retrospective analysis of treatment plans and patient conditions, to determine scoring system based on threshold values of mean heart dose (MHD) and mean LAD (MLAD) dose that would have clinical significance for development of CVDs.

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METHODS

The subjects were patients with left breast cancer treated during 2009. The Radiotherapy department of our Institute was equipped at that time with two linear accelerators, by Varian Medical Systems, Clinac series (Palo Alto, CA, USA). The computed tomography simulator was manufactured by Siemens (Munich, Germany). The treatment planning system XIO used was manufactured by Computerized Medical Systems (nowadays Elekta, Stockholm, Sweden), and the dose was calculated by the convolution algorithm. Treatment plans were generated using a 6 MV beam, which was verified according to the International Atomic Energy Agency recommendations [7, 8].

The treatment planning strategy at that time was two opposed tangential fields with enhanced dynamic wedges, and sub-fields [9].

Patients were immobilized on the Wing board® (Civco, Coralville, IO, USA) or Thorawedge® (Civco). Radiation oncologists delineated the target volume (breast), both lungs, and the heart. The LAD was not delineated at the time of treatment. Dose prescribed to the center of the breast (The International Commission on Radiation Units and Measurements reference point) was 50–60 Gy, with or without boost and supraclavicular lymph nodes, depending on the type and stage of the disease. Treatment plans were evaluated based on dose volume histograms. Patient position was verified before the first fraction on a portal imaging device and then checked weekly.

The patients in this study were invited eight years after cancer treatment, to a clinical cardiological examination. Out of the 114 patients invited, 31 attended examinations, while others either did not answer, or members of the family reported their death (three patients). Out of the subset of 31 women who responded to the cardiology examination and finally entered the study, 27 women were confirmed with CVD and only four women had never had any kind of CVD.

Patient's cardiology assessment consisted of a physical examination, an electrocardiogram, an echocardiography, an exercise stress test and included further diagnostic and therapeutic procedures. echocardiography evaluated atherosclerotic changes of aortic walls, aortic valves, left atrium dimension and volume; mitral valve; left ventricle walls and dimension, systolic (ejection fraction) and diastolic function; cavities of the right heart and systolic pressure and systolic function of the right ventricle; pulmonary artery and valve as well as pericardium. The stress tests were conducted for patients with symptoms of coronary artery disease or an irregular heart rhythm (arrhythmia). The overall conclusion of cardiologist for each patient was included in data analysis.

At the time of treatment planning in 2009, the LAD artery was not contoured. Since LAD cannot be visualized reliably on computed tomography images made for treatment planning, radiation oncologists delineated the LAD structure on the de-archived plans according to guidelines and clinical atlases, and the heart [10, 11]. The physicists re-calculated treatment plans by the clinical version of the treatment planning system XIO (Elekta) to obtain doses to these two new structures (heart and LAD).

Figures were generated by the software Origin Pro 8.0 (Northampton, MA, USA).

The patients at the hospital are treated according to the approved clinical radiation therapy protocols, by the Ethical board. Every patient signed approval for their treatment, and the cardiological study was purely volunteered. Patients examined by cardiologists got their cardiologist report, and also signed approval before any treatment was initiated based on findings from this study.

RESULTS

The patients were identified from the hospital registry, with their clinical data.

There was in total 114 left breast cancer patients irradiated during 2009, of which 92 could be successfully de-archived seven years after treatment, and returned to the treatment planning system, without any errors during the de-archiving procedure. Out of this number, 86 patients could be recalculated without an error in the treatment planning system. Six plans had unknown calculation error. Finally, 31 patients responded to the appointment with cardiologist at the time when study was conducted and were included in dose/CVD evaluation.

At the time of the treatment, age distribution was as follows: there were no patients younger than 30 and older than 80. Four patients (3.5%) of 114 patients were in their 30s, 28% of patients in their 40s, 30.7% patients in their 50s, 34.3% patients in their 60s and 3.5% of patients in their 70s.The mean age of female left breast patients during the year 2009 was 60.9 years.

The distribution of diagnosis was as follows: most of the 114 patients had ductal carcinoma (55.3%), medullar carcinoma was present in 4.4% patients, lobular carcinoma in 9.6%, mixed type in 3.5%, tubular, micropapillary and mucinous in 0.9%. In 28 patients (24.6%) there were no data on the type of carcinoma.

When the tumor grade was evaluated in the group, there were 11% of patients with G1 grade, 28% of patients with G2 grade, 13% of patients with G3 grade, while no data was present for 52% of the patients.

There were no records in the hospital database about CVD risk factors associated with heart diseases prior to cancer treatment.

The chemotherapy drugs before, during and after radiation therapy were: 32% patients received two drug combination, mainly FAC and tamoxifen, 6% received three drugs, 0.9% had four drugs, while one drug was received by 32% of patients, mainly tamoxifen; 3.5% did not have chemotherapy at all, while there was no data for 24.5% of the patients in the system (most likely received chemotherapy in local hospitals). The chemotherapy agents used were as follows: fluorouracil, doxorubicin, cyclophosphamide; adriamycin and cyclophosphamide; cyclophosphamide, methotrexate, fluorouracil, tamoxifen, docetaxel, paclitaxel, bevacizumab, trastuzumab, goserelin. All medicines listed

Table 1. Doses to heart, LAD, and left lungs (min., max., and mean dose per patient and per cardiologically assessed cohort)

Mean dose range of heart (Gy)	Number of patients	Heart				LAD				Left lung			
		Avg. of mean dose (Gy)	Avg. of min. dose (Gy)	Avg. of max. dose (Gy)	Avg. volume (cm^3)	Avg. of mean dose (Gy)	Avg. of min. dose (Gy)	Avg. of max. dose (Gy)	Avg. volume (cm^3)	Avg. of mean dose (Gy)	Avg. of min. dose (Gy)	Avg. of max. dose (Gy)	Avg. volume (cm^3)
0–2 Gy	7	1.58	0.18	46.92	654.2	8.92	0.72	33.22	4.73	4.88	0.05	59.46	1321.42
2–4 Gy	8	3.07	0.27	55.94	708.9	21.56	0.95	53.97	5.23	6.22	0.11	60.46	1172.63
4–6 Gy	8	4.92	0.32	55.8	652.13	25.39	1.18	55.11	4.42	8.03	0.18	60.71	1208.6
6–14 Gy	8	8.82	0.7	56.6	897.4	30.57	2.77	55.65	6.34	8.02	0.3	57.9	1176.8
Data for all patients	31	4.6	0.37	53.81	727.17	21.63	1.4	49.49	5.18	6.78	0.16	59.63	1219.8

Avg. – average; LAD – left anterior descending artery

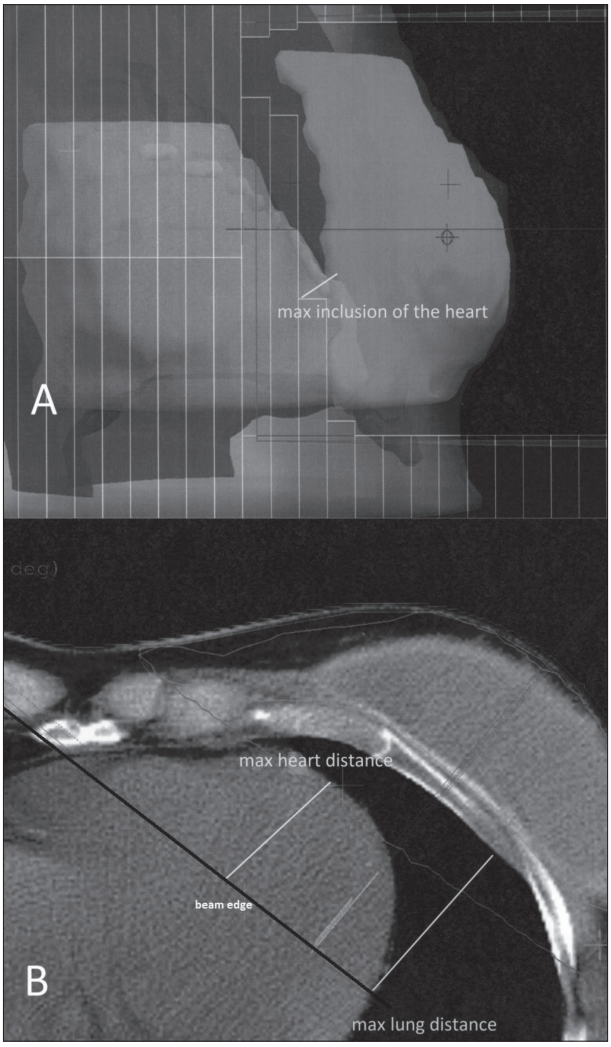

Figure 1. Beam's eye view of the heart (A) and measurements of maximum heart and lung distance (B)

(fluorouracil, doxorubicin, cyclophosphamide; adriamycin and cyclophosphamide; cyclophosphamide, methotrexate, fluorouracil, tamoxifen, docetaxel, paclitaxel, bevacizumab, trastuzumab, goserelin) have some degree of proven cardiotoxic effect [4, 12]. It has been confirmed in literature that tamoxifen cannot be associated with an increased incidence of heart diseases [13] but is correlated to an increased incidence of venous thrombosis and stroke [14].

Radiotherapy treatment

Prescribed doses to the breast were 60 Gy (66.6%) and 50 Gy (33.4%). The prescription to the supraclavicular region was 50 Gy (36%), while 20.2% of patients received an additional boost to the breast (10–12 Gy).

There were 58% patients who were treated to the breast only, 15% breast with boost, 25% breast with supraclavicular field and 2% breast, boost and supraclavicular field.

Calculated doses to heart and LAD and volumes are given in Table 1. The mean doses to the heart volume ranged among patients between 0.3 Gy and 62.4 Gy, with average of MHD of 4.6 Gy. The mean volume of the heart was 727 cm^3. As for the left lung, the maximum dose was 65.5 Gy (mean maximum 59.6 Gy), and average mean dose in the group 6.8 Gy. LAD, which was newly delineated, after the de-archiving of the treatment plans, received maximum of 62.1 Gy while the mean dose was 21.6 Gy. The mean volume of delineated LAD was 5.2 cm^3. The trend of maximum doses to LAD and heart, as well as lungs follow the increase of mean doses: higher the mean dose – higher the maximum dose.

Maximum heart distance (distance from radiation field edge to heart edge) and maximum lung distance (distance from radiation field edge to chest wall) were measured. The heart entered the irradiated volume by a mean length of 3.5 cm (but was shielded by multileaf collimator), while the left lung was included with a mean of 3.7 cm (also shielded by multileaf collimator). The heart was exposed to open beam by a mean value 1.4 cm. Measurements were done from the beam edge and presented in the Figure 1.

Cardiovascular evaluation

In total, 31 patients responded to the appointment with cardiologist. Out of this number, only four women never had any kind of heart associated diseases, while in other 27 women CVDs found were ranging from simpler hypertension to very complicated myocardial infarction (in total three patients). Out of the 31 evaluable patients, six patients had had a history of CVD before the treatment of breast cancer. Additionally, eight patients (26%) developed some form of cardiovascular disease during the first five years after treatment (phlebothrombosis, cardiomyopathy and myocardial infarction).

The data of all examined patients after cancer treatment are presented in Table 2.

Table 2. Cardiovascular diseases (CVD) in examined patients and complications developed eight years after treatment

Cardiovascular diseases (31 patients)	No. of patients with CVD*
Hypertension	14
Angina pectoris	3
Mitral valve insufficiency	2
Aortic valve stenosis	1
Tricuspid valve insufficiency	2
Venous disease	2
Hypertrophic cardiomyopathy	6
Chest pain (Stenocardia)	3
Phlebothrombosis	3
Myocardial infarction	3

*Some patients had more than one complication

The MHD of examined patients and their MLAD dose were correlated on Figure 2. The positive strong correlation ($r = 0.7772$, $p < 0.00001$) between MHD and MLAD dose was found.

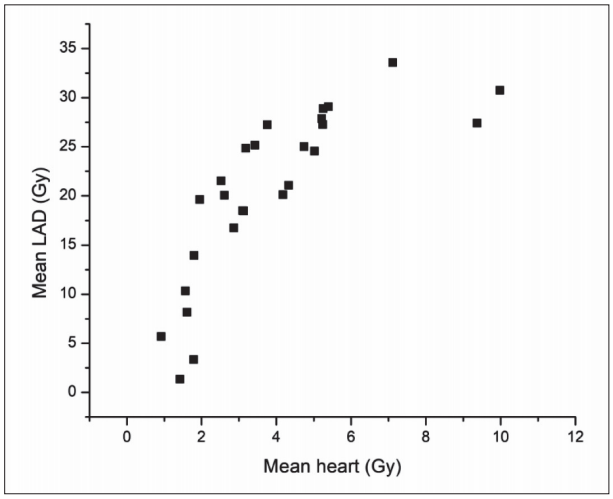

Figure 2. Correlation of mean heart dose to mean left anterior descending artery (LAD) dose for examined patients

Cardiovascular complications found in analyzed group were as follows: myocardial dysfunction and heart failure, coronary artery disease, valvular disease, arrhythmias, arterial hypertension, thromboembolic disease, peripheral vascular disease and stroke, pulmonary hypertension and pericardial complications.

Patients were graded according to the cardiotoxicity grading system given in literature-measured ejection fraction and other findings [14].

According to severity of CVD we divided all patients in four groups: Group 1 – venous disease and/or hypertension, group 2 – group 1 + arrythmia or coronary disease (angina pectoris), group 3 – group 2 + hypertrophy and/or cardiomyopathy, group 4 – group 3 + stenocardia/myocardial infarction. In the next step, we defined threshold values for MHD and MLAD for each group. Analyzing given data, we concluded that the results show a scoring system. Finally, after correlation od CVD group and mean doses values, our results have showed that patients can be divided in four scores (1–4) which is presented in Table 3.

Table 3. Scoring system developed based on complications and dose received by critical organs

Score	CVD complication groups	Mean heart dose (Gy)	MLAD artery dose (Gy)
1	Group 1: venous disease and/or hypertension	$x < 2$	$y < 10$
2	Group 2: Group 1 plus arrythmia or coronary disease (angina pectoris)	$2 < x < 3$	$10 < y < 20$
3	Group 3: Group 2 plus hypertrophy and/or cardiomyopathy	$3 < x < 5$	$20 < y < 25$
4	Group 4: Group 3 plus stenocardia/myocardial infarction	$x > 5$	$y > 25$

MLAD – mean LAD; LAD – left anterior descending artery; x – mean heart dose (Gy); y – MLAD artery dose (Gy); CVD – cardiovascular diseases

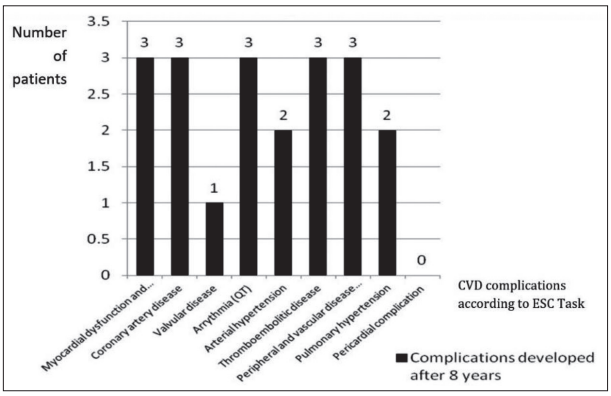

Figure 3. Cardiology complications developed in the examined group of 31 patient

Score 1 were patients with MHD < 2 Gy and MLAD < 10 Gy; score 2 – patients with MHD < 3 Gy and MLAD < 20 Gy; score 3 – patients with MHD < 5 Gy and MLAD < 25 Gy; score 4 – patients with MHD > 5 Gy and MLAD > 25 Gy. Our results showed that patients with MHD < 2 Gy and MLAD < 10 Gy had venous disease and/or hypertension; patients with MHD < 3 Gy and MLAD < 20 Gy developed venous disease and/or hypertension plus arrhythmia or coronary disease (angina pectoris); patients with MHD < 5 Gy and MLAD < 25 Gy developed venous disease, hypertension, arrhythmia, coronary disease (angina pectoris) and hypertrophy and/or cardiomyopathy; and patients with MHD > 5 Gy and MLAD > 25 Gy developed all previous diseases plus stenocardia and/or myocardial infarction, as shown in Table 3. The scoring system developed in this work is based on possible complication severity correlated to doses received by critical organs in our data, ranging from 1 to 4, and correlates with the values of doses to heart and LAD found in literature [11].

The positive correlations between mean doses to heart and CVD developed ($r = 0.9803$, $p < 0.003$), as well as

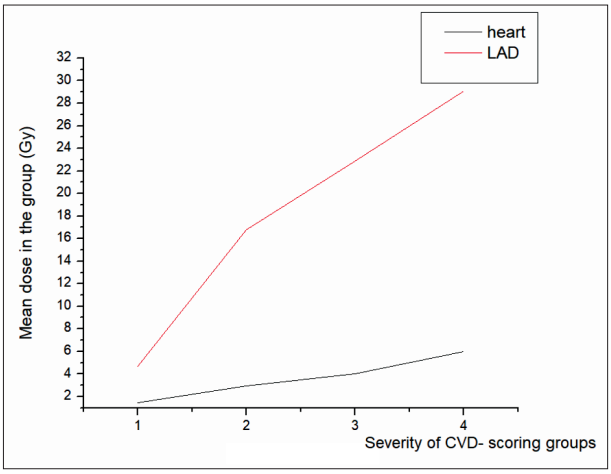

Figure 4. Correlation of the mean heart dose, mean left anterior descending artery (LAD) dose in the group and cardiovascular (CVD) complication scoring system

MLAD and clinical CVD developed ($r = 0.9803$, $p < 0.003$), significant at $p < 0.05$, was found on Figure 4.

At the time of treatment planning of these patients, only QUANTEC (Seoul, South Korea) parameters were available for treatment plan evaluation. According to QUANTEC, heart and its pericardium should be irradiated within following limits: mean dose < 26 Gy (our results in cardiologically examined group was 4.6 Gy), V30 Gy < 46% (our result 3.4%), and V25 Gy < 10% (our result 4%). LAD dose is not mentioned in QUANTEC. Lung V20 Gy according to QUANTEC should be < 30% (our result 10.4%) and mean dose with least complication probability 7 Gy (our result 6.8 Gy). Generally speaking, the average treatment planning dosimetry results in treatment plans were far below the indicated upper limits for long term cardiac or pulmonary complication probability, but contrary to the stated QUANTEC parameters, some form of CVD complications developed in majority of patients.

DISCUSSION

Breast cancer as the most common cancer in women worldwide, is curable in early stages thus survival can be long term. Since radiation therapy is an effective tool in the treatment of breast cancer, where structures in the heart, such as the LAD, are exposed to radiation, sparing the heart and its structures becomes significant issue in breast treatment planning.

It is evident from literature that the risk of major cardiovascular events becomes more pronounced five years after radiation therapy and continues to increase even for three more decades [15, 16]. Other studies reported cardiac events 10 years after initial radiotherapy treatment. The worst-case scenario is when irradiation worsens an already present CVD, or accelerates its appearance in cases where risk factors are present. Our results are in line with these findings: out of 31 examined patients, three had myocardial infarction and all of them had previous CVD before radiotherapy.

Between treatment and cardiology assessment, three patients had died, others could not be reached or could not show up for the appointment, while only 1/3 of recalculated and 1/4 of the total number of left breast patients treated in 2009, actually responded to cardiology evaluation.

There is evidence of strong correlation of dose distribution to the heart with the later developing cardiac effects which defines the increasing risk of major cardiac effect by MHD increase by 7.4% per 1 Gy of MHD [16]. The main strength of this paper is that it clearly correlates the doses received by the heart and LAD, to the cardiovascular complications developed after treatment. The cardiological assessment data of cancer survivors are now used as reference, for treatment plan strategy and evaluation. The results presented in our study correlate with published data [11, 17].

The most frequent cardiac problem reported during radiotherapy in the literature is acute pericarditis, pericardial effusion and arrhythmia [14]. In our investigation, none of our patients have reported cardiac problems during their treatment (according to hospital database reports). However, from the database of our cardiovascular clinic, there were two patients who requested clinical appointment immediately after radiation therapy and eight patients in the following five years.

During radiotherapy treatment planning and delivery, special attention should be paid to the use of cardiac shielding opportunities and modern techniques, such that the dose volume histogram reflects the need for sparing the heart and heart structures [18]. The implementation of deep inspiration breath hold (free or assisted) is the easiest way to naturally shield the heart by increasing the volume of air between the heart and chest wall where the tangential field edge is positioned [19, 20, 21], by the use of arc techniques – volumetric modulated arc therapy (VMAT) or advanced robotic accelerators [22, 23]. The optimal option for a significant decrease of dose to both lungs and heart is prone positioning but also use of immobilizing devices dedicated for both prone and supine breast radiotherapy [23, 24]. Assisted voluntary breath hold (ABC, Elekta), VMAT and both prone and supine breast irradiation are now available forms of treatment at our clinic.

Although all dosimetry parameters from the dose-volume histogram of treatment plans of examined patients were far below any clinically known limitation, it is clear that patient's heart and LAD may be severely damaged by radiation, especially if previous cardiovascular disease was registered [24]. Our results also confirm these findings. Severity degree of cardiovascular disease can be predicted according to the MHD and MLAD artery dose together, as we did in this work through the scoring system generated, but more detailed constraints are needed [25, 26]. Patients treated with radiotherapy for left-sided breast cancer, should remain in cardiology follow-up to diagnose possible cardiotoxicity [27].

The limitation of the current study is limited number of patients. Out of 114 initially selected patients, only 31 entered the final analysis. Definitive conclusions should be made after conducting prospective well-designed trial with more patients included.

The management of cardio-oncological patients requires a multidisciplinary approach to provide optimal care for patients. In that respect, these specialties will soon have to bring about new joint protocols, on management of cardio-oncological patients [28]. Propositions on management of cardiac toxicity are still under development and additional studies and research are needed, but it is recognized that a model predicting cardiology complication due to therapy is needed [29, 30]. The scoring system we proposed here serves in our institution as a predictor of CVD complications.

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Conflict of interest: None declared.

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Радиотерапија леве дојке – утицај доза на срце и леву предњу десцедентну артерију и развој кардиоваскуларне болести осам година после третмана

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САЖЕТАК

Увод/Циљ Болеснице оболеле од карцинома леве дојке, лечене радиолошком терапијом, у већем су ризику од настанка кардиоваскуларних болести (КВБ), с обзиром на то да је анатомским положајем део срца често у непосредној близини третиране регије, што у годинама после третмана може да изазове појаву кардиоваскуларних болести.

Циљ овог рада био је развој систем скоровања који идентификује болеснице са повећаним ризиком од развоја кардиоваскуларних болести, као последицу зрачења леве дојке.

Методе Болеснице које су праћене током ове студије зрачене су током 2009. године. Осам година касније позване су да учествују у студији током које су прегледане од стране кардиолога. Њихов налаз корелиран је са дозама које су током радиолошке терапије примили срце и лева предња десцедентна коронарна артерија (ЛАД).

Резултати Од 114 позваних болесница којима је током 2009. године зрачена лева дојка, 31 болесница се одазвала позиву на кардиолошки преглед. Од овог броја, шест болесница је имало кардиоваскуларну болест пре лечења малигне болести. Четири жене нису никад развиле ниједну кардиоваскуларну болест до кардиолошког прегледа, док су осталих 27 болесница развиле бар једну од КВБ, од хипертензије до инфаркта миокарда. Тежина кардиоваскуларне болести је у снажној позитивној корелацији са средњом дозом на срце и средњом дозом на ЛАД ($p = 0,003$).

Закључак Доза на критичне органе зависи од анатомије болесника, али и од радиотерапијске технике која је спроведена. Кардиоваскуларне компликације су доказана последица зрачења леве дојке. Тежина компликација зависи од односа између доза на срце и ЛАД изражена кроз систем скоровања. Систем скоровања заснован на дозама које примају срце и ЛАД поуздан је у предикцији развоја КВБ.

Кључне речи: кардиотоксичност; компјутерско планирање радиотерапије; карцином леве дојке; радиотерапија

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Tumor-mimicking musculoskeletal infectious lesions – experience of a single referral center

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SUMMARY
Introduction/Objectives Bone and soft tissue infections might mimic bone and soft tissue tumors. Therefore, differential diagnosis is important to prevent errors in treatment. This report aims to present the data of patients with indistinct clinical and radiological findings mimicking benign and malignant bone and soft tissue tumors, which were later diagnosed as inflammatory infections.
Methods A retrospective chart review of the clinical, microbiological, radiologic, and pathologic findings of patients presented with a presumed diagnosis of a possible malignant lesion was performed.
Results The study included 21 patients with a median age (IQR) of 37 (1 month – 72 years) years, and 13 (61%) patients were men. In total, 16 (76%) patients were admitted to the hospital with complaints of pain. The diagnoses were hydatid cyst, tuberculous osteomyelitis, cat-scratch disease, chronic osteomyelitis, subacute osteomyelitis, and soft tissue abscess. All patients were treated depending on the diagnosis of the lesion.
Conclusion There are chances of misdiagnosis due to shared common characteristics of tumoral and infectious lesions which might be mildly increased inflammatory markers with deeply seated non-mobile soft tissue masses and aggressive periosteal reactions and/or bone destruction patterns. So, each pseudotumoral lesion due to possible infectious causes should be histopathologically examined and correlated with other clinical and laboratory data in order to achieve a final diagnosis
Keywords: biopsy; imaging; hydatid cyst; tuberculosis; soft tissue abscess; osteomyelitis

INTRODUCTION

Bone and soft tissue sarcomas are extremely rare malignant tumors, which are mesenchymal in origin. Nearly 21% of all pediatric solid malignant tumors and less than 1% of all adult solid malignant tumors are sarcomas [1]. Despite their rarity, sarcomas represent the third leading tumor type in young populations [2].

Benign tumors are classified according to the matrix protein produced by the tumor cells, such as bone, fibrous tissue, cartilage, fat, or blood vessels [3]. Skin and soft tissue infections are characterized by microbial invasion of the layers of the skin and/or underlying soft tissues [4]. Although they are usually caused by bacterial infections, fungal, viruses, parasitic, or mycobacterial aetiologias might be detected [5].

Osteomyelitis is an inflammation of the bone and bone marrow, most caused by bacterial and rarely fungal, parasitic, or *mycobacterium* species [6].

Although data are scarce, infections of the bone and soft tissue might mimic tumoral lesions. However, the treatment approach for the two conditions is very different; hence, differential diagnosis is important to prevent errors in treatment [7, 8].

In this paper, we aimed to present the data of patients with indistinct clinical and radiological findings, mimicking benign and malignant bone and soft tissue tumors, which were later proven to be infectious lesions on biopsy.

METHODS

The study was conducted at the Istanbul Medeniyet University Medical School, Goztepe Training and Research Hospital. The patients signed voluntary consent forms, and the study complied with the ethical standards of the Helsinki Declaration. This is a retrospective study from a prospectively collected database of a tertiary referral center for musculoskeletal tumors. The data of patients with suspected bone and soft tissue tumors or bone sarcomas that were later diagnosed as bone and soft tissue infections by clinical, microbiological, radiological, and pathological methods between 2015

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and 2021 were analyzed parameters analyzed included age, sex, comorbid conditions, main symptoms on admission, history of previous infections, tuberculin skin test (TST), white blood cell (WBC) count, C-reactive protein (CRP) level, sedimentation, direct chest radiographs, computerized tomography (CT), magnetic resonance imaging (MRI) findings, preoperative diagnosis, surgical techniques, microbiological analyses, and pathological diagnoses.

RESULTS

This study included 21 patients. Table 1 presents the overall characteristics of the study population. The median age of the patients was 37 years (1 month – 72 years), and 13 (61%) were male. The most common symptom was pain; 16 patients in the study cohort were admitted to the hospital with complaints of pain.

According to the results of the investigations, four patients had tuberculous (TB) osteomyelitis, two had hydatid cyst, three had cat-scratch disease (CSD), five had subacute osteomyelitis, and one had chronic osteomyelitis. Six patients were diagnosed with soft tissue abscesses mimicking soft tissue sarcoma or soft tissue lymphoma (patient numbers 3, 7, 8, 14, 19, and 20). Two of these patients had been previously diagnosed with chondroma and leiomyosarcoma, respectively, and had undergone resection (patients 7 and 3). The patients were followed up for possible recurrences and metastases.

One patient had a soft tissue mass lesion abutting the sciatic nerve (patient number 14). A preoperative biopsy was consistent with lipoma; however, pathological examination of the excised lesion revealed accompanying chronic fungal infection. One patient with a soft tissue abscess had

Figure 1. Penumbra sign at the proximal humerus of the patient with tuberculous osteomyelitis

Table 1. Characteristics of the patients

Case	Age/ Sex	Comorbidities /Main symptom	Location	Previous history of Infectious disease /Pulmonary involvement / TST	WBC (10^3/uL) / CRP (mg/dl) / Sedimentation (mm/hour)	Pathology	Microbiological analysis	Surgery	Radiology	Treatment
1	15/ M	-/Pain	Cruris	- / - / -	11.1/ 5.82 / 44	Chronic suppurative osteomyelitis	CN	Biopsy and curettage	Medullary oedema	TEIC/DA
2	34/ F	-/Pain	Sacrum	- / - / 17mm at 72 hours	5.2 / 0.7 / 117	Granulomatous infection	CN	Open biopsy	Lytic expansile lesion	RMP/EMB/ INH/PYR
3	47/ F	-/Pain	Shoulder	- / - / -	6.79 / 0.1 / 18	Lipogranulomatous inflammation	CN	Tumoral bed excision	Soft tissue lesion	TEIC/DA/ CIP
4	1 m/ M	-/Pseudoparalysis of right extremity	Thigh	Nosocomial sepsis / - / -	15.8 / 9.22 / 98	Osteomyelitis and septic arthritis	CN	Biopsy and debridement	Lamellar type periosteal. reaction in femur	VA/AK
5	67/ M	-/Pain	Shoulder	- / + / 17 mm at 72 hours	6.8 / 10.3 / 74	Chronic granulomatous inflammation	*S. hominis M tuberculosis complex*	Biopsy and resection arthroplasty of right proximal humerus	Penumbra sign	RMP/EMB/ INH/PYR
6	35/ M	-/Sciatalgia	Gluteal area	- / - / -	6.63 / 3.94 / 25	Consistent with cyst hydatic	CN	Excisional biopsy	Irregular cystic lesion	ALB
7	49/ F	Chordoma/Pain and mass lesion	Sacrum	- / Chordoma metastases / -	8.3 / 21.3 / 20	Dense abscess	CN	Trucut biopsy	Mass lesion posterior to sacrum	CIP/SAM/ AMC
8	39/ F	-/Pain	Groin	- / - / -	6.18 / 0.1 / 25	Consistent with abscess	CN	Fine needle aspiration biopsy	Cystic lesion	TPZ/DA/CIP
9	10/ F	-/Pain	Cruris	- / - / -	10.5 / 0.1 / 40	Inflammatory granulation tissue	CN	Biopsy and curettage	Penumbra sign.	CFZ/AMC

10	9/ M	-/Pain	Symphysis pubis	- / - / -	14.3 / 6 / 117	Inflammatory granulation tissue	*S. aureus*	Curettage	Lytic lesions	TEIC
11	18/ M	-/Pain	Gluteal area	- / +/ -	10.8 / 9 / 47	Inflammatory granulation tissue	CN	Biopsy	Cortical erosive lesion.	CFZ/DA
12	10/ F	-/Pain	Ancle	- / - / -	10.4 / 1.34 / 13	Subacute osteomyelitis	CN	Biopsy and curettage	Bone lesion at distal tibia	CXM
13	28/ M	-/Pain and mass lesion	Supracondylar area	- / - / -	8.4 / 0.1 / 18	Granulomatous inflammation, abscess	CN	Excision	Soft tissue lesion	AZM
14	51/ F	Breast carcinoma / Sciatalgia	Gluteal area	- / - / -	6.89 / 6.7 / 21	Fatty necrotic areas and fungal hyphae	CN	Biopsy and excision	Space-occupying mass lesion	None
15	39/ M	-/Pain and mass lesion	Supracondylar area	- / - / -	7.1/ 6.4 / 17	Granulomatous inflammation consistent with *Bartonella henselae*	CN	Biopsy	Nodular lesion	AZM
16	65/ M	-/Pain	Lumbar region	- / - / -	16.3 / 6.54 / 87	Chronic granulomatous inflammation	*M. tuberculosis complex*	Open transpedicular vertebral biopsy	L3 vertebral lesion	RMP/EMB/ INH/PYR
17	33/ M	-/Mass lesion	Elbow	- / - / -	12.1 / 3.5 / 14	Focal dense plasma cells and lymphoid tissue	CN	Biopsy	Soft tissue lesion	AZM
18	72/ M	-/Pain	Tibia	- / - / -	11.2 / 0.1 / 12	Consistent with cyst hydatic	CN	Biopsy and curettage	Cystic mass lesion	ALB
19	63/ M	-/Mass lesion	Inguinal area	- / - / -	11.1 / 4.8 / 29	Wide suppurative inflammation with microorganisms consistent actinomyces	CN	Biopsy	Soft tissue lesion	AX
20	23/ F	-/Pain and mass lesion	Thigh	- / - / -	16.1 / 2.7 / 4	Hyperkeratosis, acanthosis and epithelial spongious changes	CN	Biopsy	Soft tissue lesion	TPZ/DA/ CIP/ DAP
21	44/ M	HCV-HBV / Pain and mass lesion	Thigh	- / - / -	6.3 / 19.23 / 83	Granulomatous infection	CN	Biopsy and curettage	Mass soft tissue lesion	RMP/EMB/ INH/PYR

WBC – white blood cells; CRP – C-reactive protein; ALB – albendazole; AK – amikacin; AMC – amoksicilin-clavulonate; AZM – azithromycin; AX – amoksicilin; CFZ – cefazole; CN – culture negative; CIP – ciproflaksosin; CXM – cefuroxime; DA – clindamycin; DAP – daptomycin; EMB – ethambutole; INH – izoniazid; PYR – pyrasinamid; RMP – rifampin; SAM – ampicilin-sulbactam; TEIC – teicoplanin; TPZ – piperacilin-tazobactam; VA – vankomicin

an infection with *Actinomyces* species, which was detected on histopathological examination.

Three patients with CSD had mass lesions on the medial side of the epitrochlear region of the elbow. All of them had a one-week-long history of a cat-scratch, and their diagnoses were confirmed on preoperative biopsy. Their symptoms completely resolved after one week of antibiotic therapy (patient numbers 13, 15, 17).

Only one patient with subacute osteomyelitis at the symphysis pubis had a positive culture which revealed Staphylococcus aureus (patient number 10). Two patients had a lesion at the proximal tibiae mimicking Ewing sarcoma and eosinophilic granuloma (patients 1 and 9), and one patient each had a lesion at the distal tibia and the ischium, mimicking eosinophilic granuloma (patients 12 and 11).

One patient with a tuberculosis lesion had a monofocal, extremely painful involvement of the right proximal humerus, mimicking a metastatic bone lesion. Resection arthroplasty was performed after preoperative biopsy and culture, which revealed *Mycobacterium tuberculosis (M. tuberculosis)* infection (patient number 5) (Figure 1). Another patient with TB had multiple lesions of the musculoskeletal system, mimicking metastatic primary bone tumor or metastatic carcinoma. The diagnoses were confirmed with a bone biopsy, which revealed granulomatous inflammation (patient number 2) (Figure 2). One patient had gradually progressive hip pain with decreased range of motion of the hip joint. His roentgenograms revealed lytic bone lesions in the femoral head and neck. MRI revealed destruction of the femoral neck and head with a soft tissue mass mimicking an abscess and a tumoral lesion with contrast enhancement. Biopsy revealed granulomatous abscesses consistent with TB. His erythrocyte sedimentation rate decreased dramatically after the initiation of anti-tuberculosis drug regimen (patient number 21). One patient had low back pain and lesions at L3 and L5, mimicking metastatic bone lesions. However, a biopsy revealed granulomatous inflammation (patient number 16). Although his TST and interferon-γ (IFN-γ) release assay tests

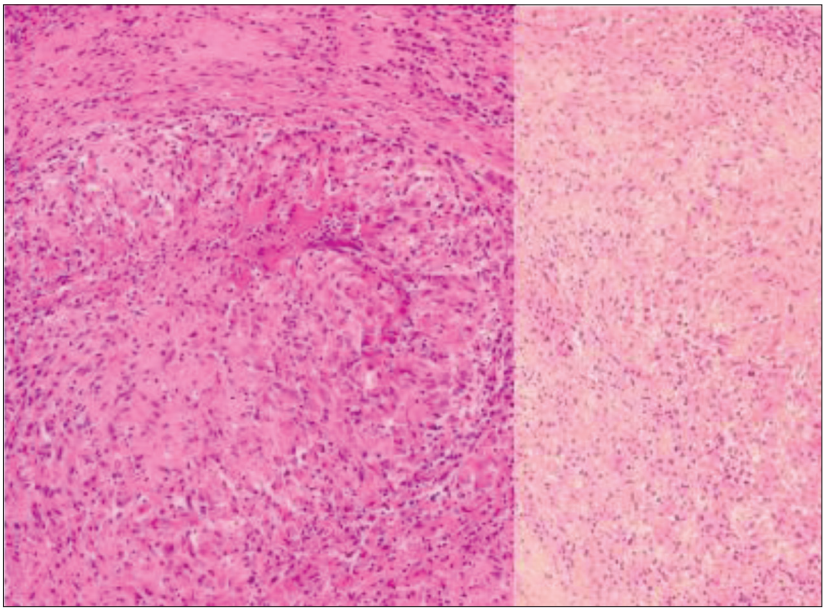

Figure 2. "Langhans" type multinuclear giant cells and lymphocytes (Hematoxylin eosin 400 ×) in granulomas consisting of epithelioid (Histological examination of serial sections revealed granuloma formation accompanied by small focal necrosis in the central area with surrounding lymphocytes; "Langhans" type giant cells were seen embedded in the fibrous stroma (in the center of the granuloma) (black arrow); in the immunohistochemical examination, CD68 was positive in histiocytes

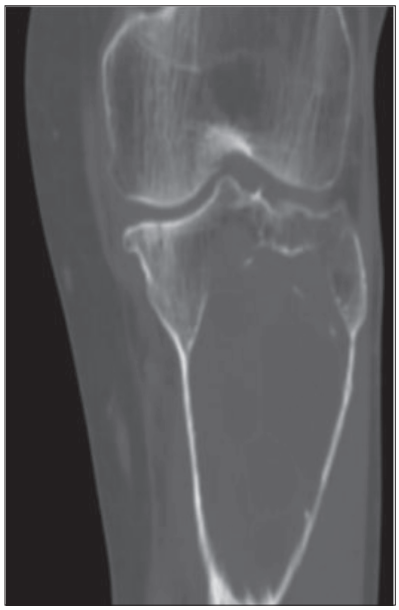

Figure 3. Cystic bone lesion at the proximal tibia due to cyst hydatid mimicking primary bone tumor or metastases

were negative, *Mycobacterium tuberculosis* was detected on the culture. Two patients with TB had completed their anti-TB medications, and two others were still under treatment.

One patient had a bone lesion on the proximal tibia, mimicking a primary bone tumor or metastases. Preoperative biopsy revealed a hydatid cyst which was removed by intralesional curettage and irrigation with hypertonic saline solution, and cement fixation was performed (patient number 18) (Figure 3). One patient had a hydatid cyst in the pelvic soft tissue anterior to the sacrum. Removal of the cysts and irrigation with hypertonic saline solution were performed (patient number 6).

DISCUSSION

In this report, we aimed to present the data of patients who had been referred to our clinic for a suspected bone or soft tissue tumor but were diagnosed with musculoskeletal infections based on their past medical history, physical examination, radiological work-up, and biopsy.

It is essential to differentiate bone and soft tissue infections from benign and malignant bone and soft tissue tumors to proceed with appropriate treatment [9]. A previous history of trauma (open fracture); previous surgeries, immune status of the patient; or presence of any associated disease affecting the immunity, such as diabetes mellitus, renal or hepatic failure, malignancy, malnutrition, alcoholism, intravenous drug use, and tuberculosis infection should be investigated [6, 10]. Any symptoms, such as, poor appetite, malaise, fever, and characteristics of the pain should be investigated. Scars of previous incisions, redness, swelling, or presence of a fistula should be noted [9, 10]. Contact with domestic animals is also important to rule out certain infections such as CSD [11].

Laboratory tests should include blood hemograms, erythrocyte sedimentation rate and CRP levels [9, 10]. Specific serological tests should be performed for the diagnosis of CSD [11], and hydatid cysts [12]. For suspected TB infections, interferon-γ (IFN-γ) release assay or TSTs should be performed [13].

Roentgenography is important for detecting periosteal reactions and bone destruction patterns [14, 15]. CT is important for visualizing details of the bone cortex, and MRI is important for the evaluation of medullary and soft tissue. A periosteal reaction occurs when tumoral lesions, infection, or trauma separate the periosteum from the bone cortex. Benign periosteal reactions usually have a uniform appearance with a single solid layer. The multi-layered (onion- skin) type is an intermediate type between solid periosteal reaction and aggressive periosteal reactions such as spiculated-sunburst or Codman's triangle [15]. Although multi-layered and other more aggressive periosteal reactions usually occur in malignant bone tumors, especially in Ewing sarcoma and osteosarcoma [15, 16], they might also be detected in benign bone lesions such as Langerhans cell histiocytosis, which is characterized by the proliferation of dendritic cells and macrophages, and even in osteomyelitis [17], as in our case with chronic osteomyelitis and septic arthritis in a premature child.

There are three major bone destruction patterns according to the Lodwick classification. Geographic bone destruction is characterized by a narrow zone of transition of the lesion, which is easily separated from the surrounding normal bone. A sclerotic margin of variable thickness encompasses the lesion in type A [18, 19]. Geographic

bone destruction usually occurs in benign or benign aggressive bone tumors and osteomyelitis, as in our patient with subacute osteomyelitis.

Moth-eaten and permeative-type bone lesions are usually accompanied by primary malignant and metastatic bone lesions [16]. Osteomyelitis might occasionally cause these two types of aggressive bone destruction patterns [16], as in our case with TB osteomyelitis. Opportunistic pathogens, especially fungal musculoskeletal infections, might be detected in patients with immunocompromised status [6], as in our case with a history of breast carcinoma and chemotherapy.

TB osteomyelitis of the bone is a rare condition caused by *Mycobacterium tuberculosis*. The bacillus usually prefers the spine and large joints due to the rich vascular supply of the vertebrae and the growth plates of the long bones [20]. Although rare, TB of the bone is an important cause of lytic bone lesions. Pain is the main symptom of bone TB. Fever and systemic symptoms might not occur until the late stages of musculoskeletal TB [20, 21].

Due to the low bacterial load in musculoskeletal tuberculosis, the possibility of detecting the *Mycobacterium* is very low, and for an accurate diagnosis of *M. tuberculosis*, multiple biopsies should be performed, and more time and attention should be paid during microscopy [21]. Biopsy revealed granulomatous osteomyelitis in all our patients with musculoskeletal tuberculosis.

Granulomatous inflammation mimicking bone and soft tissue tumors may be caused by various etiologic factors including infection, autoimmune, toxic allergic, drug and neoplastic conditions.

In cases of negative microbiological findings, differential diagnosis depends on meticulous assignment of clinical findings including laboratory test results, and examination of the histopathological specimens by an expert musculoskeletal pathologist [22, 23].

The penumbra sign is an area of a relatively hyperintense signal between the intermediate to low-signal intensity abscess cavity and the adjacent edematous or sclerotic bone marrow on unenhanced T1-weighted imaging. On histology, the cases exhibiting the penumbra sign showed a rim of active, highly vascular, inflamed granulation tissue around the abscess cavity. The penumbra sign on T1-weighted MRI in subacute and chronic osteomyelitis is very important, with high sensitivity and specificity for differentiating these lesions from musculoskeletal tumors [24, 25]. Other cases of subacute osteomyelitis with lytic bone lesions suspected to have benign or benign aggressive bone tumors were confirmed by histopathological examination because the radiographic findings of soft tissue swelling, cortical tunnelling, focal cancellous lysis, focal cortical resorption, and a periosteal reaction were a diagnostic challenge in these cases [7]. Two of our cases, one with TB osteomyelitis and the other with subacute osteomyelitis due to Staphylococcus infection, displayed the penumbra sign.

Cystic Echinococcosis (CE) occurs in humans as a result of infection by the cestodes of the genus *Echinococcus*. Characteristically, CE lesions are found in the liver and lungs; however, any part of the body might be affected [26, 27]. Skeletal lesions usually involve the vertebrae and rarely the long bones. The cestodes first settle in the epiphyseal and metaphyseal areas because of their relatively high blood supply. Here the cysts proliferate and enlarge, which then lead to a multicystic appearance. They might resemble musculoskeletal tumors with medullary and cortical destruction, and surgery is usually the first therapeutic approach [27]. Curettage and cementation with fixation were performed in our patient with a proximal tibial hydatid cyst. Primary hydatidosis of the skeletal muscle is extremely rare [28]. Excision was performed in our patient with a soft tissue hydatid cyst localized to the pelvis. We also used hypertonic solution locally during surgery in both our cases with bone and soft tissue hydatid cyst, and oral albendazole (15 mg/kg/day) was administered after surgery.

Multiorgan involvement was not observed with positron emission tomography (PET-CT) imaging performed after biopsy findings of four patients with TB osteomyelitis, two patients with hydatic cysts and five patients with subacute osteomyelitis. In cases of nonspecific histopathological findings and culture negative results, differential diagnosis was made because tumoral cells were not detected in the histopathogical specimens of our patients, but two patients (number 2 and 16) had multifocal bone involvement detected with PET-CT imaging.

Microorganisms such as *Mycobacterium tuberculosis* and *Brucella* species should not be overlooked into the etiology of pseudo tumoral infectious lesions in countries with high endemicity. Brucellosis was excluded in our patients with serological test results in addition to culture with increased sensitivity and specificity.

Musculoskeletal tuberculosis, on the other hand, is unfortunately still posing a serious problem and often causes diagnostic challenge especially in developing countries with low incomes and limited health resources. It should be noted that the TST and interferon gamma release assay tests are not highly sensitive and specific for the diagnosis of bone tuberculosis [29, 30].

In our patients with clinically suspected tuberculosis infection but negative culture and serological tests with positive granulomatous inflammation and negative tumoral cells in histopathological specimens, we started empirical antituberculosis treatment. In this process, we evaluated the clinical symptoms and laboratory findings including especially sedimentation rate with close follow- ups. In bone lesions due to TB infection, regression of SUV max values was also taken into consideration for assessment of response to the anti-TB treatment.

Ewing sarcoma is the second most common malignant bone tumor in children and young adults after osteosarcoma. The features of Ewing sarcoma and osteomyelitis might be similar on imaging, and there are numerous reports of Ewing sarcoma being misdiagnosed as osteomyelitis on radiological studies. A sharp and defined margin, optimally visualized on T1-weighted MRI, is the most significant feature differentiating the Ewing sarcoma from osteomyelitis [31]. Our patient with osteomyelitis of the

tibia had overlapping radiographic findings and MRI images with osteomyelitis and Ewing sarcoma; therefore, a biopsy was performed before the final diagnosis.

CSD is an infection that develops from the bites or scratches of flea-bearing cats and is caused by *Bartonella henselae* [11, 32]. The disease, which is one of the causes of chronic granulomatous lymphadenitis, usually resolves with a short course of antibiotics. The medial epitrochlear region is the most common location. It should be suspected, especially in young patients presenting with lymphadenopathy [32]. History of exposure to cats, serology, and biopsy are crucial for the diagnosis [11]. Three of our patients displayed signs and symptoms of CSD, and all had a history of cat scratches.

Pyomyositis might rarely be confused with soft tissue sarcoma due to the absence of signs of inflammation such as warmth and redness and might present only with mass lesions as seen in three of our cases. Clinical and laboratory panels may sometimes be not adequate for diagnosis of bone and joint infections with diverse presentations and the diagnosis may solely be obtained by histopathological analysis [33].

Pseudotumor lesions share imaging characteristics with neoplastic lesions such as periosteal reactions, bone destruction patterns, contrast enhancement patterns on MRI etc.

Meticulous attention should be paid to history of contact with animals or animal products and immune status of the patients [34].

Study limitation

The small sample size, retrospective design and heterogeneity of pathologic diagnosis are limitations of this study.

CONCLUSION

Histopathological examination of lesions due to soft tissue and bone infections should be performed to rule out tumors in cases of doubt. The cause of uncertainty is the shared common characteristics of tumoral and infectious lesions. There may be mildly increased inflammatory markers in cases with deep-seated non-mobile soft tissue masses and aggressive periosteal reactions and/or bone destruction patterns. When the biopsy results are indeterminate, a repeat biopsy is recommended.

Conflict of interest: None declared.

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Мускулоскелетне инфективне лезије које имитирају туморе – искуство једног референтног центра

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САЖЕТАК

Увод/Циљ Инфекције костију и меких ткива могу имитирати туморе костију и меких ткива. Због тога је диференцијална дијагноза важна да би се спречиле грешке у лечењу.
Циљ овог рада је да прикаже податке болесника са нејасним клиничким и радиолошким налазима који имитирају бенигне и малигне туморе костију и меких ткива, који су касније дијагностиковани као инфламаторне инфекције.

Методе Урађен је ретроспективни преглед клиничких, микробиолошких, радиолошких и патолошких налаза болесника са претпостављеном дијагнозом могуће малигне лезије.

Резултати Студија је обухватила 21 болесника средње старости од 37 (1 месец – 72 године) година, а 13 (61%) болесника су били мушкарци. Укупно 16 (76%) болесника који су се жалили на бол примљено је у болницу. Дијагнозе су биле хидатидна циста, туберкулозни остеомијелитис, болест мачјих огреботина, хронични остеомијелитис, субакутни остеомијелитис и апсцес меког ткива. Сви болесници су лечени у зависности од дијагнозе лезије.

Закључак Инфективне лезије костију и меког ткива треба подвргнути хистопатолошком прегледу да би се искључили тумори у случајевима сумње. Постоје шансе за погрешну дијагнозу због заједничких карактеристика туморских и инфективних лезија које могу бити благо повећани инфламаторни маркери са дубоко смештеним немобилним масама меког ткива и агресивним периосталним реакцијама и/или обрасцима деструкције кости.

Кључне речи: биопсија; снимање; хидатидна циста; туберкулоза; апсцес меког ткива; остеомијелитис

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Assessment of the diagnostic ability of RIFLE classification and neutrophil gelatinase-associated lipocalin biomarker in detecting acute kidney injury in newborns in the intensive care unit

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SUMMARY

Introduction/Objective This study was designed to demonstrate the association of the RIFLE classification and neutrophil gelatinase-associated lipocalin (NGAL) in predicting of newborns with acute kidney injury (AKI).

Methods This was a prospective study. We included 100 newborns suspected of having a kidney injury. These newborns were admitted to the intensive care unit (ICU) at the University Clinic of Pediatrics from the period of two years. The severity of the disease was determined by RIFLE classification. The biochemical marker NGAL was included in this study because it is an early biomarker of AKI in newborns. The statistical processing of the material was by methods of descriptive statistics.

Results The prevalence rate of AKI was 6.25%, but according to the RIFLE classification the prevalence was 8.7%. According to RIFLE classification, we reported "risk" in 36%, "injury" in 50% and "failure" in 14% of newborns with AKI. In newborns with perinatal asphyxia, kidney injury was seen in 34% and 30%, making perinatal asphyxia the most common predisposing factor. The difference in average value of the score for neonatal acute physiology with perinatal extension in newborns with AKI and the control group without AKI was confirmed significant ($p < 0.001$). Also, there was a significant difference ($p < 0.001$) between serum creatinine and urinary NGAL values, on the day they were admitted to the ICU.

Conclusion In newborns hospitalized in the ICU, AKI is a serious condition. We could identify kidney injury and follow up the progression of the disease by using RIFLE classification. The need for early diagnosis of kidney injury, in a period when the disease is not clinically manifest, in the first hours of its occurrence, is provided by NGAL.

Keywords: acute kidney injury; newborns; RIFLE classification; NGAL

INTRODUCTION

Acute kidney injury (AKI) involves a sudden impairment of kidney function, leading to an imbalance of electrolytes, fluids, and waste products [1]. This complex disorder of kidney function can present with a variety of clinical manifestations. It can manifest as a kidney injury requiring replacement therapy or as minimal kidney dysfunction. In newborns who are critically ill, AKI is a common clinical condition. The reason for this is the immaturity of the kidneys in newborns, making them sensitive to reduced kidney function [2, 3].

Due to the need of timely and appropriate diagnosis as well as assessment of the severity of kidney injury, Risk Injury Failure Loss End-stage renal disease (RIFLE) classification is used in newborns with AKI. RIFLE classification covers the following stages: risk of kidney failure, kidney injury, kidney failure, kidney function loss and end-stage kidney failure). Taking into account the immaturity of tubular cells, high body water content and presence of maternal creatinine in newborn's circulation, Bezzero et al. [4] have modified RIFLE classification to a newborns RIFLE classification. In this classification, the corrected values of serum creatinine and 24-hour urinary output are taken as criteria [4].

AKI occurs in 8–24% of newborns. The mortality rate is 10–61% [5]. Clinical conditions in newborns such as perinatal asphyxia, prematurity, congenital heart disease, sepsis, and meconium plug syndrome are factors that predispose to AKI. The risk of kidney injury in newborns hospitalized at the intensive care unit (ICU) is reduced with appropriate and timely treatment of these associated comorbidities [6, 7].

The scoring system most commonly used in newborns as a tool for predicting severity of illnesses is Score for Neonatal Acute Physiology with Perinatal Extension 2 (SNAPPE 2) score. This score needs to be performed within the first 24 hours of the newborn's admission to the ICU. The SNAPPE 2 score system is used to assess the severity of the disease and is correlated with newborns mortality in ICUs. A score above 40 is associated with a higher mortality rate [8].

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In newborns, serum creatinine as a parameter for glomerular filtration rate is not used. The reason for this is that in newborns serum creatinine shows higher values in the first 2–3 days of birth, because those values reflect the function of the mother's kidneys. Serum creatinine values in the following days they subsequently decrease [9]. Serum creatinine levels also change 48–72 hours after the onset of kidney injury. So, the serum value of creatinine is considered a late marker for kidney injury [10, 11]. Neutrophil gelatinase-associated lipocalin (NGAL) is used as a biomarker to detect kidney injury in the first two to three hours after onset. Kidney damage can be detected with the help of NGAL even before there is a decrease in urine output and before an increase in serum creatinine. That is why NGAL is the most appropriate biomarker for early detection of kidney injury in critically ill newborns.

The severity of the disease correlates with serum and urine NGAL values, and are dependent on the extent of current kidney injury. Its clinical use ensures that we make the right clinical decisions before the disease manifests itself [12, 13, 14]. The purpose of this study was to analysis the role of RIFLE classification and (NGAL) in predicting AKI in newborns.

METHODS

Study population

The study was a prospective and we included 100 newborns suspected for kidney injury admitted at the ICU, at the University Clinic of Pediatrics from the period of two years. The newborns were divided into a group with AKI, included 50 newborns, and the control group included 50 newborns without AKI. Inclusion criteria in this study were newborns up to 28 days after birth, who were admitted at the ICU due to certain pathological condition, with or without the development of AKI. In newborns less than 33 weeks of gestation, an increase in serum creatinine level above 130 μmol/l, as well as a serum creatinine value greater than 90 μmol/l in newborns older than 33 weeks of gestation was defined as AKI. Oliguria was defined as a urinary output less than 1.0 ml/kg/h. All newborns who were less than 25 weeks of gestation according to our criteria were excluded from the study.

According to the criteria for classification Neonatal RIFLE, gestational age, birth weight, gender and predisposing factors such as asphyxia, prematurity, sepsis, congenital heart diseases and meconium plague syndrome, were analyzed medical data from hospitalized newborns. With SNAPPE 2 achieved in the first 12 hours after admission at the ICU, the severity of the disease of the admitted newborns was assessed.

For all studied patients we have the approval of the institutional committee on ethics.

Study design

In the neonates included in the study, the severity of the disease was determined based on the neonatal RIFLE classification using the criteria shown in Table 1.

Table 1. Neonatal RIFLE classification criteria

Stage	Criteria	Urine output
R (risk)	> 1.5 Cr, GFR < 25%	< 1.0 ml/kg/24 h, term neonates < 1.5 ml/kg/24 h, in premature
I (injury)	> 2 Cr, GFR < 50%	< 1.0 ml/kg/24 h
F (failure)	> 3 Cr, GFR < 75%	< 0.7 ml/kg/24 h or anuria 12 h
L(loss)	AKI > 1 month	
E (end stage)	AKI > 3 month	

GFR – glomerular filtration rate; AKI – acute kidney injury

The laboratory tests were done at the Clinical Laboratory at the University Clinic of Pediatrics and in the Institute of biochemistry Faculty of Medicine, Skopje. Laboratory test which included serum creatinine, and urinary NGAL were taken first at the admission, and after 72 hours. The collected urine samples were frozen at -70 to -80°C, before being transferred to the Institute of Biochemistry.

Using the biochemical analyzer Architect c4000 Abbott (Mmol/L) and the NGAL biomarker, using the NGAL ELISA method (Bioporto, Hellerup, Denmark) ng/L, the laboratory values of serum creatinine were examined.

Statistical analysis

The statistical processing of the material was by the methods of descriptive statistics. Independent sample tests were used to determine the significance of the difference in the samples. Statistical significance was established for the values of $p < 0.05$.

RESULTS

During the study, we have evaluated 100 newborns hospitalized at the University Clinic of Pediatrics, during the two-year-long period. The AKI group included 50 newborns, and without AKI group, included 50 newborns with various pathological conditions.

In the AKI group 62% were male and 38% female. Comparable values with the control group of newborns, without AKI group, were 56% males and 44% females, retrospectively. Most of involved newborns in AKI and without AKI group were born at term (61% and 57%). In newborns with AKI, the average gestational age was 36.152 ± 4.2 weeks of gestation and 35.26 ± 2.7 weeks of gestation in control group. In newborns with AKI, the average birth weight was 2660.5 ± 458.1 grams, while in the control group was 2489.4 ± 564.8 grams. The average serum creatinine values in AKI group was 184.44 ± 103.74 μmol/l, and 77.78 ± 30.6 μmol/l in control group. The mean urine output values in AKI group was

Table 2. Demographic characteristics of newborns with acute kidney injury and non-acute kidney injury

Parameters	Acute kidney injury	Non acute kidney injury
Male	31 (62%)	28 (56%)
Female	19 (38%)	22 (44%)
Mean ± SD gestation age (week)	36.15 ± 4.2	35.26 ± 2.7
Mean ± SD birth weight	2660.5 ± 458.1	2489.4 ± 564.8
Duration of stay (days)	17.82 ± 8.4	13.38 ± 7.7
Mean ± SD serum creatinine	184.44 ± 103.74 μmol/l	76.78 ± 30.6 μmol/l
Mean ± SD urine output	1.14 ± 0.8 ml/kg/h	3.49 ± 1.5 ml/kg/h

1.14 ± 0.8 ml/kg/h and 3.49 ± 1.5 ml/kg/h in control group. Demographic characteristics of newborns with AKI and non AKI are summarized in Table 2.

In the first 12 hours of ICU hospitalization, all newborns were analyzed with the SNAPPE 2 score. The results of the score were evaluated in four categories: very severe (over 70), severe (41–70), medium (score 21–40) and light (score 1–20).

All newborns were analyzed by SNAPPE 2 score within the first 12 hours of hospitalization in NICU. The score values were evaluated in four categories: light (score 1–20), medium (score 21–40), severe (41–70) and very severe (over 70). The results showed that the value of the average score of newborns with AKI was 54.72 ± 35.3, while 38 ± 10 in control group.

The difference between the AKI group and the control group was 16.72 and it was confirmed as significant $p < 0.001$.

The distribution of newborns with AKI depending of score levels compared to control group without AKI. In 50% newborns with AKI predominate severe score level, while in control group predominate median score level in 42% of newborns show in Table 3.

Table 3. Distribution of newborns with acute kidney injury (AKI) depending of score levels compared to control group

Score	AKI / without AKI	Mean ± SD	Min–Max
Light	AKI	0	0
	without AKI	13.7 ± 3.2	9–19
Medium	AKI	31.5 ± 4.4	28–39
	without AKI	27.6 ± 5.7	21–38
Severe	AKI	54.41 ± 7	43–69
	without AKI	41.21 ± 5.8	36–51
Very severe	AKI	89.45 ± 8.3	77–95
	without AKI	80.46 ± 8.3	70–93

Distribution of predisposing factors among newborns with AKI and control group are shown in Table 4. The most common contributing condition reported in both groups (34% *vs.* 30%) was perinatal asphyxia.

The RIFLE classification, which categorizes the severity of kidney injury, was implemented in the examined AKI group and control group without AKI. We founded

Table 4. Predisposing factors in newborns with acute kidney injury and control group without acute kidney injury

Predisposing factors	Acute kidney injury N / %	Without acute kidney injury N / %
Asphyxia	17 (34%)	15 (30%)
Sepsis	13 (26%)	12 (24%)
Preterm	12 (24%)	14 (28%)
Congenital heart disease	3 (6%)	5 (10%)
Meconium plug syndrome	3 (6%)	5 (10%)

Table 5. Distribution of newborns with acute kidney injury and control group according to the RIFLE classification

RIFLE classification	Acute kidney injury N / %	without acute kidney injury N / %
Risk	18 (36%)	15 (30%)
Injury	25 (50%)	3 (6%)
Failure	7 (14%)	0

"risk" in 36% (18/50) of newborns with AKI, "injury" in 50% (25/50) and "failure" in 14% (7/50) of newborns. Additionally, using this classification, kidney injury was detected in the non AKI group too, with 30% (15/50) registering "risk" and 6% (3/50) "failure." Distribution of newborns with AKI compared to non AKI group in accordance with the RIFLE classification show in Table 5.

In 18 newborns with AKI, in whom we registered risk (R) in 78% (14/18), progression of disease occurred, with injury (I) registered in 62% (11/18) of newborns and failure (F) in 17% (3/18) of newborns. Only 19% (2/11) of the newborns with AKI in whom we reported subsequent injury (I), the experienced progressed of the disease to renal failure (F). From here, according to the standard definition of AKI in newborns the calculated prevalence was 6.25%, while according to the RIFLE classification it was 8.7%.

The results of the study, on the day of ICU admission showed that the serum creatinine level was normal in newborn with AKI and it was 76.78 ± 30.6 μmol/L and had an upward trend of 184.44 ± 103.74 μmol/L after 72 hours (Figure 1).

On the day of admission to the ICU in neonates with AKI the values of urinary NGAL the results in this study showed higher values (373.8 ± 194.9) and a slight upward trend, while on the third day after admission the results showed an additional increase (439.4 ± 254.7) $p < 0.001$ (Figure 2).

There was a significant difference $p < 0.001$ between serum creatinine and urinary NGAL values on the day of admission to the ICU.

DISSCUSION

The study presents a clinical, epidemiological investigation that evaluated in 100 newborns suspected for kidney injury admitted at the ICU at the University Clinic of Pediatrics, from the period of two years. The newborns were divided into examined and control group. The examined group included 50 newborns with AKI and the control group

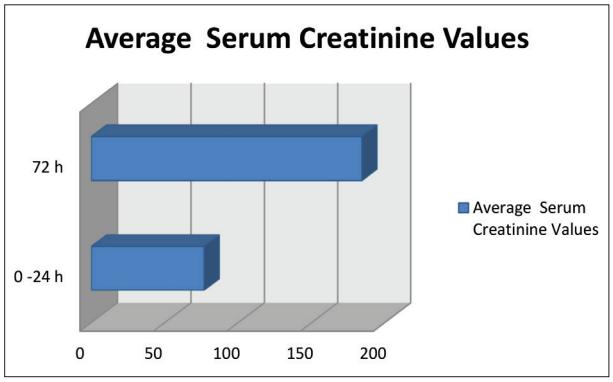

Figure 1. Serum creatinine value distribution in newborns with acute kidney injury overtime

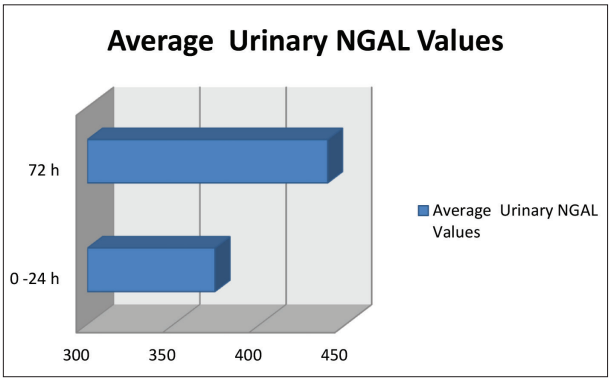

Figure 2. Urinary neutrophil gelatinase-associated lipocalin (NGAL) values distribution in newborns with acute kidney injury overtime

included 50 newborns without AKI with various pathological conditions [15].

According to the standard definition of AKI in newborns the calculated prevalence was 6.25%, which is consistent with literature data [16, 17]. A similar finding has been published from the study conducted by Vachvanichsanong et al. [18] where the prevalence of AKI in newborns was 6.3%, where in the study by Bolat et al. [19] it was 8%. There are opposite findings too. Mortazavi et al. [20] report on the prevalence of AKI of 2.7%, and Agras et al. [21] of 3.4%. We hypothesize that these differences may be due to differences in the diagnostic criteria for detecting kidney injury in newborns. According to the RIFLE classification, the prevalence of kidney injury in our study was 8.7%. This suggests that the diagnosis of AKI according to the standard classification may be missed in most newborns. Less of 36% of newborns with AKI had "risk," 50% "injury" and 14% had "renal failure." In 36% of newborns with registered risk, there was a progression of disease to injury in 62% and to "failure" in 17% of newborns. In 19% of newborns with verified "injury," the condition progressed to "renal failure." A similar finding is presented in the study by Mohkam et al. [22], in which, according to RIFLE criteria, 43% of newborns with AKI were at risk, 51% at injury and 6% at failure [23]. RIFLE classification was also applied in control group, newborns without AKI. The "risk" for AKI was present in 30% of newborns, while the "injury" in 6%. We did not record clinical progression of the condition and development of kidney injury in this group of newborns. This finding suggests that in control group, with verified "risk" and "injury," we could have overlooked the situation. We assume that the resolution of the kidney injury will come with the treatment of other indications. This suggests that the RIFLE classification in newborns can be used as a more sensitive method than the standard one in terms of diagnosis and monitoring of kidney injury [24]. There was a significant difference ($p = 0.00001$) between average SNAPP II score value in newborns with AKI compare to control group. In our study, newborns with AKI have had severe SNAPPE 2 score. The high score level was significantly associated with the severity of the disease. Critically ill newborns with AKI and other predisposing factors were significantly associated with high level score and poor prognosis. This finding correlates with the data presented in the study of Mortazavi et al. [20]. The poor prognosis was significantly higher in newborns with severe score in admission to the ICU, in whose further predisposing factors develop [25].

For all gestational ages according to gender distribution, in both groups of newborns most were male (62% and 56%). In terms of distribution by gestational age, most newborns were born term (64% and 54%). The most common predisposing factor in examined and control group perinatal asphyxia was observed in 34% and 30% of neonates, with a predominance of term male newborns, who were born with a low Apgar score in the fifth minute of life. Abu-Haweleh et al. [26] and Mortazavi et al. [20] published similar findings in their studies as well. Abu-Haweleh et al. [26] reports that 42% and Mortazavi et al. [20] reports that 30% of the newborns with AKI have had asphyxia as a dominant predisposing factor. In critically ill newborns on the day of ICU admission, in our study we found significantly higher urinary NGAL values compared to serum creatinine levels. This demonstrates that NGAL is a sensitive biomarker in the diagnosis of kidney injury. These data correlate with the data reported by Nickolas et al. [27] and Youssef et al. [28], emphasizing the role of NGAL as a sensitive marker in detecting kidney injury. Monitoring of urinary NGAL on the day of neonatal hospitalization of newborns in the ICU and 72 hours later showed an increasing trend in the average value. A high level of NGAL on initial measurement suggests that the kidney injury may have occurred prior to the newborns' hospitalization in the ICU. Namely, all critically sick newborns are transported to our unit, as a specialized tertiary center for intensive therapy. Intensive care explains the presence of renal injury in critically ill neonates in whom AKI is not yet clinically manifest. The role of urinary NGAL as an early biomarker for kidney injury is confirmed by its high values noted upon admission to the ICU. Mishra et al. [29] and Devarajan [30] in their studies also refer the ability of NGAL to early detect the newborns at risk of AKI.

CONCLUSION

AKI is a serious condition in hospitalized newborns in ICU. The severity of the disease can be assessed using the

SNAPPE 2 score. Kidney injury could be identified and disease progression monitored using the RIFLE classification. Early diagnosis of kidney injury in the first hours after its onset is made possible by NGAL, even when the disease is not clinically manifest.

Conflict of interest: None declared.

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Процена дијагностичке способности класификације РИФЛЕ и биомаркера липокалина везаног за неутрофилну гелатиназу у откривању акутне повреде бубрега код новорођенчади у јединици интензивне неге

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САЖЕТАК

Увод/Циљ Циљ студије био је да се испита улога класификације РИФЛЕ и липокалина везаног за неутрофилну гелатиназу у предвиђању акутне повреде бубрега (АПБ) код новорођенчади.

Методе У проспективној студији било је укључено 100 новорођенчади са суспектним бубрежним оштећењем примљених у јединицу интензивне неге на Универзитетској клиници за педијатрију у периоду од две године. Тежина АПБ утврђена је класификацијом РИФЛЕ. У студији је примењен липокалин везан за неутрофилну гелатиназу као рани биомаркер АПБ код новорођенчади. Материјал је статистички обрађен методама дескриптивне статистике.

Резултати Процењена преваленција АПБ била је 6,25%, док је преваленција према класификацији РИФЛЕ била 8,7%. Према класификацији РИФЛЕ, дијагностиковали смо „ризик" код 36%, „повреду" код 50% и „инсуфицијенцију" код 14% новорођенчади са АПБ. Најчешћи предиспонирајући фактор повезан са оштећењем бубрега била је перинатална асфиксија примећена код 34% и 30% новорођенчади. Потврђена је сигнификантна разлика између средње вредности скора за неонаталну акутну физиологију перинаталне екстензије код новорођенчади са АПБ и контролне групе без АПБ ($p < 0,001$). Постојала је значајна разлика ($p < 0,001$) између вредности креатинина у серуму и липокалина везаног за неутрофилну гелатиназу у урину на дан пријема у јединицу интензивне неге.

Закључак АПБ је озбиљно стање код хоспитализоване новорођенчади у јединици интензивне неге. Помоћу класификације РИФЛЕ могли бисмо идентификовати повреду бубрега и пратити напредовање болести. Биомаркер липокалин везан за неутрофилну гелатиназу пружа рану дијагнозу повреде бубрега у првим сатима настанка, када болест још увек није клинички јасна.

Кључне речи: акутна повреда бубрега; новорођенчад; класификација РИФЛЕ; липокалин везан за неутрофилну гелатиназу

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Postoperative recovery assessment after appendectomy in children – laparoscopic versus open technique

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SUMMARY

Introduction/Objective Surgery is a "gold standard" in treating the acute appendicitis in pediatric patients. The aim of the study was to determine the effect of open and laparoscopic appendectomy on postoperative recovery, return to everyday activities, and the quality of life in patients operated on for acute appendicitis.

Methods This prospective study was performed at the Institute for Children and Youth Healthcare of Vojvodina, over a period of 10 months. This study was approved by the Ethics Committee of the Institute. All patients treated for acute appendicitis by surgery were divided into two basic groups – open or laparoscopic appendectomy – and into three subgroups, depending on the degree of appendicitis. We analyzed the length of surgery, oral intake, establishing peristalsis, hospital stay, return to everyday activities, and the quality of life after surgery.

Results Laparoscopic technique was performed in 60 patients (48%), and the open method in 65 patients (52%). In 66.7% of laparoscopically treated patients, peristalsis occurred earlier ($p < 0.001$), length of hospital stay was shorter (5.95 ± 1.21 days) ($Z = -3.054$; $p = 0.002$), the total score of daily activities showed a statistically significantly better score ($Z = -7.667$; $p = 0.000$), and they achieved a high level of quality of life significantly earlier ($t = 2.773$; $p = 0.007$).

Conclusion The advantages of minimally invasive surgery in the treatment of acute appendicitis in children are reflected in the faster re-establishment of everyday functioning, faster recovery, and a good quality of life.

Keywords: appendicitis; minimally invasive surgery; return to everyday activities; quality of life; children

INTRODUCTION

Acute appendicitis is one of the most common abdominal emergencies in pediatric population. The lifetime risk of developing appendicitis is 6–8%, with a peak incidence in the teenage years [1]. The cause of appendicitis is an obstruction of the appendix, either from inflammation of the wall or a fecalith. Typical symptoms of appendicitis are acute abdominal pain, fever, nausea and vomiting. Appendicitis in pediatric patients is less likely to present in a classic manner than commonly thought [2]. Considering the stage of inflammation, appendicitis can be classified as noncomplicated and complicated. Complicated appendicitis is defined as gangrenous or perforated appendicitis, suppurative appendicitis, or appendicitis with an abscess formation, periappendicular mass, or fecal peritonitis [3]. In children, complicated appendicitis is relatively common, and the rate of perforated appendicitis varies with age, the presence of obesity, socioeconomic status, and healthcare access. High rate of postoperative morbidity in complicated cases in children require prompt and precise diagnosis, as well as adequate treatment [4].

Surgical treatment is a "gold standard" in treating this condition. Surgeons still use the open surgical technique described back in the 18th century. Rising popularity of minimally-invasive surgery in other surgical fields implemented this technique in pediatric surgeon's everyday practice. First laparoscopic appendectomy was performed by Kurt Semm in 1981 [5]. Eight years later, Thom Lobe performed this technique in a child [6]. Compared to the open technique, laparoscopic appendectomy, like any minimally invasive technique, causes less tissue trauma, which is associated with less postoperative pain, shorter hospital stay, faster recovery, and better cosmetic result.

There has been an increased interest in the conservative management of appendicitis over the last 20 years [7, 8]. Conservative (non-operative) management for carefully selected children with acute appendicitis has been described. In patients with initial appendicitis, non-operative treatment, with the use of antibiotics, could be applied, with healing in most of them. The use of antibiotics in these patients may be sufficient for cure, if there are no sure indications for surgery, such as the presence of peritonitis or signs of perforation [9, 10, 11].

Although laparoscopic appendectomy gained popularity among many surgeons, the advantage of laparoscopic appendectomy is still the subject of research [3].

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The aim of the study was to determine the effect of both methods, the open technique and laparoscopic appendectomy, on early postoperative recovery, return to everyday activities, and the quality of life in pediatric patients operated on for acute appendicitis.

METHODS

This prospective study was performed at the Clinic for Pediatric Surgery, Institute of Children and Youth Healthcare of Vojvodina, over a period of 10 months. This study was approved by the Ethics Committee of the Institute. We analyzed all patients treated for the acute appendicitis by surgery during this period. All patients with acute appendicitis, American Society of Anesthesiologists (ASA) classification (general state) I–III, whose parents had given written consent, were included in research. Patients who were classified as ASA IV and V, and without written consent, were excluded.

All patients operated on for acute appendicitis were divided into two basic groups, in relation to the surgical technique: open or laparoscopic appendectomy. Conversion, intraoperative change of laparoscopic to open access was performed due to a complex operative finding. It is important to emphasize that the decision to make a conversion is due to a proper intraoperative surgeon judgment which provides the best possible outcome for the child.

Then, all the patients operated on were divided into three subgroups, depending on the degree of appendicitis (negative, uncomplicated, and complicated appendicitis). Appendicitis with perforation or with abscess was classified as complicated appendicitis, and those remaining were classified as uncomplicated. Intraoperative assessment of the degree of appendix was performed by a surgeon macroscopically, after which all surgical specimens were transferred to the histopathology department for histopathological analysis. Age, sex, and ASA classification score were analyzed preoperatively. Type of surgery, the degree of appendicitis, and the length of surgery were analyzed during surgery. Oral intake, establishing peristalsis, length of hospitalization, restitution of daily activities using Activity Assessment Scale (AAS) modified for children, and the quality of life of patients after surgery, were analyzed after surgery.

AAS is a measure of functional activity designed as part of the postoperative period analysis. This scale measures a wide degree of activity, and in the form of a questionnaire, it is easily and quickly filled out by the patient. AAS modified for children was used to measure activity every postoperative day, during the first five days, a month, three, and six months after surgery. The patient's ability to perform activities was measured through nine categories, from behavior in hospital bed to the ability to go to school and engage in sports activities after dismissing. All items have response categories ranging 1–5. The response categories for the activities are as follows: (1) no difficulty, (2) little difficulty, (3) difficult, (4) very difficult, (5) unable to do so.

We especially observed the quality of life of patients after surgery on the basis of the child's health condition, limitations in performing daily activities, physical pain, the child's satisfaction, and anxiety. We used the modified questionnaire SF 10 for children during the period of six months after surgery.

Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics, Version 20.0 (IBM Corp., Armonk, NY, USA). Descriptive statistical methods were used. The significance of the differences between the two groups was determined using the t-test of independence or the Mann–Whitney U-test, depending on the normality of the distribution determined by the Shapiro–Wilk test. Significance between categorical variables was determined using the χ^2 test. One-factor analysis of variance (ANOVA) with repeated measurements was used to determine the difference between the quality of life after the first, third, and sixth months. Statistically significant correlations between days of hospitalization with daily activities were determined by Pearson's correlation analysis and linear regression model. The influence of age and sex on the type of surgery and the degree of inflammation as potential predictors of performing daily activity in the observed time periods was analyzed by multiple regression analysis. For daily activities in the examined time intervals, the area under the curve was determined by the receiver operating characteristic (ROC) curve analysis. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Over a period of 10 months, we operated on 125 patients, aged 2–18 years, due to acute appendicitis. Laparoscopic technique was performed on 60 patients (48%), and the open method on 65 (52%). Conversion (operative technique changed from laparoscopic to open method) was made in four patients, and they were included into the open group. There were no statistically significant differences between the treatment groups with respect to mean age, sex distribution, preoperative risk assessment, the degree of appendix inflammation. In both treatment groups, most subjects belong to ASA I category, and only one child belongs to ASA III (Table 1). The mean operative time was 65 minutes (range 25–185 minutes) when laparoscopy was used. Open technique required statistically significantly shorter period of time (mean 49.38 minutes, range 25–130 minutes; $p < 0.001$). In the majority of laparoscopically treated patients (66.7%), peristalsis occurred on the first postoperative day, whereas in 78.5% of the patients who were classically operated on it was registered one day later, which made a significant difference ($p < 0.001$). Oral feeding was initiated earlier in patients operated on laparoscopically (in 85% on the first postoperative day) compared to those operated on using open technique (on the second postoperative day in 84.6%) ($\chi^2 = 82.763$; df = 4; $p = 0.000$).

Length of hospital stay in children operated on by laparoscopy was 5.95 ± 1.21 days and by open technique it was

Table 1. Demographics data

Demographics	Laparoscopic (n = 60)	Open (n = 65)	Total (N = 125)	Significance
Sex ratio (M/F)	37/23	39/26	76/49[&]	0.859[‡]
Age (yr) (Mean ± SD)	12.17 ± 3.55	11.09 ± 3.69		0.100[†]
Degree of inflammation				
negative appendix n (%)	5 (8.3)	3 (4.6)	8 (6.4)	0.480[#]
uncomplicated n (%)	25 (41.7)	30 (46.2)	55 (44.0)	0.500[‡]
complicated n (%)	30 (50)	32 (49.2)	62 (49.6)	0.799[‡]
American Society of Anesthesiologists classification				
I n (%)	37 (61.6)	50 (76.9)	87 (69.6)	0.163[‡]
II n (%)	22 (36.7)	14 (21.5)	36 (28.8)	0.182[‡]
III n (%)	1 (1.7)	1 (1.5)	2 (1.6)	-

[&]χ^2 test (χ^2 = 6.950; df = 1; p = 0.008);
[†]t-test between groups;
[‡]χ^2 test between groups;
[#]Fisher exact test between groups

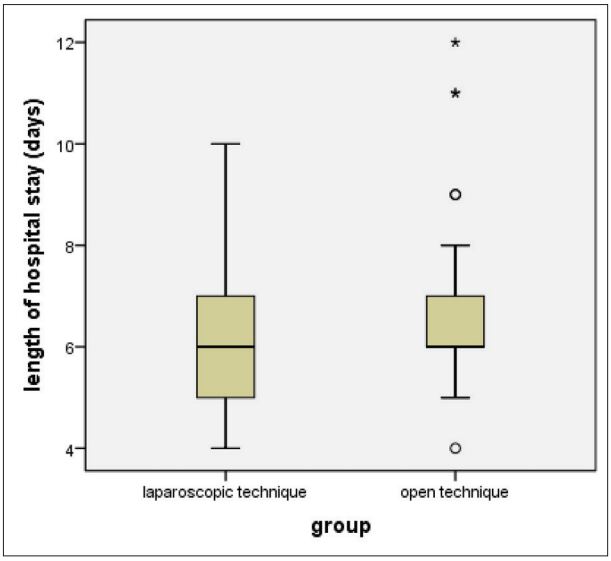

Figure 1. Distribution of length of hospitalization in days in relation to the method of surgery

6.63 ± 1.04 days, which is significantly longer (Z = -3.054; p = 0.002) (Figure 1).

AAS modified for pediatric population was used for measuring period of time needed for recovering to everyday activities. These results were compared for the first, third, seventh postoperative day, as well as for one, three, and six months after surgery. It is noticed that daily activities are established faster after minimally invasive surgery in each observed time period. The difference was statistically relevant on the first (Z = -7.783; p = 0.000) and third postoperative day (Z = -3.955; p = 0.000). The total score of daily activities showed a statistically significantly better overall score for the group of laparoscopic appendectomies (Z = -7.667; p = 0.000) (Figure 2).

We analyzed the influence of special categories such as the technique of surgery, the degree of inflammation of the appendix, sex, and age on everyday activities.

From the partial influences on the performance of everyday activities after the first and third postoperative day, the method of operation and the degree of inflammation have a statistically significant influence. From the seventh day to six months after the operation, only the technique of operation held a statistically significant (p < 0.05) effect (Table 2). Results of the Mann–Whitney U-test showed significantly better overall record of daily activities for a group of laparoscopic appendectomy (Z = -7.667; p = 0.000).

Table 2. Influence of sex, method of operation, degree of inflammation, and age of the patients on daily activities in the examined times

Parameter	Beta	Significance	R
1st day			0.715
Sex	0.107	0.100	
Group	-0.698	**< 0.001**	
Degree of inflammation	-0.125	**0.048**	
Age	0.015	0.818	
3rd day			0.430
Sex	0.028	0.735	
Group	-0.350	**< 0.001**	
Degree of inflammation	-0.239	**0.005**	
Age	-0.083	0.324	
7th day – 6th month			0.286
Sex	0.087	0.340	
Group	-0.188	**0.038**	
Degree of inflammation	-0.171	0.061	
Age	-0.089	0.323	

Values in bold are statistically significant

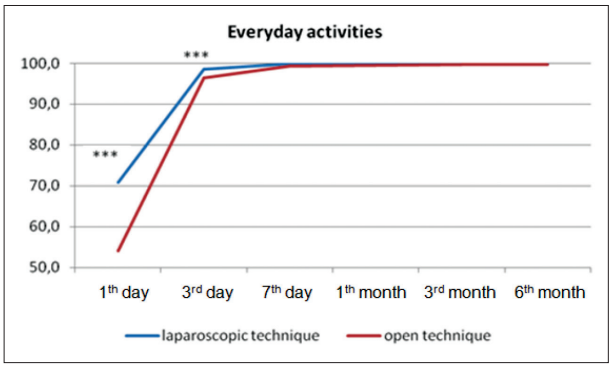

Figure 2. Total score of daily activities in the examined times for the examined groups

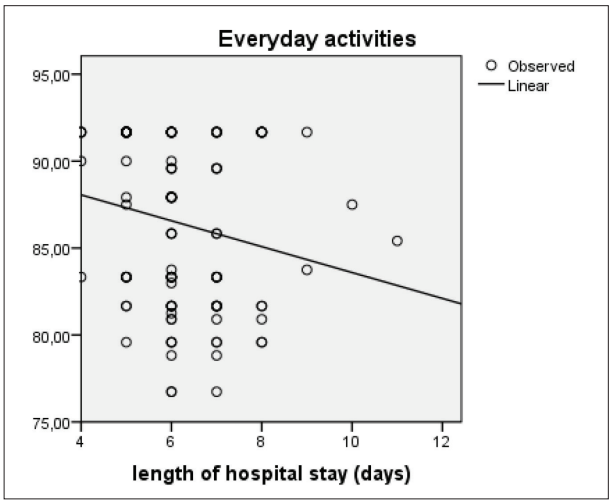

Figure 3. Influence of hospital length on performing daily activitiesto the method of surgery

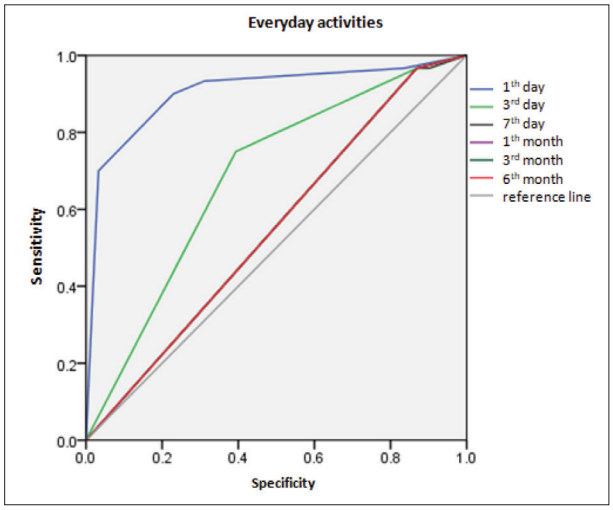

Figure 4. Receiver operating characteristic curve analysis of performing everyday activities by days

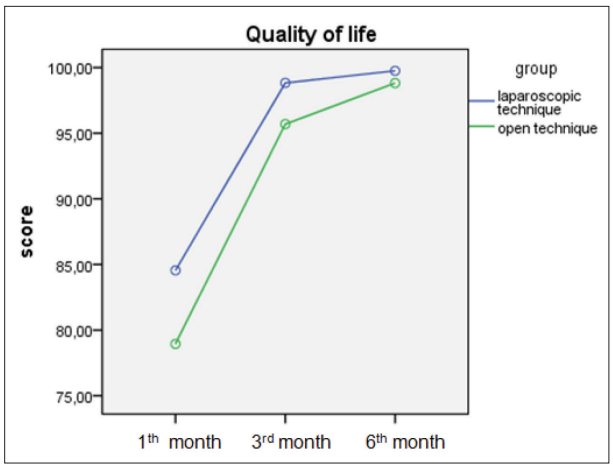

Figure 5. Quality of life at one month, three months, and six months after surgery in laparoscopic and open technique groups

The influence of the length of hospitalization on daily activities is shown in Figure 3, where a significant negative correlation was observed between daily activities ($r = -0.190$; $p = 037$) and the length of hospitalization.

ROC analysis results suggested that randomly chosen child operated on by laparoscopy performed better in everyday activities than a child operated on using open technique in 88.8% of cases on the first and in 68% of cases on the third postoperative day (area under the ROC curve – AUC = 0.888; $p = 0.000$; 95% CI = 0.825–0.951 and AUC = 0.680; $p = 0.000$; 95% CI = 0.586–0.778, respectively). There was no significant separation in other time intervals examined (Figure 4).

In all examined indicators of the quality of life, children of the laparoscopic group had a higher score. Statistically significant ($p < 0.01$) is the fact that they complained less about pain and were more satisfied. Children with acute appendicitis operated on by laparoscopy achieved a high level of the quality of life significantly earlier ($t = 2.773$; $p = 0.007$). The quality of life increased gradually over the time ($F = 7.404$; $p = 0.007$), with significantly better results in the laparoscopy group after the first and third month (Figure 5).

Over 95% of parents reported their children's quality of life as excellent six months after the surgery in all the patients. There was no difference in the quality of life between children in these two groups.

DISCUSSION

The advantages of laparoscopic surgery in relation to open surgery are described in many papers in terms of postoperative recovery, reduced pain, significantly better aesthetic result, and a total number of complications [12, 13, 14]. However, many studies have described that the length of the operation, and even the cost of treatment, are higher with the laparoscopic technique compared to the open one [15, 16]. In our study, the mean operative time was 65 minutes (range 25–185 minutes) when laparoscopy was used. The open technique required statistically significantly shorter period of time (mean 49.38 minutes, range 25–130 minutes; $p < 0.001$). Recent studies have indicated that the operative time for laparoscopic appendectomies in uncomplicated appendicitis is even shorter compared to the open technique. One study showed that the average operative time for laparoscopic appendectomies today is 41 minutes in the trainee group *vs.* 39 in the surgeons group [16]. Another meta-analysis shows that increased experience of surgeons and nursing staff indeed decreased the operating time [17].

Analyzing immediate postoperative recovery in our study, in majority of the laparoscopically treated patients (66.7%), peristalsis occurred faster, oral feeding was initiated earlier. These results, which indicate a statistically significant difference between the two groups of patients, definitely confirm the advantage of the laparoscopic approach in resolving acute appendicitis. Seqsaqa et al. [13] compared the results of open and laparoscopic appendectomy in complicated appendicitis. Patients could tolerate oral intake after 2.37 ± 0.85 days in the open appendectomy group *vs.* 1.9 ± 0.71 days in the laparoscopic appendectomy group, which was significantly faster with laparoscopic appendectomy ($p = 0.025$). Meta-analysis of Neogi et al. [3] showed that the laparoscopy group has a statistically significant shorter time taken to oral intake as compared to open appendectomy, with almost no statistical heterogeneity.

The length of hospital stay in children operated on by laparoscopy was significantly shorter. Patients of both treatment groups with complicated appendix had a statistically significantly longer hospital stay than children with negative and uncomplicated appendix. According to results of some recent studies, the length of hospitalization after laparoscopic appendectomy is significantly shorter compared to open appendectomy, which is explained by faster postoperative mobilization of the patient and recovery. In this way, costs of treatment are reduced and the return of patients to daily life activities is accelerated [3, 13]. Most authors agree that the length of hospital stay is shorter for patients operated on with laparoscopy. Median hospital stay for laparoscopic appendectomy varies among authors. Some report a median stay of three days in cases of simple

appendicitis and 5.2 days in cases of peritonitis. In other studies, it varies 2.06–4.1 days [3].

Restoring daily functioning is an important measure of patient satisfaction. The AAS is a measure of functional activity designed as part of the postoperative period analysis. This scale measures a wide range of activities in the form of a questionnaire that is easily filled out by the patient [18, 19]. This instrument was constructed to analyze the results of a multicenter study after open and laparoscopic operations for inguinal hernias [18]. McCarthy et al. [20] classified physical activities at 13 levels of physical activity, and scoring was done according to the degree of difficulty in performing them.

The questionnaire created for the purposes of this study is a modified form of the AAS questionnaire, with the necessary adaptation to children's age. Physical activities were classified to four or five levels according to the difficulty in performing them. Then, we analyzed them on the first, third, seventh postoperative day, one month, three months, and six months after the operation. In our study, it was very clearly observed that a significantly higher number of patients from the laparoscopy group did not have or had minimal difficulties in getting up, sitting, and walking compared to the group of open appendectomies. At the end of the observed period, the results of the analysis were equalized. It can be concluded on the basis of the mentioned analysis that faster recovery of children after laparoscopic appendectomy was confirmed, in relation to the therapeutic group of open appendectomies.

The effects of treatment on the functioning of patients and on their quality of life have been the subject of much research for decades [21, 22, 23]. When the postoperative course was analyzed through the prism of the quality of life of patients, the questionnaire SF-10, which was modified for the needs of our study, was especially emphasized in clinical practice. This questionnaire was completed by parents and contains 10 questions to assess physical and psychosocial categories.

Within this analysis, limitations in performing daily activities, the presence of pain, child satisfaction, and anxiety after surgery were assessed. In our study, a statistically significantly better quality of life is clearly seen in the group of children laparoscopically operated on, especially in the first three months after surgery, which is another confirmation of faster recovery and return to daily activities after laparoscopic appendectomies.

The quality of life increased gradually over the time in all the patients, but in the first and the third month after surgery it was significantly better in the group of patients operated on with the minimally invasive approach.

CONCLUSION

It is already known that laparoscopic appendectomy, like any minimally invasive technique, causes less tissue trauma, which is associated with less postoperative pain, shorter hospital stay, and better cosmetic result. The advantage of minimally invasive surgery in the treatment of acute appendicitis in children is reflected in the faster re-establishment of everyday functioning and, therefore, a faster overall recovery, resuming normal activities, and a good quality of life.

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Процена постоперативног опоравка након апендектомије код деце – лапароскопска у поређењу са класичном техником

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САЖЕТАК

Увод/Циљ Хируршко лечење представља „златни стандард" у третману акутног апендицитиса код педијатријских болесника.

Циљ студије је да се утврди ефекат отворене и лапароскопске технике на постоперативни опоравак, повратак свакодневним активностима и квалитет живота болесника оперисаних због акутног апендицитиса.

Методе Ова проспективна студија рађена је на Институту за здравствену заштиту деце и омладине Војводине током десет месеци. Студију је одобрио Етички одбор Института. Сви болесници хируршки лечени због акутног апендицитиса подељени су у две основне групе у односу на хируршку технику: отворена или лапароскопска апендектомија и у три подгрупе, у зависности од степена упале апендикса. Анализирали смо дужину операције, започињање оралног уноса, успостављање перисталтике, дужину хоспитализације, повратак свакодневним активностима и квалитет живота после операције.

Резултати Лапароскопска техника изведена је код 60 болесника (48%), а отворена метода код 65 (52%). Код већине лапароскопски оперисаних болесника (66,7%) перисталтика је успостављена раније ($p < 0,001$), хоспитализација је била краћа (5,95 ± 1,21 дана) ($Z = -3,054$; $p = 0,002$), укупни скор дневних активности показао је статистички значајно бољу укупну вредност ($Z = -7,667$; $p = 0,000$) и постигнут је висок ниво квалитета живота знатно раније ($t = 2,773$; $p = 0,007$).

Закључак Предност минимално инвазивне хирургије у лечењу акутног апендицитиса код деце огледа се у бржем поновном успостављању свакодневног функционисања, бржем опоравку и добром квалитету живота.

Кључне речи: апендицитис; минимално инвазивна хирургија; повратак свакодневним активностима; квалитет живота; деца

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Association of bacterial vaginosis with the most common sexually transmitted infections

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SUMMARY

Introduction/Objective Bacterial vaginosis (BV) is the most common vaginal dysbiosis that increases the possibility of getting sexually transmitted infections (STI). The objectives of this research are to examine association between BV and the nine most common causes of STIs (*Chlamydia trachomatis*, *Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasma* spp., *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, high-risk human papilloma viruses and herpes simplex virus types 1 and 2) and to determine if the presence of BV increases the probability of coinfection with any of the STI microorganisms.

Methods This study involved 235 patients of reproductive age. One sample swab each was collected for vaginal and cervical testing. The vaginal swabs were used for the detection of BV by the reverse transcription polymerase chain reaction (RT-PCR) test. The cervical swabs were used for the detection of the most common STIs, which were tested by four different multiplex RT-PCR tests. Pearson's χ^2 test and Fisher's probability test were used for statistical analysis of the results.

Results Comparison of the total number of STIs and the condition of the vaginal flora has shown that STIs are the most common in patients with BV (80; 89.9%). Women with BV have a higher frequency of infections with *Ureaplasma* spp. and *M. hominis*, 71 (78.9%) and 50 (44.4%), respectively. The presence of detected STI pathogens and relation with the state of vaginal flora indicate that mono infections are present most often in patients with normal flora (51; 42.1%), while coinfections are mostly present in BV patients (50; 55.6%).

Conclusion This study has confirmed the association of *M. hominis* and *Ureaplasma* spp. with BV as well as an association of coinfections with this dysbiosis. Better understanding of the association between various STIs and the status of vaginal flora is necessary to enable better diagnosis, prevention of diseases and women's health protection.

Keywords: bacterial vaginosis; sexually transmitted infections; coinfection; RT-PCR

INTRODUCTION

The vaginal microbiome consists of various microorganisms which coexist in dynamic balance, establishing complex interconnections not only among themselves, but also with a host. In healthy women of reproductive age, the vaginal microbiome predominantly contains bacteria of the genus *Lactobacillus*. These bacteria support vaginal homeostasis and prevent colonization and growth of unwanted microorganisms including Sexually transmitted infections (STI) [1, 2].

The most common imbalance of vaginal flora is bacterial vaginosis (BV). It is a microbial dysbiosis in which normal microflora, consisting of predominantly *Lactobacillus* microflora, is replaced with numerous anaerobic bacteria, herein referred to as bacterial vaginosis-associated bacteria (BVAB). Symptoms of BV are increased gray or white vaginal discharge, itching or local discomfort, although symptoms are absent in 50% of patients [3, 4].

According to the World Health Organization data, 376 million people get infected with STIs globally every year, which indicates the importance of this public health problem [5]. STIs include more than 30 bacterial, viral, and parasitic pathogens which can be transmitted via vaginal, anal, or oral sex. Some of the most common STIs are *Chlamydia trachomatis*, *Mycoplasma genitalium*, *Mycoplasma hominis*, *Ureaplasma* spp., *Trichomonas vaginalis*, *Neisseria gonorrhoeae*, and human papilloma viruses [6]. There is a great number of clinical manifestations caused by STIs in the female upper and lower reproductive tract, but some of the most common ones are as follows: pelvic inflammatory disease, cervicitis, ectopic pregnancy, miscarriage, chronic pelvic inflammatory disease, neonatal infections, genital cancer, etc. [7]. Asymptomatic STIs are quite challenging, as these infections are difficult to identify, while they are easily transmissible in a sexually active population [5].

Various studies have indicated that BV increases the chances of STIs [8, 9]. Reduction of protective *Lactobacillus* types and changes in the vaginal environment such as increased pH, or reduction of lactic acid concentration enables the survival of vaginal pathogens. BVAB produces mucin-degrading enzymes (such as sialidase), which degrade the mucosal membrane of the vaginal epithelium and cervix, considered one of the most important components of the barrier against infection. The degradation of

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mucin and glycogen may cause microabrasions and changes in epithelial cells, which can make pathogen attachment to the receptors on epithelial cells easier. In addition, during BV, the immune balance is affected in a way that causes increased levels of proinflammatory cytokines, which make women more susceptible to STIs [6].

The objectives of this research paper are to establish an association between BV and the nine most common causes of STIs (*Chlamydia trachomatis*, *Ureaplasma* spp., *Mycoplasma genitalium*, *M. hominis, Trichomonas vaginalis, Neisseria gonorrhoeae*, high-risk human papilloma virus types (HR-HPV-16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59), herpes simplex virus types 1 and 2 (HSV-1, HSV-2), and to examine if the presence of BV increases the probability of coinfection with any of the STI microorganisms.

METHODS

This cross-sectional study was conducted on women of reproductive age who attended regular gynecological examinations at the Centre for Gynecology and Human Reproduction, Military Medical Academy, Belgrade, Serbia, during the period from November 2018 to December 2019. The patients included asymptomatic cases and those with various vaginal complaints. Exclusion criteria included recent antibiotic use (> 2 weeks) prior to sample collection. All the patients provided full informed consent for participation. The research has been approved by the Ethics Board of the Military Medical Academy.

One sample swab was collected for vaginal and cervical testing each (FLOQSwab, COPAN, Murrieta, CA, USA) for molecular analysis using multiplex RT-PCR. After collection, the swabs were placed into transport medium, vortexed, and stored at -20°C until DNA extraction (DNA-sorb-AM, AmpliSens, Moscow, Russia), which was performed according to the manufacturer's instructions.

The vaginal swabs were examined for the presence and quantification as well as interrelationship between *Lactobacillus* spp., *Gardnerella vaginalis*, *Atopobium vaginae,* and total concentration of bacterial DNA using a quantitative real-time PCR (RTQ-PCR) (AmpliSensFlorocenosis/Bacterial vaginosis-FRT). Microsoft Excel (Microsoft Corporation, Redmond, WA, USA) software was used to calculate three coefficients. The coefficients RC1 = log (Lac DNA) - log (Gv + Av DNA), RC2 = log (Bac DNA) - log (Lac DNA), RC3 = log (Bac DNA - log (Gv + Av DNA) were determined by the mutual relations between those bacteria. Based on coefficients, the patients were grouped in the following categories: normal vaginal flora (RC1 > 1, *Lactobacillus* spp. is the dominant flora); intermediate flora (0.5 ≤ RC1 ≤ 1, the same number of *Lactobacillus* spp. and aerobic bacteria); BV (RC1 < 0.5, dominant *G. vaginalis* and *A. vagine*); vaginal flora of non-specific etiology (RC2 > 1, RC3 > 2, any RC1 value, small concentration of *Lactobacillus* spp., but also *G. vaginalis*, *A. vagine*).

The cervical swabs were used for the detection of STIs and were tested by four different commercial RT-PCR tests. The first one detected *C. trachomatis, Ureaplasma*, *M. genitalium, M. hominis*; the second one detected *T. vaginalis* and *N. gonorrhoeae*; the third test identified HSV-1/HSV-2; and the fourth RT-PCR was used for the detection of HR-HPV (16, 18, 31, 33, 35, 39, 45, 51, 52, 56, 58, 59). All PCR reactions were done on a PCR thermocycler (Sa-Cycler 96, Sacace Biotechnologies, Como, USA).

Statistical analysis

To assist statistical analysis, the PCR test results for BV were categorized into three groups: normal, BV, and abnormal non-BV flora. The flora of nonspecific etiology and intermediary flora were considered to be abnormal non-BV flora. A result of 'bacterial load decreased' was considered a normal finding.

Association between dependent and independent variables was tested using Pearson's χ^2 test or Fisher's probability test. Statistical hypotheses were tested at the level of statistical significance (alpha level) of 0.05. All data were processed in the software package IBM SPSS Statistics, Version 22.0 (IBM Corp., Armonk, NY, USA).

RESULTS

In total, 235 patients of reproductive age were recruited for this study with the average age being 29.39 years (± 6.685). The results (RT-PCR) for BV showed that 121 (51.4%) women had normal (healthy) flora, 90 (38.2%) had BV, nine (3.8%) were denoted as intermediate, and 15 (6.4%) had vaginal flora of non-specific etiology.

Using four different RT-PCR tests, the frequency of the most common STIs were examined. The tests detected *C. trachomatis* (16; 6.8%), *Ureaplasma* spp. (143; 60.9%), *M. hominis* (66; 28.1%), *M. genitalium* (2; 0.9%), HR-HPV 12 types (70; 29.8%), *T. vaginalis* (3; 1.3%), HSV-1 (2; 0.9%), HSV-2 (2; 0.9%), while *N. gonorrhoeae* was not detected in any sample.

Out of 235 patients, 179 (76%) had one of the STI microorganisms detected, while 56 (23.8%) were negative for all microorganisms. Simultaneous presence of more than one microorganism was detected in 93 patients (39.6%). The distribution of mono- and coinfections is presented in Figure 1.

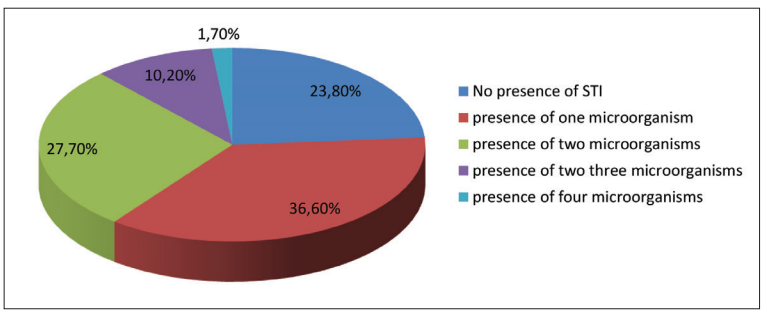

Figure 1. Distribution of mono- and coinfection

Table 1. Association between bacterial vaginosis (BV) and the presence of total sexually transmitted infections (STI)

Total number of STIs	Presence	Normal flora	BV	Intermediate	Flora of unspecified etiology	Total
	no presence	37 (30.6%)	10 (11.1%)	2 (22.2%)	7 (46.7%)	56 (23.8%)
	presence	84 (69.4%)	80 (88.9%)	7 (77%)	8 (53.3%)	179 (76.2%)
	Total	121 (51.4%)	90(38.2%)	9 (3.8%)	15 (6.4%)	235 (100%)

Table 2. Distribution of sexually transmitted infection pathogens in correlation with the state of vaginal flora

Pathogens	Normal flora	BV	Abnormal non-BV		p
			Intermediate	Flora of unspecified etiology	
C. trachomatis	5 (4.1%)	10 (11.1%)	0	1 (6.7%)	0.2
Ureaplasma spp.	63 (52.1%)	71 (78.9%)	5 (55.6%)	4 (26.7%)	< 0.01
M. genitalium	0	2 (2.2%)	0	0	0.34
M. hominis	20 (16.5)	50 (44.4%)	3 (33.3%)	3 (20.0%)	< 0.01
T. vaginalis	1 (0.8%)	1 (1.11%)	0	1 (6.7%)	0.33
N. gonorrhoeae	0	0	0	0	-
HR-HPV (6 ,18, 31,33, 35, 39, 45, 51, 52, 56, 58, 59)	34 (37%)	25 (50%)	6 (75%)	5 (45%)	0.125
HSV-1	1 (0.8%)	1 (1.1%)	0	0	1
HSV-2	0	1 (1.1%)	1 (11.1%)	0	0.3

HSV – herpes simplex virus; HR-HPV – high-risk human papilloma virus; BV – bacterial vaginosis

Table 3. Association between bacterial vaginosis and presence of mono- and coinfection

Infection	Normal vaginal flora	BV	Abnormal non-BV microbiota	Total
No presence of STI	37 (30.6%)	10 (11.1%)	9 (37.5%)	56 (23.8%)
Mono infection	51 (42.1%)	30 (33.3%)	5 (20.8%)	86 (36.6%)
Coinfection	33 (27.3%)	50 (55.6%)	10 (41.7%)	93 (39.6%)
Total	121 (51.4%)	90 (38.2%)	24 (10.4%)	235 (100%)

BV – bacterial vaginosis; STI – sexually transmitted infection

The results have shown that there is a statistically significant difference ($\chi^2 = 15.380$, $p = 0.001$) between the total number of STIs and the condition of the vaginal flora obtained by RT-PCR BV. STIs are most common in patients with BV (80; 89.9%) followed by women with intermediate flora (7; 77.8%) (Table 1).

Distribution of the presence of various STI pathogens in correlation with the state of vaginal flora is shown in Table 2. Statistical significance of correlation of STI pathogens related to the state of vaginal flora exists only with *Ureaplasma* spp. and *M. hominis,* while no such significance was identified with other pathogens. Although there is no significant difference in distribution of correlation with the state of vaginal flora, it has been confirmed that the largest percentage of STI-positive pathogens is in patients with BV.

The association between BV and the presence of mono- and coinfection is presented in Table 3. There is statistical significance between the presence of monoinfections and coinfections compared to the state of vaginal flora ($\chi^2 = 23.677$, $p < 0.001$). In most cases, women with normal flora had monoinfection (51; 42.1%), while women with BV had coinfection (50; 55.6%).

DISCUSSION

This paper employs molecular methods to investigate the prevalence of BV in women of reproductive age but also the association between vaginal dysbiosis and some of the most frequent STI pathogens. Investigations into the association of BV and STIs traditionally use Nugent score or Amsel criteria, which are the gold standard in BV diagnostic method [10–13]. We have used RT-PCR diagnostic testing for BV in the study because there is research that demonstrates shortcomings in the application of the Amsel criteria and Nugent score methods [14, 15]. AmpliSensFlorocenosis/Bacterial vaginosis-FRT in relation to Nugent score, Amsel criteria, vaginal culture, and BD MAX Vaginal panel shows the highest degree of association related to 16S rRNA genome sequencing with microbiome analysis, presented in research paper by van den Munckhof et al. [16]. The RT-PCR test used is based on detection and quantification of *G. vaginalis* and *A. vaginae*, which are important markers for BV diagnostics. On the grounds of the relationship of these two anaerobes – lactobacilli and the total number of bacteria – the test enables assessment of the status of the vaginal flora. Our results indicate that BV prevalence is 38.2% in our study population.

This research has shown that women with BV have a higher frequency of infections with *Ureaplasma* spp. and *M. hominis.* Since *M. hominis* and *Ureaplasma* spp. can be found in both healthy individuals and women with BV, there is an ongoing debate and disagreement on the detection of these pathogens. Due to this fact, the presence of *M. hominis* and *Ureaplasma* spp. in the urogenital tract is not definitive proof of infection and, as such, can be a significant clinical problem. It is considered that the identification of these two pathogens is not adequate without the assessment of the status of vaginal flora. On account of these pathogens' association with various reproductive problems (chorioamnionitis, endometritis, postpartum fever, low birth weight, and preterm delivery) we consider that their identification is of great importance, particularly if BV is present as well [17, 18].

Some STI pathogens (*C. trachomatis* and HR-HPV) have been detected in women with all three states of vaginal flora. However, we have confirmed that the highest percentage of BV patients is positive with *C. trachomatis* (11.1%). At the same time, the highest percentage of HPV-positive patients are in the intermediate vaginal flora (75%) and BV (50%) groups.

In this study, some of the pathogens, such as *M. genitalium, T. vaginalis,* HSV-1/HSV-2, have been detected in a very small percentage of swabs or have not been identified at all (*N. gonorrhoeae*). Considering these results, the opportunity to compare these STI pathogens with various states of vaginal flora has been limited.

The presence of detected STI pathogens and relation with the state of vaginal flora indicate that monoinfections are most often present in patients with normal flora (42.1%), while coinfections with two, three, or four pathogens are mostly present in BV patients (55.6%). Other research studies have also confirmed an association of coinfection and certain STI pathogens with BV [19, 20, 21]. A large number of studies examine the association between STIs and BV, but they rarely include as many pathogens as the current study.

Huge gaps in our knowledge of STI etiology remains an issue, including coinfection and its links with certain clinical manifestations. Bacterial coinfections impact significantly the process of pathogenesis and appearance of clinical manifestations [22, 23]. It is also indicative that coinfections may be present in asymptomatic patients [24]. Compared to infections, coinfections change the process of inflammation in different ways. They also provide fertile ground for the multiplication of opportunistic mycoplasma and its pathogenic effects. The mediators freed by the process of inflammation can cause stagnation in the development cycle of *C. trachomatis* and lead the process into inactive, persistent form. Urogenital mycoplasma can prolong the inflammation of the urethra even after the elimination of *C. trachomatis* if there is a resistance to antibiotic therapy. A great number of microorganisms reduce bioavailability of applied medication during the infection therapy. For this reason, it is important that further STI research targets coinfections, their pathogenesis, eradication, and efficiency of therapy [22].

In patients with abnormal flora (not related to BV), there was a high percentage of coinfections (41.7%), but also no presence of STI pathogens (37.5%). However, since we detected, while using PCR tests, a small percentage of patients with intermediate vaginal flora and flora of nonspecified etiology, our results cannot adequately confirm the association of abnormal non-BV vaginal flora and STI pathogens.

Although many longitudinal and cross-sectional studies have examined the association between BV and STI, the results are different [8, 21, 25, 26, 27]. The problem arises from the fact that BV is a polymicrobial syndrome whose diagnosis is not precise because many of these studies used Amsel criteria or Nugent score as the gold standard. On the other hand, the identification of STI pathogens depends on laboratory tests, geographic region, and characteristics of the examined populations.

The limitation of this study is that the presence of some of STI pathogens were detected in very small percentages or were not detected at all, which impedes full examination of their association with BV. Besides BV, association of anaerobic vaginitis with various STI pathogens also needs to be researched as there are few studies on this topic in the current literature [17].

CONCLUSION

This study has confirmed the association of *M. hominis* and *Ureaplasma* spp. with BV, as well as an association of coinfections with this dysbiosis. Considering that the total frequency of STI pathogens in the examined swabs is 76%, it is important to pay attention to the prevention and elimination of the spreading of STIs. It is also important to continue with education, screening, and raising awareness of STIs and the health issues they may cause. Better understanding of the association between various STIs and the status of vaginal flora is necessary to enable better diagnosis, prevention of diseases, and women's health protection.

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Повезаност бактеријске вагинозе и најчешћих узрочника сексуално преносивих инфекција

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САЖЕТАК

Увод/Циљ Бактеријска вагиноза (БВ) најчешћа је вагинална дисбиоза која повећава могућност настанка сексуално преносивих инфекција (СПИ).

Циљ овог истраживања је утврђивање повезаности БВ и девет најчешћих узрочника СПИ (*Chlamydia trachomatis, Ureaplasma spp., Mycoplasma genitalium, Mycoplasma hominis, Trichomonas vaginalis, Neisserria gonorrhoeae*, високо ризични типови хуманог папилома вируса, вирус херпес симплекса типа 1 и 2) и да ли присуство БВ повећава вероватноћу за постојањем коинфекције неким од узрочника СПИ.

Методе У студију је укључено 235 жена у репродуктивном периоду. Један вагинални и један цервикални брис коришћени су за молекуларну анализу. Вагинални брисеви коришћени су за детекцију БВ и процену вагиналне флоре уз помоћ мултиплексног квантитативног *RT-PCR* теста. Цервикални брис је коришћен за доказивање присуства сексуално преносивих патогена који су испитани са четири различита комерцијална *RT-PCR* теста. За статистичку анализу резултата коришћени су Пирсонов χ^2 и Фишеров тест вероватноће.

Резултати Поређење присуства укупног броја узрочника СПИ у зависности од стања вагиналне флоре показује да су СПИ најчешће код болесника са БВ (80; 89,9%). Жене са БВ имају повећану учесталост инфекција са *Ureaplasma spp*. и *M. hominis*, 71 (78,9%) односно 50 (44,4%). Присуство свих детектованих узрочника СПИ у односу на стање вагиналне флоре показује да су моноинфекције најчешће присутне код болесника са нормалном флором (42,1%), док су коинфекције (55,6%) највише присутне код жена са БВ.

Закључак Истраживање је показало асоцијацију *M. hominis* и *Ureaplasma spp.* са БВ као и повезаност коинфекција са овом дисбиозом. Разумевање повезаности између различитих СПИ и стања вагиналне флоре је неопходно како би се омогућила боља дијагностика и превенција болести, као и заштита здравља жена.

Кључне речи: бактеријска вагиноза; сексуално преносиве инфекције; коинфекције, *RT-PCR*

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Selective laser trabeculoplasty as adjunctive treatment in pseudoexfoliative glaucoma patients

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SUMMARY

Introduction/Objective Pseudoexfoliation syndrome is characterized by abnormal production and accumulation of fibrillar, white-gray, "dandruff-like" material in almost all ocular structures. The aim of this study was to examine effect of selective laser trabeculoplasty in pseudoexfoliation glaucoma patients.

Methods Thirty-two patients (47 eyes) with medically uncontrolled pseudoexfoliation glaucoma were enrolled. All the patients could not reach target intraocular pressure with maximal tolerated medical therapy before treatment. Selective laser trabeculoplasty was performed with about 100 non-overlapping spots. Intraocular pressure was measured one hour, seven days, four weeks, three, six, twelve, eighteen, and twenty-four months after the procedure.

Results The mean base intraocular pressure was 23.45 mmHg (SD = 3.07). Statistically significant reduction of mean intraocular pressure was observed at all follow-ups except one hour after treatment. Mean intraocular pressure after 24 months was 18.39 mmHg (SD = 1.82). Success, defined as intraocular pressure reduction from base intraocular pressure by more than 20% after 24 months, was achieved in 27 eyes (57.45%). We did not find any influence of sex and age on selective laser trabeculoplasty effects in pseudoexfoliative glaucoma patients. Baseline intraocular pressure is proved to be a reliable predictor of intraocular pressure-lowering effect, as there was a strong correlation between baseline intraocular pressure and the percentage of reduction of intraocular pressure after 24 months ($r = 0.71$, $p < 0.01$).

Conclusion Selective laser trabeculoplasty is a safe and effective method for the reduction of intraocular pressure in pseudoexfoliation glaucoma patients and should be used more often in this challenging form of glaucoma. Baseline intraocular pressure seems to be a reliable predictor of success.

Keywords: selective laser trabeculoplasty; pseudoexfoliative glaucoma; intraocular pressure

INTRODUCTION

Pseudoexfoliation syndrome is a systemic disease of the extracellular matrix with primary ocular manifestations. Syndrome is characterized by an abnormal production and accumulation of fibrillar, white-gray, "dandruff-like" material in almost all ocular structures. The pseudoexfoliative material is produced by cells of the anterior segment of the eye. This material is insoluble and floats in the aqueous humor, and is most often deposited on the structures of the eye in contact with aqueous humor, such as the lens, ciliary body, corneal endothelium, and trabecular meshwork (TM). In clinical presentation, it is usually seen on the pupillary margin, anterior capsule of the lens, and on TM. Pseudoexfoliative material can disturb the function of goblet cells in conjunctiva, which causes tear film instability and dry eye syndrome. Structure of this material is fibrillar and it contains parts of basal membranes and some enzymes [1, 2, 3].

Accumulation of this material in the TM can lead to its obstruction and to increased intraocular pressure (IOP). Pigment granules can be seen in the anterior chamber angle and they are probably released from the iris while rubbing on the fibrillar deposits on the lens. Pigment granules can participate in clogging of TM.

Pseudoexfoliation (PXF) syndrome is the most common cause of secondary glaucoma, although not all patients with pseudoexfoliation syndrome will encounter this disease [1].

It is known that PXF glaucoma is a very difficult form of glaucoma to treat [2], with high IOP and rapid unpredictable progression of the disease [4]. It is quite challenging for treatment because it often responds poorly to medical therapy. Treatment of glaucoma includes medical, laser, and surgical treatment.

Laser trabeculoplasty as a method for lowering IOP has been in use for more than 40 years. It was first introduced by Wise and Witter in 1979 [5]. At that time, argon laser was user for this method, so it was called argon laser trabeculoplasty (ALT). ALT has shown good results in lowering IOP although its mechanism of action has never been completely understood. Later it was noticed that it is often followed by scaring in the iridocorneal angle and occurrence of peripheral anterior synechia. This finding limited ALT to a single application in one patient.

First results with selective laser trabeculoplasty in lowering IOP in patients with open angle glaucoma were presented in 1995 by

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Park and Latina [6]. This method is in commercial use since 2001 [6]. For this procedure, Q-switched, frequency-doubled laser with a wavelength of 532 nm is used. Laser beam is directed at TM and duration of the pulse of laser energy is only 3 ns. Small amount of laser energy released is enough to heat and damage only pigmented cells of TM that absorbs light energy to a greater extent than surrounding non pigmented cells of TM.

The aim of this study was to analyze the effects of selective laser trabeculoplasty on patients with medically uncontrolled PXF glaucoma. We examined the significance of IOP reduction compared to baseline IOP during follow-up period of 24 months, influence of baseline IOP value to the percentage of reduction, as well as influence of demographic characteristics.

METHODS

In this prospective, nonrandomized, self-controlled, interventional cohort study 32 patients (47 eyes) with medically uncontrolled pseudoexfoliation glaucoma were enrolled to assess the response to SLT. With the approval of institutional Committee on Ethics and according to the tenets of the Declaration of Helsinki, all the patients gave their written consent at the beginning of the study.

All patients could not reach target IOP with maximal tolerated medical therapy. Patients underwent detailed ophthalmological examination before procedure: Snellen visual acuity, slit lamp examination of anterior segment, gonioscopic examination of anterior chamber angle, optic disc examination and visual field testing which confirmed diagnosis.

The study was conducted at an opthhalmological hospital, Belgrade Ophthalmological Center, Belgrade, Serbia from June 1, 2019 until June 1, 2021. All the procedures were done by one experienced ophthalmologist.

Selective laser trabeculoplasty was performed over 360 degrees of TM.

IOP was measured one hour, seven days, four weeks, three, six, 12, 18, and 24 months after the procedure. Baseline characteristics of all the patients were recorded at the beginning of the study. These include the following: age, sex, diagnosis, baseline IOP, and the number and the type of used medications.

Patient inclusion criteria were IOP above target IOP with maximal tolerated medical therapy in patients with pseudoexfoliation glaucoma. Patients had to be older than 18 years to participate in the study.

Exclusion criteria were advanced-stage glaucoma; patients who underwent any previous antiglaucoma laser or surgical procedure; eyes with previous anterior segment surgery, such as cataract extraction within the previous six months, and patients with baseline IOP > 30 mmHg with fully medicated local and per oral antiglaucomatous treatment (carbonic anhydrase inhibitors). Patients who could not be followed for at least 24 months were also excluded and their results were removed.

During four weeks before treatment, IOP was measured at least two times in all the patients in order to get the baseline IOP as a mean value of these measurements. IOP was measured using the Goldmann applanation tonometer.

Immediately before treatment, IOP was measured and after that 0.5% solution of apraclonidine was instilled in the treated eye with the aim to prevent IOP spikes [7, 8].

The procedure was performed with a topical anesthetic – benoxinate hydrochloride 0.4%. With the patient seated at the laser slit-lamp system, a Goldmann three-mirror goniolens or Latina lens was placed on the eye with methylcellulose 2%.

All eyes were treated with Ellex, Tango® SLT laser (Ellex Medical Pty. Ltd, Adelaide, Australia) a frequency-doubled, Q-switched Nd: YAG laser emitting at 532 nm with fixed pulse duration of 3 ns and a spot size of 400 μm. Treatment began at 0.8 mJ and was titrated according to the response. If cavitation bubbles appeared the laser energy was reduced by 0.1 mJ until no bubbles formed and treatment was continued at this energy level. If no cavitation bubble was observed, the pulse energy was increased by 0.1 mJ until bubble formation and then decreased as described above. Sometimes higher energy was required for the treatment of the superior, less pigmented angle. Approximately 100 adjacent, but non overlapping, laser spots were placed over 360° of the TM [8].

Immediately after the laser treatment, nepafenac eye drops were administered once in treated eye than three times daily for three days. The IOP in the treated eye was measured and recorded one hour after surgery. The same preoperative anti-glaucoma medication regimen was continued. Patients were evaluated at one hour, one week, and at one, three, six, 12, 18, and 24 months. At each visit, the visual acuity and IOP were measured, and slit-lamp examination of the anterior segment was performed. All major and minor complications and complaints were recorded and treated appropriately.

Complete success of treatment was defined as IOP reduction of 20% and more and qualified success reduction of IOP between 10% and 20%.

The data was analyzed using IBM SPSS Statistics, Version 22.0 (IBM Corp., Armonk, NY, USA), using ANOVA for repeated measures and t-test for dependent samples. A p value of less than 0.05 was considered to be statistically significant.

RESULTS

From 47 eyes of 32 patients initially enrolled, 25 patients (38 eyes) completed the study. Seven patients (nine eyes) were sent for other surgical treatment or to repetition of SLT due to high IOP during follow-up period and they were excluded from the study. Patients that did not come on all follow-ups were also excluded, and their data erased.

Average age of our patients was 73.76 (SD = 5.79) and baseline IOP was 23.13 (SD = 3.06). There were 13 men (21 eyes) and 12 women (17 eyes) in our group. Baseline characteristics of included patients are presented in Table 1.

Seven days after the treatment, mean IOP was reduced to 18.26 (SD = 2.65). One month after SLT, mean IOP dropped to 16.74 (SD = 2.06). The lowest mean IOP was recorded three months after treatment and it was

Table 1. Baseline characteristics of the patients

Characteristics		Male	Female	All
Number (%)	Patients	13 (52)	12 (48)	25
	Eyes	21 (55.26)	17 (44.74)	38
Age (years)	Mean	75.19	72	73.76
	SD	5.19	6.14	5.79
	Min	67	60	60
	Max	82	83	83
Base IOP	Mean	23.14	23.12	23.13
	SD	2.89	3.35	3.06
	Min	19	18	18
	Max	29	30	30

IOP – intraocular pressure; SD – standard deviation; Min – minimal value; Max – maximal value

15.84 mmHg (SD = 1.87) or 31.51% reduction from the baseline. After that period, mean IOP showed mild but constant raise and 24 months after treatment the mean IOP was 18.03 mmHg (SD = 1.64) ($p < 0.01$) and that was 5.11 mmHg lower than baseline, or 22.07%. It is shown in Tables 2 and 3, and Figure 1.

Table 2. Intraocular pressure values values over time (mm Hg)

IOP values	Means	SD	Min	Max
Baseline	23.13	3.06	18	30
1 hour	22.74	4.42	14	33
7 days	18.26	2.65	15	23
1 month	16.74	2.06	12	21
3 months	15.84	1.87	10	21
6 months	16.37	2.01	14	20
12 months	16.95	1.74	13	21
18 months	17.26	1.81	12	21
24 months	18.03	1.64	14	21

IOP – intraocular pressure; SD – standard deviation; Min – minimal value; Max – maximal value

Table 3. Intraocular pressure (IOP) reduction over time

Time	IOP reduction	
	mm Hg	%
1 hour	0.39	1.71
7 days	4.87	21.05
1 month	6.39	27.65
3 months	7.29	31.51
6 months	6.76	29.24
12 months	6.18	26.73
18 months	5.87	25.37
24 months	5.11	22.07

Success of treatment was defined as reduction of IOP of at least 20% from baseline IOP. Eleven eyes did not achieve IOP reduction of 20% after 24 months. When we take into account the nine eyes that were referred to surgery and 11 eyes that did not achieve IOP reduction of 20% or more, it makes 20 out of 47 eyes, or 42.55%. Success, after 24 months, was achieved in 27 eyes (57.45%).

Paired-samples t-test was used to compare the means of baseline IOP and IOP 24 months after treatment and difference is highly statistically significant ($p < 0.001$).

We also investigated the role of baseline IOP values as a predictor of SLT response in patients with pseudoexfoliative glaucoma. There was a strong correlation between the mean base IOP and the mean percentage of reduction after 24 months from treatment ($r = 0.71$, $p < 0.01$) (Table 4, Figure 2).

Table 4. Correlation between baseline intraocular pressure (IOP) and reduction percentage

		Baseline IOP	Reduction
Baseline IOP	Pearson correlation	1	0.710**
	Sig. (2-tailed)		0.000
	n	38	38
Reduction	Pearson correlation	0.710**	1
	Sig. (2-tailed)	0	
	n	38	38

**Correlation significant – $p < 0.01$

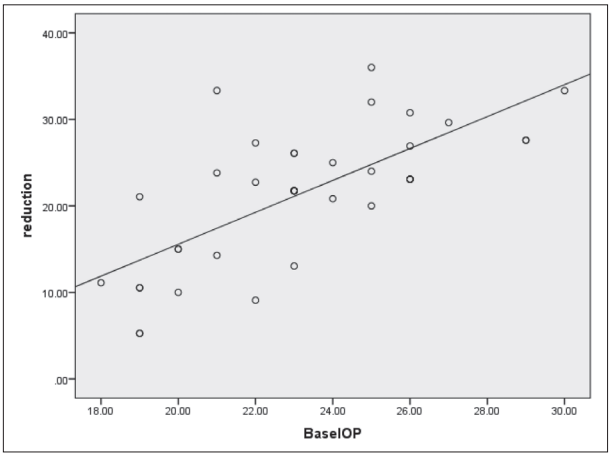

Figure 2. The influence of baseline intraocular pressure (IOP) on selective laser trabeculoplasty reduction percentage – significant correlation ($r = 0.71$, $p < 0.01$)

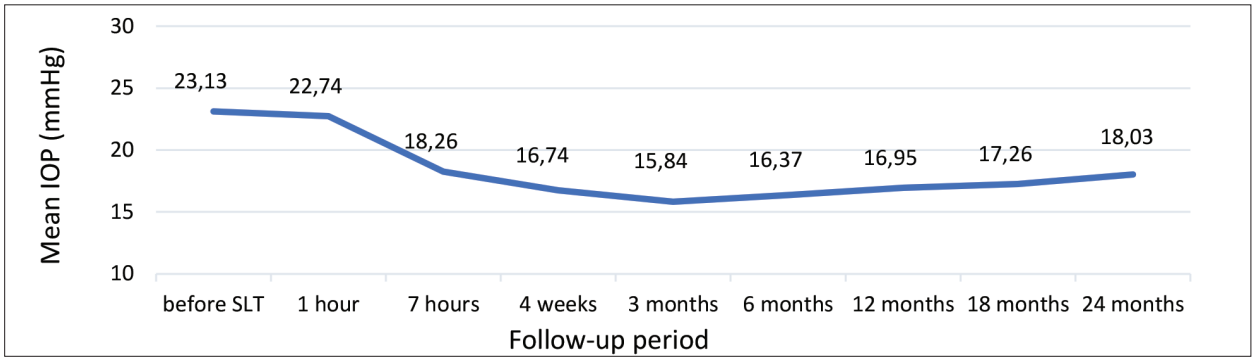

Figure 1. Mean intraocular pressure (IOP) values during the follow-up of 24 months

Most significant side effect of SLT was postoperative IOP spike, which was noticed in 10 eyes (26.31%) one hour after treatment. In six of them (15.79%), IOP spike was higher or equal with 5 mmHg. After seven days there were no eyes with IOP higher than baseline. In 11 eyes (28.95%) mild anterior chamber inflammation was present one hour after treatment, but at seven days all eyes were quiet. One patient complained at ocular discomfort, but did not require discontinuation of the treatment. There were no corneal complications.

DISCUSSION

In previous papers selective laser trabeculoplasty was proved to reduce IOP in primary open angle glaucoma as well as in pseudoexfoliative and pigmentary glaucoma. Although the percentage of reduction was similar in many reports, there were some papers that found reduction as high as 40% and others that reported less than 10% [9–18].

In our group of patients with pseudoexfoliative glaucoma, SLT was used as adjunctive treatment and we achieved mean reduction of IOP after two years of 22.07% from mean baseline IOP that was similar to expected values based on earlier papers [19–23]. At the beginning of the study it looked that reduction of IOP was even higher than compared to reduction found in primary open angle glaucoma, and it was more than 30% at three months, but at 24 months check-up it was the same and even lower than in POAG patients. Thus it seems that lowering effect of SLT in pseudoexfoliative glaucoma patients fades over time. It is possible that the reason for that is continuous releasing of pseudoexfoliative material that cloggs iridocorneal and trabecular tissue. Even though the initial IOP lowering effect is accomplished, release of pseudoexfoliative material leads to continuous increase in IOP.

As complete success we defined lowering of IOP for at least 20%. We achieved this result in 57.45% of patients after 24 months. At 12 months this result was 65.96%, which suggests faster fading of SLT IOP reduction effect in PXF glaucoma patients. IOP reduction 10–20% we defined as qualified success, which was achieved in 7.89% after 24 months, so success was accomplished in 78.95% of the eyes that have finished the study or in 63.83% of the eyes that entered the study. Sometimes, even small reduction of IOP could be significant in reaching target IOP for some patients and delaying or avoiding the need for surgical treatment and value of SLT for these patients should not be underestimated.

We did not find any difference between male and female eyes regarding the SLT response. This finding is consistent with other papers [24]. Also we did not find any influence of age on SLT effects.

Comparing all follow-ups and mean IOP it was obvious that after one month we had almost definite results and we thought that one should wait for one month to see if the SLT response was adequate for the patient, but that there was no reason to wait any longer if the result was not satisfactory.

IOP spike was the most significant side effect that occurred after treatment. It was noticed in 26.31% of the eyes and in 15.79% of the eyes it was higher or equal to 5 mmHg. Some mild anterior chamber inflammation was present in 28.95% of eyes one hour after treatment. At seven days follow-up there were no eyes with IOP higher than baseline and all the eyes were without anterior chamber inflammation. This suggest that SLT in this group of patients is a quite safe method, with no significant side effects. These results are similar to those of some other studies [25, 26, 27].

Our study confirmed that higher baseline IOP predicts higher hypotensive response and higher percentage of IOP reduction from baseline [28, 29].

In this group we did not aim for the reduction in the number of medications used because all the patients were on maximally tolerated medical therapy and did not reach target IOP, and we were looking for additional reduction of IOP, so the mean number of medications did not change before and after the study.

CONCLUSION

Selective laser trabeculoplasty is very effective in lowering IOP in medically uncontrolled patients with pseudoexfoliative glaucoma and should be used more readily in this challenging form of glaucoma. Also, it appears that effect of selective laser trabeculoplasty on those patients fades away with time. Intervention is safe and not accompanied with significant side effects. Baseline IOP seems to be a reliable predictor of IOP-lowering effect. Also, it looks like that definite IOP-lowering results can be estimated after one month.

Conflict of interest: None declared.

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Селективна ласерска трабекулопластика као адјувантна терапија код пацијената са псеудоексфолијативним глаукомом

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САЖЕТАК

Увод/Циљ Псеудоексфолијативни синдром карактерише абнормална производња и накупљање фибриларног, белосивог материјала сличног перути у готово свим окуларним структурама.

Циљ ове студије био је испитати ефекат селективне ласерске трабекулопластике код пацијената са псеудоексфолијативним глаукомом.

Методе Тридесет два пацијента (47 очију) са медикаментно неконтролисаним псеудоексфолијативним глаукомом била су укључена у студију. Сви пацијенти нису могли постићи терапијски интраокуларни притисак са максимално толерисаном медикаментном терапијом пре почетка лечења. Селективна ласерска трабекулопластика изведена је са око 100 печата који се не преклапају. Интраокуларни притисак мерен је један час, седам дана, четири недеље, три, шест, дванаест, осамнаест и двадесет четири месеца после захвата.

Резултати Средњи базни интраокуларни притисак био је 23,45 *mmHg* (*SD* = 3,07). Статистички значајно смањење средњег интраокуларног притиска уочено је код свих контрола осим једног часа након третмана. Средњи интраокуларни притисак након 24 месеца износио је 18,39 *mmHg* (*SD* = 1,82). Успех, дефинисан као смањење интраокуларног притиска од базног за више од 20% након 24 месеца, постигнут је код 27 очију (57,45%). Нисмо открили утицај пола и старости на селективне ефекте ласерске трабекулопластике код пацијената са псеудоексфолијативним глаукомом. Показало се да је почетни интраокуларни притисак поуздан предиктор ефекта снижавања интраокуларног притиска, јер је постојала снажна корелација између основног интраокуларног притиска и процента смањења интраокуларног притиска након 24 месеца ($p = 0,71$, $p < 0,01$).

Закључак Селективна ласерска трабекулопластика је сигурна и ефикасна метода за смањење интраокуларног притиска код пацијената са псеудоексфолијативним глаукомом и требало би је чешће користити у овом изазовном облику глаукома. Чини се да је основни интраокуларни притисак поуздан предиктор успеха.

Кључне речи: селективна ласерска трабекулопластика; псеудоексфолијативни глауком; интраокуларни притисак

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Evaluation of peripapillary retinal nerve fiber layer thickness in patients with primary open-angle glaucoma

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SUMMARY

Introduction/Objective The objective of the study was to determine the difference in peripapillary retinal nerve fiber layer (RNFL) thickness in patients with open-angle glaucoma (POAG) in comparison to the healthy population and according to the progression of the disease.

Methods Four groups were formed among 120 patients: group without glaucoma, early, moderate POAG, and preperimetric glaucoma group. Visual field and optical coherent tomography were performed.

Results RNFL thickness value was found to be the highest in the inferior quadrant, the second highest in the superior one, the third highest in the nasal one, and the lowest value was found to be in the temporal quadrant. The highest average value of RNFL thickness was in the superior quadrant of the healthy group (124.3 ± 17.8 μm), and the lowest one was in the temporal quadrant of the moderate group (46.5 ± 10.8 μm). Other RNFL thickness values per quadrants among groups were distributed between these two endpoints. Mean value of peripapillary RNFL thickness (AvgThic) in patients with moderate POAG was lesser than in patients with early POAG, which, in turn, was lesser than that in the healthy subjects (59.6 ± 10.6 μm *vs.* 73.4 ± 12.1 μm *vs.* 105.5 ± 11.3 μm). AvgThic in the preperimetric glaucoma group was 83.6 ± 9.2 μm. Pearson correlation showed a high positive correlation between mean deviation values and the following parameters: AvgThic, S, I, Smax, Imax, Savg, Iavg. Receiver operating characteristic curves found that the parameter with the best diagnostic ability was AvgThic, with the area of 0.803 (< 0.0005), sensitivity of 67%, and specificity of 83.3%.

Conclusion Peripapillary RNFL thickness parameters AvgThic, S, I, Smax, Savg, Iavg, Imax have an excellent ability to discriminate between healthy eyes and eyes with POAG. The parameter with the highest specificity and sensitivity is AvgThic, which makes it the best for early detection and monitoring of POAG.

Keywords: retinal nerve fiber layer; primary open-angle glaucoma; optical coherent tomography; AvgThic

INTRODUCTION

Primary open-angle glaucoma (POAG) represents a chronic, progressive, and irreversible multifactorial optic neuropathy. It is characterized by cupping of the optic disc, visual field defects, open anterior chamber angle, and, in the majority of cases, increased intraocular pressure (IOP). The progressive loss of retinal ganglion cells is the most important characteristic of POAG and it can be quantified by measuring the thickness of peripapillary retinal nerve fiber layer (RNFL) [1, 2].

During the 1970s, Hoyt et al. pointed out the importance of evaluation of RNFL thickness in the diagnosis of glaucoma [3], and along with other researchers showed that the thinning of peripapillary RNFL could even precede the visual field defects as the first sign of glaucoma pathology [4, 5]. Optical coherence tomography (OCT), as a highly objective and reproducible imaging method, was developed at the Massachusetts Institute of Technology by David Huang et al. in 1991 [6]. It represents a non-invasive, non-contact, trans-pupillary method for scanning the retinal structures layer by layer and it is used to analyze different retinal diseases [6]. OCT produces images of high resolution and is able to identify diffuse and focal RNFL defects that occur in glaucoma [7]. Numerous studies have shown that OCT measurement of peripapillary RNFL thickness and macular zone thickness is an excellent method for the diagnosis of glaucoma. However, RNFL thickness has shown itself as a better indicator in glaucoma evaluation [8, 9]. The purpose of this study was to determine the difference in peripapillary RNFL thickness in patients with preperimetric glaucoma and POAG in comparison to the healthy population, as well as to determine the difference in thickness of peripapillary RNFL according to the progression of the disease. By accurately determining these differences, we wanted to define the ability of every RNFL thickness parameter in early detection and monitoring of patients with POAG.

METHODS

This research was a prospective and observational type of study. Based on the inclusion

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criteria, patients were included in the study and they underwent a complete ophthalmologic examination, visual field analysis, and OCT of the peripapillary RNFL.

A total of 120 patients over the age of 18 years were included in this study. Pathology of only one eye of each patient was analyzed. In cases where both eyes of the patient met the inclusion criteria, the eye included in the study was randomly selected. Based on the clinical findings, the following four groups were formed with the specified inclusion criteria.

Group 1 (control – healthy group): 30 patients without glaucoma or other eye conditions, with best corrected visual acuity ≥ 0.9, IOP 10–21 mmHg, normal cup-to-disc ratio (C/D), and normal visual field finding, regardless of sex, race, and ethnic background.

Group 2 (early glaucoma group): 30 patients with POAG, with characteristic defects of the optic disc and RNFL, with a mean deviation (MD) lower than -2dB and higher than -6dB in standardized automated perimetry (Hodap classification), with characteristic glaucomatous visual field defects, without other eye conditions, without anamnestic data about previous laser or surgical intervention on the examined eye, with best corrected visual acuity ≥ 0.5, regardless of gender, race and ethnic background.

Group 3 (moderate glaucoma group): 30 patients with POAG, with characteristic defects of the optic disc and RNFL, with MD lower than -6 dB and higher than -12 dB in standardized automated perimetry (Hodapp classification), without other eye conditions, without anamnestic data about previous laser or surgical interventions on the examined eye, with best corrected visual acuity ≥ 0.5, regardless of sex, race, and ethnic background.

Group 4 (preperimetric glaucoma group): 30 patients with characteristic changes in the optic nerve head that represent glaucoma neuropathy, without functional outbreaks. The standard automated perimetry showed normal values of MD (from -2 dB to +2 dB), with the best corrected visual acuity ≥ 0.9, regardless of the IOP.

Visual field was performed on the Humphrey Visual Field Analyser (Carl Zeiss Meditec Inc. – Humphrey Systems, Dublin, CA, USA), using the Threshold C24-2 testing protocol by SITA-FAST strategy. A reliable visual field (VF) test was defined as one with less than 20% fixation loss, and false-positive and false-negative rates of less than 33%. We used two of VF global indices, MD and pattern standard deviation (PSD) in this study. The VF test was obtained within four weeks before the OCT scans.

All peripapillary RNFL thickness measurements were done on the Stratus OCT 3000, Carl Zeiss Meditec apparatus, honoring the fast-scanning protocol for optical disc and peripapillary RNFL. Afterwards, an automatic analysis was applied using the previously implemented software version (3.0 Stratus OCT analysis software). OCT measurements were made along a circle concentric with the optic disc at a radius of 1.73 mm, using a scanning mode that samples 512 data points (RNFLThickness 3.4 acquisition protocol). Maximum two scans of the peripapillary zone were used (with three consecutive scans), provided that the quality of the scan was equal or higher than 7. For data analysis, we chose the better one of the two scans.

The study protocol was approved by the institutional ethics committee.

In our research, the following descriptive statistics were used: arithmetic mean, standard deviation, median, quartiles, frequencies, and percentages. Means comparison analysis of paired parameters between the groups was evaluated by one-way ANOVA including the Levene's homogeneity of variance test. Post-hoc adjustment for multiple comparisons was performed by the Games–Howell method if variances in groups were not equal, and by the Tukey's honest significant difference test when variances in groups were equal. The connection between RNFL thickness and visual field parameters was characterized by bivariate correlation analysis computing the Pearson correlation coefficients. The Pearson correlation coefficients with absolute values ≥ 0.5 suggesting a strong association with $p < 0.01$ were accepted as statistically significant. Receiver operating characteristic (ROC) curves were used to describe the accuracy of each OCT parameter to differentiate the glaucoma from the healthy group. The diagnostic sensitivity and specificity were examined with the area under the ROC curve (AUC). The results were analyzed using the SPSS for Windows software, Version 11.5 (SPSS Inc., Chicago, IL, USA) and relations were considered significant if p value was < 0.05.

RESULTS

The demographic characteristics of the patients enrolled in the study are presented in Table 1. The four study groups were homogeneous in the number, but not in the sex of the subjects. There was a predominance of female patients in Groups 1, 2, and 4, while in Group 3 there were more male than female patients (57% vs. 43%). Overall, there were 73 (60.83%) female and 47 (39.17%) male patients, with an average age of 55.9 ± 13.7 years.

Table 1. Demographic characteristics of the patients

Group		1	2	3	4	Σ
No. of patients		30	30	30	30	120
Sex	Male	8 (26.6%)	13 (43.3%)	17 (56.6%)	9 (30%)	47 (39.1%)
	Female	22 (73.3%)	17 (56.6%)	13 (43.3%)	21 (70%)	73 (60.8%)
Age		50.7 ± 12.7	60.1 ± 13.1	64.1 ± 10.1	51.8 ± 9.5	55.9 ± 13.7

According to the age analysis, the youngest group of patients was Group 1, with the average of 50.7 ± 12.7 years, and the oldest group was Group 3, with average of 64.1 ± 10.1 years.

The distribution of the patients according to the age group is shown in Figure 1.

The majority of the patients belonged to the 50–59 years age-group (36; 30%), followed by 60–69 years (33; 26.6%), while the smallest number (7; 6.6%) belonged to the group of under 30 years of age. In the eldest group (70+ years of age) there were 17 (14%) patients.

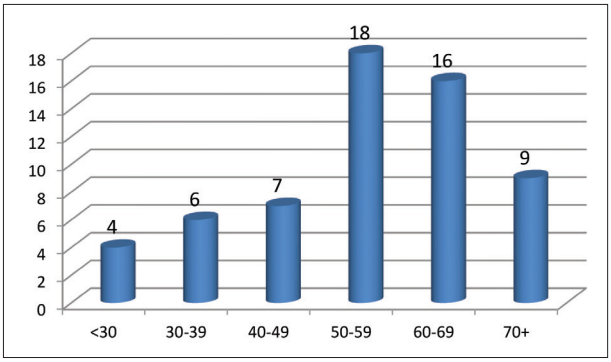

Figure 1. Graphical presentation of the patients according to age groups

The mean values of visual field parameters (MD, PSD) and RNFL quadrant thickness for each study group are presented in Table 2.

The results of the RNFL thickness distribution by quadrants showed the highest values in the healthy group, followed by the preperimetric group, early POAG group, and the moderate POAG group. The highest average value of RNFL thickness was in the upper quadrant of the healthy group (124.3 ± 17.8 μm), and the lowest average value of RNFL thickness was in the temporal quadrant of the moderate POAG group (46.5 ± 10.8 μm). Other RNFL thickness values per quadrants are distributed between these two endpoints.

The mean values for all parameters of RNFL thickness and statistical differences for each study group are presented in Table 3.

For the parameters Max–Min, Smax, Imax, Savg, Iavg, and AvgThic, the highest average values are in the healthy group, slightly lower in the preperimetric group, lower still in the early glaucoma group and the lowest values are in the moderate glaucoma group. All these parameters show very high statistically significant differences between the groups ($p < 0.001$). Since AvgThic is the most commonly used parameter, its mean value for Group 1 was 105.5 ± 11.3 μm, for Group 4 it was 83.6 ± 9.2 μm, for Group 2 73.4 ± 12.1 μm, for Group 3 59.6 ± 10.6 μm, and represents the parameter with the highest statistical significance of differences between the groups.

The relationships between VF global indices and RNFL thickness parameters were evaluated by the Pearson correlation analysis for all the groups and presented in Table 4.

Table 2. Differences in mean values of mean deviation, pattern standard deviation, and retinal nerve fiber layer quadrants thickness

Parameters	Group 1	Group 2	Group 3	Group 4	p1	p2	p3
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD			
MD	-0.29 ± 0.7	-4.18 ± 1.27	-9.89 ± 1.74	-0.8 ± 1.01	< 0.001	< 0.001	0.027
PSD	1.4 ± 0.53	4.34 ± 1.72	9.08 ± 2.02	1.92 ± 0.76	< 0.001	< 0.001	0.002
S	124.3 ± 17.8	84.6 ± 18.4	68.9 ± 19.5	91.6 ± 14.2	< 0.001	< 0.001	< 0.001
N	90.5 ± 22.5	62.7 ± 15.4	54.5 ± 16.8	59.8 ± 19.5	< 0.001	< 0.001	< 0.001
I	133.4 ± 15.3	87.2 ± 22.4	69.2 ± 23.9	113.8 ± 19.1	< 0.001	< 0.001	< 0.001
T	73.73 ± 13.71	55.9 ± 16.12	46.5 ± 10.85	70.63 ± 20.52	< 0.001	< 0.001	0.494

p1– p-value between Groups 1 and 2;
p2 – p-value between Groups 1 and 3;
p3 – p-value between Groups 1 and 4

Table 3. Differences in mean values of retinal nerve fiber layer thickness parameters

Parameters	Group 1 Mean ± SD	Group 2 Mean ± SD	Group 3 Mean ± SD	Group 4 Mean ± SD	p1	p2	p3
Imax/Smax	1.1 ± 0.1	1 ± 0.32	1.1 ± 0.5	1.2 ± 0.2	0.234	0.084	0.02
Smax/Imax	0.9 ± 0.1	1.1 ± 0.4	1.2 ± 0.6	0.8 ± 0.2	0.258	0.034	0.088
Smax/Tavg	2.2 ± 0.4	2.2 ± 0.74	2.1 ± 0.7	1.9 ± 0.5	0.836	0.196	0.008
Imax/Tavg	2.3 ± 0.3	2.1 ± 0.62	2.1 ± 0.8	2.3 ± 0.6	0.102	0.105	0.85
Smax/Navg	1.8 ± 0.4	1.9 ± 0.62	1.8 ± 0.6	2.4 ± 1.1	0.756	0.941	0.057
Max–Min	128.3 ± 15.1	96.6 ± 25.1	83.3 ± 25.7	121.1 ± 19.3	< 0.001	< 0.001	0.111
Smax	160.1 ± 17.2	117.2 ± 25.1	94.1 ± 28.6	128.5 ± 21.7	< 0.001	< 0.001	< 0.001
Imax	169.8 ± 14.5	113 ± 25.7	95.1 ± 29.7	150.1 ± 22.1	< 0.001	< 0.001	< 0.001
Savg	124.3 ± 17.8	84.1 ± 17.7	68.7 ± 18.8	92.1 ± 15.5	< 0.001	< 0.001	< 0.001
Iavg	135.6 ± 18.1	87 ± 21.7	69.5 ± 23.5	113 ± 17.7	< 0.001	< 0.001	< 0.001
AvgThic	105.5 ± 11.3	73.4 ± 12.1	59.6 ± 10.6	83.6 ± 9.2	< 0.001	< 0.001	< 0.001

p1– p-value between Groups 1 and 2;
p2 – p-value between Groups 1 and 3;
p3 – p-value between Groups 1 and 4

Table 4. Pearson correlation testing between Groups 2 and 3, 1 and 4 in relation to the retinal nerve fiber layer thickness parameters

Parameters	S	N	I	T	Imax/ Smax	Smax/ Imax	Smax/ Tavg	Imax/ Tavg	Smax/ Navg	Max– Min	Smax	Imax	Savg	Iavg	Avg. Thic.
Pearson corr. (2–3)	0.418	0.207	0.421	0.397	-0.093	-0.086	0.071	0.021	0.106	0.276	0.422	0.348	0.432	0.406	0.571
p	0.001	0.112	0.001	0.002	0.482	0.515	0.591	0.873	0.421	0.033	0.001	0.006	0.001	0.001	0.0005
Pearson corr. (1–4)	0.717	0.595	0.498	0.09	-0.303	0.223	0.238	0.025	-0.314	0.208	0.633	0.472	0.698	0.539	0.734
p	0.001	0.001	0.001	0.494	0.019	0.087	0.067	0.85	0.015	0.111	0.001	0.001	0.001	0.001	0.0005

Table 5. Receiver operating characteristic curves, cut-off, sensitivity, and specificity

Parameters	Surface area	Cut-off	Sensitivity (%)	Specificity (%)	p
S	0.736	75	63.3	73.3	0.002
I	0.733	76	70	73.3	0.002
T	0.676	54	80	53.3	0.019
Max–Min	0.637	/	/	/	0.069
Smax	0.727	102.5	60	76.7	0.002
Imax	0.678	96	63.3	80	0.018
Savg	0.728	75	60	73.3	0.002
Iavg	0.736	76.5	73.3	73.3	0.002
AvgThic	0.803	63.9	70	83.3	< 0.0005

It was found that there is a high positive correlation between the MD values and the following RNFL thickness parameters: AvgThic, S, I, Smax, Imax, Savg, Iavg. A low positive correlation between the MD values and Max–Min parameter was demonstrated. Other parameters do not show statistically significant correlation, and their change during the MD value change is not significant. This statistical analysis showed that the RNFL parameters that have a high statistical correlation with MD values among different groups are parameters that change with glaucoma progression, but they are also parameters that occur at the outset of POAG even in preperimetric phase of the disease. The AvgThic parameter has the highest statistical significance relations with MD values ($p < 0.0005$).

The ROC curves area for parameters were calculated to discriminate glaucomatous from healthy eyes. The surfaces determined by ROC curves, cut-off, sensitivity and specificity, and p-values for each individual parameter are displayed in Table 5.

By comparing the surface area under the ROC curve, it can be concluded that the parameter which has the best diagnostic ability is AvgThic, with AUC of 0.803 (< 0.0005), the sensitivity of 67% and the specificity of 83.3%. The following parameters are S and Iavg with the same surface area under the ROC curve of 0.736 (< 0.002). For the parameter S, the sensitivity was 63.3% and the specificity was 73.3%, while for the parameter Iavg sensitivity was 73.3% and the specificity 73.3%. For parameter I, the surface area of the curve was 0.733 ($p = 0.002$), the sensitivity was 70%, and the specificity was 73.3% (Figure 2). ROC curve shows that parameter T has pure ability to discriminate glaucomatous from healthy eyes.

DISCUSSION

Even though sex is not considered a risk factor for POAG, Framigham, Barbados, Blue Mountains, and other studies have shown that a greater number of males than females suffer from POAG [9, 10]. Analysis of the sex structure of our 120 study participants shows that the majority of participants were females (60.8%). Only the moderate POAG group had more males (56.6%). However, if we look only at the patients with early and moderate POAG, there is an equal number of males and females. The average age in the whole sample was 55.9 ± 13.7 years. The eldest group was the moderate POAG group with an average of 64.1 ± 10.1 years.

Analysis of the mean values of the MD visual field parameter showed a statistically significant decrease from the healthy, through the preperimetric and early glaucoma group to the moderate POAG group. Testing of differences between MD and PSD values among study groups, ANOVA, and post-hoc analysis showed that there was a statistically significant difference between groups 1, 2, and 3 ($p < 0.001$), except between the healthy and the preperimetric group ($p = 0.384$), which suggests that these groups cannot be distinguished according to the parameters of the visual field but this can be done using OCT analysis. The investigation done by Li et al. [10], as well as some other researchers, has shown a high correlation between MD values and the stage of POAG [11, 12, 13].

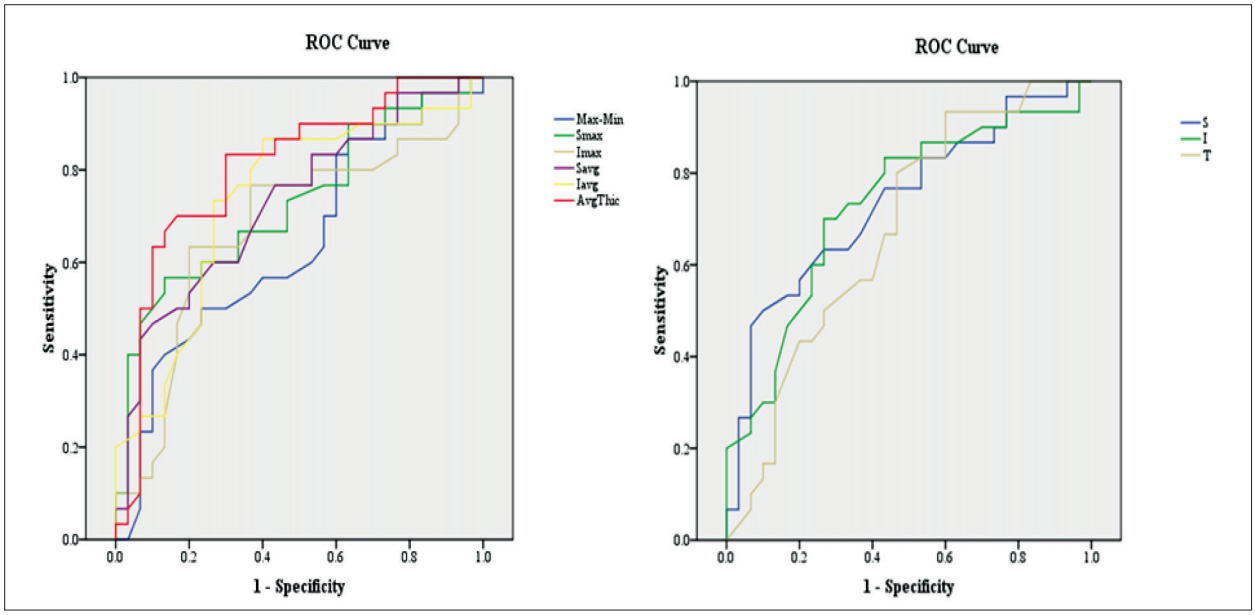

Figure 2. Receiver operating characteristic curves for the retinal nerve fiber layer thickness parameters

The mean value of RNFL thickness for the healthy group in our study was 105.5 ± 11.3 μm, which was the highest value compared to the other groups. The lowest value of RNFL thickness was in the moderate POAG group (59.6 ± 10.6 μm). RNFL thickness value decreases with the progression of POAG, which was confirmed by the statistical analysis of the AvgThic parameter differences between groups ($p < 0.0005$). Scientific studies of Patel et al. [9]. and Komaratih [11] have shown similar values of RNFL thicknesses for healthy populations, varying 90–128 μm, and they also found that thickness of RNFL in patients with POAG and preperimetric glaucoma are statistically significantly lower compared to the healthy population.

The results obtained by OCT measuring of RNFL thickness per quadrants showed the same distribution in all study groups. RNFL thickness value was found to be the highest in the inferior quadrant, the second highest in the superior quadrant, the third in the nasal quadrant, while the value was the lowest in the temporal quadrant. Taking into account all values, the greatest RNFL thickness was found in the healthy group and the lowest in the moderate POAG group. Research by Aydogan et al. [12] has shown that the average RNFL thickness in healthy individuals is 112.7 ± 8.7 μm, for temporal quadrant the thickness is 82.3 ± 9.6 μm, for superior the thickness is 139.9 ± 18 μm, for nasal thickness is 83 ± 10.6 μm, and for the inferior quadrant thickness is 145.9 ± 14.6 μm. Patel et al. [9] have published that the thinning of RNFL by quadrants follows the progression of glaucomatous disease. The high congruence between the results of our research and other researchers, both in average values and quadrant thickness distribution, confirms the applicability of the ISNT rule in all stages of glaucoma [9–12].

Stefanova et al. [13] reported that both inferior and superior RNFL quadrants are the specific glaucomatous sites for early POAG damage, which was also confirmed by the study done by Singh et al. [14], which analyzed OCT findings among the healthy group of 50 subjects and 55 patients with early POAG. In the study that involved 98 healthy individuals, 285 patients with ocular hypertension, and 66 patients with glaucoma, Mayoral et al. [15] have found that the RNFL thickness across the quadrants decreases from healthy, over OHT patients to POAG patients. This means that as the disease progresses, the RNFL thickness decreases by quadrants.

Detection of an early-stage glaucoma was confirmed by the study done by Komaratih [11], as well as by Li et al. [10], who recommended that the best parameter for recognition of an early POAG is AvgThic parameter. Yalvac et al. [16] conducted an interesting study of patients with ocular hypertension using Stratus OCT. The patients were divided into three groups: at low, medium, and high risk of developing POAG. The best parameters for differentiating the risk level of glaucoma development were Iavg and Imax. Thereby, they emphasized the lower part of the RNFL as the site of pathological knockout and the place where the earliest POAG occurs. Guedes et al. [17] studied the ability of early detection of glaucoma by the OCT apparatus. They compared the changes that occur in the thickness of the macular zone and the peripapillary RNFL zone and concluded that in the competition of numerous parameters, the average thickness of RNFL is by far the best at detecting patients with early glaucoma. They hinted that there are almost 100% ganglia retinal cells in the peripapillary zone, and in the macular zone their number is about 50%, and the parameters of the thickness of RNFL are better for determining glaucoma than the parameters of the macular region. The area of ROC curve for AvgThic was 0.93 in the above-mentioned study, which was higher than the results obtained in our study (0.803). Our study showed that quadrant S has the highest, quadrants I and N high ability for discriminating between the healthy and preperimetric glaucoma patients.

ROC curves were calculated and constructed to discriminate healthy from glaucomatous eyes. A study by Stagg and Medeiros [18] showed the areas under the ROC curves for discriminating POAG from normal eyes were 0.89 for global RNFL and 0.75 for global MRW ($p = 0.006$). Similarly, according to this study, the best parameter of the RNFL thickness group is AvgThic with the largest area under the ROC curve of 0.803, the cut-off value of 63.9, sensitivity of 67%, and specificity of 83.3%. Hsieh et al. [19] reported that the largest area below the ROC curve were with AvgThic, quadrant I, and quadrant S. Singh et al. [14] also confirmed that the surface of the ROC curve is the largest for AvgThic and quadrant S parameter (area = 0.963, area = 0.943), and a slightly smaller area in the case of quadrant I, but with high values of sensitivity (89%) and specificity (81%). These results are almost the same as those in our study, which underlines the importance of the parameters AvgThic, S, Smax, I, and Iavg for the earliest possible diagnosis of glaucoma.

CONCLUSION

In summary, peripapillary RNFL thickness parameters AvgThic, S, I, Smax, Savg, Iavg, and Imax have excellent ability to discriminate between healthy eyes and eyes with POAG. However, the parameter with the highest specificity and sensitivity is the AvgThic parameter, which makes it the best for early glaucoma detection and monitoring of POAG. Finally, the determination of thickness of peripapillary RNFL in patients with POAG using OCT represents the method which distinguishes between patients with POAG, preperimetric glaucoma, and healthy population, hence it can be used in glaucoma diagnostics and follow-up. We believe the current high precision and reliability of OCT parameters can be even better, and perhaps the answer lies in future studies of related influences of OCT parameters through mathematical models.

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A part of this work can be found at the Research Square platform as a preprint.

Conflicts of interest: None declared.

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Евалуација дебљине перипапиларног слоја ретиналних нервних влакана код болесника са примарним глаукомом отвореног угла

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САЖЕТАК

Увод/Циљ Циљ овог рада је био одредити разлику у дебљини перипапиларног слоја ретиналних нервних влакна (СРНВ) код болесника са примарним глаукомом отвореног угла у поређењу са здравом популацијом, и према прогресији болести.

Методе Свих 120 болесника су подељени у четири групе: болесници без глаукома, са почетним, са средње узнапредовалим и са преперимеријским глаукомом. Комплетан офталмолошки преглед, видно поље и оптичка кохерентна томографија су урађени код сваког болесника.

Резултати Дебљина СРНВ је највећа у доњем квадранту, мања у супериорном, још мања у назалном, најмања у темпоралном квадранту. Највећа просечна дебљина СРНВ била је у горњем квадранту у групи здравих испитаника (124,3 ± 17,8 микрона), а најмања у темпоралном квадранту групе са средње узнапредовалим глаукомом (46,5 ± 10,8 микрона). Остале вредности дебљине СРНВ по квадрантима распоређене су између ове две крајње тачке. Параметар средња дебљина код болесника са средње узнапредовалим глаукомом био је мањи него у групи са почетним, који је био мањи него код групе здравих испитаника (59,6 ± 10,6 према 73,4 ± 12,1 према 105,5 ± 11,3 микрона). Параметар средња дебљина у преперимеријској групи је 83,6 ± 9,2 микрона. Пирсонова анализа показала је високу позитивну корелацију глобалних индекса видног поља и следећих параметара: средња дебљина, супериорне и инфериорне максималне и средње вредности. Упоређивањем РОК крива, параметар са највећом дијагностичком способношћу је средња дебљина, са површином од 0,803, осетљивошћу 67% и специфичношћу 83,3%.

Закључак Параметри дебљине перипапиларног СРНВ – средња дебљина, супериорне и инфериорне максималне и средње вредности – имају одличну способност разликовања здравих болесника од оних са примарним глаукомом отвореног угла. Параметар са највећом специфичношћу и осетљивошћу је средња дебљина, те је најбољи за рано откривање и праћење примарног глаукома отвореног угла.

Кључне речи: слој ретиналних нервних влакана; примарни глауком отвореног угла; оптичка кохерентна томографија; средња дебљина нервних влакана

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Research on the influence of prenatal exercises on anthropometric and vascular parameters in pregnant women

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SUMMARY

Introduction/Objective The frequency of a sedentary lifestyle during pregnancy increases. This contributes to gestational weight gain and has a negative impact on health.
This study researched the impact of prenatal exercise on gestational weight gain, blood pressure, and microcirculation in pregnant women who exercised and those who did not exercise.

Methods The study included 70 pregnant women with a normal pregnancy, who attended a psychophysical preparation program for childbirth for eight weeks. The control group (n = 35) attended theoretical classes on childbirth, and the experimental group (n = 35) attended prenatal exercises as well. Gestational weight gain, blood pressure, and nailfold capillary density were determined and compared between the two groups.

Results Gestational weight gain of 19.94 kg in non-exercising pregnant women was significantly greater than the gestational weight gain of 11.65 kg in pregnant women who exercised. Pregnant women who did not exercise had an increase in systolic (by 15.56 mmHg) and diastolic pressure (by 16.08 mmHg), which is significantly higher compared to pregnant women who exercised. In this group, systolic pressure increased by 2.5 mmHg, while the diastolic one did not change. A significant difference in the nailfold capillary density at the end of the prenatal program has not been determined.

Conclusion Prenatal exercise of moderate-intensity has a positive effect on gestational weight gain and the level of blood pressure in pregnant women. The nailfold capillary density has not differed significantly after the prenatal program in pregnant women who have exercised and in those who have not.

Keywords: physical activity; pregnancy; gestational weight gain; blood pressure; microcirculation; microvessel density measurement

INTRODUCTION

The incidence of a sedentary lifestyle during pregnancy increases, ranging from 64.5% to 91.5%, and tends to increase in the third trimester of pregnancy. This contributes to the occurrence of obesity in pregnancy, gestational weight gain (GWG), and maintaining postpartum body weight, and has a negative impact on the health of pregnant women. In recent years in Europe, the prevalence of obesity in the entire population has increased epidemically and in pregnant women is around 25% [1–5]. The American Institute of Medicine presented guidelines and recommendations for GWG. GWG for normally fed pregnant women [body mass index – (BMI) 18.50–24.99 kg/m^2], is 11.5–16 kg [6].

Studies have shown that pregnant women who have a sedentary lifestyle are 1.5 times more likely to gain weight during pregnancy compared to pregnant women who exercise [7, 8]. However, concerns about the safety of exercise in pregnancy seem to persist. In studies, there is a large difference in interventions and exercise intensity in pregnancy [9].

Excessive weight gain during pregnancy is a risk factor for gestational diabetes and pregnancy-induced hypertension (PIH). During pregnancy, the circulatory system is largely adapted to meet the needs of the mother and fetus [10, 11]. In a normal pregnancy, blood pressure gradually increases during the second and third trimesters, while peripheral vascular resistance decreases, resulting in maintaining blood pressure values in the normal range. Pregnant women who do not exercise are three times more likely to develop hypertension compared to those pregnant women who exercise [10–15]. There is little data on the effect of exercise in the prenatal period on circulatory characteristics in normal pregnancy. This study researched the effect of prenatal exercise on GWG, blood pressure, and microcirculation

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in pregnant women who exercised regularly, compared to pregnant women who did not exercise regularly.

METHODS

The prospective, randomized study included 70 pregnant women (n = 70) in the northern part of Bosnia and Herzegovina. The study was performed at the university setting of the University of Banja Luka, Faculty of Medicine, and at the Sveti Vračevi Čelinac Health Center, from December 2020 to August 2021. Trial registration: Clinicaltrials.gov identifier – NCT05001906. At the gynecological appointment healthy singleton pregnant women, confirmed by a gynecologist, were assigned to the control and experimental group with a simple randomization process using a list of random numbers. Respondents, aged 20–40 years, with BMI < 25 kg/m^2, joined the birth preparation program from the 20th to the 32nd week of gestation. BMI before pregnancy was determined by dividing body weight in kilograms by squared body height in meters (kg/m^2) [16]. Respondents who agreed to participate in the research voluntarily signed an informed consent form. The Ethics Committee of the Faculty of Medicine in Banja Luka approved the study.

Pregnant women were excluded from the study if bleeding appeared during the prenatal program in the second or third trimester, rupture of the amniotic sac, preeclampsia, or PIH, and intrauterine growth restriction in the current pregnancy, or anemia.

The prenatal program lasted for eight weeks and consisted of theoretical classes on childbirth and prenatal exercises. The control group (n = 35) attended theoretical classes three times a week, and the experimental group (n = 35) attended prenatal exercises, as well. According to the American College of Obstetricians and Gynecologists recommendations a prenatal exercise program was created to improve the condition of the pregnant woman, strengthen the extremities, the area of the abdomen, back, pelvic girdle and improve circulation [6]. Breathing exercises were conducted. During the research, pregnant women followed the general recommendations of exercise: they did not exercise additionally in other places, ate normally, dressed lightly, and avoided high heat, the room temperature of the exercise room did not exceed 24°C. Pregnant women exercised three times a week for 45 minutes. The exercise program was conducted by the prenatal instructor and supervised by a physiatrist. Training started and ended with breathing and relaxation techniques for about 10 minutes, warm-up consisted of moderate walking for about five minutes, followed by strength and stretching exercises for about 30 minutes. Pregnant women exercised in standing, sitting, kneeling, and sideways position, with or without props (balls, straps, weights, etc.). Exercises performed in a supine position with bent knees during this study did not last more than five minutes, thus avoiding the reduction of venous flow and hypotension [6]. The exercises were performed alone or in pairs, with another pregnant woman or partner, in two to three sets, with 10–12 repetitions.

At the beginning and end of the study GWG, blood pressure, and nailfold capillary density were analyzed. The blood pressure was measured using a standard mercury manometer before prenatal exercises, at the beginning (between 20 and 32 weeks of gestation), and after eight weeks of prenatal exercises (between 28 and 40 weeks of gestation).

During prenatal exercises, the exercise load of pregnant women was monitored based on the subjective feeling of load using the Borg rating of perceived exertion scale. The applied physical activity was of medium intensity for which the score of perceived effort should be 13–14 (moderately difficult) out of a total of 6–20. The range of 6–20 is actually an analogy with the resting heart rate (60) and at maximum load (200), [2]. Pregnant women were explained that when they subjectively felt "moderately difficult" they should stop doing the exercise. It was also used "talk test". It is believed that as long as a pregnant woman can talk during exercise, she is probably not overburdened or tired [7].

In this study, nailfold capillaroscopy was performed with a Leica Z4 stereomicroscope and a digital camera. Pregnant women sat with their left hand placed on a table at heart level and a drop of immersion oil was placed on the nailfold in order to improve the image resolution. The density of capillary loops of the nailfold was assessed as the number of capillary loops per 1 millimeter of the distal row of nailfold capillary. Nailfold capillary loops of the fourth finger of the left hand of all pregnant women were analyzed. The morphometry of the nailfold capillaries was performed using ImageJ Software (ImageJ, Bethesda, MA, USA).

Statistical analysis was performed with the use of the licensed version of the SPSS 20 software (IBM Corp., Armonk, NY, USA). The obtained data were analyzed by methods of descriptive statistics. The t-test for paired samples, with the appropriate level of significance $p < 0.05$, was used to test the significance of the difference between the two arithmetic means.

RESULTS

The control and experimental groups were equalized in relation to the life age, the gestational age, and the BMI of the pregnant women before pregnancy (Table 1).

Table 1. The average life age of pregnant women, gestational age at the time of participation in the study, and body mass index of pregnant women before pregnancy

Parameters	Age of pregnant women (years)	Gestational age of pregnant women (gestational weeks)	BMI (kg/m^2)
Pregnant women who did not exercise	29.72 ± 3.24	28.97 ± 3.38	22.23 ± 1.84
Pregnant women who did exercise	30.12 ± 3.31	29.35 ± 3.56	21.32 ± 1.87

The results are presented as mean values ± standard deviation

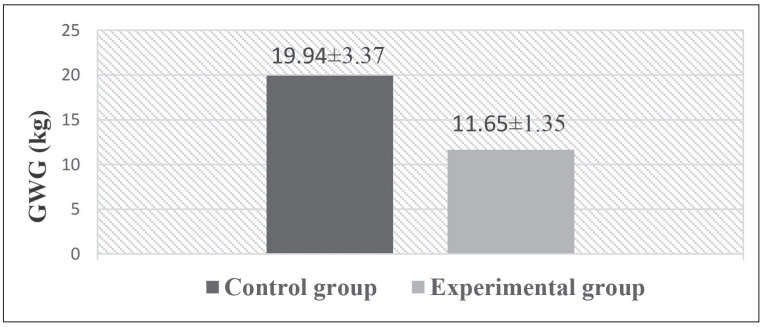

Figure 1. Gestational weight gain in pregnant women who did not exercise and in pregnant women who exercised; the results are presented as mean values ± standard deviation

Table 2. Systolic and diastolic blood pressure in the control group and the experimental group, at the beginning and end of the prenatal program

Parameters		Beginning of the prenatal program	End of the prenatal program	p value
Pregnant women who did not exercise	Systolic blood pressure (mmHg)	111.67 ± 9.41	127.22 ± 13.44	p < 0.001
	Diastolic blood pressure (mmHg)	71.11 ± 7.85	87.19 ± 17.97	p < 0.001
Pregnant women who did exercise	Systolic blood pressure (mmHg)	108.68 ± 9.79	111.18 ± 9.77	p = 0.039
	Diastolic blood pressure (mmHg)	68.38 ± 9.43	68.24 ± 8.25	p = 0.887

The results are presented as mean values ± standard deviation

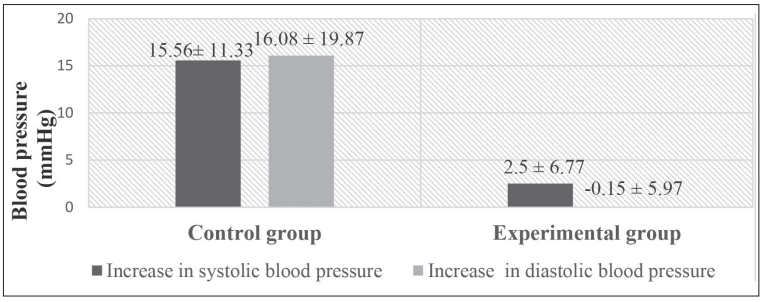

Figure 2. Increase in systolic and diastolic blood pressure after eight weeks of monitoring in pregnant women who performed prenatal exercises and in pregnant women who did not perform prenatal exercises; the results are presented as mean values ± standard deviation

In the control group after eight weeks of attending theoretical classes on childbirth, the GWG was 19.94 ± 3.37 kg [mean value (MV) ± standard deviation (SD)] (Figure 1). The smallest weight gain in this group was 13 kg and the largest 29 kg. In the experimental group, after eight weeks of attending prenatal exercises, the GWG was 11.65 ± 1.35 kg (Figure 1). The smallest weight gain in this group was 9 kg and the largest 15 kg.

Using a t-test, a significant difference in the increase in GWG was found between pregnant women who did not exercise (MV = 19.94, SD = 3.37) and pregnant women who exercised (MV = 11.65, SD = 1.35), ($t = 13.658$, $p < 0.001$). The difference in GWG (MV = 8.30) between the two groups of pregnant women is large ($\eta^2 = 0.70$). Pregnant women who did not exercise had significantly higher GWG.

In the control group, at the beginning of the theoretical classes on childbirth, systolic blood pressure was 111.67 ± 9.41 mmHg (Table 2). The lowest recorded value of systolic blood pressure was 90 mmHg, and the highest value was 120 mmHg. At the end systolic blood pressure was 127.22 ± 13.44 mmHg. The lowest recorded value was 90 mmHg, and the highest value was 150 mmHg, which is a hypertensive value. Four pregnant women developed hypertension. A significant large difference was found in the systolic blood pressure determined in the first measurement time and the second measurement time ($t = -8.241$, $p < 0.001$; $\eta^2 = 0.66$) (Table 2). The increase in systolic blood pressure in the control group was 15.56 mmHg (Figure 2).

In the control group at the beginning of the theoretical classes, diastolic blood pressure was 71.11 ± 7.85 mmHg (Table 2). The lowest recorded value of diastolic blood pressure was 60 mmHg, and the highest value was 80 mmHg. At the end of the classes, diastolic blood pressure was 87.19 ± 17.97 mmHg. The lowest recorded value was 60 mmHg, and the highest value was 110 mmHg. There is a significant large difference in diastolic blood pressure found in the first and second measurement time ($t = -4.857$, $p < 0.001$; $\eta^2 = 0.40$). The increase in diastolic blood pressure in the control group was 16.08 mmHg (Figure 2).

In the experimental group at the beginning of prenatal exercise, systolic blood pressure was 108.68 ± 9.79 mmHg (Table 2). The lowest recorded value of systolic blood pressure was 90 mmHg, and the highest value was 130 mmHg. At the end of prenatal exercise, systolic blood pressure was 111.18 ± 9.77 mmHg. The lowest value of systolic blood pressure was 90 mmHg, and the highest value was 130 mmHg. In the experimental group there was a significant increase in systolic blood pressure (mean difference = 2.50 mmHg) with systolic pressure remaining within normal limits, that is hypertension did not develop ($t = -2{,}153$, $p = 0.039$). The difference is of medium intensity ($\eta^2 = 0.12$) (Figure 2). In the experimental group, it was observed that some pregnant women had to slow down with exercise because they felt tired and weak.

In the experimental group, at the beginning of exercise, the MV of diastolic blood pressure was 68.38 ± 9.43 mmHg (Table 2). The lowest determined value of diastolic blood pressure was 60 mmHg, and the highest was 80 mmHg. At the end of exercise, diastolic blood pressure was 68.24 ± 8.25 mmHg (Table 2). The lowest recorded value was 60 mmHg, and the highest value was 85 mmHg. A significant difference in the MV of diastolic blood pressure in the first and second measurement time was not found ($t = 0.144$, $p = 0.887$). Diastolic blood pressure was within normal limits (Figure 2).

In pregnant women who exercised, the increase in systolic blood pressure of 2.50 mmHg was significantly

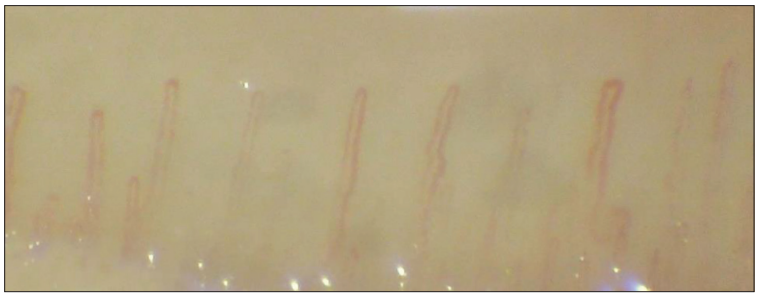

Figure 3. Nailfold capillary loops

Table 3. Nailfold capillary density in the control group and the experimental group, at the beginning and end of the prenatal program

Parameters	Nailfold capillary density (capillary/mm)	
	Pregnant women who did not exercise	Pregnant women who exercised
Beginning of the prenatal program	7.29 ± 1.21	7.05 ± 1.36
End of the prenatal program	7.29 ± 0.98	7.05 ± 1.36

The results are presented as mean values ± standard deviation

less compared to the increase in systolic blood pressure of 15.56 mmHg in pregnant women who did not exercise ($t = -5.892$, $p = 0.0001$) (Figure 2). In pregnant women who exercised, there was no significant increase in diastolic blood pressure after eight weeks of follow-up, while in non-exercising pregnant women there was an increase in diastolic blood pressure of 16.08 mmHg, which is a significant difference ($p < 0.0001$) (Figure 2).

The density of nailfold capillary loops was analyzed. Nailfold capillary loops had a hairpin shape and were organized in rows (Figure 3). In non-exercising pregnant women nailfold capillary density was 7.29 ± 1.21 capillaries/mm at the beginning of the study and 7.29 ± 0.98 capillaries/mm at the end of the study (Table 3). The nailfold capillary density in non-exercising pregnant women did not change after eight weeks of gestation ($p < 0.001$).

In pregnant women who exercised nailfold capillary density was 7.05 ± 1.36 capillaries/mm at the beginning of the study and 7.05 ± 1.36 capillaries/mm at the end (Table 3). The density of nailfold capillary loops in pregnant women who exercised did not change after eight weeks of pregnancy ($p < 0.001$).

DISCUSSION

In this study pregnant women who exercised had significantly less GWG. A study by Vargas-Terrones et al. [8] showed that prenatal exercise and diet during pregnancy reduced GWG. Non-exercising pregnant women were 1.5 times more likely to gain weight during pregnancy [15]. da Silva et al. [9] found that an exercise program reduced GWG. In pregnant women different types of prenatal exercises have reduced GWG [17]. Multiple factors can affect GWG, including type and/or intensity of exercise, lifestyle, diet, lifestyle alignment, and socioeconomic and environmental factors [8]. Weight gain is caused by an imbalance between energy intake and energy spending. In this study, pregnant women who exercised, ate normal, but did not have a sedentary lifestyle, and therefore, had higher energy spending. The weight of pregnant women during the prenatal program can be controlled and possible complications for the health of the mother and baby can be prevented.

Weight gain increases the risk of developing hypertension during pregnancy. Blood pressure gradually increases during the second and third trimesters [14]. Physical activity may reduce the risk of developing hypertensive disorders and functional status depends on individual patient performance [18, 19, 20].

In this study, at the end of theoretical classes, non-exercising pregnant women had a significant increase in systolic blood pressure of 15.56 mmHg, and diastolic blood pressure of 16.08 mmHg. In pregnant women who exercised, a significant increase in systolic blood pressure of 2.50 mmHg was found, and diastolic blood pressure did not change significantly.

In pregnant women who exercised, the increase in systolic blood pressure after eight weeks of 2.50 mmHg was significantly less than the increase in systolic blood pressure in non-exercising pregnant women. In pregnant women who exercised, there was no significant increase in diastolic blood pressure after eight weeks of follow-up, which is a significant difference compared to non-exercising pregnant women in whom there was an increase in diastolic blood pressure of 16.08 mmHg.

In pregnant women who exercise there were improvements in blood pressure [15, 21]. Boparai et al. [14] showed that pregnant women who exercised, showed after intervention an attenuated increase in arterial pressure compared to the control group. Pregnant women who have not exercised are three times more likely to develop hypertension compared to pregnant women who have exercised [15, 21]. The potential reason why pregnant women who exercised had a lower systolic and diastolic blood pressure increase than the non-exercising pregnant women, could be the beneficial effects of exercise in the prevention of excessive GWG. Obesity is associated with hypertension. Weight gain leads to an increased risk of developing hypertension during pregnancy. Pregnancy is a unique opportunity to improve health outcomes for both mother and child. Physical activity in pregnancy had beneficial effects on anxiety and blood pressure [22, 23, 24].

During pregnancy, the macrocirculation and microcirculation of pregnant women adapt. Data on the microcirculatory adaptation of pregnant women are scarce, mostly due to technical limitations [10].

In this study, the density of nailfold capillary loops in non-exercising pregnant women and those who exercised did not change after eight weeks of pregnancy. The average density of nailfold capillary in non-exercising pregnant women, at the beginning of the study was 7.29 capillaries/mm, and at the end was 7.29 capillaries/mm. The density

of nailfold capillary in pregnant women who exercised at the beginning of exercise was 7.05 capillaries/mm, and at the end 7.05 capillaries/mm.

In studies, the average capillary density ranges 7.3–10.3 capillaries/mm in healthy adults [25, 26, 27]. In previous research during pregnancy was observed an increase in capillary density and neoangiogenesis, and a reduction in capillary dilations [10]. Monitoring of microcirculatory changes can be viewed as a method that helps us to record the changes that precede the onset of clinical disease. Decreased capillary density has been demonstrated in individuals with hypertension [28]. Studies have revealed that the method of nailfold capillaroscopy facilitates the identification of women who have a more pronounced tendency to PIH because of the structural rarefaction of capillaries. In the control group, where a significant increase in blood pressure was found after eight weeks of follow-up, there was no decrease in capillary density. It is possible that the increase in blood pressure is reflected more in other characteristics of nailfold capillaries, such as the shape or length of capillaries, and further research is needed.

The described birth preparation program was developed for the purpose of promoting physical activity during pregnancy as a healthy lifestyle, reducing anxiety, and improving the self-control of pregnant women in childbirth.

Identifying risk factors for future cardiovascular diseases and early assessment of the circulatory status of pregnant women can lead to earlier lifestyle changes and disease prevention.

CONCLUSION

Prenatal exercise of moderate intensity has a positive effect on GWG. Pregnant women who did not exercise had a significantly greater increase in GWG. Prenatal exercise of moderate-intensity has a positive effect on blood pressure. In pregnant women who exercised, the increase in blood pressure was less compared to the increase in blood pressure in pregnant women who did not exercise. After eight weeks of the prenatal program, the difference in the capillary density of the nailfold was not determined in pregnant women who exercised and pregnant women who did not exercise.

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Истраживање утицаја пренаталних вежби на антропометријске и васкуларне параметре трудница

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САЖЕТАК

Увод/Циљ Учесталост седалачког начина живота током трудноће се повећава. То доприноси гестацијском повећању телесне тежине и има негативан утицај на здравље.
У овој студији се истраживао утицај пренаталних вежби на гестацијско повећање телесне тежине, крвни притисак и микроциркулацију код трудница које су вежбале и оних које нису вежбале.

Методе Испитивање је укључило 70 трудница са уредном трудноћом, које су осам недеља похађале програм психофизичке припреме за порођај. Контролна група (n = 35) похађала је теоретску наставу о порођају, а експериментална група (n = 35) похађала је и пренаталне вежбе. Утврђени су гестацијско повећање телесне тежине, крвни притисак и густина капилара кожног набора нокта и упоређени између две групе.

Резултати Гестацијско повећање телесне тежине од 19,94 *kg* код трудница које нису вежбале је значајно веће од гестацијског повећања телесне тежине од 11,65 *kg* код трудница које су вежбале. Труднице које нису вежбале су имале значајно веће повећање систолног притиска (за 15,56 *mmHg*) и дијастолног притиска (за 16,08 *mmHg*) у односу на труднице које су вежбале. У овој групи систолни притисак се повећао за 2,5 *mmHg*, док се дијастолни није мењао. Значајна разлика у густини капилара кожног набора нокта на крају пренаталног програма није утврђена.

Закључак Пренатално вежбање умереног интензитета има позитиван утицај на гестацијско повећање телесне тежине и на висину крвног притиска трудница. Густина капилара кожног набора нокта није се значајно разликовала након пренаталног програма код трудница које су вежбале и код оних које нису.

Кључне речи: физичка активност; трудноћа; гестацијско повећање телесне тежине; крвни притисак; микроциркулација; мерење густине микроваскуларних судова

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

Anxiety and depression in individuals with and without cancer during the early COVID-19 pandemic period

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SUMMARY

Introduction/Objective The COVID-19 disease, which has turned into an important public health problem, has negatively affected individuals not only physically but also psychologically.
The aim of this study is to examine the anxiety and depression status of cancer patients, comparing to individuals with non-cancer chronic diseases and healthy individuals during the COVID-19 pandemic period.
Methods This cross-sectional study was conducted during the COVID-19 pandemic period between May 8 and June 25 2020 with 1107 people in Turkey. The mean age of the participants in the study was 36.41 ± 12.80. The sample comprised three groups: cancer group (n = 262), chronic disease control group (n = 250), and healthy control group (n = 595). The data were collected with the Descriptive Characteristics Form and Hospital Anxiety and Depression Scale.
Results The anxiety and depression scores were found to be higher in patients with cancer comparing to the other two groups. Having cancer was predictive of higher anxiety and depression score explaining 13% of the anxiety scores variance and 17% of the depression scores variance. Patients with the non-cancer chronic diseases, and healthy participants had anxiety and depression mean scores in the range of the borderline level.
Conclusion These findings suggest that depression and anxiety rates among patients with cancer are specifically higher comparing to patients with other chronic diseases and healthy individuals. The levels of anxiety and depression in the latter two groups should also be paid attention to during the pandemic. This study may have important practical implications in terms of the need for psychological screening.
Keywords: COVID-19; anxiety; depression; cancer; chronic disease

INTRODUCTION

The COVID-19 disease, which is a significant health threat for the entire human population, is more severe in people with chronic diseases [1]. The COVID-19 pandemic has turned into an important public health problem due to considerable risks for physical health, but has also been negatively affecting individuals psychologically. Recent studies have demonstrated that anxiety and depression rates have increased in the society during the COVID-19 pandemic [2, 3]. Factors such as fear of illness and death, uncertainty of the pandemic process, and uncertain daily life, all cause psychological stress in individuals [4]. During the pandemic, stress and anxiety especially develop in individuals who have chronic diseases [5]. In literature, it has been stated that cancer patients have experienced high levels of depression and anxiety [6]. To our knowledge, no comparative studies investigating the anxiety and depression status of individuals with cancer, individuals with non-cancer chronic diseases, and healthy controls have been encountered.

Cancer patients are immunosuppressed due to malignancy itself or treatments such as chemotherapy, radiotherapy and surgery, and this makes them vulnerable to infections [1]. The concerns of cancer patients about getting infected and sick, hospitalization and death are higher than the general population [7]. Factors such as social isolation, fear of sickness, and death of other patients increase the probability of depression in cancer patients. Reasons such as being in a high risk group, having to go to hospitals, which are the places with a high risk of infection, and thinking about the possible harms of their treatment may negatively affect the psychological functioning of cancer patients [4]. On the other hand, the focus of the entire society on the intense events of the pandemic, on disease, hospitalization and death, may be something that cancer patients and the rest of the individuals have in common now, distracting the cancer patients from their more personal perspective of problems, and possibly alleviating their negative feelings.

Individuals with chronic diseases are at higher risk of anxiety and depression compared to the healthy population [8]. This is very important as it may impair adherence to the treatment and directly worsen disease outcomes by adding psychological stress to physiological

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stress [9]. Cancer patients are a more vulnerable patient group among those with chronic diseases. Decreased adherence to the treatments and delayed follow-up screenings can lead to irreversible negative outcomes [10]. In extraordinary situations like pandemic, it is important to know the psychological status of high-risk individuals, as well as of the general population [11]. Since COVID-19 can affect individuals with chronic diseases in multiple ways, it has been reported in literature that providing adequate care and support is a matter that specifically needs attention [8]. COVID-19-related anxiety influenced the decision-making processes of the patients [12]. Cancer patients expressed that they were deprived of social support as they could not talk to healthcare providers during the COVID-19 period [13]. It is important to determine the distress status of the cancer patients. Online screening programs are gaining significance within the scope of isolation measures.

Studies have been carried out to determine the anxiety and depression level of the society during COVID-19; however, there are few studies comparing the cancer patients and the healthy population [14, 15]. There are contradictory data regarding whether the anxiety and depression rates of cancer patients are higher than the healthy population during the pandemic [16, 17]. In a study from China, 23.4% of the cancer patients had depression and 17.7% had anxiety during the pandemic, but only 1.6% of these patients received psychological support [18]. Determination of specific groups may help with developing intervention programs. In this sense, it is significant to identify the anxiety and depression status of individuals with cancer, individuals with non-cancer chronic diseases and healthy individuals. Cancer patients who avoid going to the hospital even because of their treatments can be screened in terms of depression and anxiety with an online questionnaire. In this context, we anticipate that our work will set an example.

The aim of the study described in this paper was to evaluate and compare the anxiety and depression status of cancer patients, individuals with non-cancer chronic diseases and healthy individuals during the early COVID-19 period with an online screening program.

METHODS

Study design

This cross-sectional study was conducted in Istanbul, Turkey during the COVID-19 pandemic period between May 8 and June 25, 2020.

Participants

The study sample comprised three groups, the group of patients with cancer (C), the group of patients with other chronic diseases (CD), and the group of healthy subjects (H). A simple random sampling method performed by a computer was used in selecting the participants from 7,000 individuals whose information was available at the University of Health Sciences Sultan 2. Abdülhamid Han Training and Research Hospital automation system. The computer program enumerates the items in the sampling frame, determines the automatically produced random numbers, and presents the selected items to the researcher in writing or digitally. The sample groups were randomly selected from the groups of cancer patients (records of the oncology department), of patients with non-cancer chronic diseases (records of the internal medicine clinic) and of those without any diseases (individuals with general health screening code, who were referred to the hospital for various reasons such as recruitment, or military service application).

To assess the eligibility for the study, the participants' diagnoses recorded in the electronic medical records were examined. Patients with chronic diseases were eligible if they had diabetes mellitus, hypertension, neurological diseases, asthma, chronic obstructive pulmonary disease, other pulmonary diseases, cardiovascular diseases, or hypothyroidism. Patients with conditions other than the aforementioned ones who had the long-term use of medication and/or were followed up for any chronic disease (rheumatic disease, endocrinal disorders, liver disease, kidney diseases) were also eligible (classified as other chronic diseases).

Existing diseases were determined according to the diagnoses written according to the ICD-10 classification. The patients with any diagnosed psychiatric disorder (anxiety and depressive disorders, bipolar disorder, psychosis, obsessive-compulsive disorder, etc.), intellectual disability, Alzheimer's disease or other forms of dementia were excluded from the study. The patients were also excluded from the study if they reported having these comorbidities within the descriptive characteristics form. Finally, the patients who had both cancer and other comorbid chronic disease were excluded from the study as well.

To determine the sample size, a power analysis was conducted in the G-Power 3.1 program (G-Power, Aichach, Germany) by taking into consideration the values of the data obtained from a similar study in the literature [19]. The t-test for independent groups, Cohen's d coefficient used. The sample size was calculated to achieve a power of 95% at a significance level of 0.05. The power calculation indicated that the required sample size was 1120 people. The initial sample comprised 1141 individuals, out of which 21 were excluded from the study due to having psychiatric disorders, and 13 individuals not completing the questionnaire. The final sample comprised 1107 participants: group C (n = 262), group CD (n = 250), and group H (n = 595). The mean age of the individuals who participated in the study was 36.41 ± 12.80, and 82.7% of the participants (n = 916) were female. Comorbidity data of the patients are shown in Table 1.

Instruments

The participants completed a self-report questionnaire consisting of the Descriptive Characteristics Form and the Hospital Anxiety and Depression Scale (HADS).

Table 1. Distribution of descriptive characteristics

Age (years)	Min–Max (Median)	18–80 (36)
	Mean ± SD	36.41 ± 12.80
		N (%)
Gender	Female	916 (82.7)
	Male	191 (17.3)
Marital Status	Married	734 (66.3)
	Single	373 (33.7)
Non-Cancer Chronic Disease	Diabetes mellitus	53 (4.8)
	Hypertension	77 (7)
	Neurological disease	21 (1.9)
	Asthma, COPD or other lung diseases	82 (7.4)
	Cardiovascular disease	13 (1.2)
	Hypothyroidism	37 (3.3)
	Other*	163 (14.7)
Cancer	No	845 (76.3)
	Active	53 (4.8)
	Cured, still receiving treatment	93 (8.4)
	Cured, not receiving treatment	116 (10.5)
Group	H	595 (53.7)
	CD	250 (22.6)
	C	262 (24.7)

*More than one disease was noted
COPD – chronic obstructive pulmonary disease; H – healthy control group; CD – non-cancer chronic disease group; C – cancer group

Descriptive characteristics form

In the form developed by the researchers, there is a total of six questions, which investigate the participants' age, gender, marital status, number of children, comorbidity, and additionally, state of the condition for cancer patients (active disease, in remission and still receiving treatment, in remission and having completed the treatment). Patients who received various preventive treatments (such as long-term hormone therapy for some cancers, trastuzumab treatment for breast cancer etc.) were classified in remission and still receiving treatment group despite their cancer disease being in remission.

Hospital Anxiety and Depression Scale

HADS is a self-report questionnaire designed to screen the symptoms of anxiety and depression in individuals with medical diseases other than psychiatric illnesses [20]. It was created by Zigmond and Snaith [20], and its Turkish version validity and reliability study was carried out by Aydemir et al [21]. The four-point Likert type scale, which consists of 14 items, has two subscales (anxiety scale, and depression scale). Higher scores on each scale indicate a higher level of anxiety or depression (more severe symptoms). The scores of 0–7 refer to the normal levels, 8–10 to the borderline levels, and the score of 11 or above to the high, abnormal levels of anxiety or depression. Cronbach α value was found to be 0.85 for the anxiety subscale, and 0.78 for the depression subscale by Aydemir et al [21]. In the current study, the Cronbach α value of the anxiety, and depression subscale were 0.86 and 0.78, respectively.

Procedure

The study was approved by the University of Health Sciences Hamidiye Ethics Committee (approval number 20/133, on May 5, 2020) before the study commenced. The patients were called by phone and they confirmed their initial willingness to participate in the study. Individuals who initially agreed to participate, were then informed about the purpose of the study, the procedure, data privacy, and the voluntary nature of participation. Individuals read and signed the informed consent forms online. The study was conducted in compliance with the "Ethical principles for medical research involving human subjects" of the Helsinki Declaration. The online questionnaire was completed by participants through the Survey Monkey platform. Confidentiality was secured by completely deactivating electronic records and IP address records. This study was registered at the Protocol Registration and Results System (Clinicaltrials.gov PRS) with the registration number NCT04698044.

Statistical Analysis

For statistical analyses, Number Cruncher Statistical System (NCSS) 2007 (Kaysville, UT, USA) was used. The data of the study were analyzed with descriptive statistical methods (mean, standard deviation, median, frequency, rate, minimum, maximum). In the comparison of quantitative data, the student's t-test was used for the two-group comparisons of normally distributed continual variables. One-way ANOVA test was used for the comparison of three and more groups with normal distribution of continuous variables. Bonferroni post-hoc analysis was used to determine the difference between groups. Pearson's χ^2 test was used for the comparison of the categorical data. The predictive effects of having cancer (1), noncancer chronic disease (2) or being healthy (3) on anxiety and depression scores were evaluated using the multiple linear regression analysis. Significance was evaluated at $p < 0.05$.

RESULTS

Anxiety and depression scores and level categories in groups of participants

The descriptive parameters of HADS scores in the total sample are presented in Table 2. Table 3 presents the comparison of scale for anxiety (HADS-A) and depression (HADS-D) scores according to the cancer disease status of the individuals. HADS-A and HADS-D scores of

Table 2. Hospital Anxiety and Depression Scale subscale and total score distribution

Disorders	Min–Max (Median)	Mean ± SD
Anxiety	0–21 (9)	8.88 ± 4.38
Depression	0–21 (8)	8.56 ± 4.07
Total	0–40 (17)	17.44 ± 7.80

Table 3. Mean Hospital Anxiety and Depression Scale (HADS) scores according to cancer disease status

Parameters			HADS	
			Anxiety	Depression
Cancer	**No (n = 845)**	Min–Max (Median)	0–21 (9)	0–20 (8)
		Mean ± SD	8.75 ± 4.48	7.63 ± 4.06
	Yes (n = 262)	Min–Max (Median)	1–21 (9)	0–19 (7)
		Mean ± SD	14.4 ± 4.21	12.45 ± 4.12
	Test value		t: -1.831	t: 0.135
	p		***[b]0.035***	***[b]0.008***
Cancer	**Active (n = 53)**	Min–Max (Median)	1–17 (10)	0–21 (8)
		Mean ± SD	14.92 ± 4.02	12.12 ± 4.27
	In remission, still receiving treatment (n = 93)	Min–Max (Median)	1–21 (9)	0–18 (8)
		Mean ± SD	14.85 ± 4.14	11.45 ± 4
	Cured, having completed treatment (n = 116)	Min–Max (Median)	2–18 (9)	0–19 (7)
		Mean ± SD	13.48 ± 3.87	12.98 ± 4.1
	Test value		F:0.683	F:1.522
	p		***[a]0.506***	***[a]0.220***
Participant groups	**H (n = 595)**	Min–Max (Median)	0–21 (8)	0–20 (7)
		Mean ± SD	8.28 ± 4.46	8.34 ± 4.18
	CD (n = 250)	Min–Max (Median)	0–20 (9)	0–20 (8)
		Mean ± SD	9.87 ± 4.35	8.41 ± 4.09
	C (n = 262)	Min–Max (Median)	1–21 (9)	0–19 (7)
		Mean ± SD	14.4 ± 4.2	12.45 ± 4.12
	Test value		F:8.969	F:4.495
	p		***[a]0.001*** C > CD > H*	***[a]0.004*** C > CD* C > H*

aOne-way ANOVA
bStudent's t-test
*Bonferroni Correction was used.
H – healthy control group; CD – non-cancer chronic disease group; C – cancer group

Table 4. Mean Hospital Anxiety and Depression Scale (HADS) scores according to non-cancer chronic diseases

Chronic diseases		HADS	
		Anxiety	Depression
Diabetes mellitus (n = 53)	Min–Max (Median) Mean ± SD	2–20 (10) 10.52 ± 4.47	1–20 (9) 9.19 ± 4.55
Hypertension (n = 77)	Min–Max (Median) Mean ± SD	0–20 (9) 9.3 ± 4.86	0–21 (8) 8.29 ± 4.44
Neurological diseases (n = 21)	Min–Max (Median) Mean ± SD	1–17 (9) 8.83 ± 4.39	1–21 (8) 8.62 ± 4.91
Asthma/COPD/other lung diseases (n = 82)	Min–Max (Median) Mean ± SD	2–20 (10) 10.39 ± 4	1–21 (9.5) 9.34 ± 4.38
Cardiovascular diseases (n = 13)	Min–Max (Median) Mean ± SD	5–17 (8) 8.92 ± 3.73	2–12 (8) 8.08 ± 3.2
Hypothyroidism (n = 37)	Min–Max (Median) Mean ± SD	0–19 (9) 7 ± 4.2	0–15 (8) 7.6 ± 4.08
Other chronic diseases (n = 163)	Min–Max (Median) Mean ± SD	1–20 (9) 9.93 ± 4.42	0–20 (8) 8.26 ± 4.14

COPD – chronic obstructive pulmonary disease

group C were found to be statistically significantly higher compared to those without cancer. The anxiety and depression scores of group C were found to be significantly higher than anxiety and depression scores in the groups CD and H. While the mean anxiety score of group CD was significantly higher than that of group H (p = 0.032), no significant difference was observed in terms of mean depression scores (p = 0.61). The mean values of HADS-A and HADS-D scores for the groups CD and H were higher than the normal cut-off value (borderline level).

The HADS-A and HADS-D scores of patients with different non-cancer chronic diseases are presented in Table 4. Anxiety and depression scores of the individuals were found to be higher than the normal cut-off values in all chronic diseases except for hypothyroidism.

When we analyzed the HADS scores as categorical variables, according to the proposed cut-offs, about a half of the group C had high levels of anxiety (50.76%) and depression (50%). The distribution of anxiety and depression categorical levels of the participants was shown in Table 5. In cancer patients, the rates of those with high anxiety and high depression scores were higher than in the CD and H groups. In addition, the rates of those with high anxiety and high depression scores in the CD group were higher than in the H group.

Predictive models of anxiety and depression scores

To determine the predictive factor of anxiety and depression factor in having cancer, having chronic disease and being healthy, a multiple linear regression models were applied, with the disease conditions (study group) as the predictors, and anxiety and depression scores as the outcome variables. Having cancer was a significant predictor of higher anxiety and depression, with the participants' status of having cancer explaining the anxiety score variance by 13% and the depression score variance by 17% (Table 6).

DISCUSSION

Beyond medical risks, the pandemic has led to enormous psychological and social effects in the whole population [11]. In this study conducted during the pandemic, the mean anxiety and depression scores of the individuals were found to be borderline or above (depending on the study group) and these rates raise attention. Anxiety and depression seem to be commonly observed during the COVID-19 period. In a study conducted in Wuhan, it was revealed that depression was observed in 48.3% and anxiety in 22.6% of participants in the general population during the COVID-19 pandemic [22]. The stress levels of the individuals were found to be high in the studies conducted in the United States and Turkey, and our study results are in line with these findings [23, 24].

In Turkey, the first case was observed on March 11, 2020, schools were closed, work programs were changed,

Table 5. Anxiety and depression levels (based on cut-offs) in the cancer group, chronic disease control group and healthy control group

Levels		Healthy control group	Chronic disease control group	Cancer group	Test value
		n (%)	n (%)	n (%)	*p*
Anxiety	Normal	263 (44.2)$_a$	72 (28.8)$_b$	68 (25.9)$_b$	χ^2: 5.453
	Borderline	150 (25.2)$_a$	68 (27.2)$_a$	61 (23.28)$_a$	***0.001****
	High	182 (30.6)$_a$	110 (44.0)$_b$	133 (50.76)$_c$	
Depression	Normal	318 (53.4)$_a$	73 (29.2)$_b$	71 (27)$_b$	χ^2: 8.125
	Borderline	157 (26.4)$_a$	73 (29.2)$_a$	60 (22.9)$_a$	***0.003****
	High	120 (20.2)$_a$	104 (41.6)$_b$	131 (50)$_c$	

*χ^2 test; Bonferroni correction was used, different letters next to the frequencies indicate columns that differ significantly

Table 6. Multiple linear regression analysis of participants anxiety and depression scores

Anxiety score					CI	
Parameters	β	SE	t	p	lower	upper
Constant	2.896	0.052				
C[1] versus CD[2]	0.365	0.132	2.263	0.036	0.095	0.456
C versus H[3]	0.269	0.116	2.362	0.023	0.063	0.598
Model r = 0.365 R^2 = 0.145 Adjusted R^2 = 0. 136 F = 8.65 p = 0.003						
Depression score						
Constant	3.012	0.143				
C[1] versus CD[2]	0.313	0.112	0.123	0.006	0.023	0.456
C versus H[3]	0.462	0.102	0.236	< 0.001	0.102	0.456
Model r = 0.445 R^2 = 0.195 Adjusted R^2 = 0. 173 F = 8.96 p = 0.001						

CI – confidence interval; C – cancer group (1); CD – non-cancer chronic disease group; (2); H – healthy control group (3)

cafes/restaurants were closed, lockdowns were implemented, and people were advised to stay at home within the scope of the measures taken for the purpose of protection [25]. Launching the quarantine implementation caused many changes in people's social lives, which affected people psychologically [2]. Unlike previous pandemics, the intense use of social media today has caused the news of illness and death, the photos and videos of diseased persons, to be constantly present in lives of individuals. All of these factors have a great potential to provoke additional anxiety and depression [26].

Moreover, HADS-A and HADS-D levels of the individuals with various chronic diseases were examined, and these scores were found higher than the normal cut-off values in all diseases except for hypothyroidism. In China Center for Disease Control and Prevention's report, cardiovascular disease, diabetes mellitus, chronic respiratory diseases, hypertension and cancer were found to be associated with the increase in death risk [27]. Therefore, having these diseases may give more precaution and fear about COVID-19 than healthy individuals in this period, which can lead to more overall anxiety and depression in this group. Anxiety and depression levels of individuals were found high in chronic diseases in several studies, and it has been emphasized that these people need to be supported psychologically during this period [8]. In our study, the anxiety scores of the individuals with non-cancer chronic diseases were higher than those of the healthy individuals, whereas there was no significant difference in terms of the mean depression scores. Given that our study was a cross-sectional exploration in the early period of pandemic, this may have been related to anxiety occurring earlier as a response to acute events, whereas depression would be expected in a longer follow-up period [28].

When it comes to group C, our study showed significantly higher anxiety and depression scores in this group comparing to individuals without cancer. Furthermore, with multiple regression analysis, we showed that the status of having cancer explained the variance of anxiety scores by 13% and depression scores by 17%. These findings are in line with pre-pandemic studies showing anxiety and depression considerably associated with this disease. Depression was found to be four times higher in cancer patients than in non-cancer patients [29] and anxiety was found to be common in cancer patients as well [6]. However, during the pandemic, the study results are heterogenous. In the study where Ng et al. [16] compared cancer survivors and healthy controls, less distress was observed in cancer survivors during the pandemic. In the study conducted by Gallagher et al. [17], an increased depression risk was identified in those with breast and prostate cancers and leukemia, but there was no increased risk in other cancers. In the study conducted by Qian et al. [14], HADS-A and HADS-D scores were stated to be borderline or high in half of cancer patients during the COVID-19 pandemic. Approximately 70% of these patients needed mental support [14]. In addition to the assessment of earlier studies, our study further explored the anxiety and depression in subgroups of group C – those with active disease, those in remission and were still receiving treatment, and those in remission who have completed their treatment. Anxiety and depression scores were high in all three groups, potentially implying the need of carefully assessing distress in both cancer survivors and those with an active disease.

Management of cancer patients with COVID-19 is difficult during the outbreak. Stress, anxiety and depression worsen the clinical outcomes of cancer [24–27]. In the management of cancer patients, psychological evaluation is quite important, and there is a need for emergency screening and intervention programs.

During the social isolation period, people have begun to spend more time on the Internet and use social media more often as a way of communication. The Word Health Organization published short suggestions on the website under the heading "hashtagHealthyAtHome-Mental Health," consisting of daily routine, time to be informed and social contact. The Beijing University has prepared a handbook to cope with stress and other psychological problems to protect public mental health [30]. Each country should develop psychological screening and intervention programs in compliance with their socioeconomic status, culture and beliefs during and after the pandemic period. Screenings

and interventions may have importance for the entire population, and cancer patients and those with chronic diseases may be perceived as a specific at-risk group.

Reaching people, informing them accurately and providing support to them via psychological online support lines, handbooks and social media are just some of the interventions to be applied. It is recommended to create online patient support programs and to teach patients the methods of coping with anxiety and depression. Questionnaires can be first applied, and then diagnostic and interventional programs involving online interviews and detailed tests can be developed. The check up and treatment visits of the patients can be an opportunity to be screened for anxiety and depression with the HADS questionnaire. Medical staff should pay attention during the pandemic period in terms of psychological approach to the patients. They should provide calming, informative and motivating interventions.

Limitations

This study contains several limitations. Firstly, this was a cross-sectional study, which restricts the possibility of analyzing the causality of effects. The fact that the pre-pandemic levels of anxiety and depression among participants were not measured, the interpretation of the effects of the pandemic on anxiety and depression is limited. Secondly, even though the scales with acceptable, validity and reliability were used to determine the anxiety and depression status of the individuals, these were self-report scales, whereas psychiatric assessment would provide more reliable evaluation (which was not done due to the quarantine). The participants with an underlying psychiatric disorder that has not yet been diagnosed could potentially have been present in the study, though, this factor was equally possible in all three groups. Thirdly, the socio-economic factors that affected anxiety and depression levels of the individuals could not be evaluated and controlled for. Furthermore, women's participation was higher than men, and the effects of gender were not adjuster for in this study, which limits the generalizability of the results. Similarly, other potential confounding factors were not controlled for, such as family history of psychiatric disorders, history of adverse childhood experiences or traumatic events in the adulthood, social support (family, partner, friends, colleagues, community), as well as other known risk factors for anxiety and depression.

Despite all these limitations, our study results may speak for the importance of psychological screening in accordance with isolation measures during the pandemic, and point out to the potential at-risk groups of patients. The strengths of our study are the larger study sample compared to similar studies [6, 9, 14, 16], as well as the unique timing of lockdown that may have provided specific important information in relation to the COVID-19 pandemic. Furthermore, this is the first study evaluating the level of anxiety and depression in cancer patients in comparison to both healthy population, and patients with non-cancer chronic diseases. Finally, anxiety and depression were evaluated in cancer patients dependently on the current status of disease and treatment.

CONCLUSION

In times of increasing use of telehealth programs due to the pandemic, our study speaks in favor of the importance of online psychological evaluation, especially among the patients with serious diseases such as cancer. These findings showed that the level of depression and anxiety is borderline or high in various groups of participants (healthy individuals, patients with non-cancer chronic diseases and cancer patients) during the COVID-19 pandemic, but specifically higher in patients with a serious risk of complications if infected, such as the cancer patients. To that end, it is important to pay attention to anxiety and depression levels of all individuals, and especially cancer patients, and to empower them with timely and necessary interventions to enhance their psychological welfare, primary disease outcomes and quality of life.

Conflict of interest: None declared.

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Анксиозност и депресија код особа са онколошким обољењима и без њих током раног периода пандемије ковида 19

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САЖЕТАК

Увод/Циљ Болест ковид 19, која се претворила у важан јавноздравствени проблем, негативно је утицала на појединце не само физички већ и психички.

Циљ ове студије је да испита анксиозност и депресију болесника оболелих од рака у поређењу са особама са хроничним обољењима без рака и здравим особама током периода пандемије ковида 19.

Методе Ова студија пресека спроведена је током периода пандемије ковида 19 од 8. маја до 25. јуна 2020. године на 1107 испитаника у Турској. Просечна старост учесника у студији била је 36,41 ± 12,80 година. Узорак се састојао од три групе: група болесника са онколошким болестима (n = 262), контролна група болесника са другим хроничним болестима (n = 250) и здрава контролна група (n = 595). Подаци су прикупљени помоћу Обрасца дескриптивних карактеристика и Болничке скале за процену анксиозности и депресије.

Резултати Утврђено је да су скорови анксиозности и депресије виши код болесника са онколошким болестима у поређењу са друге две групе. Присуство онколошке болести је било значајан предиктор виших скорова анксиозности и депресије, и објашњавало је 13% варијансе скорова анксиозности и 17% варијансе скорова депресије. Болесници са другим хроничним болестима и здрави испитаници имали су средње вредности анксиозности и депресије на граничном нивоу.

Закључак Добијени налази указују на то да стопе симптома депресије и анксиозности код болесника са онколошким болестима могу бити веће у поређењу са болесницима са другим хроничним болестима и здравим особама. На стопе анксиозности и депресије у последње две групе такође треба обратити пажњу током пандемије. Ова студија може имати важне практичне импликације у смислу потребе за психолошким скринингом.

Кључне речи: ковид 19; анксиозност; депресија; рак; хронична болест

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

Lymphangioleiomyomatosis and Langerhans cell histiocytosis – two case reports from our practice

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SUMMARY

Introduction Lymphangioleiomyomatosis and pulmonary Langerhans cell histiocytosis are the most common pulmonary cystic diseases. Although they differ in pathogenesis, they share several features. The aim of this paper is to present the similarities and differences between these diseases, as well as to describe two cases from our practice.

Outlines of cases The patient with lymphangioleiomyomatosis (43 years old) had pulmonary changes detected during a regular examination within the underlying disease – tuberous sclerosis. Four years after starting therapy with everolimus, she was still respiratory asymptomatic, a slight radiological deterioration of cystic changes was registered, the diffusion capacity was declining (by 12%).

The second patient (23 years old) was admitted due to bilateral radiological lung changes and symptoms in the form of dry cough, quick fatigue, and chest pain. Pathohistological examination of the transbronchial biopsy showed numerous large-core histiocytes, immunohistochemically positive for CD1a and S100, so it was concluded that it was Langerhans cell histiocytosis. Cessation of smoking was advised. The follow-up examinations showed withdrawal of symptoms and an orderly finding of lung function, chest high-resolution computed tomography indicated slight regression of changes. In the meantime, the patient gave birth to a healthy child, the pregnancy and prenatal period were uneventful.

Conclusion These diseases are extremely rare and in cooperation with other specialties should be distinguished from diseases that mimic lung cysts.

Keywords: lymphangioleiomyomatosis; pulmonary Langerhans cell histiocytosis; cystic lung disease

INTRODUCTION

Diffuse cystic lung diseases are a group of disorders of different pathophysiological mechanism of occurrence, which is characterized by the presence of multiple lung cysts [1]. There are conditions that mimic lung cysts, both in clinical and radiological terms, and these are emphysema, bronchiectasis, cavitation, honeycombing, localized pneumothorax [2]. Individual cysts in the lungs may be present in healthy individuals, as well as a result of aging, previous infections, or trauma. In the past decade, owing to the development of high-resolution computed tomography (HRCT), the level of knowledge about these diseases has increased and disease evaluation is performed based on the radiological appearance of cysts (shape, size, wall thickness, and distribution) [1, 3].

Lymphangioleiomyomatosis (LAM) and pulmonary Langerhans cell histiocytosis (PLCH) are the most common pulmonary cystic diseases. Although they differ in pathogenesis, they share several features. Both diseases act as neoplastic disorders, have a cystic radiological pattern, affect young people in generative period, and can have extrapulmonary involvement.

The aim of the paper is to present the similarities and differences between these diseases as well as to describe two cases from our practice.

REPORTS OF CASES

The patient with lymphangioleiomyomatosis

Female patient (43 years old) was referred to the Institute of Pulmonary Diseases of Vojvodina in 2016 by a competent neurologist to clarify the etiology of bilateral diffuse pulmonary changes, detected during regular systematic physical examination within the underlying disease – TSC, which was diagnosed in her fourth month of life. Within her underlying disease, the patient had occasional epileptic seizures of the petit mal type, mild mental retardation, changes in the skin of the face and torso, previously ultrasound-verified tumor-altered structures of the kidneys and uterus (myomas).

Upon admission, the patient was asymptomatic; auscultatory finding was normal. X-ray of the chest showed no pathological changes on the lungs. HRCT verified bilateral and diffusely increased density of the lung parenchyma, which showed smooth thickening of the interlobular septa and a large number of clearly demarcated, randomly distributed hypodense

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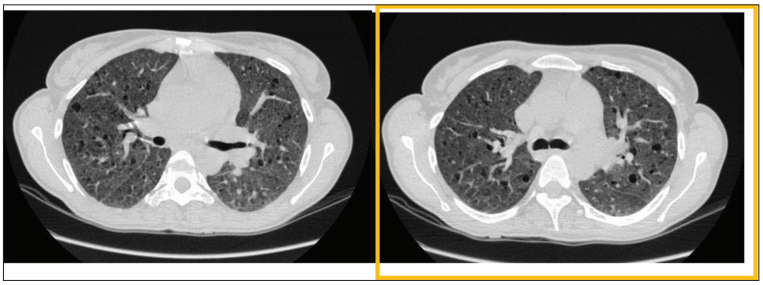

Figure 1. Chest high-resolution computed tomography of the patient with lymphangioleiomyomatosis before therapy

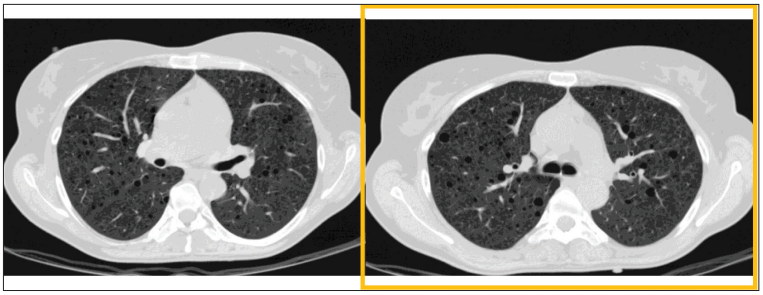

Figure 2. Chest high-resolution computed tomography of the patient with lymphangioleiomyomatosis after four years of therapy

Table 1. Findings of spirometry, diffusion capacity, and six-minute walk test at the first and control examination in a patient with lymphangioleiomyomatosis

Parameter	First examination		Control	
	[l]	%	[l]	%
FVC	3.25	89.29	3.66	103.7
FEV 1	2.75	87.58	2.93	96.38
FEV1/FVC		84.62		80.05
DLCO	7.61	82.09	6.35	69.93
6MWT	420 m	57%	350 m	47%

FVC – forced vital capacity; FEV1 – forced expiratory volume in one second; DLCO – diffusion capacity; 6MWT – six-minute walk test

(cystic) smooth-walled lesions of various shapes and diameters (up to 16 mm in diameter) (Figure 1). The pulmonary gas exchange was preserved at rest, while during exercise of two floors oxygen dropped by 1.3 kPa. The spiroplethysmographic finding was normal. During the six-minute walk test the patient walked 420 m (57%). Mild pulmonary hypertension (right ventricular systolic pressure – RVSP 35 mmHg) was verified by echocardiography (ECHO). Based on the clinical and radiological findings, the patient was diagnosed with LAM within tuberous sclerosis (TSC) complex. The patient started using everolimus (5 mg/day) in consultation with a neurologist and an immunologist.

The patient reported four years after the first examination (she had not reported for the advised annual check-ups at her own initiative). She denied respiratory problems, auscultatory finding was normal. HRCT indicated a discrete increase in the number of multifocal thin-walled cystic changes (Figure 2). The spirometric finding was normal, the diffusion capacity was declining compared to the first examination (Table 1). The ECHO finding was stationary. The use of everolimus was continued.

The patient with Langerhans cell histiocytosis

The patient (23 years old) was admitted to the Institute of Pulmonary Diseases of Vojvodina in 2018 due to bilateral radiological lung changes and symptoms (dry cough, chest pain and quick fatigue) that appeared two weeks before admission. She was a smoker (5 packs/year), without comorbidities.

The X-ray of the chest verified reticular changes in the upper and middle lung fields on both sides. Chest HRCT indicated bilateral diffuse, more pronounced in the upper and middle parts, thin-walled cystic lesions of various sizes, up to 15 mm in size (Figure 3). Spiroplethysmographic finding indicated a slightly reduced diffusion capacity (64%). The gas exchange was normal. Pulmonary hypertension (RVSP 43 mmHg) was registered on the ECHO. Pathohistological examination of the transbronchial biopsy showed numerous large-core histiocytes, immunohistochemically positive for CD1a and S100. It has been concluded that it is PLCH and additional examination (ultrasound and CT of the abdomen, endocrinological examination) ruled out systemic spread of the disease. The therapy included advice on the cessation of smoking, which the patient did.

The six-month follow-up examinations showed a good general condition of the patient and an orderly finding of lung function. In the meantime, the patient gave birth to a healthy boy, the pregnancy and perinatal period were uneventful. Chest HRCT three years after diagnosis (Figure 4) indicated slight regression of the bilateral cystic changes. ECHO showed an improvement, RVSP was within the reference limits. Diffusion capacity was not done due to the COVID-19 pandemic (the Institute of Pulmonary Diseases of Vojvodina was in the COVID system).

The paper was approved by the Ethics Board of the Institute for Pulmonary Diseases of Vojvodina and written consent was obtained from the patients for the publication of this case report and any accompanying images.

DISCUSSION

Pulmonary LAM is a disease involving the proliferation of smooth muscle cells of the blood and lymph vessels of the pulmonary interstitium, which leads to the formation of thin-walled cysts, pulmonary hemorrhage, and the involvement of lymph nodes can lead to chylous pleural effusions [4].

PLCH is a disease of former or current smokers, where cigarette smoking triggers abnormal proliferation and migration of dendritic cells, followed by the activation of the immune system, which lead to the formation of peribronchiolar nodules and later lung cysts [1]. These two diseases are reclassified from interstitial diseases into

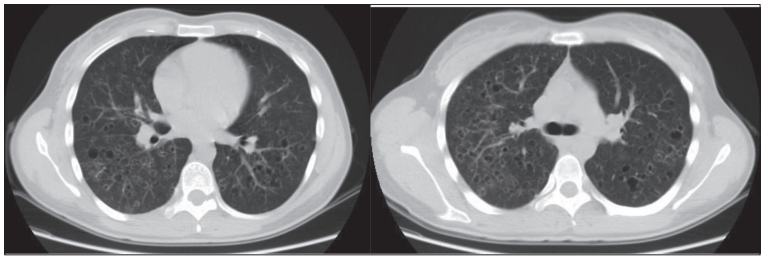

Figure 3. Initial chest high-resolution computed tomography of the patient with pulmonary Langerhans cell histiocytosis

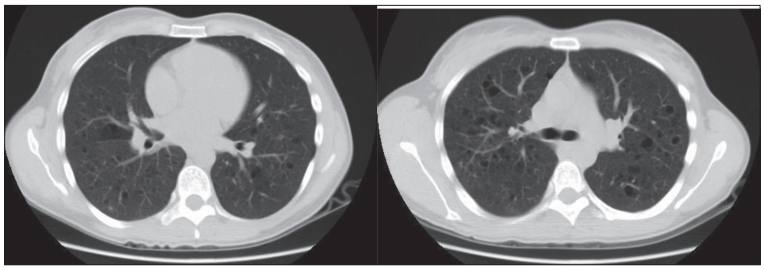

Figure 4. Chest high-resolution computed tomography of the patient with pulmonary Langerhans cell histiocytosis after three years

tumors – LAM belongs to perivascular epitheloid cell tumors (PEComa), classified as low-grade connective tissue neoplasms, and PLCH is classified as a bone marrow-derived dendritic cell tumor according to the World Health Organization [4].

Both diseases can occur in isolated or systemic form. LAM exists in a sporadic form (s-LAM) or within the tuberous sclerosis complex (TSC/LAM). Likewise, Langerhans histiocytosis may have its own isolated-pulmonary form (PLCH), or multisystem form (MS-LCH) [1].

These diseases are extremely rare. The annual incidence of PLCH is 2.2 per million, while the incidence of LAM is 1–7 per million inhabitants [3]. There are no official data for either Serbia or the region [5, 6]. PLCH is equally represented by sexes, s-LAM occurs only in women, a small percentage of men with TSC (13%) will develop LAM [4, 7]. Age at diagnosis is similar for both diseases (32–35 years) [4, 7]. Smoking is 90–100% present in PLCH, while LAM occurs mainly in non-smokers [4, 7]. Our patients were females in generative period (the PLCH patient was 23, the LAM patient was 43 years old). The patient with PLCH was a smoker; the patient with LAM was a non-smoker.

In clinical terms, both diseases may be asymptomatic when detected as an incidental finding, or may be accompanied by nonspecific symptoms (cough and/or difficulties in breathing), while acute shortness of breath is a consequence of pneumothorax [3]. In our cases, the patient with LAM was asymptomatic, previously diagnosed with TSC, which urged further diagnosis. The patient with PLCH had dry cough, chest pain and fatigue symptoms.

The radiograph in both diseases shows the changes primarily in the upper and middle thirds of the lungs, in the form of discrete cystic changes of the interstitium. HRCT is essential in diagnosing and monitoring both diseases. According to the criteria of the European Respiratory Society for the diagnostic algorithm of LAM, the finding of HRCT with the data on previously proven TSC may be sufficient to give a diagnosis, as was the case with our patient. HRCT findings in LAM describe multiple (more than 10), diffusely distributed, thin-walled (up to 2 mm), round, clearly demarcated, air-filled cysts with preserved or increased lung volume without other interstitial damage except for the possible presence of micronodular epithelial hyperplasia in TSC [7].

The HRCT findings of PLCH patients depend on the stage of the disease. In the early phase of the disease, it is dominated by centrilobular nodules, 1–10 mm in diameter. In the later stages of the disease, thin-walled cysts of different sizes develop. In most patients, a combination of nodular and cystic parenchymal lesions is registered. Both nodules and cysts follow the apicobasal distribution, they are larger and more numerous in the upper than in the lower lung parts [8].

Spirometry tests can be normal in early stages (both LAM and PLCH patients); however, as diseases progress, airflow obstruction and decreased lung diffusion capacity occur [1]. Our patient with LAM had a normal spiroplethysmographic finding, while 6MWT distance was reduced. The PLCH patient had slightly decreased diffusion capacity. With regard to pregnancy, a study showed that it is safe in woman with PLCH and not associated with deterioration of pulmonary function or blood oxygenation [9]. The pregnancy and perinatal period in our patient were uneventful.

PLCH cells (Langerhans cells) are 12–15 μm in diameter, with eosinophilic cytoplasm. Immunohistochemical staining of these cells showed the expression of CD1a, S100 protein and CD207 (langerin) [10, 11, 12]. The characteristic finding consists of granulomas composed of the Langerhans cells described above and infiltrates of inflammatory cells (eosinophils, lymphocytes, neutrophils). Granulomas can occur in any organ (most commonly in the skin, bones, the pituitary gland, the liver, and the lungs).

Current therapy for LAM are mTOR inhibitors – sirolimus and everolimus [13]. Sirolimus is indicated in patients with FEV1 ≤ 70%, chylous effusions, or a rapidly progressive form of the disease [1]. The drug sirolimus is registered under the name Rapamune® (Pfizer Manufacturing Deutschland GmbH, Freiburg im Breisgau, Germany), and everomilus under the name Afinitor® (Novartis Pharma Stein AG, Stein, Switzerland) in the Republic of Serbia. In addition to mTOR inhibitors, supportive therapy in the form of bronchodilators and oxygen is recommended, as well as respiratory rehabilitation [3]. Regular immunization against the seasonal flu is necessary, as well as the application of pneumococcal vaccine. As far as lifestyle is concerned, smoking and diving are prohibited [1]. Since our patient was previously diagnosed with TSC, the decision on therapy (everolimus) was made in agreement with a neurologist and immunologist.

Smoking cessation plays a major role in the treatment of PLCH, leading to stabilization and even regression of the disease [14]. However, in one-third of patients, the disease progresses even after smoking cessation. Numerous chemotherapeutic protocols have been tested so far, but with little success [14]. In our case, smoking cessation led to slight radiological regression and ECHO improvement.

For both diseases, pleurodesis is advised after the first episode of pneumothorax, and lung transplantation is indicated in the progressive form of the disease.

These diseases are extremely rare, far less frequently described in the literature compared to numerous other pulmonary diseases and should be distinguished from diseases that mimic lung cysts (emphysema, bronchiectasis, honeycomb lungs). A good cooperation of several specialists is necessary to establish the diagnoses of either LAM or PHLC.

Conflict of interest: None declared.

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Лимфангиолејомиоматоза и Лангерхансова хистиоцитоза – два приказа болесника из наше праксе

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САЖЕТАК

Увод Лимфангиолејомиоматоза и плућна Лангерхансова хистиоцитоза су две најчешће плућне цистичне болести. Премда се разликују у патогенези, имају неколико заједничких карактеристика.

Циљ рада је био описати сличности и разлике између ових болести, као и приказати два случаја из наше праксе.

Прикази болесникâ Болесници са лимфангиолејомиоматозом (43 г.) плућне промене су откривене током редовних контрола основне болести – туберозне склерозе. После четири године лечења еверолимусом она је и даље респираторно асимптоматична, са благом радиолошком прогресијом цистичних промена и падом капацитета дифузије (за 12%). Друга болесница (23 г.) хоспитализована је због обостраних плућних промена и симптома у виду сувог кашља, убрзаног замарања и болова у грудном кошу. Патохистолошким прегледом трансбронхијалне биопсије уочени су бројни хистиоцити, имунохистохемијски позитивни на *CD1a* и *S100*, те је закључено да се ради о Лангерхансовој хистиоцитози. Саветован је престанак пушења. На контролним прегледима болесница је била асимптоматична, налази плућне функције су били уредни, компјутеризована томографија високе резолуције грудног коша указивала је на благу регресију промена. У међувремену болесница је родила здраво дете, трудноћа и пренатално доба су протекли уредно.

Закључак Ове болести су изузетно ретке и у сарадњи са другим специјалностима треба их разликовати од болести које имитирају плућне цисте.

Кључне речи: лимфангиолејомиоматоза; плућна Лангерханосова хистиоцитоза; цистичне плућне болести

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

Resection of Haglund's deformity using the arthroscopic method with the three-portal technique

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SUMMARY

Introduction Haglund's deformity represents a reactive enlargement of the posterosuperior aspect of the calcaneus and often causes pain that can significantly disturb everyday activities. If the patient's condition does not improve after six months of non-surgical treatment, surgical treatment could be taken into consideration. Although Haglund's deformity is successfully treated by endoscopic calcaneoplasty with a two-portal technique, we decided to apply a slightly newer technique – arthroscopic surgery with a three-portal technique.

Case outline A patient with clinically and radiographically confirmed Haglund's deformity was operated on using arthroscopic calcaneoplasty with a three-portal technique. The postoperative recovery was uneventful and full weight-bearing was allowed after four weeks. A complete return to sports activities was allowed after four months.

Conclusion Arthroscopic calcaneoplasty with the three-portal technique proved to be a safe and cost-effective surgical method in the treatment of Haglund's deformity. There is still not enough data in the literature regarding this type of surgical treatment, but the good postoperative functional results are extremely encouraging since this surgical technique enables faster recovery and fewer postoperative complications.

Keywords: endoscopic calcaneoplasty; hindfoot; Haglund disease; retrocalcaneal bursitis

INTRODUCTION

Haglund's deformity (HD) represents a reactive enlargement of the posterosuperior aspect of the calcaneus, often followed by the inflammation of the retrocalcaneal bursa and insertional tendinopathy of the Achilles tendon [1, 2].

For the precise diagnosis of HD, a clinical examination and a lateral-view radiograph are usually sufficient. HD is successfully treated non-surgically, but if the patient's condition does not improve after six months of non-surgical treatment, surgical treatment can be taken into consideration [2, 3]. Operation can be performed as open or arthroscopically assisted surgery. Both methods usually include the removal of retrocalcaneal bursa and the resection of the ossification in the posterosuperior aspect of the calcaneus [2, 3, 4]. An "open" surgery bears a higher risk of postoperative complications, which include difficulties in wound healing, hyperkeloid scars, larger postoperative swelling and hematoma, skin paresthesias near the postoperative scar, neurovascular injuries, avulsions of the Achilles tendon attachment, greater possibility of the iatrogenic fracture of a heel bone and the postoperative Achilles tendon shortening, which causes decreased foot dorsiflexion [2, 4, 5, 6]. Thus, for the last three decades, arthroscopically assisted surgery of the ankle represents the surgical method of choice in the treatment of this deformity [4].

CASE REPORT

The patient was a 27-year-old male, a recreational basketball player with pain and swelling in the posterior aspect of the right heel, with difficulties in walking. The symptoms had been lasting for three months affecting his normal life and sports activities.

Examination showed typical symptoms for HD, local swelling and redness of the posterior aspect of the heel, palpatory painful bony prominence in the mentioned area, with painful sensitivity on both sides of the Achilles tendon insertion. Tendon palpation did not cause any pain. The lateral radiograph of the foot revealed the enlargement of the posterosuperior aspect of the calcaneus, with the positive parallel pitch lines, which is the most important radiographic sign [7]. Due to clear clinical and radiographic presentation, MRI of the foot was not performed.

The patient was advised against wearing tight shoes and using 1 cm of padding placed under the right leg heel was suggested to him, as well as, practicing the Achilles tendon stretching on daily basis, using anti-inflam-

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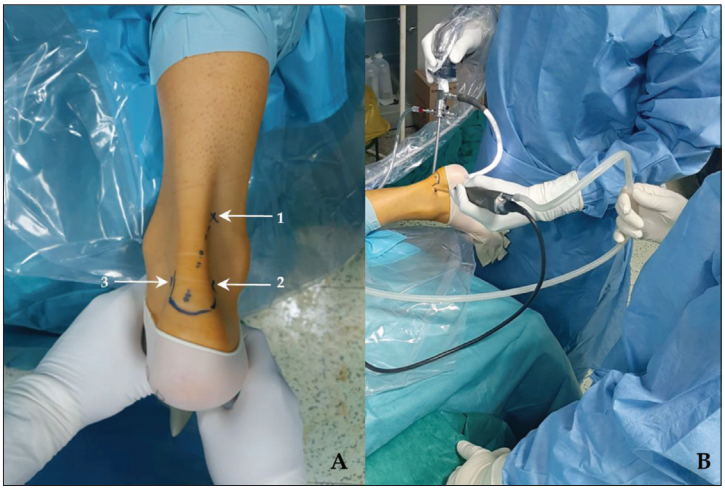

Figure 1. A) 1 – proximal posterolateral portal; 2 – distal posterolateral portal; 3 – distal posteromedial portal; B) operation procedure

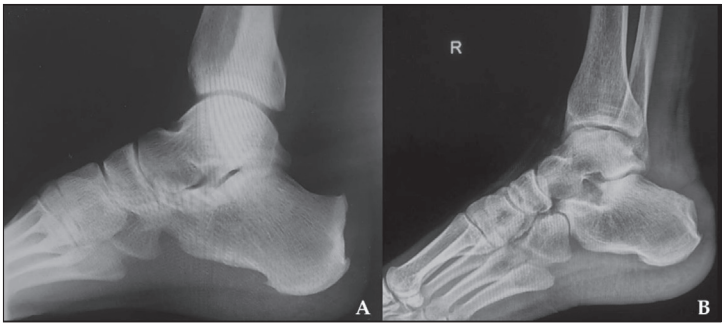

Figure 2. A) Pre-operative lateral radiograph; B) postoperative lateral radiograph

matory drugs and physical therapy. We did not decide to administer local corticosteroid therapy due to the possibility of Achilles tendon rupture, which was published in some earlier studies [6, 8, 9]. After the unsuccessful eight-month-long non-surgical treatment, we decided to perform the arthroscopic surgery with a three-portal technique, a slightly newer technic that has been in use for less than a decade [4, 9].

The endoscopic procedure was performed with the patient in a prone position under spinal anesthesia. The foot was positioned over the edge of the table, allowing a full range of motions of the ankle joint. A tourniquet was applied at the thigh. Preoperatively, bony prominences were marked, which served as landmarks for portals' placement.

The first two portals, the distal posteromedial portal (DPMP) and distal posterolateral portal (DPLP), were standard portals for hindfoot endoscopy. They were positioned at the level of the tip of the lateral malleolus, immediately adjacent to the Achilles tendon, and served as working portals. The third portal was proximal posterolateral portal (PPLP) and it served for the insertion of an optical instrument, visualization of the retrocalcaneal area and the ankle. The portal was positioned directly outward the Achilles tendon, 5 cm cranially to its attachment. Subcutaneous tissue was separated by a mosquito clamp, in order to enter caudally into the retrocalcaneal area by a blunt trocar. In this way, a passage was made for an undisturbed, safe insertion of an endoscope into the ankle (Figure 1).

By inserting the endoscope through the PPLP, synovitis and retrocalcaneal bursitis were diagnosed. Excision of inflamed retrocalcaneal bursa and synovitis was performed using a shaver 4.5 mm in diameter (Arthrex GmbH, Munich, Germany) through the DPMP, which also enabled better ankle visualization. A contact between a bone spur at the posterosuperior aspect of the calcaneus with the Achilles tendon was discovered during the foot dorsiflexion. Using distal portals, the bone spur was removed by an abrader. The extent of bony resection was judged dynamically by performing a full range of passive motions of the ankle during the resection itself until there was no longer contact between the calcaneus and the Achilles tendon. Special attention was paid to avoiding iatrogenic Achilles tendon injury. Loose intraarticular bodies and tissue debris were removed. With the foot in full plantar flexion, a space was obtained for the insertion of the optical instrument immediately behind the Achilles tendon attachment, which made it possible to evaluate the local condition. Neither pathological substrate nor the signs of insertional tendinopathy were found.

After the tourniquet had been released and the ankle had been irrigated with saline solution, a hemostasis check was performed. The portals were closed with single sutures. Elastic bandage was applied on the operated leg for the first three postoperative days in accordance with recommendations of van Dijk et al. [4]. Standard perioperative thromboprophylaxis, antibiotic, and pain management therapy were administered.

On the first postoperative day, passive movements of plantar and dorsiflexion up to the pain threshold were started and the patient was discharged with advice for crutch-assisted walking and non-weight bearing on the operated leg. Leg elevation after activities was advised for the period of the first seven days, and avoiding conventional footwear for next eight weeks.

The first check-up was scheduled two weeks after the surgery. The patient was allowed partial, increment weight bearing on the operated leg in the following period, and functional physical therapy was suggested. Four weeks after the surgery, full weight bearing on the operated leg was allowed. After three months, the patient was allowed to gradually return to physical activities, and full return to sports was allowed after four months (Figure 2). The final check-up was carried out six months after the surgery. The patient did not report any previously presented symptoms even after performing sports activities.

This case report was approved by the institutional ethics committee, and written consent was obtained from the patient for the publication of the report and any accompanying images.

DISCUSSION

Since it was first described in detail in 1928, HD has represented a therapeutic challenge even for experienced surgeons. It is usually treated non-surgically, but when there is no improvement after six months, surgical treatment is considered. One of the main problems of the surgical treatment of HD is the amount of resection of the bony prominence. Excessive bone resection is the cause of greater post-operative problems in comparison to insufficient resection [10]. This excessive resection can cause disinsertion of the Achilles tendon and the calcaneus fracture [11]. Nesse and Finsen [10] stated that excessive resection can cause pain in the posterior aspect of the heel and ankle stiffness. Nonetheless, there are no clearly defined guidelines about the amount of bone resection during an endoscopic calcaneoplasty [1].

Open surgery usually includes either the resection of the posterosuperior aspect of the calcaneus or the wedge osteotomy of the calcaneus [10, 11]. Open surgery is a well-accepted method with good to excellent results [6, 12, 13, 14]. In cases with an accumulation of calcium salts inside the Achilles tendon or in the extremely painful insertional tendinopathies, "open" surgery has its advantages compared to the arthroscopic one [2, 15]. The most frequently used surgical approaches are the lateral and Achilles tendon-splitting approach. There are reports that open calcaneoplasty demonstrated a greater risk of the Achilles tendon attachment weakening, especially with osteoporotic bone, which can cause its avulsion during intensive rehabilitation [16]. Open surgery bears greater risks of post-operative complications, and the risks significantly decreased by the introduction of arthroscopically assisted surgery of an ankle [11, 17].

Arthroscopically assisted surgical treatment of the HD is followed by minor postoperative complications, smaller incision and scar, minor blood loss, smaller post-operative swelling and hematoma, sparing soft tissues, thus faster and easier recovery [3]. Since van Dijk performed the first arthroscopic resection of the posterosuperior aspect of the calcaneus in 1991, calling it endoscopic calcaneoplasty, this surgical procedure has been more and more accepted as a therapeutic procedure [11]. Arthroscopic surgery enables precise local decompression; thus, excessive bone resection is avoided, in addition to lesser risk of Achilles tendon avulsion [1]. Ortmann and McBryde [17] reported a case of Achilles tendon rupture three weeks after arthroscopic calcaneoplasty. Amount of the bone resection is shown to be lesser in arthroscopic calcaneoplasty [1, 6].

Initially, endoscopic procedures had been performed only through two portals, in a prone or supine position, which has been well documented [4, 11, 16, 17]. Arthroscopic calcaneoplasty through two portals have certain limitations. The small distance between DPMP and DPLP, with the need for frequent portal switching for adequate visualization and bony resection, causes technical difficulties, increases the risk of both iatrogenic injury and instrument damage, and prolongs the time of surgery [9].

Arthroscopic calcaneoplasty with the three-portal technique has been used for the past decade due to the better visibility of retrocalcaneal bursa and bony prominence, the enlarged area for arthroscopic manipulation, and minimization of the risk of iatrogenic lesions [9]. The first two portals are standard posteromedial and posterolateral portals [18]. Our third portal was PPLP and it was used for visualization, positioned immediately lateral to the Achilles tendon, 5 cm cranially from its attachment, according to the surgical technique described by Wu et al [9]. The proximal posteromedial portal was not used due to the possibility of injury to the posterior tibial neurovascular structures [5]. Different radiographic measurements are used in the preoperative estimation of the amount of bony resection in HD [6]. The ideal preoperative planning does not exist, since every radiographic measurement has its disadvantages [1]. In our case, in addition to the preoperative positive parallel pitch line, the amount of a bony resection was determined intraoperatively by dynamic tests. Under the direct control of an arthroscope, we resected the bony prominence, until there was no longer contact with the Achilles tendon through a full range of ankle motion. The same method was used by other authors, and despite the positive postoperative parallel pitch lines in three operated heels, they had good results and satisfied patients [9].

According to published studies, in the early postoperative period, we advised the leg elevation for seven days, immediate postoperative passive and active motions up to the pain threshold, and avoidance of conventional footwear for the first eight weeks [9]. Due to the inexperience and the fact that we were getting familiar with the procedure, we kept our patient non-weight bearing on the operated leg for the first two weeks after the surgery. The avoidance of sports activities in the first three months was in accordance with the recommendations of the aforementioned studies [7, 9].

There were no iatrogenic injuries, intraoperative, or postoperative complications. We are aware that the postoperative period of monitoring is relatively short, but the good functional result and satisfied patient encourages us to continue with this surgical procedure and try to further refine and improve the surgical technique and the postoperative management.

The analysis of a larger number of patients and a longer period of monitoring would provide us with more relevant data, which would indicate if the arthroscopic calcaneoplasty with the three-portal technique is an adequate and cost-effective surgical method in the treatment of HD. Nevertheless, this paper gives insights into the possibilities of arthroscopic surgery of HD, especially with the three-portal technique.

Conflict of interest: None declared.

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Ресекција Хаглундовог деформитета помоћу артроскопске технике са три портала

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САЖЕТАК

Увод Хаглундов деформитет представља реактивно увећање постеросупериорног аспекта калканеуса и често узрокује бол који може значајно реметити свакодневне активности. Ако се стање болесника не побољша после шестомесечног неоперативног лечења, у разматрање се узима хируршко лечење. Иако се Хаглундов деформитет успешно лечи ендоскопском калканеопластиком применом технике кроз два портала, одлучили смо да применимо нешто новију технику – артроскопску хирургију применом технике са три портала.

Приказ болесника Болесник са клинички и радиографски потврђеним Хаглундовим деформитетом оперисан је артроскопском калканеопластиком применом технике са три портала. Постоперативни опоравак протекао је без компликација и ход са пуним ослонцем је дозвољен после четири недеље. Потпуни повратак спортским активностима био је дозвољен после четири месеца.

Закључак Артроскопска калканеопластика применом технике са три портала показала се као сигурна и исплатива хируршка метода у лечењу Хаглундовог деформитета. У литератури још увек нема довољно података о овој врсти хируршког лечења, али су добри постоперативни функционални резултати изузетно охрабрујући јер ова хируршка техника омогућава бржи опоравак и мање постоперативних компликација.

Кључне речи: ендоскопска калканеопластика; стопало; Хаглундова болест; ретрокалканеални бурзитис

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

Occipitocervical fusion as treatment of instability in Chiari malformation

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SUMMARY

Introduction Occipitocervical (OC) fusion is a method for fixation of the OC junction when there is instability of that segment**.** Arnold Chiari malformation is a congenital disorder where cerebellar tonsils descend through the foramen magnum, which can lead to corticomedullar compression and formation of a syrinx. While treating this condition, for the purpose of decompression, the foramen magnum is expanded, which can potentially harm the stability of the OC junction.

Case outline We are presenting the case of a 16-year-old female who was surgically treated (suboccipital craniectomy and decompression) because of Arnold Chiari malformation type I. One-year post-op multislice computed tomography verified a slight enlargement of basion-axial and basion-dens intervals, with signs of spinal cord compression. Surgery was performed – OC fusion, canal decompression on C1 and C2 levels with a plate on occipital bone and screws placed in the third, fourth, and fifth cervical vertebra.

Conclusion OC fusion is a complex surgical procedure (vital neurovascular structures), but it is a reliable method for treatment of instability of the OC junction.

Keywords: occipitocervical instability; fusion; Arnold Chiari

INTRODUCTION

Occipitocervical (OC) fusion is a surgical method for fixation of the OC junction when there is instability of the OC segment caused by a variety of conditions (trauma, rheumatoid arthritis, congenital anomalies, neoplasm, iatrogenic instability due to decompression) [1].

Arnold Chiari malformation is a congenital disorder where cerebellar tonsils descend through the foramen magnum, which can lead to corticomedullar compression and creation of syrinx. It can also be associated with numerous anomalies (syringomyelia, spina bifida, hydromyelia, kyphosis, scoliosis and tethered cord syndrome), as well as hereditary syndromes [2].

To this date the treatment protocol is debatable, with some authors preferring conservative treatment to surgery [3], but available literature is in favor of surgical treatment [4, 5, 6].

While treating this condition, for the purpose of decompression, the foramen magnum is expanded which can potentially harm the stability of the OC junction [7].

CASE REPORT

We are presenting the case of a 16-year-old female who was surgically treated (suboccipital craniectomy and decompression) because of Arnold Chiari malformation type I. She was initially hospitalized because of repetitive episodes of weakness in both arms and legs, that lasted for a few minutes and disappeared spontaneously. They first appeared six months prior to hospital admission. After careful examination she was diagnosed with Chiari malformation type I that was magnetic resonance (MRI) verified – a prolapse (herniation) of the cerebellar tonsils through the foramen magnum by 20 mm, with compression of the spinal cord. The ventricular system was in an orderly position and shape, without signs of hydrocephalus. In addition to this obvious malformation, the existence of syringomyelia (starting from C5 and caudally) and malformation of the base of the skull in terms of platybasia and an abnormal angle between the medulla oblongata and mesencephalon were observed (Figure 1).

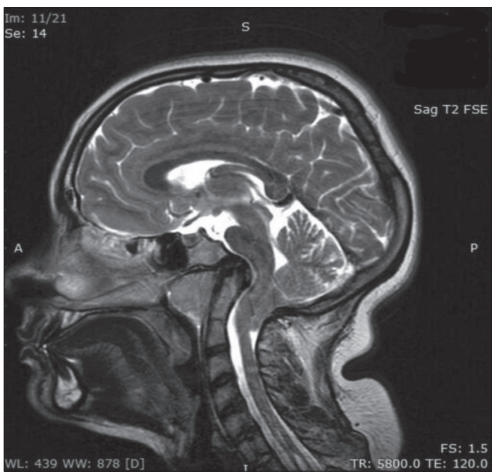

Figure 1. The first magnetic resonance imaging showing herniation of cerebellar tonsils through the foramen magnum (20 mm) with spinal cord compression

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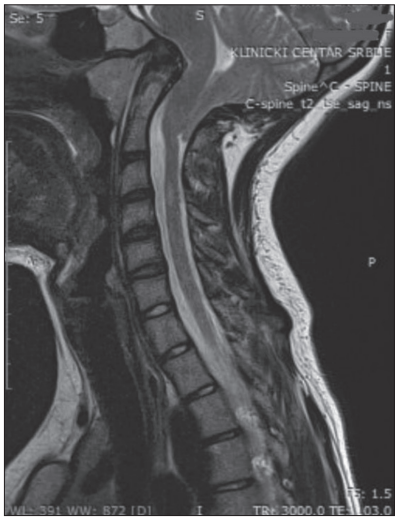

Figure 2. The first follow up magnetic resonance imaging, before the first surgery

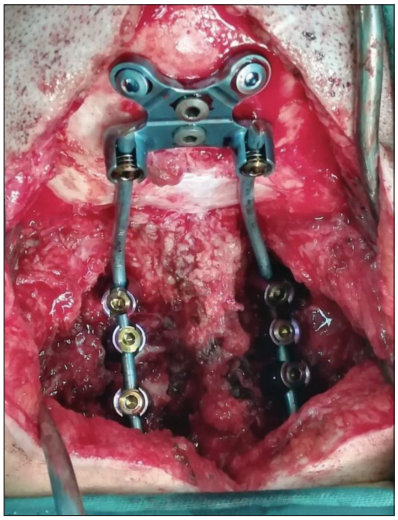

Figure 3. Intraoperative finding – occipital plate and screws

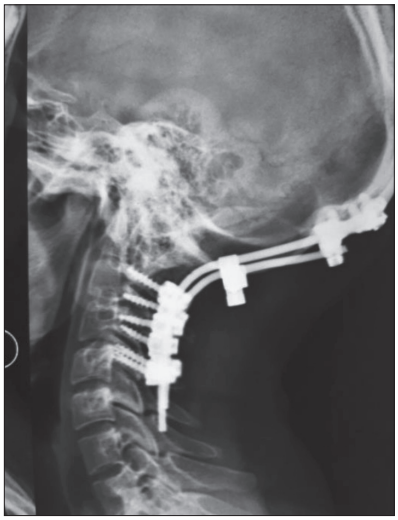

Figure 4. Postoperative X-ray

Follow up nuclear MRI was performed (Figure 2) following the first hospitalization and the findings were unchanged and the neurosurgical procedure (suboccipital craniectomy and decompression) was performed. Approximately one-year post-op the patient presents with following symptoms: headache, left arm paresthesia eventually followed by left side hemiparesis. New multislice computed tomography and MRI scans were obtained and showed progression of syringomyelia expanding cranially to syringobulbia, decompression in posterior cranial fossa was still intact. Two months later the symptoms persisted and new MRI scans were obtained – in comparison with former scans progression of hydrosyringomyelia was observed, as well as expanded central medullar canal with oedema. Conservative treatment was tried with antiedema therapy, but there was no clinical improvement. The patient was hospitalized again and another MRI evaluation was performed, and neurosurgeon and orthopedic spinal surgeon indicated OC stabilization.

Surgery was performed – OC fusion, canal decompression on C1 and C2 levels with a plate on occipital bone and screws placed in the third, fourth and fifth cervical vertebra. Neuromonitoring was used throughout the whole surgery (Figure 3 and 4).

Postoperatively the patient has no significant symptoms, with notable reduction of pain and paresthesia of the left arm. Movements of cervical spine are limited, in accordance with the stated surgery. The patient is feeling well and she is back to her daily activities.

The study was done in accordance with the institutional Committee on Ethics.

DISCUSSION

OC fusion is a surgery indicated for treatment of craniocervical junction instability caused by a variety of different pathologies (congenital, traumatic, degenerative, inflammatory, infective, or neoplastic) [1, 8].

This surgery represents a huge challenge for the operator and his team, considering that C0–C1–C2 is the most mobile portion of the spine, and it is the portion of the spine that must resist force in eight axes of rotation [9].

Each patient that presents with craniocervical junction instability, whatever the cause, must be carefully and minutely assessed, appropriate diagnostic methods used. Only then – while taking into account clinical presentation (functional stability, neurological status, accompanying symptoms), as well as MRI/CT findings – after preoperative planning the decision about the right treatment should be made [10].

The advances made in the field of neuroradiology have made it possible to understand biomechanics and structure of this region, especially considering substantial number of anatomical variations of vascular and neural structures [11]. Today, these radiologic findings are crucial in preoperative planning, and intraoperative visualization.

In 1900s, OC instability was considered inoperable and a terminal condition, and in the last 90 years there was a large number of surgical techniques developed to perform OC fusion [12].

Despite of the chosen method, the main goal is the same – restore and maintain alignment, decompress neural elements and provide good conditions for the bone fusion to occur.

The spine surgeon performing OC fusion must be well aware of spinal biomechanics and anatomy, and he must be familiar with the procedures to achieve decompression, alignment immobilization and fusion. Also, the surgeon should be aware of the perioperative risks and complications rates that are significant, and the readmission rates following OC fusion – that although lower in elective OC fusion surgeries are not negligible [13, 14].

Taking into account the aforementioned, it is clear that indicating this surgery is a big decision and the surgeon must consider all of the methods available as well as evaluate each patient individually before deciding this is the best solution.

While official surgical guidelines are yet to be established advancements are being made and teams consisting of experts in diagnosing and treating this condition were put together to discuss this matter [15]. Most authors agree that a decision should be made based on the clinical condition of the patient [5–8]. In their research, Asghar Ali Turabi et al. [16] concluded that OC fusion along with decompression surgery, had better outcome than decompression surgery alone. In order to be able to make the best patient-oriented decision, larger studies focusing on treatment outcomes are necessary as the available studies have shown that outcomes vary relative to a number of factors like chosen surgical procedure, symptoms duration and syringomyelia [17].

In our experience, OC fusion is a complex surgical procedure (involving vital neurovascular structures), demanding a multidisciplinary approach (spinal surgeon, neurosurgeon, anesthesiologist) but it is a reliable method for treatment of instability of the OC junction. Every case should be carefully considered, and if decided that OC fusion is the appropriate method of treatment, it requires detailed planning.

Conflict of interest: None declared.

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Окципитоцервикална фузија у лечењу нестабилности код Кјаријеве малформације

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САЖЕТАК

Увод Окципитоцервикална (ОЦ) фузија је метода фиксације ОЦ прелаза када постоји нестабилност тог сегмента. Арнолд–Кјаријева малформација је конгенитални поремећај где долази до миграције церебеларних тонзила кроз форамен магнум, што може довести до цервикомедуларне компресије и стварања сиринкса. Приликом лечења овог поремећаја, због декомпресије се шири форамен магнум, што може довести до нестабилности ОЦ прелаза.

Приказ болесника Приказујемо случај болеснице старости 16 година са Арнолд–Кјаријевом малформацијом типа I која је хируршки лечена (субокципитална краниектомија и декомпресија). Годину дана после операције на мултислајсној компјутеризованој томографији је верификовано благо увећање интервала *базион-аксис* и *базион-денс*, са знацима компресије кичмене мождине. Учињен је хируршки захват – ОЦ фузија, декомпресија спиналног канала на нивоима *C*1 и *C*2, плоча је позиционирана на окципиталну кост, док су шрафови пласирани у пршљенове *C*3, *C*4 и *C*5.

Закључак ОЦ фузија је сложена хируршка процедура (анатомска позиција виталних неуроваскуларних структура), али представља поуздану методу лечења нестабилности ОЦ прелаза.

Кључне речи: окципитоцервикална нестабилност; фузија; Арнолд–Кјари

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

Ocrelizumab associated late-onset neutropenia in the patient with multiple sclerosis – case report and literature review

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SUMMARY

Introduction Ocrelizumab is a recombinant humanized monoclonal antibody that selectively depletes CD20-expressing B cells, which is approved for the treatment of the relapsing and primary progressive multiple sclerosis (MS). It is extremely rarely associated with late-onset neutropenia (LON), as an adverse event.

Case outline We describe a case, from the Treatment Registry of the Clinic of Neurology, University Clinical Center of Serbia, Belgrade, of a transient, asymptomatic LON detected in a naïve relapsing–remitting MS patient, six-months after treatment with ocrelizumab.

Conclusion Having in mind all the presently available data, which indicate that rarely occurring LON on ocrelizumab is asymptomatic and transient in the majority of cases, we assume that it may be suggested that only in patients with complaints suggesting the presence of possible infection, additional complete blood count monitoring should be mandatory, exclusively at that moment, apart from the precisely defined regular follow-up.

Keywords: late-onset neutropenia; ocrelizumab; multiple sclerosis

INTRODUCTION

Ocrelizumab is a recombinant humanized monoclonal antibody that selectively depletes CD20-expressing B cells [1]. The precise mechanisms of action of ocrelizumab are not fully elucidated, but it has been demonstrated that this molecule has no influence on plasma cells or neutrophils [2]. In March 2017, it has been approved by the United States Food and Drug Administration, and in January 2018 by the European Medicines Agency, for the treatment of both relapsing (R) and primary progressive (PP) multiple sclerosis (MS). Until May 2021, more than 200,000 people have been treated globally with ocrelizumab [1]. The most commonly reported adverse effects in clinical trials were infusion-related reactions, infections, and in a small proportion of subjects, malignancies [1].

Late-onset neutropenia (LON), is defined as an absolute neutrophils count (ANC) < 1.5 × 10^9/L that develops in more than four weeks after the last drug administration, preceded by a normal neutrophils count, without other identifiable causes [2, 3, 4]. In the postmarketing surveillance period, ocrelizumab-induced late-onset neutropenia (LON) was rarely reported [2–6]. LON was transient in all of those patients, and they all continued with ocrelizumab treatment after neutropenia resolved.

We describe a case of a transient, asymptomatic LON which developed in a naïve relapsing–remitting (RR) MS patient after treatment with ocrelizumab.

CASE REPORT

A 25-year-old female patient was diagnosed with RRMS, after second, severe, motor relapse in December 2019. The diagnosis was based on brain MRI that revealed a large number of T2-weighted supra- and infratentorial lesions, with one gadolinium-enhancing lesion, and oligoclonal bands present exclusively in the cerebrospinal fluid. The patient had autoimmune thyroiditis, without other illnesses or use of other drugs. Several years prior to establishing MS diagnosis, she suffered from Epstein–Barr virus infection. At that time, because of the abnormal complete blood count, sternal puncture was performed, which did not indicate any abnormalities in the bone marrow aspirate. On August 6, 2020, she started the treatment with the first two doses of intravenous infusions of ocrelizumab (600 mg in total). Preinfusion blood counts were normal and the patient did not have any signs or symptoms of infection. Six months later, on February 3, 2021, isolated neutropenia (ANC = 1.3 × 10^9/L) was observed in the laboratory results, without other changes in the blood count (hemoglobin 128 g/l, MCV 9.6 fL, white blood cells 3.6 × 10^9/l, platelets 191 × 10^9/l). Routine biochemical analysis, test

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Table 1. Previously reported cases of ocrelizumab associated with late-onset neutropenia

Reference	Age (years) sex	MS phenotype	Previous MS therapy	Date of the I/II dose of OCR	Date of the III dose of OCR	Date, laboratory results	Clinical presentation at time of LON	Treatment related to neutropenia	Date of recovery
[2]	35 F	RRMS	GA, INF-ß-1a, DMF	/	January, 2018	April 3, 2018 WBC = 3.5 ALC = 0.3 ANC = 0	mucositis, lethargy, fever	cefepime acyclovir MP filgrastim	April 6, 2018 WBC = 19 ALC = 14.8 ANC = 1.1
[3]	26 F	RRMS	/	October, 2018	April, 2019	August 1, 2019 WBC = 1.1 ALC = 0.3 ANC = 0 AMC = 0.8	aphthous stomatitis, headache, fever, lethargy	ceftriaxone acyclovir	August 3, 2019 WBC = 4.6 ALC = 1.8 ANC = 1.3 AMC = 1.3
[4]	21 F	RRMS	DMF, RTX (April 2016, January 2017)	March, 2019	/	July 8–12, 2019 ANC = 0.3 > 0.1	/	Lidaprim acyclovir MP	July 19, 2019 ALC = > 1.5 September, 2019 ALC = 5.6
[5]	34 M	PPMS	/	N/A	N/A	42 days post initial infusion of ocrelizumab	fever, abdominal tenderness – neutropenic enterocolitis	broad-spectrum of intravenous antibiotics G-CSF	in 5 days
[6]	38 M	PPMS	/	3.5 years before LON	N/A	January 29, 2020 WBC = 3.7 ALC = 0.8 ANC = 0 AMC = 2.8	fever, chills, painful swelling of the left great toe, generalized weakness, vesicular lesions in the mouth	broad-spectrum of intravenous antibiotics, acyclovir, G-CSF	January 31, 2020 WBC = 8 ALC = 1 ANC = 2.7 AMC = 3.9
Current case, 2021	25 F	RRMS	/	August, 2020	February 26, 2021	February 3, 2021 WBC = 3.6 ALC = 1.4 ANC = 1.4 AMC = 0.2	/	/	February 24, 2021 WBC = 5.6 ALC = 2.3 ANC = 2.7 AMC = 0.6

MS – multiple sclerosis; RRMS – relapsing–remitting multiple sclerosis; PPMS – primary progressive multiple sclerosis; ALC – absolute lymphocyte count; AMC – absolute monocyte count; ANC – absolute neutrophil count; all values are $\times 10^3/\mu L$ ($10^9/L$); DMF – dimethyl fumarate; F – female; GA – glatiramer acetate; G-CSF – granulocyte colony-stimulating factor; INF-ß-1a – interferon beta 1a; LON – late-onset neutropenia; MP – methylprednisolone; OCR – ocrelizumab; RTX – rituximab; WBC – white blood cells

panel for autoimmune diseases including autoimmune thyroiditis, did not reveal any pathological findings. Due to the above-mentioned data from medical history, on February 10th, 2021, sternal puncture was repeated and analysis of the bone marrow aspirate indicated normal bone marrow, characterized by normal cellularity and appearance of granulocytic lineage, as well as the absence of dysplastic features or interrupted differentiation. Based on the finding of the bone marrow aspirate, and absence of other proven causes of neutropenia – such as relevant data in the patient's medical history, absence of any other complaints or physical findings, lack of other laboratory deviations – or concomitant medication possibly causing abnormalities in the blood count, diagnosis of LON was established. In accordance with the registered level of LON as ANC = 1.3×10^9/L, close monitoring of blood count twice weekly was indicated without application of granulocyte colony-stimulating factor. After three weeks of follow-up, the patient was asymptomatic with complete recovery of LON (ANC = 2.7×10^9/L) and ocrelizumab administration was continued as previously scheduled on February 26, 2021.

The study was done in accordance with the institutional Committee on Ethics.

DISCUSSION

Ocrelizumab is a recombinant anti-CD20 monoclonal antibody that has proven its efficacy and safety in pivotal controlled clinical trials (OPERA I, OPERA II, ORATORIO) for RMS and PPMS [1]. In the OPERA I and II, neutropenia in the RMS patients treated with ocrelizumab (14.7%) occurred significantly less frequently compared to interferon beta-1a patients (40.9%) [1]. Comparison of PPMS ocrelizumab patients (13%) with patients on placebo (10%), related to the development of neutropenia, did not demonstrate major differences [1]. In all of those patients, neutropenia was transient, and thus the ocrelizumab administration was continued.

We present the first case of LON associated with ocrelizumab at the Clinic of Neurology of the University Clinical Center of Serbia, in Belgrade. The diagnosis, follow-up, and treatment of LON were conducted in accordance with the

current recommendations for the diagnostics and treatment of neutropenia. As of March 30, 2021, 139 patients with RMS and PPMS have been included in the Treatment Registry for highly effective disease-modifying therapies for MS, established at the Clinic of Neurology. Until now, in the postmarketing surveillance, there are five reported cases of LON associated with ocrelizumab (Table 1). Female sex and RRMS were the most common demographic and clinical characteristics in these patients. In line with our case, three reported patients, one with RRMS and two with PPMS, were treatment-naïve [3, 5, 6]. In the two remaining cases, disease-modifying therapy had been already administered prior to ocrelizumab. Therefore, this interaction may have contributed to the development of LON [2, 4]. At least three to six months from the last dose of ocrelizumab was necessary for LON to be developed. Bone marrow biopsy performed in our patient, and one recently reported case, did not suggest any primary bone marrow dysfunction [6]. Cohen [2], Zanetta et al. [3], and Rauniyar et al. [6] have described symptoms of possible infection due to LON in their MS patients, which completely resolved after treatment with antibiotics, acyclovir [2, 3], and human granulocyte colony-stimulating factor [6]. Additionally, a reported case of a 34-year-old male who developed neutropenic enterocolitis, treated with administration of broad-spectrum intravenous antibiotics, had a complete recovery [5]. It has been described that with rituximab, B cell-depleting drug with very similar mechanism of action, rates of infections due to LON ranged 0–20% [7].

Our patient had no complaints, and thus the antibiotics were not applied. In the clinical trials with ocrelizumab, in 13% of patients' neutropenia was transient, without associated infection [1]. Reported untreated cases resolved spontaneously within 6–20 days [2]. Only three patients with LON due to ocrelizumab, published to date, received human granulocyte colony-stimulating factor [2, 5, 6]. Ocrelizumab administration was continued in all cases, except in the patient with neutropenic enterocolitis, where ocrelizumab was not scheduled at the moment of publication [5].

LON associated with ocrelizumab has unpredictable time of appearance, transient course and low prevalence. Therefore, the treatment with ocrelizumab does not necessitate the development of the new guidelines for regular complete blood count monitoring during therapy. Currently available data suggest that LON may be asymptomatic and, extremely rarely, associated with severe clinical manifestations. Having all the aforementioned in mind, we suggest that blood count monitoring should be mandatory immediately only in patients on ocrelizumab with complaints suggesting possible infection.

Conflict of interest: None declared.

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Неутропенија касног почетка удружена са применом окрелизумаба код болеснице са мултиплом склерозом – приказ болесника и преглед литературе

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САЖЕТАК

Увод Окрелизумаб је рекомбинантно хуманизовано моноклонско антитело које доводи до селективне деплеције лимфоцита *CD-20 B*, и које је одобрено за лечење релапсне и примарно прогресивне мултипле склерозе. Касна неутропенија је изузетно ретко удружена са окрелизумабом, као нежељени догађај.

Приказ болесника Приказана је болесница из Терапијског регистра Клинике за неурологију Универзитетског клиничког центра Србије, у Београду, са транзиторном, асимптоматском неутропенијом касног почетка, која је детектована код претходно нелечене болеснице са релапсно ремитентном мултипле склерозом, шест месеци после примене окрелизумаба.

Закључак На основу досадашњих доступних података који указују на то да је неутропенија касног почетка индукована окрелизумабом, у већини случајева асимптоматска и транзиторна, могло би се закључити да је додатно праћење крвне слике, ван стандардизованог протокола, неопходно само у случају сумње на постојање инфекције.

Кључне речи: неутропенија касног почетка; окрелизумаб; мултипла склероза

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

Two cases of uneventful pregnancies following the treatment of choriocarcinoma

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SUMMARY

Introduction Gestational trophoblastic disease represents a distinguished group of disorders that are derived from placental trophoblastic tissue aroused from abnormal fertilization. Choriocarcinoma is a malignant human chorionic gonadotropin-producing epithelial tumor arising from villous trophoblast. The choice of the chemotherapy regime is based on the International Federation of Gynecology and Obstetrics stage and World Health Organization score of the disease.

The objective of this article is to show that successful pregnancy is possible even after treatment of high-risk gestational trophoblastic neoplasia.

Outlines of cases We present two successfully treated patients who achieved pregnancy and delivered healthy babies in term.

Conclusion Gestational trophoblastic neoplasia has become the most curable malignant disease since the introduction of chemotherapy, which is effective and well-tolerated, and allows fertility preservation in high-proportion of women.

Keywords: gestational trophoblastic neoplasia; choriocarcinoma; pregnancy; fertility

INTRODUCTION

Gestational trophoblastic disease (GTD) represents a distinguished group of disorders that are derived from placental trophoblastic tissue aroused from abnormal fertilization [1]. The malignant forms of GTD are known as gestational trophoblastic neoplasia (GTN) [2]. Choriocarcinoma is a malignant hCG-producing epithelial tumor arising from villous trophoblast. It is characterized by myometrial invasion, and histologically by specific trophoblastic hyperplasia and anaplasia, absence of formed chorionic villi, and hemorrhage with central necrosis [1]. Human chorionic gonadotropin (hCG), a glycoprotein hormone, is an excellent biomarker of disease progression, response to therapy, and post-treatment follow-up instrument [3].

Before the era of chemotherapy these tumors were highly lethal, usually due to the inability to control hemorrhage within the tumor or metastatic site. Indeed, choriocarcinoma is considered one of the most chemosensitive solid tumors, with the cure rates reaching 95%. The choice of the chemotherapy regime is based on the International Federation of Gynecology and Obstetrics (FIGO) stage (Table 1) and World Health Organization score of the disease [4]. The scoring system prognostic factors are the patient's age, antecedent pregnancy, hCG levels, tumor mass size, metastases, and previously failed chemotherapy [5] (Table 2). Since the majority of patients are women of reproductive age, fertility preservation and the outcome of future pregnancies represent one of the major post-treatment issues. Clinic for Gynecology and Obstetrics, University Clinical Centre of Serbia represents the national center for the treatment of GTD. This national center was formed in 2015 by the Ministry of Health of the Republic of Serbia and since then 50 patients have been treated, 18 of them had malignant form of the disease, 13 of which have been treated with single- and five with multi-agent therapy.

Table 1. International Federation of Gynecology and Obstetrics stages of the disease

Stage I	Disease confined to the uterus
Stage II	GTN extends outside of the uterus but is limited to the genital structures (adnexa, vagina, broad ligament)
Stage III	GTN extends to the lungs with or without known genital tract involvement
Stage IV	All other metastatic sites

GTN – gestational trophoblastic neoplasia

Here we present two patients successfully treated with multi-agent chemotherapy, who subsequently became pregnant and delivered healthy infants.

REPORTS OF CASES

Case 1

A 24-year-old patient was admitted in December 2015, two weeks after the uterine curettage in a regional hospital due to bleeding and beta-hCG > 250,000 IU/L three months

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Table 2. The scoring system prognostic factors

Scores	0	1	2	4
Age (years)	< 40	≥ 40	-	-
Antecedent pregnancy	Mole	Abortion	Term	-
Interval from index pregnancy (months)	< 4	4 – < 7	7 – < 13	≥ 13
Pre-treatment serum hCG (IU/ml)	$< 10^3$	10^3 – $< 10^4$	10^4 – $< 10^5$	$\geq 10^5$
Largest tumor size (including uterus)	-	3 – < 5 cm	≥ 5 cm	
Site of metastases	Lung	Spleen, kidney	Gastrointestinal	Liver, brain
Number of metastases	-	1–4	5–8	> 8
Previous failed chemotherapy	-	-	Single drug	2 or more drugs

hCG – human chorionic gonadotropin

after the delivery (emergency Caesarean section in term pregnancy due to fetal asphyxia). The newborn died few hours after the delivery. The expert pathologist confirmed the diagnosis of choriocarcinoma. Beta-hCG value was 362,958 IU/L. Radiological staging [computed tomography (CT) and magnetic resonance imaging (MRI)] showed a heterogenous mass measuring 61 × 83 × 77 mm in the uterus with the full-thickness myometrial invasion, and probable parametrial invasion, as well as the vaginal metastatic lesion measuring 15 × 12 × 20 mm. The GTD clinical board determined FIGO stage II high-risk disease (FIGO score = 7) and the patient was scheduled for the EMA-CO (etoposide, methotrexate, actinomycin D, cyclophosphamide, vincristine) chemotherapy. The patient received nine cycles of chemotherapy until the normalization of the beta-hCG values followed by additional two consolidation cycles. During the post chemotherapy surveillance period no increase in beta-hCG values were noticed over a period of three years. Three years after the last chemotherapy cycle the patient spontaneously conceived. The pregnancy course was uneventful. At the 39th gestational week a scheduled Caesarean section was done, and the patient delivered a healthy male infant weighing 3760 g, Apgar score at five minutes was 9. The placenta appeared normal on gross morphological examination, and was sent for histopathologic examination, which showed no signs of gestational trophoblastic neoplasia. The beta-hCG values were controlled and reported negative six weeks after the delivery.

Case 2

In April 2017 the 31-year-old patient was hospitalized due to prolonged postpartum bleeding, 40 days after an uneventful vaginal term delivery. Uterine curettage was done in the regional hospital two weeks prior to admission and pathology indicated choriocarcinoma. The initial beta-hCG values were 276,070 IU/L. The expert pathologist confirmed the diagnosis of choriocarcinoma. Thoracal CT showed three metastatic lesions measuring up to 30 mm and several satellite lesions ranging 5–10 mm. Abdominal and pelvic MR imaging detected only myomas.

The GTD clinical board determined FIGO stage III and high-risk (FIGO score = 9) disease and EMA-CO chemotherapy commenced. Nine therapy cycles (until the normalization of the beta-hCG level) and two consolidation cycles were administered. The levels of beta-hCG remained negative for 27 months. In November 2019, the patient spontaneously conceived. The pregnancy course was uneventful. Elective Caesarean section was done in the 38th gestational week and the patient gave birth to a healthy female infant weighing 3170 g, with the Apgar score at five minutes of 9. Both the macroscopical and microscopical histological examination of the placenta were normal. Beta-hCG level was negative six weeks after the delivery.

These reports have been approved by the institutional ethics committee, and written consent was obtained from the patients for the publication of the reports and any accompanying images.

DISCUSSION

Choriocarcinoma is a pregnancy-associated tumor and can arise after any type of pregnancy; about 50% follow molar pregnancy, and the other half occurs with similar frequency after a spontaneous abortion or ectopic pregnancy, or after a term or preterm gestation [1]. The diagnosis of choriocarcinoma and other malignant entities within GTD can be challenging, with abnormal vaginal bleeding and elevated bhCG values being the hallmarks of the clinical presentation [3]. In the two cases described, choricarcinoma was preceded by term pregnancies. Both patients complained of prolonged postpartal vaginal bleeding, and the diagnosis was confirmed histologically after the uterine curettage.

The advent of chemotherapy has changed the prognosis of GTN dramatically. The therapy is instituted based upon the FIGO risk score, which takes into account the age of the patient, the type of and the time interval from the antecedent pregnancy, value of the pretreatment hCG, the number and sites of the metastases, the size of the largest tumor mass, and the response to the prior chemotherapy, with high-risk patients (FIGO score ≥ 7) being treated with multi-agent protocol. The multi-drug chemotherapy scheme of choice for the treatment of high-risk GTN is a combination of etoposide, methotrexate, actinomycin D, cyclophosphamide, and vincristine (EMA/CO), first introduced in 1979 [6]. Both of our patients were high-risk, so they were treated with multi-agent chemotherapy (EMA-CO).

Given the fact that most GTN patients are women of reproductive age, with excellent prognosis and long-term survival rates after the chemotherapy treatment, the attention is directed towards long term effects of chemotherapy on the ovarian function, future fertility, risk of premature menopause, and possible mutagenic and teratogenic effects [7, 5]. Ovarian function may be influenced by the chemotherapeutical agents. It has been shown that the levels of

Anti-Mullerian hormone, the marker of ovarian reserve, are decreased after the administration of etoposide-based chemotherapy for the GTN, in comparison to the patients with hydatiform mole that did not receive chemotherapy [8]. Transient amenorrhea, another sign of the disturbed ovarian function as a consequence of chemotherapy, is occasionally seen in patients with GTN [9, 5]. In the majority of women, generally, the normal menstrual cycle is recovered and there is no significant fertility compromise. Our two patients recovered their respective menstrual cycles 30–40 days after the end of treatment. The conception rate in treated patients varies 69–86% and is comparable to that of the general population [7]. Although some studies questioned increased miscarriage rates among patients who previously received chemotherapy for GTN, it was mainly associated with the conception within the first year after completed treatment [10, 11]. The first important aspect is the timing of the pregnancy. Our patients were suggested to use some of contraceptive methods in order to postpone pregnancy at least one year after treatment. As the disease recurrence monitoring is based on the hCG surveilance, an increase of this hormone associated with pregnancy may compromise adequate follow-up. There is a concern of the direct teratogenic effect of the chemotherapeutic agents. Knowing the duration of the oocyte maturation cycle, it may be concluded that the effect of chemotherapy lasts at least three months. Indeed, results of a Japanese study reported an increased risk of abnormal pregnancy outcomes (spontaneous abortion, stillbirth, repeat mole) in patients who conceived within six months of completing chemotherapy for GTN [12].

Therefore, one year appears to be a reasonable time interval from the treatment completion to the next pregnancy; it allows for the timely detection of the early recurrences and minimizes the teratogenic risk. Both single- and multi-agent chemotherapy can be safely administered to patients with a desire for childbearing [5]. In most studies, women who registered live birth were mostly of younger age (< 40 years) [11]. Both of our patients were also of younger age. Most women with gestational trophoblastic neoplasia are cured, but there is still a small and rare group refractory to all standard chemotherapy regimens – a condition called ultra-high risk GTN. For this group of patients, high-dose chemotherapy with peripheral blood stem cell support can be an option but recovery of ovarian function is very rare, and actually there have been no pregnancies described. In the group of low-risk GTN pregnancy the rate is similar to the general population. At our clinic, out of 18 GTN patients, five were resistant to single-agent therapy and the others were not, which matches the data according to which about three-quarters of patients are actually low-risk [13].

In conclusion, except for the risk of ovarian reserve damage, and, rarely, possible premature ovarian failure, multiagent chemotherapy can be safely administered to patients desiring future childbearing. The pregnancy should be deferred for at least one year after the treatment completion. Gestational trophoblastic neoplasia has become the most curable malignant disease since the introduction of chemotherapy, which is effective and well-tolerated, and allows fertility preservation in high-proportion of women, as it is shown in our two cases [14, 15].

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Два случаја некомпликоване трудноће после лечења хориокарцинома

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САЖЕТАК

Увод Гестацијска трофобластна болест представља групу поремећаја који настају из плацентног трофобластног ткива порекла абнормалне оплодње. Хориокарцином је малигни епителни тумор који производи хумани хориони гонадотропин и настаје из вилозног трофобласта. Избор режима хемиотерапије заснива се на стадијуму болести Међународне федерације за гинекологију и акушерство и скору болести према Светској здравственој организацији.

Циљ овог рада је да покаже да је успешна трудноћа могућа и после лечења високо ризичних гестацијских трофобластних неоплазија.

Прикази болесникâ Представљамо две успешно лечене болеснице које су затруднеле и родиле здраве бебе у термину.

Закључак Гестацијска трофобластна неоплазија је постала најизлечивија малигна болест од увођења хемиотерапије, која је ефикасна и добро толерисана и омогућава очување фертилитета код великог броја жена.

Кључне речи: гестациона трофобластна неоплазија; хориокарцином; трудноћа; фертилитет

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HISTORY OF MEDICINE / ИСТОРИЈА МЕДИЦИНЕ

The First National Hygiene Exposition in Belgrade in 1933

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SUMMARY
The paper is about the First National Hygiene Exposition in Belgrade in 1933. It was one of the most significant events and an important part of the cultural policy in the Kingdom of Yugoslavia at that time. It was also the last one in a series of great events under the high patronage of King Aleksandar I Karađorđević. In order to make research in the novelties the exposition introduced, the thus far unpublished archival material has been studied along with the situation drawings of the complex and the restaurant, photo documentation and the exposition presentation in the newspapers of the time, as well as the published material.
The aim of the paper is to emphasize the significance of the exposition, its dominant health and education concept with regard to social improvements for the benefit of the general public, all in the context of the period and under conditions it was organized. The paper also aims at presenting a comprehensive view of the exposition impact on the history of the Serbian and Yugoslav medicine, as well as the modernization of the society on the whole.
The paper also includes the hitherto unpublished archival material, plans, photographs, brochures' front pages and so on.
Keywords: history of medicine; Serbia; Kingdom of Yugoslavia; health and cultural policy; popular events; temporary architecture

INTRODUCTION

When the Great War had ended, an aspiration dating from the 19th century was realized – a unity of the south Slavic nations into one state, the Kingdom of Serbs, Croats, and Slovenes (Kingdom of SHS, i.e., Yugoslavia). So now, one state gathered the peoples who had hitherto lived in various cultural environments. The political act of uniting those nations could not just erase the differences between them, which ranged from economic ones to various national experiences, religions and confessions, cultural traits, mentalities, as well as differences in the level of literacy and education as the most important measure of social advancement.

There are two prominent figures featuring in the foundations of the public health of Serbia, who had always maintained that the population should be actively involved in all the subjects regarding country healthcare and society prosperity. Dr. Vladan Đorđević (1844–1930), was a figure whose visionary activities were reflected in making two reforming acts: the Public Sanitary Fund Act (1880) and the Sanitary Profession Management and Preserving Public Health Act (1881). Those acts held provisions on preventive measures of great importance not only for advancement of health in the Kingdom of Serbia, but also for the newly formed Kingdom of SHS [1, 2]. The other was Dr. Milan Jovanović Batut (1847–1940), at that moment appointed chief physician and chief of staff of the Kingdom of Montenegro Sanitary Department. His task was to improve the healthcare system in Montenegro and he examined all the school children in order to educate the population about the significance of general health. On behalf of the Serbian government, Dr. Đorđević invited him to return to Serbia and he was sent to abroad for further specialization [3]. Having completed all the additional studies in Berlin, Munich, Paris, and London (1882–1885), Dr. Jovanović Batut returned to Serbia, starting the healthcare service while working on setting up the medical service, opening hospitals, pharmacies, summer resorts, sanatoriums. He was resolute in striving to realize his visionary ideas, and among other things, he pushed for the Great School of Belgrade to become a university (working on a University Act, 1902–1905) and for founding the Faculty of Medicine [4].

Since the social policy of the Kingdom of Serbia was not properly regulated at the state level, physicians and philanthropists, encouraged setting up numerous civic associations aiming at public health improvement [5]. So, at that time, two, for us particularly important societies were established: the Society for the Preservation of Public Health (Society) and the Maternity Society which would mostly contribute to opening the First National Hygiene Exhibition (the 1st NHE) three decades later in Belgrade.

The Society was established in Belgrade in 1902 at the initiative of Dr. Radivoj Vukadinović, Dr. Vladimir Popović, and Dr. Slobodan Ribnikar, and wholeheartedly supported by the Serbian Medical Society, modelled after the

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similar societies in England for purposes of comprehensive work on the preservation of public health and education in the field. The Society's work was regulated by a Society Rule Book set up by Dr. Jovanović Batut [4, 6]. The health of mothers and children was the priority of the Society. Consequently, at the initiative of Dr. Radivoje Vukadinović, a Summer Resort for Sickly Children was opened in Košutnjak (1904) and two more in Šabac and Kragujevac [7]. From 1906, the Society published a magazine *Zdravlje* (*The Health*, a journal of doctors educating the population), in Sombor, with Dr. Jovanović Batut as the editor-in-chief. Also, in 1905, he suggested a Medical Museum to be established, providing a list of exhibits that should be collected for an exhibition. And that was the material that the Society would present at the *Balkan Exposition* in London (1907), where they would win the Grand Prix and be presented in an English medical magazine, *The Lancet*. Supported by the Serbian Red Cross Society, the Medical Museum was opened in 1912, at the Palace Russia (today the Moscow Hotel) on Terazije. Organizing numerous activities such as opening the first School for Rural Housewives (modelled after similar ones in Germany, 1906), the Society contributed greatly to educating women, including an aspect of struggle for emancipation [7, 8, 9].

And it was the women's societies and magazines, besides the school system, that greatly helped advance the women's status in Serbia in the 19th and 20th centuries. The women's societies had a humanitarian and educational character, spreading literacy and culture, thus supporting the overall social development [10]. One of those societies, with which the Society collaborated closely and whose activities were part of setting up the 1st NHE, was the Maternity Society, established in 1904 in Belgrade. The activities of the Maternity Society introduced numerous novelties in the domain of health and social care and fostering orphans and children without parental care. In addition, schools for midwives and midwife service were set up. The work of the Society was regulated in the Maternity Society Rule Book, stating concrete tasks such as establishing a collective home for infants and children under seven years of age, taking care of the children without parental care [5, 11]. And while the Society struggled to build its own home, the Military Hospital complex (1904–1909) and the General State Hospital complex (1901–1907) were built in Vračar [12]. Soon, in 1911, the Society for Supporting Orphans and Destitute Children donated a parcel of land to the Society, on the corner of Vojvode Milenka street (today Tiršova) and Kralja Milutina street in Belgrade so that they could erect their own Children's Home. Unfortunately, it was not to be, due to the upcoming war.

Just before the First World War (WWI), Dr. Jovanović Batut witnessed how his principles were being accepted with enthusiasm even outside the Kingdom of Serbia. One of his true followers in Croatia was Dr. Andrija Štampar (1888–1958). The two doctors of modern visions and broad knowledge and interests (with an age difference of 41 years) started collaborating from 1913 when Dr. Jovanović Batut vehemently supported Dr. Štampar's initiative to establish the Croatian society for the preservation of public health in Zagreb, similar to that of Serbia (1902) [4, 7, 13, 14]. Like Dr. Đorđević used to have a good ear to recognize talent of his younger colleague, Dr. Jovanović Batut in the late 19th century strongly encouraged him in his work. Immediately after the WWI when the new state was formed, Dr. Jovanović Batut also recognized a young, agile and broadly educated Croatian physician Dr. Štampar, as the one who would bring in new ideas and reform the health policy.

Health circumstances, laws and societies in the Kingdom of Serbs, Croats and Slovenes /Yugoslavia

The newly formed Kingdom of SHS (1918–1929) started its life in the WWI aftermath, with the destroyed demographic and economic foundations and huge losses in the population topped with various epidemics. Based on the pre-war experiences and the extraordinary war circumstances that required special health care arrangements, a struggle for improving overall public health and healthcare institutions in the Kingdom of SHS started. Under such circumstances, when fighting and curbing epidemics was underway, and when the housing issues needed to be resolved, a new healthcare organization was emerging, the one that started with preventions and treatments. It was also active in the field of health propaganda, education about health and raising public awareness about health in general. Social policy that was aligned with the European tendencies was becoming a significant political segment in the life of the new kingdom. It was believed that great social reforms would lead to a better and more just life of its subjects. The social policy was equaled to the communal one, and the only difference between them was just in the level and scope of work [15]. To that end, the government established a separate Ministry of Public Health, which thus far had been a part of other ministries – it was established in 1918 and was fully functional from 1920. At Dr. Jovanović Batut's suggestion, Dr. Štampar was appointed head of the Department of Racial, Public, and Social Hygiene, Department of Hygiene for short [16]. His priority tasks were implementing the already drafted social-medical plan, work on developing and advancing laws on healthcare, and trying to merge separate ministries of public health and social policy into one [1]. Right after the Ministry of Public Health was established, the Chief Medical Council was formed and the Permanent Epidemics Commission, chair by Dr. Jovanović Batut. At the initiative and under organization of the Commission, bacteriological labs were being opened all over the country, then institutes and clinics for studying and curbing malaria, clinics for treating sexually transmitted diseases (STDs), those for treating tuberculosis, public health centers and rural health centers. Regarding systematic healthcare for children, school clinics were opened, and the first Institute for Mother and Child Healthcare in Ljubljana, as well as a school for medical nurses in Belgrade, Zagreb, and Ljubljana. In addition, there was a great number of health resorts and recovery centers, as well as medical museums in Belgrade, Zagreb, Sarajevo, Ljubljana, Novi Sad, and Tuzla.

In the 1919–1929 period, the healthcare policy and medical services, managed by the Hygiene Section

(from 1929 called the Hygiene Section of the Medical Department), were reorganized by new regulations only to continue to develop according to the needs and technical circumstances in the country. All the facilities were making a "network of workshops," as Dr. Konstantinović called them, of social and medical activities and their organization [17]. Then higher education in the medical field was introduced in the new country – the Faculty of Medicine in Belgrade, which was founded in 1920 [18, 19]. Then two new important acts were passed: *Labourers' Insurance Act* and *Health Co-ops Act*, which was highly encouraging in regulating and improving workers' and farmers' healthcare. At Dr. Gavrilo Kojić's (1889–1927) initiative, healthcare co-ops were established (1922), healthcare organizations that were quite unique in the world at that time, and which would serve as a model for similar co-ops in Bulgaria, Romania, Poland, and all the way to America, India, and China [1, 20].

There were also private initiatives. Numerous pre-war voluntary associations of social and health aid developed and were based mostly on the principles established by the Society. The latter continued their work under a new name, the Yugoslav Society for the National Health Preservation (1922–1938), and in 1938 it was transformed into the Main Education Co-op [10]. The Maternity Society with their Orphanage (1922–1925) also continued with their activities within the Yugoslav Women's Association [5, 11]. There were also international institutions very active in the same field, which expressed their interest in supporting the healthcare policy reforms in the Kingdom of SHS. The most significant support came from the Rockefeller Foundation (establishing and erecting the School of Public Health in Zagreb in 1927; renovation of the Central Hygiene Institute in Belgrade in 1925–1926) and the League of Nations Health Organization. The support was most prominent in the field of eradicating infectious and endemic diseases, as well as enhancement of healthcare, sciences and education [1, 21].

State social mechanisms were being developed gradually also in the domain of urban planning and building the housing stock for the social-health centers and institutions as part of the kingdom's overall building policy. In 1921–1922, the state called for an international competition for master plan for the capital, as a basis for making the 1923 Belgrade Master Plan (adopted in 1924). When a Building Act and all its regulations became effective (1931–1934), municipalities started making construction and levelling plans [22].

The Hygiene Department's scope of activities included various remedial works which would improve the overall healthcare circumstances. With that regard, there was a key link between improving conditions in cities and the housing issues, along with enhancing the housing stock for the social-medical institutions.

With the 6 January Dictatorship (January 6, 1929 – September 3, 1931), the Kingdom of Yugoslavia emerged (October 3, 1929), consisting of nine counties and a special tenth region included Belgrade with Zemun and Pančevo. Hoping that the national conflicts and differences would be resolved soon enough by creating a unified nation, there were activities in all the fields, pushing a new ideology of a Yugoslav nation. New societies, associations and organizations, then magazines and daily papers were being established, while the old societies were invited to continue with their activities in the spirit of a new national and ideological concept [23].

Setting up the First National Hygiene Exposition in Belgrade in 1933

As previously pointed out, that was the political and social environment where the 1st NHE was opened in the capital city of the Kingdom of Yugoslavia – from August 19, to September 20, prolonged until September 25, 1933. It was an extraordinary medical and cultural event and an important part of an overall cultural policy in the country [24]. Organization and realization of such a substantial multidisciplinary function with public health at its core required the entire country to be engaged in the activities, continual work on advertising, as well as serious funding. Under the patronage of King Aleksandar I Karađorđević, the exposition was set up by the Yugoslav Society chaired by the General Board President and the first director of the Central Medical Institution, Dr. Stevan Z. Ivanić (1884–1948). The Honorable Board consisted of all the ministers, all faculties' deans, county leaders and other county representatives and those of other societies and associations (Figure 1).

The exposition General Board president was a university professor and one of the founders of the Faculty of Medicine in Belgrade, Dr. Aleksandar Kostić (1893–1983),

Figure 1. The King of Yugoslavia Aleksandar I Karađorđević and Queen Marija Karađorđević (born Princess of Romania) strolling through the exhibition at the Faculty of Engineering building, accompanied by Dr. A. Kostić, interpreting the exhibits (right) and Dr. S. Ivanić (in the background) (Belgrade City Museum, SGI2/1 2231)

Figure 2. Memorial Book cover (Museum of Science and Technology – the Serbian Medical Society Museum department)

Figure 3. The King of Yugoslavia Aleksandar I Karađorđević and Queen Marija Karađorđević (born Princess of Romania) at the entrance to the Faculty of Engineering with Dr. A. Kostić, and a minister I. Pucelj, Dr. S. Ivanić and Dr. B. Pirc (left to right) (Belgrade City Museum, SGI2/1 2234)

Figure 4. Exposition catalogue cover (private archive)

the vice-president was Dr. Bogoljub Konstantinović (1896–1944), the head of the Central Medical Institution Social-Medical Department and the secretary was Dr. Bojan Pirc (1901–1991). Also, the School of Public Health, Zagreb took part in the exposition preparations. Overall support was bestowed by the county boards of Ljubljana, Zagreb, Banja Luka, Split, Cetinje, Sarajevo, Novi Sad, Niš, and Skopje, then by all the medical centers in the country, as well as the health co-ops associations, Maternity Society, the Kingdom of Yugoslavia Red Cross, sober associations and sports societies [24].

As stated in the brochure published in 1934, the aim of the exposition as a "health festival of the entire country" was to *spread education on health as a prerequisite for the national health betterment, and to show the nation new industrial and craft products that support healthy life* [24] (Figure 2). Significant incentive for the exposition was the participation of the Kingdom of Yugoslavia at the Second International Hygiene Exposition in Dresden in late June 1920, as well as the Grand Gymnast Show held in Belgrade in June 7–29, 1930. The sport event promoted the Yugoslav nations' strength through sports, which helped to proclaim the ideology of integrated *Yugoslavism* in the national health narrative contained in the 1st NHE under a slogan *For Better Health and Better Future Generations* [24, 25].

In a newly erected imposing building of the Faculty of Engineering of Belgrade (1925–1931), in an area of over 3000 m^2, the most representative popular and professional section of the exposition was set up. The undeveloped land, the so-called *Running Course*, located between Bulevar Kralja Aleksandra, Grobljanska (today Ruzveltova) and Kraljice Marije streets, the area of 40,000 m^2 was used for exposition.

The works on setting up the exposition and the exhibits in the faculty building started on August 5, 1933, assisted by the curator at the Medical Museum and a secretary of the county board of Zagreb, Mladen Širola. The exposition had 18 sections housed in 17 faculty rooms and antechambers, halls and corridors which as separate units from the ground floor to the second floor led the visitors to educational rounds of clear and comprehensive knowledge on the ways of preserving health (Figure 3). The ground floor covered occupational medicine, food and diet, sports, demography, personal hygiene, healthcare service organization, hospitals and first aid. The first-floor covered health education, medical manikins, tourism, spas and climatic health resorts, healthcare services, medical science, mother and child hygiene, schoolchildren health and protection, fighting alcoholism, tuberculosis and STDs. The second floor covered dwelling place hygiene, settlement hygiene – remedial work (with over 50 miniature models of apartments, houses and entire settlements), infectious diseases, malaria, hygiene, and veterinary medicine. Unable to

Figure 5. Front page of a brochure that was handed out at the exposition (private archive)

Figure 6. Exhibits (Belgrade City Museum, SGI2/1 2106)

present all the collected exhibits, the exposition board created an exposition card system, covering the entire medical educational material delivered for the exposition [26]. Approach to the exposition organization was quite innovative and modern – one of the event feature was a real nursery for the children from the Belgrade Danube Nursery 2; the visitors had an opportunity to have a guide; free brochures on STDs were being handed out under a title *The Two Great Evils* by Dr. Bojan Pirc; There were also short popular talks on hygiene, then film projections and ultimately a postal room with its own stamp that read the 1st NHE (Figures 4, 5, and 6).

Having obtained all the necessary permits from the City of Belgrade administration, the Exposition Board started the works on preparing the land and erecting structures for the exterior section of the exposition, all according to a design by Svetozar S. Miletić [27]. The ground works, sewage system and water lines were constructed by engineer Mirko Bradilović, and a contractor engineer Aleksa Turbina was in charge of building the structures. Engineer Petar Čepurnjak was a supervisor over all the engineering works [28].

The exposition external section was divided into a commercial part and an entertainment part (Figure 7). The entire construction site area was equipped with sewage and water piping, and landscaping in English style. The construction started with erecting a wooden restaurant building with terrace, just behind the faculty building (Figure 8). Then, on a lower level three main rectangular exposition pavilions with a porch were erected. They were made of wooden structure on a concrete foundation. The pavilions held 67 exhibitors, displaying mostly pharmaceutical and medical products, equipment for apartments, then various household sanitary and cosmetic products. (Figures 9 and 10) Another nine exhibitors were house in 15 smaller pavilions grouped in the central section of the area, around a big potable water tap, where all the pedestrian paths converged [28].

In order to provide direct communication between the various exposition sections in the faculty building and the restaurant behind it, two wooden bridges connected the interior exhibition with the restaurant roof terrace. From terrace opened up a view over the exposition pavilions on a lower level and all the way towards the Danube and the Banat plains (Figure 11). Lingering on the restaurant terrace was made additionally pleasant with available telephone service and a radio station playing music and announcing various information [28].

The entertainment section was a sort of fair organized in collaboration with the Maternity Society and supported by the Belgrade Danube Nursery 2, and the Society for the Protection of Blind Girls. The fairground had swings and carousels, a theatre, park areas, an area for scouts, car rides and a jungle gym for kids. Its central spot was a water fountain. There were also two smaller tap water faucets and toilets (Figure 12).

Although the pavilions had been erected for the exposition, thus of temporary character, they remained in place even after the exposition closed, and were used as science and lectures workshops as per need of the Faculty of Engineering. The pavilions stayed there until a mechanical engineering laboratory was erected in Grobljanska street (1940). On the other hand, the commercial and entertainment section, the small fairground not only proved as useful but they actually provided a spark for the Society of Organizing Fairs and Exhibitions (1923) and their initiative to found the Belgrade Fairground in 1937 [29].

The entire work on promoting the 1st NHE was taken quite seriously. The entire visual material was done according to the drawings of a renowned artist M. Marković. Finally, the endeavors and efforts put in that medical and educational event were crowned when a Memorial Book was published, whose editors were Dr. Kostić, Dr. Konstantinović and Dr. Pirc. The exposition setup and its entire concept as a purposeful medium for enhancing

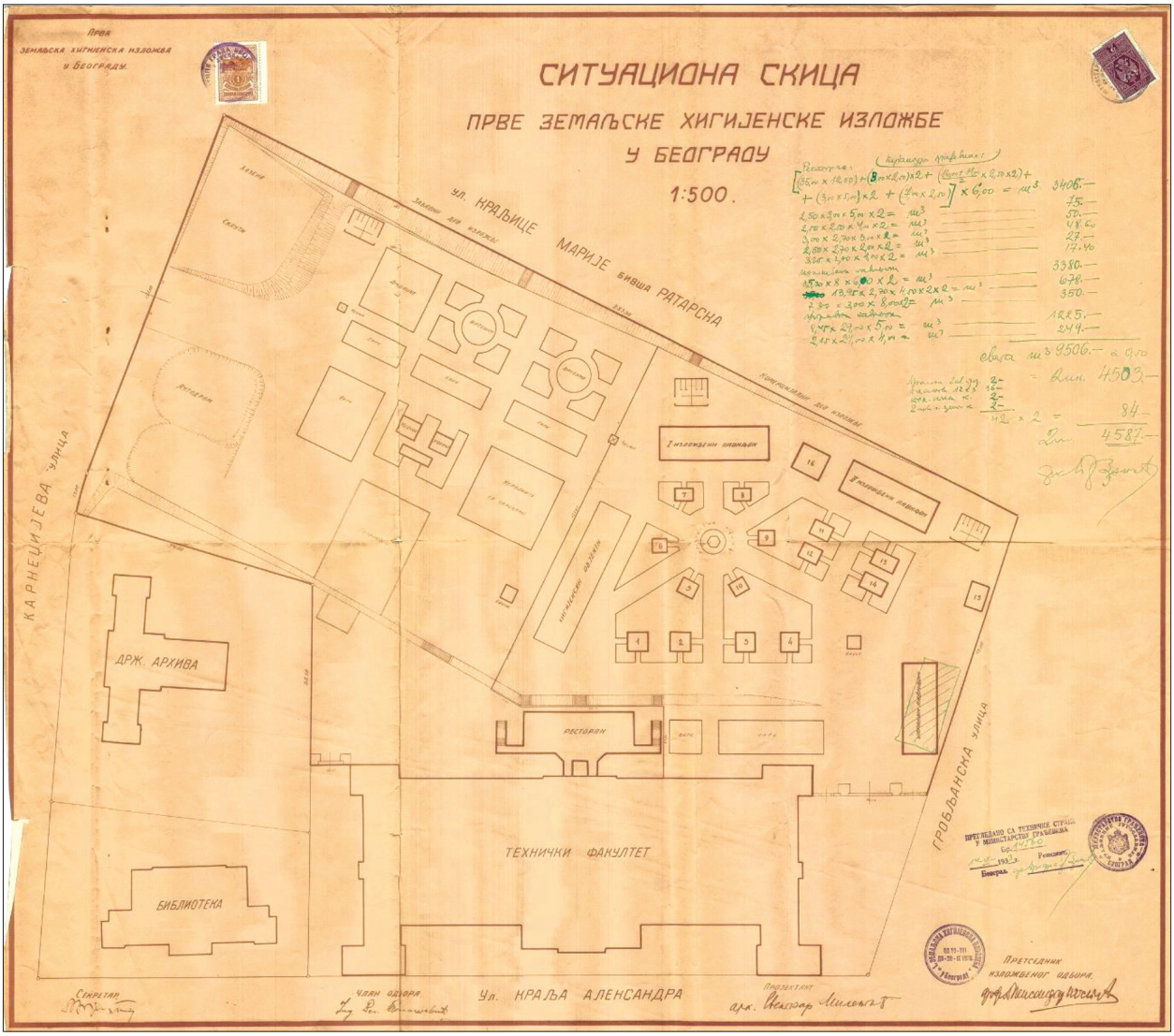

Figure 7. Exposition complex situation drawing (Historical Archive of Belgrade, OGB-TD, F11-15-1933)

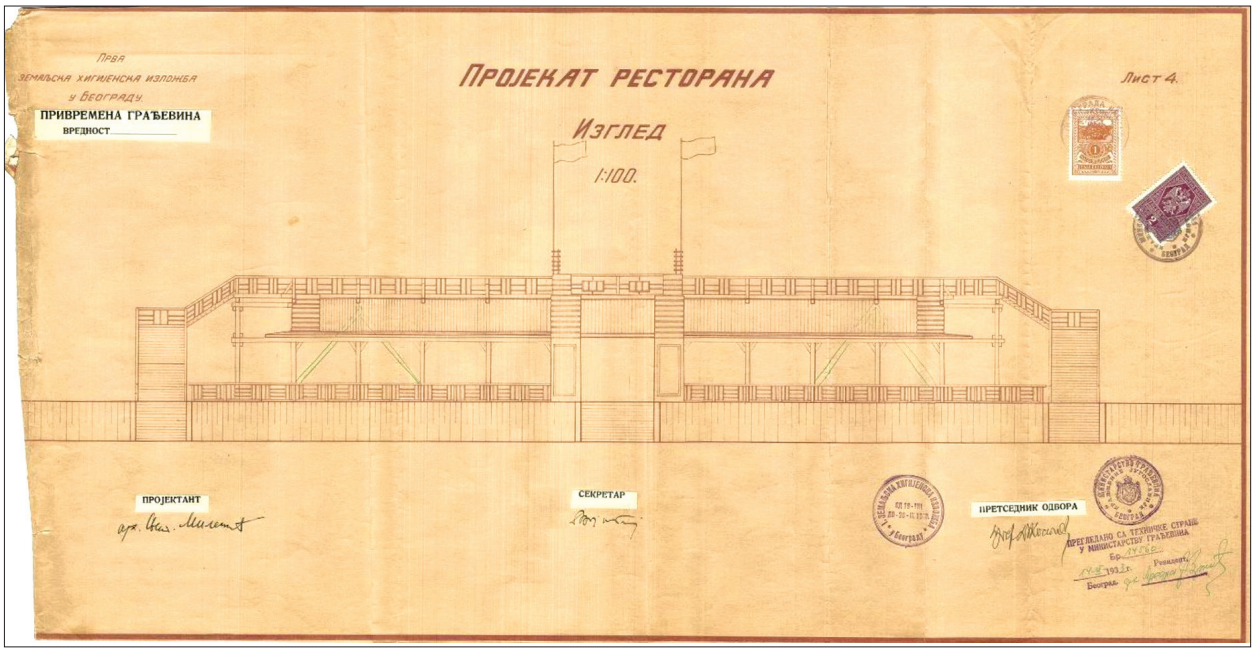

Figure 8. Restaurant design (Historical Archive of Belgrade, OGB-TD, F11-15-1933)

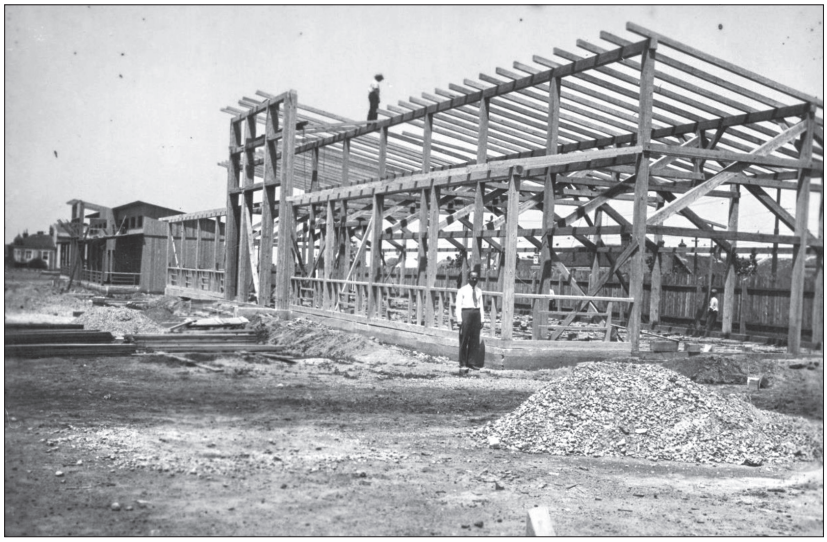

Figure 9. Construction of the pavilion (collection of Miloš Jurišić)

Figure 10. Visitors outside the pavilions (collection of Miloš Jurišić)

Figure 11. View of the restaurant and the exposition commercial section (collection of Miloš Jurišić)

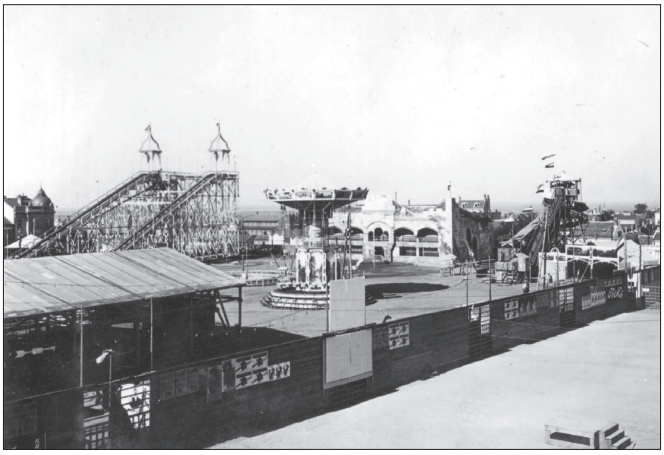

Figure 12. View of the exposition entertainment section (collection of Miloš Jurišić)

knowledge on the importance of preserving national health proved as highly successful both from the aspect of turnout of over 100,000 visitors and the aspect of its mobility. At an initiative of the Exposition Board and according to the King's wishes, the exposition was to continue its mission as a touring exhibition [30], but those plans fell through when King Aleksandar was assassinated in Marseille on October 9, 1934.

CONCLUSION

As a "country's health parade," the 1st NHE was truly an ode to an original and effective healthcare system based on ideas of the relatively new science at the time – social medicine, which required changes to be made in how the society relates to health and diseases, on the one hand, and in the domain of educational and industrial circumstances, on the other. And how important the exposition event really was could be witnessed in the printed autograph facsimile of the King Aleksandar's introductory word on the Memorial Book first pages, below a monogram with a crown and his photograph highlighting his patronage: *Providing health education, we have to create a new awareness of hygiene in the population – it can be achieved only by clear examples and active work with people – the hygiene exposition has shown us the right way to accomplish this* [24].

Besides huge success this medical and educational event had on the national level, along with its both professional and entertaining character, its significance is also reflected in strong encouragement in realizing the idea of building the Belgrade Fairground as a new international commercial center of the Balkans and a major link between the developed European countries and the developing ones of southeast Europe and Asia.

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Прва земаљска хигијенска изложба у Београду 1933. године

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САЖЕТАК

Кроз рад је приказана Прва земаљска хигијенска изложба у Београду 1933. године, један од најзначајнијих здравствених догађаја и важан део културне политике тадашње Краљевине Југославије, али и последња у низу великих манифестација под високим покровитељством краља Александра I Карађорђевића. Како би се истражиле новине које је она донела, анализирана је до сада необјављена архивска грађа, ситуациона скица комплекса и пројекат ресторана, фотодокументација, као и прикази изложбе у дневној штампи и објављеним публикацијама.

Циљ рада је да укаже на изузетну важност изложбе, доминантно здравствено-просветитељске концепције, са слухом за социјална унапређења у интересу народа, посматрано у контексту времена и условима у којима је настала. Тежило се што свеобухватнијем сагледавању њеног утицаја на историју српске и југословенске медицине и модернизацију друштва у целини.

У прилогу рада су дати до сада необјављена архивска грађа, планови, фотографије, насловне стране пратећих брошура итд.

Кључне речи: историја медицине; Србија; Краљевина Југославија; здравствена и културна политика; популарне манифестације; ефемерна архитектура

Пре подношења рукописа Уредништву часописа „Српски архив за целокупно лекарство“ (СА) сви аутори треба да прочитају Упутство за ауторе (*Instructions for Authors*), где ће пронаћи све потребне информације о писању и припреми рада у складу са стандардима часописа. Веома је важно да аутори припреме рад према датим пропозицијама, јер уколико рукопис не буде усклађен с овим захтевима, Уредништво ће одложити или одбити његово публиковање. Радови објављени у СА се не хонорарашу. За чланке који ће се објавити у СА, самом понудом рада Српском архиву сви аутори рада преносе своја ауторска права на издавача часописа – Српско лекарско друштво.

ОПШТА УПУТСТВА. СА објављује радове који до сада нису нигде објављени, у целости или делом, нити прихваћени за објављивање. СА објављује радове на енглеском и српском језику. Због боље доступности и веће цитираности препоручује се ауторима да радове свих облика предају на енглеском језику. У СА се објављују следеће категорије радова: уводници, оригинални радови, претходна и кратка саопштења, прикази болесника и случајева, видео-чланци, слике из клиничке медицине, прегледни радови, актуелне теме, радови за праксу, радови из историје медицине и језика медицине, медицинске етике, регулаторних стандарда у медицини, извештаји са конгреса и научних скупова, лични ставови**,** наручени коментари, писма уреднику, прикази књига, стручне вести, *In memoriam* и други прилози. Оригинални радови, претходна и кратка саопштења, прикази болесника и случајева, видео-чланци, слике из клиничке медицине, прегледни радови и актуелне теме, публикују се искључиво на енглеском језику, а остале врсте радова се могу публиковати и на српском језику само по одлуци Уредништва. Радови се увек достављају са сажетком на енглеском и српском језику (у склопу самог рукописа). Текст рада куцати у програму за обраду текста *Word*, фонтом *Times New Roman* и величином слова 12 тачака (12 *pt*). Све четири маргине подесити на 25 *mm*, величину странице на формат А4, а текст куцати с двоструким проредом, левим поравнањем и увлачењем сваког пасуса за 10 *mm*, без дељења речи (хифенације). Не користити табулаторе и узастопне празне карактере (спејсове) ради поравнања текста, већ алатке за контролу поравнања на лењиру и *Toolbars*. За прелазак на нову страну документа не користити низ „ентера“, већ искључиво опцију *Page Break*. После сваког знака интерпункције ставити само један празан карактер. Ако се у тексту користе специјални знаци (симболи), користити фонт *Symbol*. Подаци о коришћеној литератури у тексту означавају се арапским бројевима у угластим заградама – нпр. [1, 2], и то редоследом којим се појављују у тексту. Странице нумерисати редом у доњем десном углу, почев од насловне стране.

При писању текста на енглеском језику треба се придржавати језичког стандарда *American English* и користити кратке и јасне реченице. За називе лекова користити искључиво генеричка имена. Уређаји (апарати) се означавају фабричким називима, а име и место произвођача треба навести у облим заградама. Уколико се у тексту користе ознаке које су спој слова и бројева, прецизно написати број који се јавља у суперскрипту или супскрипту (нпр. ^{99}Tc, *IL*-6, O_2, $Б_{12}$, *CD*8). Уколико се нешто уобичајено пише курзивом (*italic*), тако се и наводи, нпр. гени (*BRCA1*).

Уколико је рад део магистарске тезе, односно докторске дисертације, или је урађен у оквиру научног пројекта, то треба посебно назначити у Напомени на крају текста. Такође, уколико је рад претходно саопштен на неком стручном састанку, навести званичан назив скупа, место и време одржавања, да ли је рад и како публикован (нпр. исти или другачији наслов или сажетак).

КЛИНИЧКА ИСТРАЖИВАЊА. Клиничка истраживања се дефинишу као истраживања утицаја једног или више средстава или мера на исход здравља. Регистарски број истраживања се наводи у последњем реду сажетка.

ЕТИЧКА САГЛАСНОСТ. Рукописи о истраживањима на људима треба да садрже изјаву у виду писаног пристанка испитиваних особа у складу с Хелсиншком декларацијом и одобрење надлежног етичког одбора да се истраживање може извести и да је оно у складу с правним стандардима. Експериментална истраживања на хуманом материјалу и испитивања вршена на животињама треба да садрже изјаву етичког одбора установе и треба да су у сагласности с правним стандардима.

ИЗЈАВА О СУКОБУ ИНТЕРЕСА. Уз рукопис се прилаже потписана изјава у оквиру обрасца *Submission Letter* којом се аутори изјашњавају о сваком могућем сукобу интереса или његовом одсуству. За додатне информације о различитим врстама сукоба интереса посетити интернет-страницу Светског удружења уредника медицинских часописа (*World Association of Medical Editors – WAME; http://www.wame.org*) под називом „Политика изјаве о сукобу интереса“.

АУТОРСТВО. Све особе које су наведене као аутори рада треба да се квалификују за ауторство. Сваки аутор треба да је учествовао довољно у раду на рукопису како би могао да преузме одговорност за целокупан текст и резултате изнесене у раду. Ауторство се заснива само на: битном доприносу концепцији рада, добијању резултата или анализи и тумачењу резултата; планирању рукописа или његовој критичкој ревизији од знатног интелектуалног значаја; завршном дотеривању верзије рукописа који се припрема за штампање.

Аутори треба да приложе опис доприноса појединачно за сваког коаутора у оквиру обрасца *Submission Letter*. Финансирање, сакупљање података или генерално надгледање истраживачке групе сами по себи не могу

оправдати ауторство. Сви други који су допринели изради рада, а који нису аутори рукописа, требало би да буду наведени у Захвалници с описом њиховог доприноса раду, наравно, уз писани пристанак.

ПЛАГИЈАРИЗАМ. Од 1. јануара 2019. године сви рукописи подвргавају се провери на плагијаризам/аутоплагијаризам преко *SCIndeks Assistant* – Cross Check (iThenticate). Радови код којих се докаже плагијаризам/аутоплагијаризам биће одбијени, а аутори санкционисани.

НАСЛОВНА СТРАНА. На првој страници рукописа треба навести следеће: наслов рада без скраћеница; предлог кратког наслова рада, пуна имена и презимена аутора (без титула) индексирана бројевима; званичан назив установа у којима аутори раде, место и државу (редоследом који одговара индексираним бројевима аутора); на дну странице навести име и презиме, адресу за контакт, број телефона, факса и имејл адресу аутора задуженог за кореспонденцију.

САЖЕТАК. Уз оригинални рад, претходно и кратко саопштење, преглед литературе, приказ случаја (болесника), рад из историје медицине, актуелну тему, рад за рубрику језик медицине и рад за праксу, на другој по реду страници документа треба приложити сажетак рада обима 100–250 речи. За оригиналне радове, претходно и кратко саопштење сажетак треба да има следећу структуру: Увод/Циљ рада, Методе рада, Резултати, Закључак; сваки од наведених сегмената писати као посебан пасус који почиње болдованом речи. Навести најважније резултате (нумеричке вредности) статистичке анализе и ниво значајности. Закључак не сме бити уопштен, већ мора бити директно повезан са резултатима рада. За приказе болесника сажетак треба да има следеће делове: Увод (у последњој реченици навести циљ), Приказ болесника, Закључак; сегменте такође писати као посебан пасус који почиње болдованом речи. За остале типове радова сажетак нема посебну структуру.

КЉУЧНЕ РЕЧИ. Испод Сажетка навести од три до шест кључних речи или израза. Не треба да се понављају речи из наслова, а кључне речи треба да буду релевантне или описне. У избору кључних речи користити *Medical Subject Headings – MeSH (http://www.nlm.nih.gov/mesh)*.

ПРЕВОД НА СРПСКИ ЈЕЗИК. На трећој по реду страници документа приложити наслов рада на српском језику, пуна имена и презимена аутора (без титула) индексирана бројевима, званичан назив установа у којима аутори раде, место и државу. На следећој – четвртој по реду – страници документа приложити сажетак (100–250 речи) с кључним речима (3–6), и то за радове у којима је обавезан сажетак на енглеском језику. Превод појмова из стране литературе треба да буде у духу српског језика. Све стране речи или синтагме за које постоји одговарајуће име у нашем језику заменити тим називом. Уколико је рад у целости на српском језику, потребно је превести називе прилога (табела, графикона, слика, схема) уколико их има, целокупни текст у њима и легенду на енглески језик.

СТРУКТУРА РАДА. Сви поднаслови се пишу великим масним словима (болд). Оригинални рад и претходно и кратко саопштење обавезно треба да имају следеће поднаслове: Увод (Циљ рада навести као последњи пасус Увода), Методе рада, Резултати, Дискусија, Закључак, Литература. Преглед литературе и актуелну тему чине: Увод, одговарајући поднаслови, Закључак, Литература. Првоименовани аутор прегледног рада мора да наведе бар пет аутоцитата (као аутор или коаутор) радова публикованих у часописима с рецензијом. Коаутори, уколико их има, морају да наведу бар један аутоцитат радова такође публикованих у часописима с рецензијом. Приказ случаја или болесника чине: Увод (Циљ рада навести као последњи пасус Увода), Приказ болесника, Дискусија, Литература. Не треба користити имена болесника, иницијале, нити бројеве историја болести, нарочито у илустрацијама. Прикази болесника не смеју имати више од пет аутора.

Прилоге (табеле, графиконе, слике итд.) поставити на крај рукописа, а у самом телу текста јасно назначити место које се односи на дати прилог. Крајња позиција прилога биће одређена у току припреме рада за публиковање.

СКРАЋЕНИЦЕ. Користити само када је неопходно, и то за веома дугачке називе хемијских једињења, односно називе који су као скраћенице већ препознатљиви (стандардне скраћенице, као нпр. ДНК, сида, ХИВ, АТП). За сваку скраћеницу пун термин треба навести при првом навођењу у тексту, сем ако није стандардна јединица мере. Не користити скраћенице у наслову. Избегавати коришћење скраћеница у сажетку, али ако су неопходне, сваку скраћеницу објаснити при првом навођењу у тексту.

ДЕЦИМАЛНИ БРОЈЕВИ. У тексту рада на енглеском језику, у табелама, на графиконима и другим прилозима децималне бројеве писати са тачком (нпр. 12.5 ± 3.8), а у тексту на српском језику са зарезом (нпр. 12,5 ± 3,8). Кад год је то могуће, број заокружити на једну децималу.

ЈЕДИНИЦЕ МЕРА. Дужину, висину, тежину и запремину изражавати у метричким јединицама (метар – *m*, килограм (грам) – *kg* (*g*), литар – *l*) или њиховим деловима. Температуру изражавати у степенима Целзијуса (°*C*), количину супстанце у молима (*mol*), а притисак крви у милиметрима живиног стуба (*mm Hg*). Све резултате хематолошких, клиничких и биохемијских мерења наводити у метричком систему према Међународном систему јединица (*SI*).

ОБИМ РАДОВА. Целокупни рукопис рада који чине – насловна страна, сажетак, текст рада, списак литературе, сви прилози, односно потписи за њих и легенда (табеле, слике, графикони, схеме, цртежи), насловна страна и сажетак на српском језику – мора износити за оригинални рад, рад из историје медицине и преглед литературе до 5000 речи, а за претходно и кратко саопштење, приказ болесника, актуелну тему, рад за праксу, едукативни чланак и рад за рубрику „Језик медицине“ до 3000 речи; радови за остале рубрике могу имати највише 1500 речи.
Видео-радови могу трајати 5–7 минута и бити у формату *avi, mp4*(*flv*). У првом кадру филма мора се навести: у наднаслову Српски архив за целокупно лекарство, наслов рада, презимена и иницијали имена и средњег слова свих аутора рада (не филма), година израде. У другом кадру мора бити уснимљен текст рада у виду апстракта до 350 речи. У последњем кадру филма могу се навести имена техничког особља (режија, сниматељ, светло, тон, фотографија и сл.). Уз видео-радове доставити: посебно текст у виду апстракта (до 350 речи), једну фотографију као илустрацију приказа, изјаву потписану од свег техничког особља да се одричу ауторских права у корист аутора рада.

ПРИЛОЗИ РАДУ су табеле, слике (фотографије, цртежи, схеме, графикони) и видео-прилози.

Свака табела треба да буде сама по себи лако разумљива. Наслов треба откуцати изнад табеле, а објашњења испод ње. Табеле се означавају арапским бројевима према редоследу навођења у тексту. Табеле цртати искључиво у програму *Word*, кроз мени *Table–Insert–Table*, уз дефинисање тачног броја колона и редова који ће чинити мрежу табеле. Десним кликом на мишу – помоћу опција *Merge Cells* и *Split Cells* – спајати, односно делити ћелије. Куцати фонтом *Times New Roman*, величином слова 12 *pt*, с једноструким проредом и без увлачења текста. Коришћене скраћенице у табели треба објаснити у легенди испод табеле. Уколико је рукопис на српском језику, приложити називе табела и легенду на оба језика. Такође, у једну табелу, у оквиру исте ћелије, унети и текст на српском и текст на енглеском језику (никако не правити две табеле са два језика!).

Слике су сви облици графичких прилога и као „слике“ у CA се објављују фотографије, цртежи, схеме и графикони. Слике означавају се арапским бројевима према редоследу навођења у тексту. Примају се искључиво дигиталне фотографије (црно-беле или у боји) резолуције најмање 300 *dpi* и формата записа *tiff* или *jpg* (мале, мутне и слике лошег квалитета неће се прихватати за штампање!). Уколико аутори не поседују или нису у могућности да доставе дигиталне фотографије, онда оригиналне слике треба скенирати у резолуцији 300 *dpi* и у оригиналној величини. Уколико је рад неопходно илустровати са више слика, у раду ће их бити објављено неколико, а остале ће бити у е-верзији чланка као *PowerPoint* презентација (свака слика мора бити нумерисана и имати легенду).
Видео-прилози (илустрације рада) могу трајати 1–3 минута и бити у формату *avi, mp4*(*flv*). Уз видео доставити посебно слику која би била илустрација видеоприказа у *e*-издању и објављена у штампаном издању. Уколико је рукопис на српском језику, приложити називе слика и легенду на оба језика.

Слике се у свесци могу штампати у боји, али додатне трошкове штампе сносе аутори.

Графикони треба да буду урађени и достављени у програму *Excel*, да би се виделе пратеће вредности распоређене по ћелијама. Исте графиконе прекопирати и у *Word*-ов документ, где се графикони означавају арапским бројевима према редоследу навођења у тексту. Сви подаци на графикону куцају се у фонту *Times New Roman*. Коришћене скраћенице на графикону треба објаснити у легенди испод графикона. У штампаној верзији чланка вероватније је да графикон неће бити штампан у боји, те је боље избегавати коришћење боја у графиконима, или их користити различитог интензитета. Уколико је рукопис на српском језику, приложити називе графикона и легенду на оба језика.

Цртежи и схеме се достављају у *jpg* или *tiff* формату. Схеме се могу цртати и у програму *CorelDraw* или *Adobe Illustrator* (програми за рад са векторима, кривама). Сви подаци на схеми куцају се у фонту *Times New Roman*, величина слова 10 *pt*. Коришћене скраћенице на схеми треба објаснити у легенди испод схеме. Уколико је рукопис на српском језику, приложити називе схема и легенду на оба језика.

ЗАХВАЛНИЦА. Навести све сараднике који су допринели стварању рада а не испуњавају мерила за ауторство, као што су особе које обезбеђују техничку помоћ, помоћ у писању рада или руководе одељењем које обезбеђује општу подршку. Финансијска и материјална помоћ, у облику спонзорства, стипендија, поклона, опреме, лекова и друго, треба такође да буде наведена.

ЛИТЕРАТУРА. Списак референци је одговорност аутора, а цитирани чланци треба да буду лако приступачни читаоцима часописа. Стога уз сваку референцу обавезно треба навести *DOI* број чланка (јединствену ниску карактера која му је додељена) и *PMID* број уколико је чланак индексиран у бази *PubMed/MEDLINE*.

Референце нумерисати редним арапским бројевима према редоследу навођења у тексту. Број референци не би требало да буде већи од 30, осим у прегледу литературе, у којем је дозвољено да их буде до 50, и у метаанализи, где их је дозвољено до 100. Број цитираних оригиналних радова мора бити најмање 80% од укупног броја референци, односно број цитираних књига, поглавља у књигама и прегледних чланака мањи од 20%. Уколико се домаће монографске публи-

кације и чланци могу уврстити у референце, аутори су дужни да их цитирају. Већина цитираних научних чланака не би требало да буде старија од пет година. Није дозвољено цитирање апстраката. Уколико је битно коментарисати резултате који су публиковани само у виду апстракта, неопходно је то навести у самом тексту рада. Референце чланака који су прихваћени за штампу, али још нису објављени, треба означити са *in press* и приложити доказ о прихватању рада за објављивање.

Референце се цитирају према Ванкуверском стилу (униформисаним захтевима за рукописе који се предају биомедицинским часописима), који је успоставио Међународни комитет уредника медицинских часописа (*http://www.icmje.org*), чији формат користе *U.S. National Library of Medicine* и базе научних публикација. Примере навођења публикација (чланака, књига и других монографија, електронског, необјављеног и другог објављеног материјала) могу се пронаћи на интернет-страници *http://www.nlm.nih.gov/bsd/uniform_requirements.html*. Приликом навођења литературе веома је важно придржавати се поменутог стандарда, јер је то један од најбитнијих фактора за индексирање приликом класификације научних часописа.

ПРОПРАТНО ПИСМО (*SUBMISSION LETTER*). Уз рукопис обавезно приложити образац који су потписали сви аутори, а који садржи: 1) изјаву да рад претходно није публикован и да није истовремено поднет за објављивање у неком другом часопису, 2) изјаву да су рукопис прочитали и одобрили сви аутори који испуњавају мерила ауторства, и 3) контакт податке свих аутора у раду (адресе, имејл адресе, телефоне итд.). Бланко образац треба преузети са интернет-странице часописа (*http://www.srpskiarhiv.rs*).

Такође је потребно доставити копије свих дозвола за: репродуковање претходно објављеног материјала, употребу илустрација и објављивање информација о познатим људима или именовање људи који су допринели изради рада.

ЧЛАНАРИНА, ПРЕТПЛАТА И НАКНАДА ЗА ОБРАДУ ЧЛАНКА. Да би рад био објављен у часопису *Српски архив за целокупно лекарство*, сви аутори који су лекари или стоматолози из Србије морају бити чланови Српског лекарског друштва (у складу са чланом 6. Статута Друштва) и измирити накнаду за обраду чланака (*Article Processing Charge*) у износу од 3000 динара. Аутори и коаутори из иностранства су у обавези да плате накнаду за обраду чланака (*Article Processing Charge*) у износу од 35 евра. Уплата у једној календарској години обухвата и све наредне, евентуалне чланке, послате на разматрање у тој години. Сви аутори који плате ову накнаду могу, уколико то желе, да примају штампано издање часописа. Треба напоменути да ова уплата није гаранција да ће рад бити прихваћен и објављен у *Српском архиву за целокупно лекарство*. Обавеза плаћања накнаде за обраду чланка не односи се на студенте основних студија и на претплатнике на часопис.

Установе (правна лица) не могу преко своје претплате да испуне овај услов аутора (физичког лица). Уз рукопис рада треба доставити копије уплатница за чланарину и претплату / накнаду за обраду чланка, као доказ о уплатама, уколико издавач нема евиденцију о томе. Часопис прихвата донације од спонзора који сносе део трошкова или трошкове у целини оних аутора који нису у могућности да измире накнаду за обраду чланка (у таквим случајевима потребно је часопису ставити на увид оправданост таквог спонзорства).

СЛАЊЕ РУКОПИСА. Рукопис рада и сви прилози уз рад достављају се искључиво електронски преко система за пријављивање на интернет-страници часописа: *http://www.srpskiarhiv.rs*

НАПОМЕНА. Рад који не испуњава услове овог упутства не може бити упућен на рецензију и биће враћен ауторима да га допуне и исправе. Придржавањем упутства за припрему рада знатно ће се скратити време целокупног процеса до објављивања рада у часопису, што ће позитивно утицати на квалитет чланака и редовност излажења часописа.

За све додатне информације, молимо да се обратите на доле наведене адресе и број телефона.

АДРЕСА:
Српско лекарско друштво
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The papers are always submitted with Summary in both English and Serbian, included in the manuscript file. The text of the manuscript should be typed in *MS Word* using the *Times New Roman* typeface, and font size 12 pt. The text should be prepared with margins set to 25 mm and onto A4 paper size, with double line spacing, aligned left and the initial lines of all paragraphs indented 10 mm, without hyphenation. Tabs and successive blank spaces are not to be used for text alignment; instead, ruler alignment control tool and *Toolbars* are suggested. In order to start a new page within the document, *Page Break* option should be used instead of consecutive enters. Only one space follows after any punctuation mark. If special signs (symbols) are used in the text, use the *Symbol* font. References cited in the text are numbered with Arabic numerals within parenthesis (for example: [1, 2]), in order of appearance in the text. Pages are numbered consecutively in the right bottom corner, beginning from the title page.

When writing text in English, linguistic standard American English should be observed. Write short and clear sentences. Generic names should be exclusively used for the names of drugs. Devices (apparatuses, instruments) are termed by trade names, while their name and place of production should be indicated in the brackets. If a letter-number combination is used, the number should be precisely designated in superscript or subscript (i.e., 99Tc, IL-6, O2, B12, CD8). If something is commonly written in italics, such as genes (e.g. BRCA1), it should be written in this manner in the paper as well.

If a paper is a part of a master's or doctoral thesis, or a research project, that should be designated in a separate note at the end of the text. Also, if the article was previously presented at any scientific meeting, the name, venue and time of the meeting should be stated, as well as the manner in which the paper had been published (e.g. changed title or abstract).

CLINICAL TRIALS. Clinical trial is defined as any research related to one or more health related interventions in order to evaluate the effects on health outcomes. The trial registration number should be included as the last line of the Summary.

ETHICAL APPROVAL. Manuscripts with human medical research should contain a statement that the subjects' written consent was obtained, according to the Declaration of Helsinki, the study has been approved by competent ethics committee, and conforms to the legal standards. Experimental studies with human material and animal studies should contain statement of the institutional ethics committee and meet legal standards.

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The authors should enclose the description of contribution to the article of every co-author individually (within the Submission Letter). Funding, collection of data or general supervision of the research group alone cannot justify authorship. All other individuals having contributed to the preparation of the article should be mentioned in the *Acknowledgment* section, with description of their contribution to the paper, with their written consent.

PLAGIARISM. Since January 1, 2019 all manuscripts have been submitted via SCIndeks Assistant to Cross Check (software iThenticate) for plagiarism and auto-plagiarism control. The manuscripts with approved plagiarism/auto-plagiarism will be rejected and authors will not be welcome to publish in Serbian Achieves of Medicine.

TITLE PAGE. The first page of the manuscript (cover sheet) should include the following: title of the paper without any abbreviations; suggested running title; each author's full names and family names (no titles), indexed by numbers; official name, place and country of the institution in which authors work (in order corresponding to the indexed numbers of the authors); at the bottom of the page: name and family name, address, phone and fax number, and e-mail address of a corresponding author.

SUMMARY. Along with the original article, preliminary and short communication, review article, case report, article on history of medicine, current topic article, article for language of medicine and article for practitioners, the summary not exceeding 100–250 words should be typed on the second page of the manuscript. In original articles, the summary should have the following structure: Introduction/Objective, Methods, Results, Conclusion. Each segment should be typed in a separate paragraph using boldface. The most significant results (numerical values), statistical analysis and level of significance are to be included. The conclusion must not be generalized, it needs to point directly to the results of the study. In case reports, the summary should consist of the following: Introduction (final sentence is to state the objective), Case Outline (Outline of Cases), Conclusion. Each segment should be typed in a separate paragraph using boldface. In other types of papers, the summary has no special outline.

KEYWORDS. Below the summary, 3 to 6 keywords or phrases should be typed. The keywords need not repeat words in the title and should be relevant or descriptive. *Medical Subject Headings – MeSH* (*http://www.nlm.nih.gov/mesh*) are to be used for selection of the keywords.

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