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ORIGINAL ARTICLES / ORIGINALNI RADOVI

Every year, on February 11th, the scientific community marks the International Day of Women and Girls in Science. On this day, the contributions of women and girls to science and innovation are celebrated, as well as breaking down persistent gender barriers. The outbreak of the COVID-19 pandemic has clearly demonstrated the significant role of women researchers in different stages of the fight against COVID-19: from advancing the knowledge on the virus, to developing the testing techniques, and, finally, to creating the vaccine against the virus. Accordingly, this year's celebration of the International Day of Women and Girls in Science has the motto "Women Scientists at the Forefront of the Fight Against COVID-19".

This is one more opportunity for promoting the full and equal inclusion of women and girls in scientific research worldwide.

Svake godine 11. februara, naučna zajednica obeležava Međunarodni dan žena i devojaka u nauci. Na ovaj dan se slavi doprinos žena i devojaka nauci i inovacijama, kao i rušenje postojećih rodnih barijera. Izbijanje pandemije COVID-19 jasno je ukazalo na značajnu ulogu žena istraživača u različitim fazama borbe protiv COVID-19: od unapređenja znanja o virusu, do razvoja tehnika za testiranje i, na kraju, do stvaranja vakcine protiv virusa. Shodno tome, ovogodišnja proslava Međunarodnog dana žena u nauci ima moto „Žene naučnice na čelu borbe protiv COVID-19".

Ovo je još jedna prilika za promociju punog i ravnopravnog uključenja žena i devojaka u naučnoistraživački rad širom sveta.

ORIGINAL ARTICLE

UDC: 616.31
DOI: https://doi.org/10.2298/VSP190115032V

The effect of hydroxyapatite and growth factors on reparative dentine formation in the therapy of injured pulp

Efekat hidroksiapatita i faktora rasta na stvaranje reparativnog dentina u terapiji ledirane pulpe

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Abstract

Background/Aim. The studies of hydroxyapatite (HAp) and growth factors as the materials used for direct pulp capping have produced conflicting results for both the issue of the inflammatory response and the issue of calcified bridge formation. Hap/poly (lactide-co-glycolide) (HAp/PLGA) is a bioresorbable polymer with demonstrated good characteristics as the carrier for the bone morphogenetic protein necessary in bone tissue regeneration. The role of growth factors in dental tissue reparation (in both reactionary and reparative dentinogenesis) represents the new foundation and provides a different approach to dental pulp treatment. Growth factors – transforming growth factor beta-1 (TGF-β-1) – directly induce morphological and functional differentiation of neo-odontoblasts. The aim of this study was to investigate the effect of calcium HAp/PLGA and growth factors (TGF β-1) in the formation of a calcified tissue – dentine bridge – on the teeth of our experimental model. **Methods.** In this experimental study, rodent (rabbit) teeth were used as the animal model. After the trepanation of pulp space with sterile steel drills, the pulp was capped with calcium HAp/PLGA (experimental group I; n = 60); calcium HAp/PLGA combined with TGF β-1 growth factor (experimental group II; n = 60), and there was a control group of intact teeth (n = 20). The experiment was performed in general anesthesia. The animals were kept alive for 1, 3, and 6 months. The extracted teeth were adequately prepared for scanning electron microscopy. **Results.** Scanning electron microscopy demonstrated that the number of teeth with calcified tissue in the form of dental bridges in the HAp/PLGA+TGF β-1 group, 6 months after the treatment, was statistically significantly greater (66.67%) than after 3 months (26.67%), at the statistical significance level of $p < 0.05$. **Conclusion.** Direct pulp capping covers the artificially exposed dental pulp and makes possible the formation of a dentine bridge (a tubular structure composed of reparative dentine) in the period of 3 months.

Key words:
dental pulp; dental pulp capping; dentine; rabbits; minerals.

Apstrakt

Uvod/Cilj. Studije o hidroksiapatitu (HAp) i faktorima rasta kao materijalima za direktno prekrivanje pulpe daju potpuno oprečne rezultate i po pitanju zapaljenskog odgovora i po pitanju formiranja kalcifikovanog mosta. Kalcijum hidroksiapatit/poli (laktid-ko-glikolid) je bioresorbilni polimer koji je pokazao dobra svojstva kao nosač koštanog morfogenog proteina neophodnog za regeneraciju koštanog tkiva. Uloga faktora rasta u reparaciji zubnih tkiva (bilo da se radi o reaktivnoj ili reparativnoj dentinogenezi) daje nove osnove i drugačiji pristup tretmanu pulpe. Faktori rasta – transformišući faktor rasta beta-1 (TGF β-1) direktno indukuju morfološku i funkcionalnu diferencijaciju neoodontoblasta. Cilj ove eksperimentalne studije bio je da se ispita uticaj kalcijum HAp/poli (laktid-ko-glikolida) (HAp/PLGA) i faktora rasta TGF β-1 u stvaranju kalcifikovanog tkiva – dentinskog mosta na zubima eksperimentalnog modela. **Metode.** U eksperimentalnoj studiji kao animalni model korišćeni su zubi glodara (kunića). Nakon trepanacije pulpnog prostora sterilnim čeličnim svrdlom, pulpa je prekrivena kalcijum HAp/PLGA (I eksperimentalna grupa zuba, n = 60), kalcijum HAp/PLGA u kombinaciji sa faktorom rasta TGF β-1 (II eksperimentalna grupa, n = 60),

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kontrolna grupa zuba (intaktni zubi, n = 20). Eksperiment je obavljen u opštoj anesteziji. Životinje su održavane u životu 1, 3 i 6 meseci. Ekstrahovani zubi su pripremljeni za posmatranje skenirajućom elektronskom mikroskopijom. **Rezultati.** Skenirajućom elektronskom mikroskopijom dokazano je da je broj zuba sa kalcifikovanim tkivom u vidu dentinskog mosta bio veći 6 meseci nakon tretmana u odnosu na 3 meseca nakon tretmana. **Zaključak.** Direktnim prekrivanjem pulpe dolazi do zatvaranja artificijelno otvorene pulpe i stvaranja dentinskog mosta reparatornog dentina tubularne strukture u toku perioda posmatranja od 3 meseca.

Ključne reči:
zub, pulpa; zub, pulpa, prekrivanje; dentin; zečevi; minerali.

Introduction

Direct pulp capping, as one of the essential endodontic modalities, is often used as a therapeutic procedure for dental pulp vitality preservation. It is usually defined as a treatment on exposed pulp tissue, where the pulp wound is covered (capped) with materials that stimulate reparative dentine formation [1]. Since the capping material comes into direct contact with the pulp tissue, it plays a key role in this treatment [2, 3].

In the selection of materials for vital pulp therapy, the following material properties should be sought: antibacterial action, ability to induce mineralization, adequate sealing of the pulp space in order to prevent the entry of bacteria from the mouth cavity [4]. Some studies have investigated the use of biomaterials such as hydroxyapatite (HAp) in dental pulp treatments within the technique of direct pulp capping or amputation of the crown portion of the dental pulp [5, 6]. The results suggested that their use in the observation period of 3 months speeds up the process of healing, i.e. the formation of dentine bridge and continued dental root growth. HAp is one of the most frequently used calcium phosphate bioceramics with osteoconductive properties. Since its structure is similar to bone minerals, it is capable of forming direct bonds with the bone tissue. HAp has got several clear advantages: it is well-accepted and incorporable into the host bone, but it also provides a solid base for new bone growth [7]. Its biocompatibility is excellent, and its surface layer has a key role in the formation, growth, and maintenance of the tissue/biomaterial bond [8]. Moreover, it does not contain any proteins and consequentially does not induce any allergic reactions or immune system responses [9, 10]. On the other hand, HAp has very poor mechanical properties [11].

Synthetic HAp belongs to the group of non-resorbable ceramic biomaterials. It could perhaps successfully replace bone tissue, facilitate new bone formation and exert an osteoconductive effect [12–15].

Poly (lactide-co-glycolide) (PLGA) is a copolymer of lactide and glycolide registered by the Food and Drug Administration as a material which can be used in medicine and pharmacy, and it belongs to the class of biodegradable and biocompatible polymers [14, 16–19].

Pulp capping with HAp-based materials requires the use of a mechanically more resilient material over the medicament, and only then a better quality of the dentine-HAp interface becomes prominent [19]. Novel insights in the role of growth factors in dental tissue reparation, in both reactive and reparative dentinogenesis, could represent the basis of different pulp treatment [20].

Growth factors are biological mediators that regulate key processes in tissue reparation, including cell proliferation, differentiation, extracellular matrix synthesis, and angiogenesis [21].

There have been attempts to use HAp and growth factors for the same purposes, although with a low success rate. A number of these investigations are still ongoing and attract much attention [22, 23].

Transforming growth factor beta-1 (TGF β-1) is a member of the superfamily of homologous disulfide-bound hemodynamic proteins, regulating proliferation and differentiation of normal and transforming cells. Human TGF β-1 is a 25.0 kDa protein that contains 2 identical polypeptide chains of 112 amino acids interconnected by one disulfide bond [24].

Growth factors are present in the dentine matrix, and they can play an important role in mediating pulp responses to an injury or restorative procedure. Since they may be released during the tooth decay process, this could represent the basis of a novel biological approach to dental tissue reparation [25–28].

The aim of this study was to investigate the dentinogenetic effectiveness of bioactive materials calcium HAp/PLGA and growth factor TGF β-1 in reparative dentine formation in the cases of injured pulp during the standard procedure of dental pulp capping.

Methods

The experimental study took place at the Institute of Biomedical Research, Faculty of Medicine in Niš, and at the Faculty of Medicine in Priština, temporarily seated in Kosovska Mitrovica, with the approval of the Ethics Committee of the Faculty of Medicine in Niš (number: 05-603/1 of 2011).

The experiment included five chinchilla rabbits that were 6 months old and of mean weight of 3–4 kg. The animals were anesthetized by intramuscular Zoletil 100 administration (Virbac S.A. 1ère avenue 2065 M - L.I.D. 06516 Carros, France) at a dose of 10 mg/kg of body weight and ketamine hydrochloride (1–4.5 mg/kg body weight). After the induction of anesthesia and placement of cofferdam rubber insulation, the teeth were cleaned using 70% ethanol. Small cavities were created on the occlusal surfaces of the teeth with small round drills. The cavities were washed with saline for the removal of debris, created during cavity

preparation. After the trepanation of pulp space, the lesions were covered with biomaterial and growth factor, and cavities were definitively closed with glass-ionomer cement and amalgam. For the purpose of this study, we used calcium HAp/PLGA and autogenic TGF β-1.

The teeth were divided into three groups: experimental group I (n = 60), composed of the left lower jaw teeth, where calcium HAp/PLGA biomaterial was applied; experimental group II (n = 60), composed of the left upper jaw teeth, where calcium HAp/PLGA was applied in combination with TGF β-1; calcium HAp/PLGA biomaterial served as a carrier, 80:20 (0.5 g), manufactured by the ITN SANU, Belgrade; intact right upper jaw teeth and right lower jaw teeth served as our control group (n = 20).

After this phase of the study, our animals were kept alive for 1, 3, and 6 months. After these periods of time, they were sacrificed with a lethal dose of ketamine hydrochloride. Jawbones were disarticulated and each tooth was individually extracted. Material preparation involved tooth storage in sterile saline at 40°C without any fixation agents.

Occlusal surfaces of dental crowns, 2–3 mm thick, were cut in a circular manner using the finest diamond fissure bur. Dental roots were cut longitudinally using separating discs, producing longitudinal separation into the oral and vestibular surfaces. In order to eliminate superficial debris produced by cutting, the samples were washed in distilled water and dried with compressed air. Occlusal surfaces were separated first using the separation pliers, and then the roots were longitudinally separated along the already prepared grooves. Each half of the sample was placed onto an appropriate mount; the samples thus fixed were gold vapour treated in a vacuum evaporator and viewed under scanning electron microscopy JEOL-JCM-5300.

The entry and tabular data representation were done using the MS Office Excel 2007 software package, and calculations were performed using the SPSS, 15.0 version. The results of the statistical analysis were presented in tables.

The differences in the observed parameters, both between the groups and within them, in different intervals of time, were established using the Mantel-Haenszel χ^2-test or Fisher's test of the exact probability of the null hypothesis (when some of the expected frequencies were less than 5).

Results

In total, 140 teeth of 5 sacrificed experimental animals (rabbits) were used in the study – 20 teeth (4 teeth × 5) in the control group, 60 teeth (12 teeth × 5) in the experimental group I, and 60 teeth (12 teeth × 5) in the experimental group II.

As the measures of effect of the studied material types and treatment modalities, we tried to observe the formation of new hard dental tissue – dentine bridges (reparative dentine) in the studied specimens. This parameter was monitored using scanning electron microscopy 1, 3, and 6 months after the treatment.

The presence of dentine bridges in experimental groups with direct pulp capping and the control group (using scanning electron microscopy) is presented in Table 1.

Comparing the number of teeth among the studied groups, it was established that the number of teeth with formed dentine bridges in the HAp/PLGA+TGF β-1 group, 6 months after the treatment, was statistically significantly greater (66.67%) than after 3 months (26.67%), at the statistical significance level of $p < 0.05$.

Table 1

The presence of dentine bridges in the experimental groups with direct pulp capping and in the control group

Group	n	Period (months)		
		1	3	6
Control	20	0 (0.00%)	0 (0.00%)	0 (0.00%)
TGF β-1 + HAp/PLGA	60	9 (26.67%)	17 (33.33%)*	29 (66.67%)†‡
HAp/PLGA	60	5 (13.33%)	9 (26.67%)	15 (33.33%)

n – number of teeth; TGF β-1 – transforming growth factor beta-1; HAp/PLGA – hydroxyapatite/poly (lactide-co-glycolide).
***$p < 0.01$ vs. control group; †$p < 0.05$ vs. the same group one month after the treatment; ‡$p < 0.001$ vs. control group.**

Comparing the number of teeth among the studied groups in the same time intervals, it was found that 3 months after the treatment, the number of teeth with dentine bridges in the TGF β-1 + HAp/PLGA group was statistically significantly greater than in the control group ($p < 0.01$). Six months after the treatment, the number of teeth with dentine bridges in the TGF β-1 + HAp/PLGA group (66.67%) was statistically significantly greater than among controls at an even higher level of statistical significance ($p < 0.001$). In the same period, comparing the TGF β-1 + HAp/PLGA and HAp/PLGA groups 6 months after the treatment, it was established that the number of teeth with dentine bridges in the TGF β-1 + HAp/PLGA group was twice the number observed in the HAp/PLGA group (66.67% vs. 33.00%), but a statistically significant difference was not established due to small sample size.

Results of the SEM analysis

After an observation period of one month, the results of SEM analysis with direct pulp capping after the application of HAp/PLGA showed the formation of fibrodentine of atubular structure (Figure 1), and after TGF β-1 and HAp/PLGA application, the results showed a regular structure of reparative dentine from the cavum towards the periphery (Figure 2).

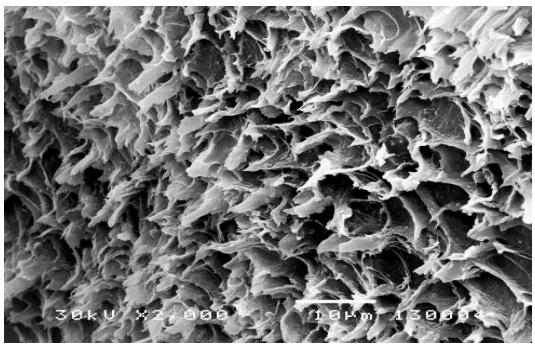


Fig. 1 – Calcium hydroxyapatite/poly (lactide-co-glycolide): atubular irregular dentine (fibrodentine) – scanning electron microscopy finding (magnification is labeled inside the figure).

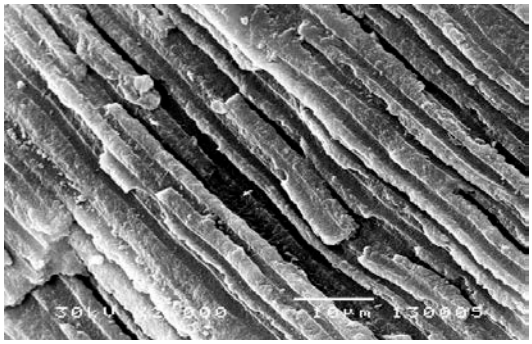


Fig. 2 – Transforming growth factor beta-1 and calcium hydroxyapatite/poly (lactide-co-glycolide): regular structure of reparative dentine at the periphery – scanning electron microscopy finding (magnification is labeled inside the figure).

After an observation period of three months, the results of SEM analysis with direct pulp capping after the application of HAp/PLGA revealed the formation of fibrodentine of atubular structure (Figure 3), and after TGF β-1 and HAp/PLGA application, the results showed the presence of newly formed dentine with numerous channels and dentine bridge formation – tubular dentine (Figure 4).

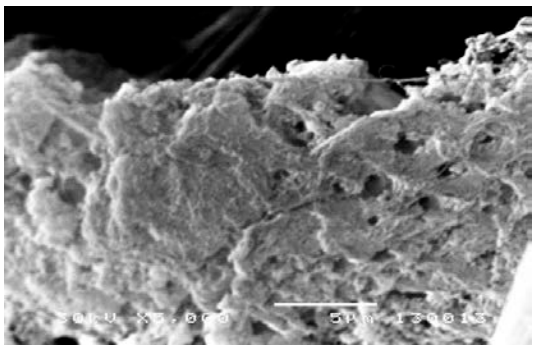


Fig. 3 – Calcium hydroxyapatite/poly (lactide-co-glycolide): a detail of the dentine bridge (fibrodentine with atubular structure finding) – scanning electron microscopy finding (magnification is labeled inside the figure).

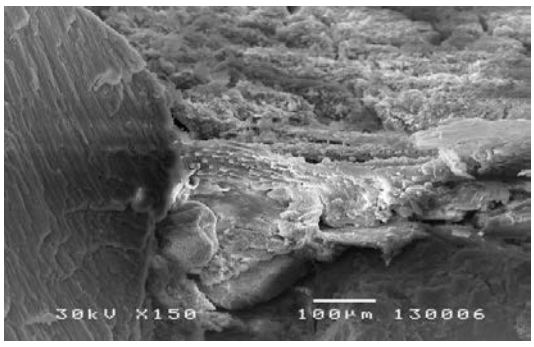
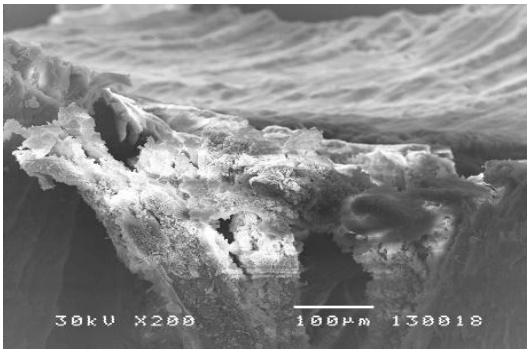
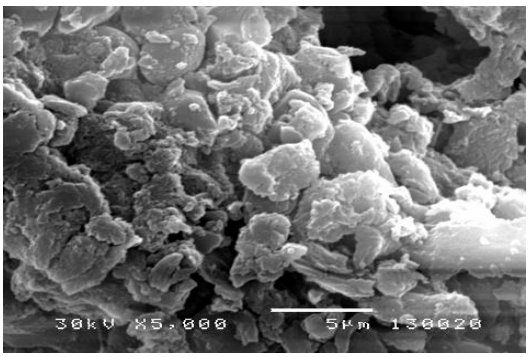


Fig. 4 – Transforming growth factor beta-1 and calcium hydroxyapatite/poly (lactide-co-glycolide): dentine bridge (tubular dentine) – scanning electron microscopy finding (magnification is labeled inside the figure).

After an observation period of six months, the results of SEM analysis with direct pulp capping after the application of HAp/PLGA showed the presence of dentine bridges, amorphous fibrodentine, dentine bridges with large hydroxyapatite crystals, fibrodentine rich in bioactive proteins (Figure 5), and after TGF β-1 and HAp/PLGA application, the results indicated the formation of dentine bridges with tubular structure, reparative dentine, calcified dentine, calcified Tomes fibers and calcified pulp within dental roots (Figure 6). Figure 7 shows regular dentine in an intact tooth.



a)



b)

Fig. 5 – Calcium hydroxyapatite/poly (lactide-co-glycolide): a) dentine bridge (amorphous dentine); b) dentine bridge structure (amorphous fibrodentine) – scanning electron microscopy finding (magnifications are labeled inside the figures).

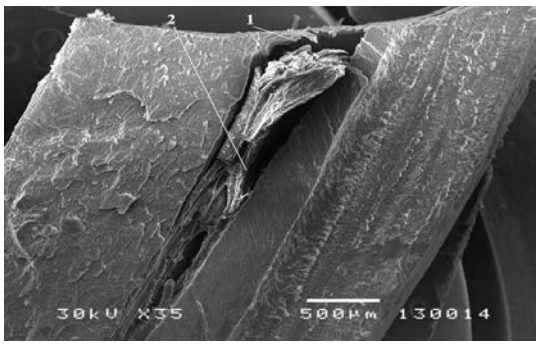


Fig. 6 – Transforming growth factor beta-1 and calcium hydroxyapatite/poly (lactide-co-glycolide): 1) dentine bridge with tubular structure; 2) portion of the calcified pulp within dental root canal – scanning electron microscopy finding (magnification is labeled inside the figure).

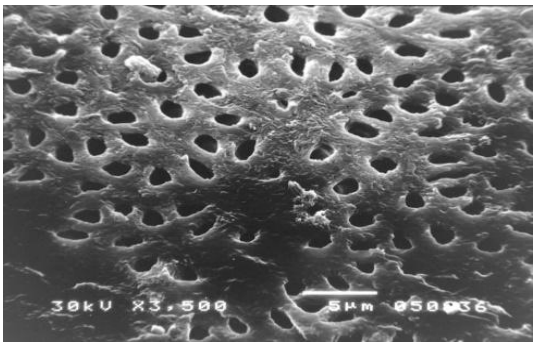


Fig. 7 – Regular dentine (an intact tooth) – scanning electron microscopy finding (magnification is labeled inside the figure).

Discussion

Direct pulp capping is a therapeutic procedure of tooth vitality preservation, whereby adequate materials are applied onto the exposed pulp tissue in order to stimulate reparative dentine formation [1]. Since the material used for direct pulp capping comes into direct contact with the pulp tissue, it, therefore, plays a key role in this kind of treatment [24].

Our study dealt with the advantages of applying hydroxyapatite calcium HAp/PLGA alone or combined with growth factors. A high biological potential of the pulp, including optimal conditions for the tissue function, with adequate vascularization and absence of inflammation, was the primary criterion in the interpretation of obtained results. The tested materials were applied in accordance with the manufacturers' manuals. HAp/PLGA powder was mixed with saline in order to obtain the consistency easy to manipulate [29].

In the *in vitro* and *in vivo* studies, the biocompatibility of HAp/PLGA, composite beta carotene/PLGA, and its effect on dental pulp cells have been demonstrated. Histological analysis has demonstrated the presence of cellular infiltration and dentine bridge formation after 60 days. In all studied groups, fibroblast development and growth and survival of macrophages were identified [30, 31].

In recent years, growth factors and their role in the initiation of reparatory processes in pulp injury have attracted much attention, which was partially the subject of our study as well. These bioactive molecules promote proliferation and differentiation of cells, matrix synthesis, and angiogenesis. Growth factors are necessary for tissue regeneration, and they have an important role in inducing angiogenesis, i.e. oxygenation and supply of nutrients essential for biological functioning.

Numerous authors have identified various difficulties in clinical manipulation, application, and retention of the materials at the site of application [30]. As the potentially suitable growth factor carriers, calcium-phosphate-based materials have been suggested, like those in our study, the porous structure of which enables gradual release and diffusion of growth factors. In our study, HAp was shown to be a good growth factor carrier.

Since synthetic biomaterials have been shown to be successful in the restoration of bone tissue, with their well-known biocompatibility and bioconductivity, the intention of this study was to investigate the use of HAp as a synthetic biomaterial and growth factors in direct pulp capping.

The results obtained by Tziafas et al. [32] have shown that the use of some of the growth factors, especially TGF β, stimulates odontoblast differentiation and leads to the release of endogenous growth factors contained in the organic dentine matrix, which further stimulates dentinogenesis [33, 34].

The results of the study by Popović Bajić et al. [35] have shown the highest regularity in the organization of deeper pulp layers in the zones of the thickest dentine bridges. These results have also shown the farthest deviation from the statement concerning platelet-rich fibrin in direct pulp capping in animals compared to other materials. In their study, they proved the formation of calcified tissue in the pulp in all the samples, which partly agreed with our research. However, it is still unclear which growth factor concentration in platelet-rich fibrin is optimal for the processes of reparation and regeneration.

In all their cases, Hebling et al. [36] have demonstrated dentine bridge formation with direct pulp capping using autogenous growth factors combined with a HAp-based material. In two observation periods, increased dentine bridge thickness was noticed, although without statistically significant differences, compared to the groups where HAp alone was applied. In this study, similar results were obtained: in a shorter observation period, dentine bridge formation occurred more rapidly and more regularly in the samples in which HAp combined with growth factors was applied, although without any statistically significant differences. At the end of a 12-month observation period, the results were the same for both HAp-treated samples and those treated with HAp combined with thrombocyte-rich plasma, suggesting that growth factors produced more rapid healing, i.e. dentine bridge formation, which agreed with our results.

Numerous studies have stressed the importance of adequate cavity sealing after the therapy and prevention of superimposed bacterial contamination, which was provided

for this study by the application of glass-ionomer cement and amalgam for cavity sealing [37].

Formation, quality, and thickness of the calcified bridge, presence of inflammatory cells, and pulp tissue preservation are all important indicators of tissue response in the therapy with direct pulp capping [38]. According to Accorinte et al. [39], these parameters have been considered relevant and used for histological assessment of treatment success with the nanomaterials tested in this study. The formation of the dentine bridge at the interface of pulp and material used for direct capping is an issue for further discussion since a dentine bridge is not necessarily the sign of dental pulp preservation (health). The presence of dentine bridges may be interpreted as a sign of healing or as a reaction (response) to irritation. In this study, it was interpreted as a sign of biocompatibility and bioactivity of the material, which agrees with other authors' opinions [26, 28]. Dentine matrix is not just a scaffold serving to mobilize and support the development of mineralized tissue; it is also the pool of growth factors excreted by odontoblasts and pulp fibroblasts [40]. These growth factors hypothetically produce signals for proliferation, differentiation, and recruitment of pulp cells at the sites of injured pulp tissue and initiate tissue regeneration [41, 42].

In all the experimental samples of our study, 1, 3, and 6 months after the treatment, a thin layer of calcified tissue – dentine bridge – was observed. These follow-up intervals matched those in the studies in which hard tissue formation was observed as early as 2 weeks after the treatment [28, 33].

Finally, it should be mentioned that these findings are the result of a healthy dental pulp response, without any inflammation, and that the performance of these materials in the presence of inflamed pulp will be assessed in further studies. Some authors have reported the absence of dentine bridges, while others report well-calcified bridges after the period of three months [43, 44].

In the study by Nowicka et al. [45], it has been histologically confirmed that Biodentin® (a calcium-silicate based material), applied as direct pulp capping of intact teeth planned for extraction, leads to the formation of calcified tissue and dentine bridges in 50% of samples after the period of 6 weeks.

Conclusion

Based on all of the above, a conclusion may be drawn that calcium HAp/PLGA combined with TGF β-1 yields better results after both shorter observation period (3 months) and longer period of time (6 months) compared to HAp/PLGA alone, which has been demonstrated as a good growth factor carrier.

It is reasonable to consider with all the necessary precautions the clinical use of growth factors, especially TGF β, which has been reported to be able to induce differentiation of the second generation of odontoblast cells.

TGF β directly induces morphological and functional differentiation of neo-odontoblasts. However, the clinical use of TGF β may lead to the "doubling" of its unchanged positive action. The clinical use of TGF β also involves paying special attention to the means of molecule transport, response dosing, and control of the degree of reparation processes, molecule half-life, and possible immune reactions.

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Follow-up dental examination a day after apicoectomy using the store-and-forward method

Kontrolni postoperativni pregled dan posle apikotomije „store-and-forward" metodom

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Abstract

Background/Aim. Although apicoectomy is performed routinely and yields excellent results, a close patient observation during the postoperative course is desirable in order to avoid possible complications. The aim of this study was to investigate the adequacy of postoperative control visits a day after surgery using the store-and-forward telemedicine method compared to the clinical in-person controls. **Methods.** A total of 122 apicoectomies were performed during 115 dental surgery interventions. The follow-up dental examination a day after apicoectomy consisted of reviewing extraoral and intraoral photographs on the internet and reviewing responses to the questionnaire. After that, patients were examined in-person. Cohen's kappa („store-and-forward" method) coefficient, diagnostic sensitivity (SE), sensitivity (SP), and efficiency (EFF) were determined. Statistical comparisons were performed using the Z-test, and nonparametric characteristics were tested using McNemar's χ^2-test at the statistical significance cut-off value of $p = 0.05$. **Results.** There were 106 (92%) patients seen at the control dental examination. The agreement between in-person and store-and-forward telemedicine method was found in 104 (98%) cases. The obtained agreement values (K = 0.85, S = 0.99, SP = 0.86, EFF = 0.98) indicated an almost complete diagnostic agreement. **Conclusion.** Based on the internet transmission of patients' digital photographs and accompanying patient medical history records, the study showed that the in-person control dental examinations a day after surgery can be successfully replaced with the remote store-and-forward method of telemedicine.

Key words:
apicoectomy; dental root; dentistry; oral surgical procedures; sensitivity and specificity; telemedicine.

Apstrakt

Uvod/Cilj. Mada se apikotomija rutinski izvodi i daje veoma uspešne rezultate, neophodno je kod pacijenta adekvatno ispratiti postoperativni tok zbog eventualnih komplikacija. Cilj ovog istraživanja bio je da se ispita pouzdanost postoperativne kontrole dan posle intervencije „store-and-forward" metodom u odnosu na „in-person" kontrolu na klinici. **Metode.** Urađene su ukupno 122 apikotomije tokom 115 hirurških operacija. Kontrolni pregled dan posle apikotomije urađen je pregledom ekstraoralnih i intraoralnih fotografija pacijenta preko interneta i pregledom datih odgovora na upitnik, kao i naknadnim pregledom pacijenta „in-person". U cilju procene pouzdanosti „store-and-forward" metode određeni su parametri saglasnosti sa "in-person" metodom: Cohen-ov kappa (κ) koeficijent, dijagnostička senzitivnost (SE), specifičnost (SP) i efikasnost (EFF). Statističko poređenje vršeno je Z-testom, a testiranje neparametarskih obeležja McNemar-ovim χ^2 testom za prag značajnosti od $p = 0.05$. **Rezultati.** Na kontrolni pregled javilo se 106 (92%) pacijenata. Slaganje između „in-person" direktnog pregleda i „store-and-forward" metode telemedicine nađeno je kod 104 (98%) pacijenata. Dobijene vrednosti saglasnosti bile su κ = 0,85, SE = 0,99, SP = 0,86, EFF = 0,98, što ukazuje na skoro potpunu dijagnostičku saglasnost. **Zaključak.** Bazirana na internet prenosu digitalnih fotografija pacijenta i pratećih anamnestičkih podataka, ova studija je pokazala da se kontrolni pregled „in-person", dan posle izvršene apikotomije, može adekvatno zameniti udaljenim „store and forward" telemedicinskim pregledom.

Ključne reči:
apikotomija; zub, korenski kanal; stomatologija; hirurgija; oralna, procedure; senzitivnost i specifičnost; telemedicina.

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Introduction

An ideal method of telemedicine would be almost identical to the one described several thousand years ago: an individual with a disease, regardless of his/her whereabouts, contacts the physician (his/her words reaching the physician faster than light), and the physician (using a magic cure) heals the patient from a large distance[1]. Although having been sought for thousands of years, we are still thriving to reach the ideal method.

Dental treatment, which has been greatly improved in the recent decades, has made the preservation of many natural teeth possible with minimal collateral damage [2]. Apicoectomy (periapical surgery) is a combined method, representing the last line of defence of a natural tooth before extraction. Apicoectomy is indicated when non-surgical approaches of endodontics are unable to preserve the tooth, and it is performed routinely in appropriate cases [3, 4]. Apicoectomy itself is an oral surgery approach that removes the apical portion of the dental root and the adjacent tissue all the way to the healthy tissue, with a success rate ranging from 65% to 95.2%, depending on various factors, such as the type of indication for surgery, tooth type, patient's age, etc. [5–7].

Apicoectomy is usually performed under local anaesthesia, in a single surgical session, after which postoperative therapy is always prescribed to the patient and he/she is discharged from the clinic. Certain discomforting sensations are often felt after the surgery, usually implying postoperative pain, swelling, difficulty swallowing, and similar [8]. A control dental follow-up examination is therefore necessary, for which an appointment has to be made. At this examination, a dental surgeon usually establishes local and general findings after surgery. Although a level of discomfort is usual and expected after apicoectomy, some minor or major postoperative complications may occur as well. Based on the aforementioned control examination, an insight is made, and decisions are agreed upon on the continuation, change, or supplementation of the prescribed postoperative therapy [9–15]. Because of the importance of the follow-up of the postoperative course, this control dental follow-up examination is strongly recommended a day after periapical surgery [16].

Our study aimed to investigate the validity of the remote postoperative control examination a day after dental apicoectomy, using the store-and-forward method.

Methods

In the period from 2016 to 2018, this experimental randomized study, performed at the Faculty of Medicine in Kosovska Mitrovica, Serbia, included 97 randomly selected patients of both genders, aged 14 to 77 years (mean age, 37 years; range, 14–77 years). The study was approved by the Ethics Committee of the Faculty of Medicine in Kosovska Mitrovica. There were 34 men and 63 women. There were 122 apicoectomies in total, out of which 70 were performed in the upper jaw and 52 in the lower jaw, 37 (53%) incisors and canines, [25 (36%) premolars and 8 (11%)] molars, [23 (44%) incisors and canines, 22 (42%) premolars and (13%)] molars. There were 60 (49%) incisors and canines in total, 47 (39%) premolars and 15 (12%) molars. The reasons for apicoectomy were as follows: a) periapical disease of the permanent tooth after the failure of endodontic treatment; b) periapical disease of the tooth which had been prosthetically or conservatively managed and the removal of which was not easily feasible; c) radiotransparent lesion of 8 mm or more in size; d) forced root canal filling or the presence of a foreign body which could not be removed in an orthograde fashion; and e) other indications (the patient insisting on endodontic-surgical management in a single session, dental root fracture in the apical third, etc.) [17].

There were 131 dental roots with performed apicoectomy, out of which 107 (82%) were filled in an orthograde and 16 (12%) in a retrograde fashion. Reintervention was done in 8 (6%) cases.

There were 115 apicoectomies, out of which sulcular flap was used in 25 (22%) cases, triangular flap in 21 (18%), trapezoidal flap in 25 (22%), semilunar flap in 25 (22%), submarginal scalloped flap in 6 (5%), submarginal straight flap in 10 (9%), and vertical flap in 3 (3%) cases (acc. Eskici) [18].

Out of 115 performed apicoectomies, control follow-up examination was done in 106 (93%) cases a day after surgery, while in 9 (7%) cases, the patients did not show up for the follow-up examination. The control examination a day after apicoectomy was performed in the following way. Patients were seen by a dentist who did not perform the periapical surgery. Three extraoral photographs were taken of the patient's head: one facial and two *en face* bilaterally, as well as one intraoral photograph focusing on the area of the apicoectomy, with lips and cheeks retracted by an assistant. The photographs were taken using Samsung S7 Galaxy EDGE mobile phone (SM-G935F), measuring 2595 × 1458 pixels, with a horizontal and vertical resolution of 150 dpi and 24 bits and sRGB colour representation, in jpg format. The photographs were taken using the flashlight, regardless of the lighting conditions in the examination room. The distance between the patient and the camera was 5–10 cm for the intraoral photo and 30–50 cm for the extraoral photo. Patients were also asked to respond to a questionnaire that consisted of the following seven questions: How are you today? Do you have any pain? Do you take your prescribed therapy regularly? Is your swelling enlarging or reducing? Was there any bleeding? Are there any discomforts or similar complaints? If there are, name and describe them? Other comments? [8, 10–13,16].

The photographs taken were uploaded to a web server [the web server represented an internally developed ASP.NET Internet application, at the web address *teleapicoectomy.xpa3.com*, the access to which was authorized, authenticated, and protected using a 256-bit SSL (Secure Sockets Layer) security protocol]. The web server application made a recording for the postoperative follow-up control examination, written down in the Microsoft SQL Server 2014 Express database. Three

individual jpg files were uploaded and recorded onto the SSD server disk, with each file getting a unique name based on the generation of GUID value [19] and jpg file extension. The names of the files generated were thus written into the database table, serving as a reference for subsequent access to these files. Into the other database fields, responses to the questionnaire were entered, as well as other associated service information (upload information, such as date, time, user, and similar). Data collection and the upload were thus completed.

A digital telemedical control examination followed after that. An oral surgeon accessed the server application at the web address *teleapicoectomy.xpa3.com*, and after authentication and authorization, under a 256-bit security protocol, examined the patient virtually, based on the four photographs and the questionnaire responses presented to him/her on the HTML page of the browser. A click on the photograph opened it in a separate window – it was possible to enlarge or additionally manipulate the photo in other ways. Based on the performed digital examination, the oral surgeon established the teledentistry status of the patient in question, i.e. his/her postoperative diagnosis. After the digital follow-up control examination, the oral surgeon directly (in-person) examined the patient in the dental examination chair for about 10 min. Thereafter, the oral surgeon established the postoperative status of the patient again for postoperative diagnosis.

The degree of diagnostic accuracy was determined in accordance with the following scale: correct – if the telemedicine postoperative diagnosis was identical to the primary diagnosis or was an acceptable differential diagnosis, and incorrect – if the telemedicine postoperative diagnosis completely differed from the primary diagnosis, or the diagnosis was not established at all.

Statistical data processing and the analysis of the obtained results was performed using the Med Calc version 18.6 for Windows and DAG (diagnostic and agreement statistics software) [20]. The agreement between examinations was obtained as the ratio of the number of examinations with an agreement and the total number of examinations. Sensitivity (SE), specificity (SP), and efficiency (EFF) were also measured. The degree of the obtained agreement between examinations using the method of telemedicine was established using the Cohen's kappa (κ) coefficient. Kappa coefficient for the confidence interval of 95% was presented in accordance with the Landis and Koch scale (Table 1). Statistical significance of the differences between the correct and incorrect diagnoses, accuracy, SE and SP, and comparisons of all the obtained values were performed using the Z-test, and testing for nonparametric characteristics, which was done using McNemar's chi-squared (χ^2) test (contingency table 2×2) at the significance threshold of $p = 0.05$.

Table 1

Kappa coefficient and degree of diagnostic agreement (Landis and Koch)

Kappa coefficient	Degree of agreement
< 0	No agreement
0.01–0.20	Slight agreement
0.21– 0.40	Sufficient agreement
0.41–0.60	Moderate agreement
0.61–0.80	Considerable agreement
0.81–0.99	Almost complete agreement
1	Complete agreement

Results

Out of 115 apicoectomies, 106 (92%) were subjected to the follow-up control examination a day after the intervention, while 9 (8%) did not (Table 2).

Oral surgeons reported general and local findings in 99 (94%) cases out of 106 cases using both methods (telemedicine and in-person), with the previously prescribed postoperative therapy to be continued without any changes. Out of that, the findings were identical (in order) in 98 (92%) cases, while in one case, telemedicine findings showed continuance and compliance with the prescribed therapy, and in-person findings showed that postoperative antioedematous therapy could be withdrawn. Furthermore, in one case, telemedicine indicated the need for antibiotic and antioedematous therapy to be increased, and the in-person method showed that the findings were in order, without the need to correct the therapy.

In 7 (7%) cases, both methods indicated a need for changing the postoperative therapy. Nevertheless, 6 out of 7 (86%) cases were identical, while 1 (14%) case was

Table 2

General data on patients and apicoectomies

Parameters	Overall, n/N (%)	Men, n/N (%)	Women, n/N (%)
Patients	97/97 (100)	34/97 (35)	63/97 (65)
Apicoectomies	115/115 (100)	38/115 (3)	77/115 (67)
Apicoectomies subjected to control examination			
yes	106/115 (92)	35/106 (33)	71/106 (67)
no	9/115 (8)	3/9 (33)	6/9 (67)

n – number of cases; N – total number.

Table 3

Agreement between two methods (in-person and telemedicine) in relation to prescribed postoperative therapy after control examination

Parameters	In-person n/N (%)	Telemedicine n/N (%)
Analyzed cases	99/106 (93)	99/106 (93)
prescribed therapy	98/99 (99)	98/99 (99)
identical findings	1/99 (1)	1/99 (1)
Additional treatments suggested	7/99 (7)	7/99 (7)
removal of one or more sutures	1/7 (14)	1/7 (14)
drain placement	1/7 (14)	1/7 (14)
correction of antibiotic therapy	6/7 886)	7/7 (100)
correction of antioedematous therapy	3/7 (43)	2/7 (29)

n – number of cases; N – total number.

differently assessed by different methods. The difference was reflected in different assessments of the size and characteristics of postoperative oedema and in the consequential correction of postoperative therapy (Table 3).

Out of 106 control follow-ups, the agreement of in-person direct examination and the store-and-forward method was found in 104 (98%) cases (Table 4). The obtained agreement values were as follows: kappa = 0.85, SE = 0.99, SP = 0.86, EFF = 0.98, indicating an almost complete diagnostic agreement (Table 5). Diagnostic differences between these two methods were not statistically significant.

Table 4

Agreement (kappa)

Telemedicine	In-person		
	0	1	
0	98	1	99 (93.4%)
1	1	6	7 (6.6%)
	99 (93.4%)	7 (6.6%)	

Weighted Kappa: 0.84704; Standard error: 0.10617; 95% confidence interval: 0.63896 – 1.00000.

Table 5

Agreement statistics

Sensitivity = 0.99 (95% CI: 0.95–1.00)
Specificity = 0.86 (95% CI: 0.42–1.00)
Efficiency (Correct classification rate) = 0.98 (95% CI: 0.93–1.00)
Cohen's Kappa = 0.85 (95% CI: 0.64–1.06)
This kappa indicates almost perfect agreement.
Test of Ho: Kappa = 0: z = 8.72, *p* = 0.0000 t.t.t.
Observed agreement = 0.98 (95% CI: 0.93–1.00)
Chance agreement = 0.88 (95% CI: 0.00–0.00)
Positive agreement = 0.99 (95% CI: 0.98–1.00)
Negative agreement = 0.86 (95% CI: 0.66–1.05)

CI – confidence interval.

Discussion

The focus of our research was the real possibility of a remote follow-up of the patient's recovery a day after oral surgery, apicoectomy. Based on the internet transmission of digital photographs of the studied patients and their responses to the questionnaire, our study showed that the in-person follow-up examination could be successfully replaced with the remote store-and-forward telemedical examination, with considerable time-saving effect for both the patient and his/her dentist.

In fact, the primary concern of a dentist after apicoectomy is the postoperative recovery of the patient. This involves the exclusion of postoperative complications, or if they still occur, their timely diagnosis and adequate therapeutic management. Timely and proper postoperative diagnosis prevents the progress of possible postoperative complications and speeds up the patient's recovery all the way until he/she is completely healed [16]. Our study demonstrates that the telemedical approach can be used to follow up the patient and to adequately assess the need for postoperative therapy correction. It should be mentioned that there were no misdiagnoses of postoperative complications or the need for additional treatment corrections when the telemedical approach was used. In cases when telemedical postoperative control examination after apicoectomy indicates complication development or the need for therapy correction, oral surgeons may react remotely and correct the therapy or, if needed, refer the patient for hospital treatment.

The concept represents an advancement of the initial idea that teledentistry is primarily intended to help dentists manage patients remotely [21]. In fact, in an age of smartphones with quality cameras widely available and widespread internet access, an idea readily comes to mind that patients may take a couple of selfies, write a few comments about their condition, and thus report their postoperative status to the dentist without leaving their home (naturally, with some appropriate instructions or perhaps a step-by-step tutorial).

We were unable to find any studies in the literature investigating the possibility of using telemedicine in the follow-up after oral surgery (nor after dental treatments for that matter). However, there are several studies dealing with the postoperative recovery of patients in other medical disciplines. A study investigating the online postoperative recovery of general surgery patients of the Vanderbilt University Medical Center, and patients who underwent elective laparoscopic cholecystectomy, laparoscopic ventral hernia repair, umbilical hernia repair, or inguinal hernia repair, showed that for 68% of doctors and patients, online follow-up of postoperative recovery was equal to the visits to the clinic, while 24% of doctors and patients preferred visits to the clinic, and 8% preferred online examinations [22]. A

systematic review protocol analysis of 1,413 studies of postoperative follow-up of discharged patients identified 7 studies dealing with a potential replacement of follow-up clinic visits with phone communication or online video-conference calls. The study found a high degree of satisfaction of both patients and doctors and a high degree of success of the telemedical approach as an alternative to postoperative clinical examination [23]. Telemedicine can be successfully used in covering intensive care unit (ICU) beds during the postoperative period[24]. Telerehabilitation is recommended for patients after total knee arthroplasty, showing better results compared to the face-to-face rehabilitation approach [25]. Telemedicine has definitely come forth as a future method in the postoperative follow-up of surgically treated patients.

On the other hand, dentistry has its specific aspects. The reliability of transferring and reviewing digital photographs that illustrate the status of the mouth cavity and teeth for the purpose of diagnosing numerous dental and mouth cavity conditions has been confirmed in a number of studies [26–30].

Telemedical follow-ups after apicoectomy have numerous associated benefits for both the patient and his/her oral surgeon. Some of them are as follows: a newly operated patient does not have to go through the trouble of visiting their oral surgeon for control follow-up examination (in many cases patients are at remote locations, and then they can continue recovering without the need for transportation). Expenses are then reduced and valuable time is saved for both the patient and his/her surgeon [31]. Oral surgeons may plan their operations for the last days of the week, and follow up their patient's recovery *via* the Internet even out of the office. Patients are allowed to travel after the surgery (they are not bound by the obligation to visit the clinic), to continue with their professional activities, etc.

In the near future, a complete shift to the telemedical approach could be envisaged for patient follow-up visits after apicoectomy, with establishing photography standards and defining precisely the questionnaire on patient's condition after this oral surgery.

Future research should be directed towards the telemedical control of the postoperative course in other routine dental surgery treatments, primarily complicated dental extractions, surgical dental extractions, and out-patient recovery follow-up in cases of odontogenic infections [28, 29]. The need for in-person control examinations will be considerably reduced, relieving from this burden both the patient and his/her dentist in the days immediately following oral surgery.

Conclusion

Comparing the follow-up examinations using the store-and-forward and in-person methods a day after apicoectomy, the obtained results showed an almost complete agreement, suggesting that this telemedical approach could be safely used to perform the above follow-up examinations.

Declaration of conflicting interests

The author(s) declare no conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Mammaglobin expression in tissue as a predictor of breast carcinoma aggressiveness

Ekspresija mamaglobina u tkivu kao prediktora agresivnosti karcinoma dojke

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Abstract

Background/Aim. Human mammaglobin is considered to be one of the most significant markers of hematogenous dissemination of breast carcinoma. This paper aimed to indicate the important role of peritumoral tissue as an active participant in the tumorigenesis process and the concentration/expression of mammaglobins in the peritumoral tissue as a significant prognostic factor. **Methods.** This research included 64 female patients with primary breast carcinoma during the five-year follow-up period. To determine the concentration of mammaglobin A in samples of carcinoma tissue and peritumoral tissue, Enzyme-linked immunosorbent assay (ELISA) test was used, and for the determination of relative gene expression of mammaglobin A, quantitative reverse transcription-polymerase chain reaction (qRT-PCR) was used. **Results.** The concentration of mammaglobin A was increased in both the carcinoma tissue and peritumoral tissue with an increase in tumor size, number of affected lymph nodes, number of metastases, while relative expression of mammaglobin A was statistically significantly higher in carcinoma tissue than in peritumoral tissue, regardless of the tumor size, number of affected lymph nodes, number of metastases and tumor type. The concentration of mammaglobin A was higher in peritumoral tissue than in tissue of ductal carcinoma, while in the case of lobular carcinoma the concentration of mammaglobin A was higher in carcinoma tissue than in peritumoral tissue. **Conclusion.** Mammaglobin A concentration in peritumoral tissue higher than 0.6704221 ng/mL, and in carcinoma tissue higher than 0.5784426 ng/mL, as well as mammaglobin A relative gene expression in carcinoma tissue higher than 1.003, were determined as cut-off values. These values may identify patients who are at higher risk of metastatic disease, which would be treated with early radical adjuvant treatment.

Key words:
breast neoplasms; mammaglobin a; neoplasm invasiveness; neoplasm metastasis; prognosis.

Apstrakt

Uvod/Cilj. Humani mamaglobin je jedan od naznačajnijih markera hematogene diseminacije karcinoma dojke. Cilj rada bio je da se ukaže na važnu ulogu peritumorskog tkiva kao aktivnog učesnika u procesu tumorogeneze i koncentracije/ekspresije mamaglobina u peritumorskom tkivu kao značajnog prognostičkog faktora. **Metode.** Ovom studijom su bile obuhvaćene 64 bolesnice sa primarnim karcinomom dojke tokom perioda od pet godina. Za određivanje koncentracije mamaglobina A u tkivu karcinoma i peritumorskom tkivu korišćen je *enzyme-linked immunosorbent assay* (ELISA) test, dok je za određivanje relativne genske ekspresije ovog molekula korišćen *quantitative reverse-transcription-polymerase chain reaction* (qRT-PCR). **Rezultati.** Koncentracija mamaglobina A je rasla kako u tkivu karcinoma, tako i u peritumorskom tkivu sa porastom veličine tumora, broja zahvaćenih limfnih čvorova, broja metastaza, dok je relativna ekspresija mamaglobina A statistički bila značajno viša u karcinomu nego u peritumorskom tkivu, bez obzira na veličinu tumora, broj zahvaćenih limfnih čvorova, broj metastaza i tumorski tip. Koncentracija mamaglobina A je bila viša u peritumorskom tkivu nego u tkivu duktalnog karcinoma, dok je u slučaju lobularnog karcinoma koncentracija mamaglobina A bila veća u karcinomu, nego u peritumor-

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skom tkivu. **Zaključak.** Koncentracije mamaglobina A u peritumorskom tkivu veće od 0,6704221 ng/mL i u tkivu karcinoma veće od 0,5784426 ng/mL, kao i relativna genska ekspresija mamaglobina A u tkivu karcinoma veća od 1,003 su "cut-off" vrednosti na osnovu kojih se mogu identifikovati bolesnici koji su pod povećanim rizikom od razvoja metastatske bolesti i koji se mogu tretirati ranim radikalnim adjuvantnim lečenjem.

Ključne reči:
dojka, neoplazme; mamaglobin a; neoplazme, invazivnost; neoplazme, metastaze; prognoza.

Introduction

Breast carcinoma (BC) is the leading cause of cancer death in the USA, with over 230,000 estimated new cases in 2014 and 40,000 estimated deaths [1]. Despite the achieved advances in treating breast carcinoma by applying numerous hormonal, genetic, and molecular markers, such as estrogen receptor (ER), progesterone receptor (PR), human epidermal growth factor receptor-2 (HER2), Ki67, etc., high rates of mortality and morbidity are obviously related to this disease. Therefore, further study in this field is necessary with the aim of finding new markers as predictors of disease aggressiveness [2–4]. Breast cancer was classified into invasive ductal carcinoma (over 80% of total BC), invasive lobular carcinoma (10% of total BC), and other BC histological types that are not so common (10% of total BC) according to pathohistological features [5]. Improvement of medical achievements led to individualization of therapy, i.e. treatment selection tailored to individual patients [6].

One of the specific breast cancer markers is the uteroglobin protein called human mammaglobin. This protein is detected both in normal breast tissue and in breast cancer. Detected blood levels are increased in cancer and have prognostic significance [7, 8]. Human mammaglobin was first detected in 1994 by Watson and Fleming [9] using the polymerase chain reaction (PCR) method. In addition to breast tissue, this uteroglobin protein occurs in two subtypes B1 and B2, detected in ovarian carcinoma [10]. In the literature, other names used for human mammaglobin are also MAM, UGB3, SCGB2A1, MMG, and MGB [11]. Human mammaglobin has been an important predictor for bone metastases in breast cancer [12]. The mRNA expression of mammaglobin may be multiplied in breast cancer versus non-malignant breast tissue [13]. The overexpression of mammaglobin is probably caused by a complex mechanism on the level of transcription [14].

Span et al. [15] demonstrated that mRNA expression of mammaglobin A could be used for individualization of postoperative adjuvant treatment planning. Human mammaglobin (hMAG) was also used to distinguish different breast carcinoma subtypes [16]. It is positively expressed in 80% of the intraductal carcinoma and 90% of invasive ductal carcinoma [17]. The expression of human mammaglobin is in correlation with a high grade of breast cancer [18].

There is no consensus in the literature on the association of human mammaglobin levels and the prognosis of the course of the disease [19]. Nunez-Villar et al. [20] showed a correlation of human mammaglobin with less aggressive forms of the disease. Many efforts have been made to detect mRNA mammaglobin in lymph nodes, blood, and bone marrow in patients with breast carcinoma. The peculiarity of hMAG lies in its almost sole existence in mammary tissue and mammary carcinoma. In addition, the elevated expression in carcinomas and its association with tumor grades renders it an excellent marker for diagnosis and prognosis [21].

BC early detection screening and other detection methods are still being studied. It is reported that expression of hMAG is mostly related to breast carcinoma tissue, and hMAG is defined as one of the first relatively mammary-specific markers [13]. There are many studies in the literature that are related to mammaglobin level of the peripheral blood in BC patients, but there are not so many studies describing mammaglobin level in cancer tissue. Studies concerning mammaglobin level of peritumoral tissue are quite rare [22–24].

Methods

This study presents a clinical observational cohort study along with an experimental study based on human origin material *in vitro*. The experimental research was carried out in the Laboratory of Cell and Molecular Biology, Biology and Ecology Institute, Faculty of Medical Sciences, University of Kragujevac. Samples (carcinoma tissue and peritumoral tissue) were taken in corporation with the General and Thoracic Surgery Clinic and Anatomic Pathology Department of the Clinical Centre of Kragujevac. This study was approved by the Ethics Committee of the Clinical Centre of Kragujevac (no. 01-4990) and was carried out in accordance with the Declaration of Helsinki. All patients were given written information about the study details.

Chemicals and reagents

Phosphate-buffered saline (PBS) was provided by Gibco, the USA; chloroform, ethanol, and isopropanol were provided by Serva Company, Germany. Human Mammaglobin-A ELISA kit and monoclonal antibody Anti-Human Antibody were provided by My BioSource, inc. San Diego, CA, the USA.

QuantiTect Reverse Transcription Kit and PCR Kit (Sensiscript Reverse Transcriptase Kit - RT) were provided by Qiagen, Hilden, Germany. The PCR water and TRIzol were provided by Ambion, the USA. Gene expression Kit KapaSYBR® Green PCR Master Mix was

provided by KAPA Biosystems, Boston, the USA. PCR primers were provided by Eurofins Genomics, Ebersberg.

Criteria for involving the patients in the study

In this study, we analyzed carcinoma and peritumoral tissue. The study included patients with early diagnosed breast carcinoma. All the patients were examined by the Tumor board meeting of the Clinical Centre of Kragujevac and then subjected to the appropriate surgical intervention. After the examination that had been carried out by the Tumor board meeting, tissue samples were taken willingly from patients. During the surgeries carried out at the General and Thoracic Surgery Clinic, Clinical Centre of Kragujevac, breast carcinoma specimens (n = 64) and peritumoral tissue specimens (n=64) were collected. The carcinoma tissue samples appeared to be different in size depending on carcinoma size, and the peritumoral macroscopic unchanged tissue samples were taken to 3 cm from the macroscopic carcinoma margin depending on the size of the excised breast tissue. All specimens were histopathologically examined and verified by the Anatomic Pathology Department of the Clinical Centre of Kragujevac. Specimens were stored at -196 °C until analysis. They were evaluated including the following parameters: histological type of the tumor, the grade of the disease (Nottingham Histological Scores), the condition of the lymph nodes, estrogen, progesterone, and HER2/neu receptors status. The specimens were evaluated according to the American Joint Committee on Cancer (AJCC) protocol [25, 26].

The study did not include patients who underwent neoadjuvant treatment preoperatively. The patients with a previous history of breast carcinoma, along with the patients with metastatic deposits, were excluded from the study. The study did not affect treatments generally conducted at the Clinical Centre of Kragujevac and established on the principles of good clinical practice.

Samples were measured and homogenized on ice in 500 μL cold lysis buffer for 0.01g of sample. IKA Homogeniser IKA®-Werke GmbH & Co. KG, Germany, and Ultrasonic homogenizers Sonopuls, Bandelin electronic GmbH & Co. KG, Germany were used. Lysis buffer contained 31.25 mM Triss-HCl pH 6.8, 2% SDS, 10% glycerol, and dH2O was added up to 100 mL. After centrifugation at 10 000 RPM at 4°C, 10 min, the supernatant was isolated, and it presented the cell lysate. In this way, the proteins from carcinoma and peritumoral tissue were isolated. The Lowry method was used to determine protein concentrations [27].

Determining human mammaglobin-A concentration in carcinoma tissue

The human mammaglobin-A levels in carcinoma tissue were quantified using Human mammaglobin-A ELISA kit and monoclonal antibody Anti-Human Antibody (My BioSource, inc. San Diego, CA, the USA) according to the manufacturer's instructions.

Relative expression of messenger ribonucleic acid (mRNA) mammaglobin gene

Total RNA from the carcinoma and peritumoral tissue was isolated using the phenol-chloroform method by Chomeczynski and Sacchi [28]. Concentrations and purity of RNA were measured on a biophotometer (Eppendorf BioPhotometer plus). $A_{260}/_{280}$ and $A_{260}/_{230}$ ratios were monitored to assess any possible contamination by protein, organic solvents, salts, carbohydrates, etc. The samples were stored at -80 °C until analysis. The RNA template is first converted into complementary DNA (cDNA) using a reverse transcriptase (RT) [29].

Quantitative mRNA analysis

Quantitative reverse transcription-polymerase chain reaction (qRT-PCR) cDNA was used for gene expression analysis. Master mix (Universal KAPA SYBR FAST qPCR Master Mix 2X) is designed for high-performance real-time PCR containing everything necessary except primers, cDNA specimens, and Rox Low dye, which were added. All qPCR experiments were performed using the Applied Biosystems, quantitative Real-Time system (Applied Biosystems 7500/7500 Real-Time PCR Software v2.0). Each reaction (a 20 μL reaction mixture) contained 10 μL SYBR Green PCR Master Mix, 1 μL forward and reverse primer (5 pmol/μL) and 2 μL cDNA and 7 μL nuclease-free water. A PCR negative control containing nuclease-free water instead of cDNA and a 2RT control containing 2RT reaction instead of cDNA were included. The thermal cycling conditions included an initial denaturation step at 95 °C for 10 min, followed by 40 cycles at 95 °C for 30 seconds, 60 °C for 30 seconds, and 72 °C for 30 seconds. To analyze the qPCR results, we used the relative quantification method, which is based on the expression levels of a target gene versus reference genes (housekeeping gene).

There are 2 replicates in each combination of genes. Relative quantification of gene expression was normalized to the β-actin mRNA expression level. The gene-specific qRT-PCR primers were as follows:

Primer	Forward sequence	Reverse sequence
β-actin	5′-AAGCAGGAGTATGACGAGTCCG-3′	5′-GCCTTCATACATCTCAAGTTGG-3′
Mammaglobin-A	5′-CAG CGG CTT CCT TGA TCCTTG-3′	5′-ATA AGA AAG AGA AGG TGT GG-3′

To calculate the expression of a target gene in relation to a reference gene, we used the $2^{-\Delta CT}$ method [30].

Statistical analysis

All data are presented as the mean ± SEM (standard error of the mean). The normality of distribution was tested by the Kolmogorov-Smirnov test. The two-tailed Student's *t*-test, ANOVA test, or the nonparametric Mann–Whitney rank-sum test were used depending on the normality of distribution. Moreover, the χ^2 test was used

for categorical variables. A binary logistic regression model was used to evaluate prediction between two variables. A receiver operating characteristic (ROC) curve analysis was employed to assess the diagnostic capabilities of the variables for predicting distant metastasis. The results were considered significantly different when $p < 0.05$. The data were analyzed using SPSS version 20, statistical package.

Results

Clinical and pathological characteristics of breast cancer patients

The levels of mammaglobin in carcinoma tissue and peritumoral tissue were observed, and their prognostic value was analyzed. Correlation between mammaglobin level in carcinoma tissue and peritumoral tissue and certain clinical and pathological characteristics were also the object of the study. Clinical and pathological characteristics of the patients are described in Table 1 .

The average age of patients was 58.95 ± 11.24 years. The median age of patients was 60.5 years. Most patients (61/64, 96%) were older than 50 years. Fifty five (86%) patients had ductal carcinoma compared to 9 (14%) patients with lobular carcinoma, and this ratio was statistically significant (χ^2 test, $p < 0.01$). A sparing operation was performed in 29 (45.3%) patients who had primary breast cancer of less than 3 cm, compared to 35 (54.7%) patients in whom mutilating surgery was performed. Adjuvant chemotherapy had 46 (72%) patients. Postoperative radiotherapy was used in 43 (67%) patients.

Mammaglobin A concentration in carcinoma and peritumoral tissue in breast carcinoma patients

The concentration of mammaglobin A grew both in carcinoma tissue and peritumoral tissue with an increase in tumor size, the number of affected lymph nodes, the number of metastases, and tumor grade (Figure 1).

The concentration of mammaglobin A was higher in peritumoral tissue than in carcinoma tissue in ductal carcinoma, while in the case of lobular carcinoma, the concentration of mammaglobin A was higher in carcinoma than in peritumoral tissue (Figure 2).

Table 1

Clinical, pathological, and immunohistochemical and tumor, node, metastasis (TNM) characteristics of breast cancer patients

Characteristics	Number of patients, n (%)
Age of patients (years)	
< 50	3 (4)
> 50	61 (96)
Total number of specimens	128 (100)
peritumoral tissue (PT)	64 (50)
carcinoma tissue (CT)	64 (50)
Histological grade	
low grade (G1 or well-differentiated)	8 (12)
intermediate grade (G2 or moderately differentiated)	36 (56)
high grade (G3 or poorly differentiated)	20 (32)
high grade (G4 or undifferentiated)	0 (0)
Histological type	
invasive ductal carcinoma	55 (86)
invasive lobular carcinoma	9 (14)
Receptor status	
ER+	38 (60)
ER-	26 (40)
PR+	29 (46)
PR-	35 (54)
HER+	31 (48)
HER-	33 (52)
The size of the tumor (cm)	
≤ 2 (T1)	27 (42)
2–5 (T2)	3 (4)
> 5 (T3)	34 (54)
tumor of any size grown into the chest wall (T4)	0 (0)
Regional lymph nodes (N)	
no regional lymph node metastasis (N0)	4 (6)
metastasis to movable ipsilateral axillary lymph node(s) (N1)	31 (48)
metastasis to ipsilateral axillary lymph node(s) fixed to one another or to other structures (N2)	29 (46)
Distant metastasis (M) developed during a 5-year period	
no distant metastasis (M0)	23 (36)
distant metastasis (M1)	33 (52)
presence of distant metastasis cannot be assessed (Mx)	8 (12)

ER – estrogen receptor; PR – progesteron receptor; HER – human epidermal growth factor receptor.

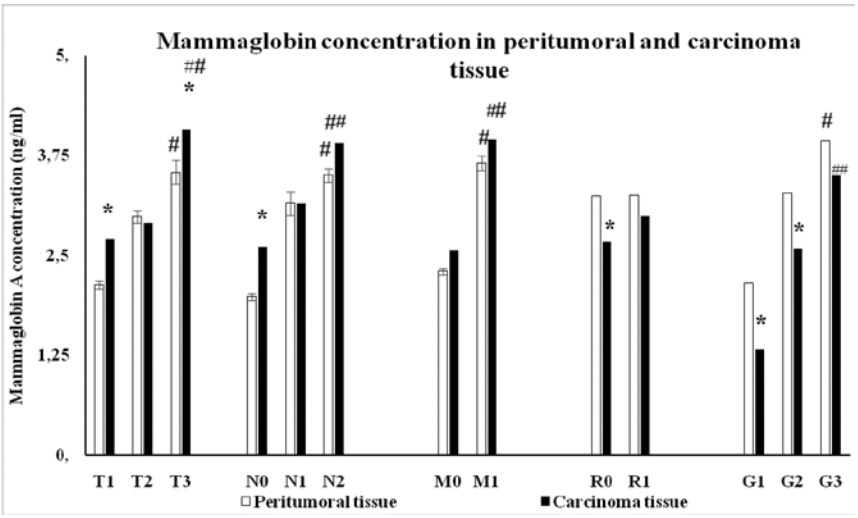

Fig. 1 – Mammaglobin A concentration in carcinoma and peritumoral tissue in breast carcinoma patients related to tumor, node, metastasis (TNM) classification system, histological tumor grade (G1-G3), and status of resection margins (R0 and R1).
T1 – tumor ≤ 2 cm; T2 – tumor 2-5 cm; T3 – tumor > 5 cm; N0 – no regional lymph node metastasis; N1 – metastasis to movable ipsilateral axillary lymph node(s); N2 – metastasis to ipsilateral axillary lymph node(s) fixed to one another or to other structures; M0 – no distant metastasis; M1 – distant metastasis; R0 – no cancer cells seen microscopically at the primary tumor site; R1 – cancer cells present microscopically at the primary tumor site; G1 – low grade; G2 – intermediate grade; G3 –high grade.
The results are given as the mean value ± SE for the examined parameter number of samples (N); *$p < 0.05$ statistically significant difference between carcinoma tissue and peritumoral tissue; #$p < 0.05$ statistically significant difference between peritumoral tissue of different patients; ##$p < 0.05$ statistically significant difference between carcinoma tissue of different patients. Student's *t*-test and ANOVA were used, and $p < 0.05$ was regarded as a statistically significant difference.

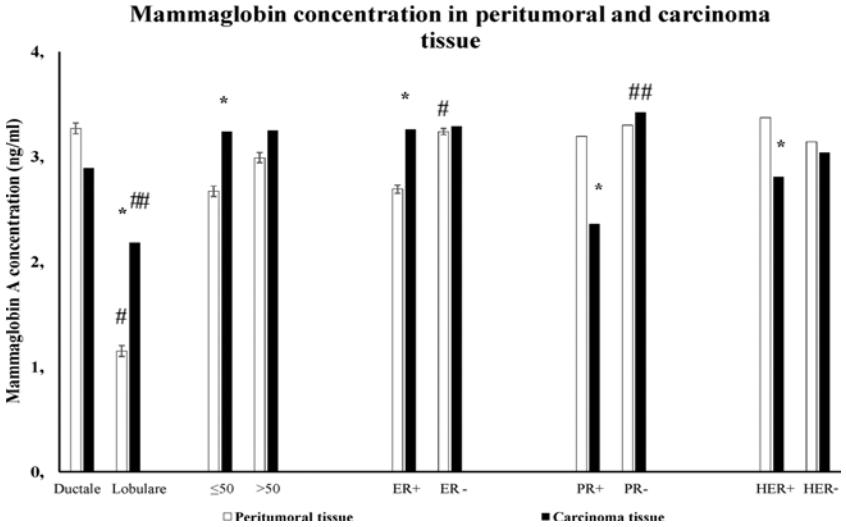

Fig. 2 – Mammaglobin A concentration in carcinoma and peritumoral tissue in breast carcinoma patients related to pathohistological tumor type, patient's age (≤ 50, > 50), hormone receptor [estrogen receptor (ER) and progesterone receptor (PR)], and human epidermal growth factor receptor 2 (HER) status.
The results are given as the mean value ± SE for the examined parameter. *$p < 0.05$ statistically significant difference between carcinoma tissue and peritumoral tissue; #$p < 0.05$ statistically significant difference between peritumoral tissue of different patients; ##$p < 0.05$ statistically significant difference between carcinoma tissue of different patients. The Student's *t*-test was used, and $p < 0.05$ was regarded as a statistically significant difference.

Analysis of mammaglobin A gene expression in carcinoma and peritumoral tissue in breast carcinoma patients

Relative expression of mammaglobin *A* was statistically significantly higher in carcinoma than in peritumoral tissue, regardless of the histological type of tumor, patient's age, hormone receptors, or HER status (Figure 3).

Relative expression of mammaglobin A was statistically significantly higher in carcinoma than in peritumoral tissue, regardless of tumor size, number of affected lymph nodes, number of metastases, and tumor grade (Figure 4).

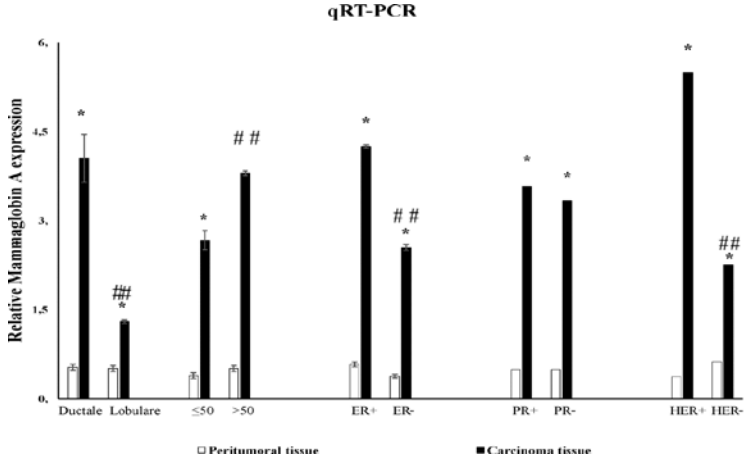

Fig. 3 – Relative mammaglobin A gene expression in carcinoma and peritumoral tissue in breast carcinoma patients related to pathohistological tumor type, patient's age (≤ 50, > 50), hormone, receptor receptor [estrogen receptor (ER) and progesterone receptor (PR)] and human epidermal growth factor receptor 2 (HER) status. The results are given as the mean value ± SE for the examined parameter; *$p < 0.05$ statistically significant difference between carcinoma tissue and peritumoral tissue; #$p < 0.05$ statistically significant difference between peritumoral tissue of different patients; ##$p < 0.05$ statistically significant difference between carcinoma tissue of different patients.

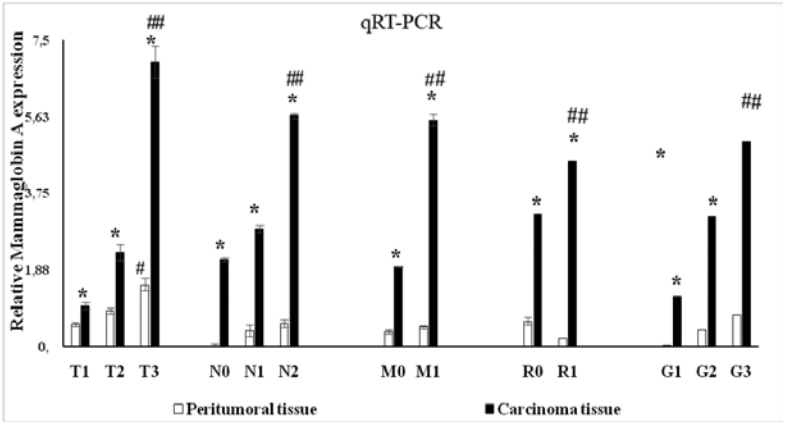

Fig. 4 – Mammaglobin A gene expression in carcinoma and peritumoral tissue in breast carcinoma patients related to tumor, node metastasis (TNM) system classification, positive resection margins (R0 and R1), and histological tumor grade (G1-G3).

T1 – tumor ≤ 2 cm; T2 – tumor 2-5 cm; T3 – tumor > 5 cm; N0 – no regional lymph node metastasis; N1 – metastasis to movable ipsilateral axillary lymph node(s); N2 – metastasis to ipsilateral axillary lymph node(s) fixed to one another or to other structures; M0 – no distant metastasis; M1 – distant metastasis; R0 – no cancer cells seen microscopically at the primary tumor site; R1 – cancer cells present microscopically at the primary tumor site; G1 – low grade; G2 – intermediate grade; G3 –high grade.

The results are given as the mean value ± SE for the examined parameter number of samples (N); *$p < 0.05$ statistically significant difference between carcinoma tissue and peritumoral tissue; #$p < 0.05$ statistically significant difference between peritumoral tissue of different patients; ##$p < 0.05$ statistically significant difference between carcinoma tissue of different patients. Mammaglobin gene expression is significantly higher in carcinoma tissue (Mann-Whitney Test, U = 754, $p = 0.001$).

Prognostic significance of mammaglobin A concentration in carcinoma and peritumoral tissue in breast carcinoma patients

As shown in Figure 5, mammaglobin concentration in peritumoral tissue had a propensity for distant metastasis (binary logistic regression, $p = 0.024$). Mammaglobin borderline value in carcinoma tissue was 0.67 ng/mL for sensitivity 0.58 and specificity 0.59.

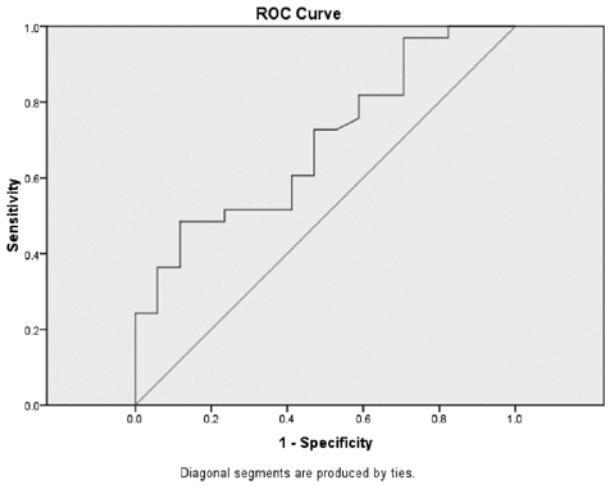

Fig. 5 – Receiver operating characteristic (ROC) curve: mammaglobin A concentration in peritumoral tissue in breast carcinoma patients.

Mammaglobin concentration in carcinoma tissue had a propensity for distant metastasis (binary logistic regression, $p = 0.025$). Mammaglobin borderline value in carcinoma tissue was 0.578 ng/mL for sensitivity 0.67 and specificity 0.65 (Figure 6).

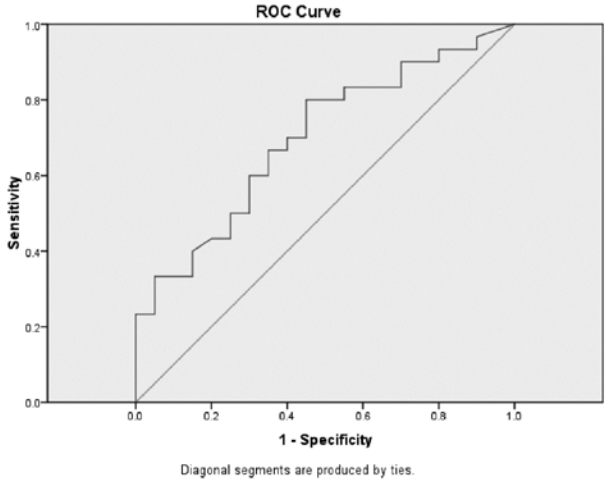

Fig. 6 – Receiver operating characteristic (ROC) curve: mammaglobin A concentration in carcinoma tissue in breast carcinoma patients.

As indicated in Figure 7, mammaglobin gene expression in peritumoral tissue had no significant influence on the occurrence of distant metastasis (binary logistic regression, $p = 0.307$).

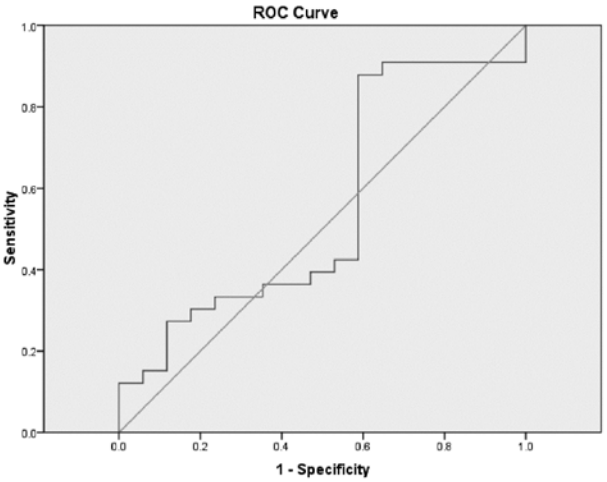

Fig. 7 – Receiver operating characteristic (ROC) curve: mammaglobin A gene expression in peritumoral tissue in breast carcinoma patients.

Mammaglobin gene expression in carcinoma tissue identified by PCR method had a propensity for distant metastasis (binary logistic regression, $p = 0.043$) (Figure 8). Mammaglobin gene expression borderline value in carcinoma tissue was 1.003 for sensitivity 0.73 and specificity 0.76.

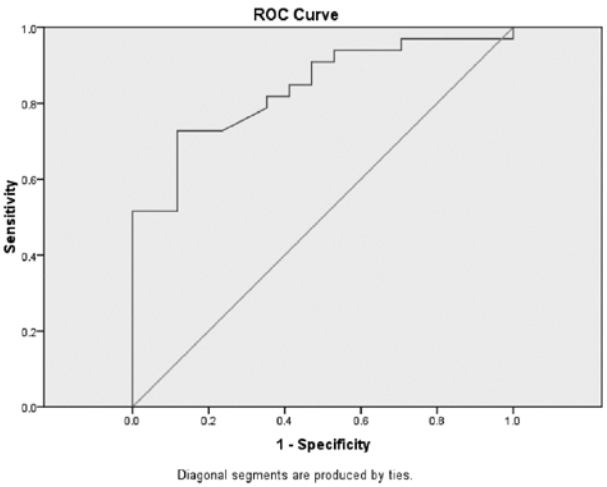

Fig. 8 – Receiver operating characteristic (ROC) curve: mammaglobin-A gene expression in carcinoma tissue in breast carcinoma patients

Discussion

Examination of high mobility group A (hMGA) mRNA levels of patients' peripheral blood results in 38.2% sensitivity, 100% specificity, 100% positive prognostic value (PPV), and 61.8% negative prognostic value (NPV) [31]. There have been many studies describing mammaglobin level in patients' serum but not so many studies related to the mammaglobin level in carcinoma tissue; studies examining mammaglobin level in peritumoral tissue are very rare. The peritumoral tissue is a relatively new research topic, and recent studies have presented its important role in breast

cancer formation and development [32]. One of the studies that investigated mammaglobin levels in peritumoral, as well as tumoral tissue in breast cancer patients, is the study of Zafracas et al. [11]. They found that mammaglobin was abundantly expressed in both malignant and normal breast tissues. Our goal was to analyze the gene and protein expression of mammaglobin in carcinoma tissue and peritumoral tissue. We also managed to determine specific values of these parameters in carcinoma tissue and peritumoral tissue that appeared to be of high prognostic value.

The goal of modern oncology is personalized therapy, which presents the optimal method for a patient [33]. This study contributes to personalized therapy research, dealing with the analysis of the potential correlation between mammaglobin expression in carcinoma tissue and peritumoral tissue and certain clinical and pathological characteristics specific for each patient. We also managed to define specific values of mammaglobin levels (cut-off values) in carcinoma tissue and peritumoral tissue, having statistically proved prognostic values related to some of the most important prognostic parameters (e.g. distant and lymph nodes metastasis) for the outcome [34].

According to the studied data, serum concentrations of mammaglobin were 0.07–9.6 ng/mL compared to 0–0.07 ng/mL of the control group [35]. Our study showed that there was no statistically significant difference in mammaglobin concentrations in carcinoma and peritumoral tissue. ELISA test was used to determine this difference. We got the values 2.4 ng/mL–3.8 ng/mL, which was higher than the range of healthy persons, 0–0.07 ng/mL, pointing out the prognostic value of mammaglobin concentrations in tissues.

Data in the studies related to the serum concentration of mammaglobin have been contradictory. Zehentner et al. [36] claimed that ELISA test data showed that mammaglobin level was not dependant on the disease stage. The ROC curve showed the values of 1.71 ng/mL of cut-off; the test was considered to be positive when values of mammaglobin were higher than the given ones [36]. In our study, the ROC curve showed that mammaglobin concentration value in peritumoral tissue can be used as a prognostic factor of distant metastasis [area under curve (AUC) = 0.693, $p = 0.027$]. Moreover, the ROC curve showed that mammaglobin concentration value in carcinoma tissue could be used as a prognostic factor of distant metastasis (AUC = 0.698, $p = 0.019$).

However, Bernstein et al. [37] claimed that patients at stages I–III had mammaglobin values of 0.9–1.4 ng/mL, and at stage IV the value of 2.3 ng/mL. There was a strong positive correlation between mammaglobin values and carcinoma size; patients with a tumor of large diameter had higher serum concentrations of mammaglobin. Our results match these data. There was an increased level of mammaglobin in carcinoma tissue and peritumoral tissue in patients with a larger breast tumor. As for the serum concentrations, results of our study showed concentrations of 2.6 ng/mL at T1 stage up to those of 3.8 ng/mL at T3 stage. Values in peritumoral tissue were significantly lower than those in carcinoma tissue – 2.4 ng/mL at T1 up to 3,6 ng/mL at T3. We did not find similar studies while searching through the available databases; therefore, it was impossible to compare the results. To our knowledge, this is the first study of this kind that dealt with determining mammaglobin tissue concentration.

Our results showed that there is a gradual increase of mammaglobin protein expression in carcinoma tissue and peritumoral tissue, with a higher possibility of lymphatic metastasis. For peritumoral tissue, the values were in the range of 2.3 ng/mL–3.7 ng/mL, and for carcinoma tissue, 2.6 ng/mL–3.8 ng/mL concerning N0 and N2 disease stages, respectively. These differences are statistically significant, giving tissue mammaglobin concentrations a prognostic role. These results correspond with the ones we found in other studies. Liu et al. [38] cited statistically higher mammaglobin concentration in patients with positive lymph nodes. Tafreshi et al. [39] demonstrated that the level of mammaglobin was significantly higher in affected lymph nodes compared with healthy lymph nodes and showed that breast cancer targeted agent based on mammaglobin could be used for the non-invasive, *in vivo* detection of cancer altered axillary lymph nodes.

The mean value of serum concentration of mammaglobin in patients with metastatic breast carcinoma was 9.38 ng/mL (7.9 ng/mL in the control group). Sensitivity was 68%, and specificity 88.8%. Slight differences may appear because of different antibodies that were used in various studies [36]. Our results showed that the mean value of mammaglobin determined by protein expression in patients with metastatic disease was 2.4–3.75 ng/mL in peritumoral and 2.55–3.8 ng/mL in carcinoma tissue. Determining protein expression with ELISA test shows prognostic value related to distant metastasis in BC patients. Furthermore, we defined specific cut-off values of mammaglobin concentration, which indicate distant metastasis occurrence risks. This value was 0.6704221 ng/mL in peritumoral tissue, and 0.5784426 ng/mL in carcinoma tissue. We did not find similar studies while searching through the available databases; therefore, it was impossible to compare the results.

As for the tumor grade, our study showed that increased mammaglobin protein expression in carcinoma tissue and peritumoral tissue affects the tumor grade. A higher concentration of mammaglobin can affect tumor metastasis in distant organs. Therefore, determining the protein expression of mammaglobin can have a prognostic value. Similar results can be found in a few studies that examined mammaglobin expression in carcinoma tissue using the method of immunohistochemistry. We determined both protein and gene expression in carcinoma and peritumoral tissue. Rehman et al. [40] also noticed increased mammaglobin concentrations in carcinoma tissue while changing the tumor grade and size.

Our results showed that protein expression of mammaglobin in carcinoma tissue and peritumoral tissue was higher in patients with ductal tumors than in those with lobular tumors. The results of some other studies were

different. Watson and Fleming [7] and Nunez-Villar et al. [20] found no significant difference in mammaglobin expression considering histological types of breast cancer. On the other hand, there are studies like ours that have confirmed increased protein expression in ductal tumors [40]. This confronts with the study by Bhargava et al. [41]. Their study showed that infiltrated lobular carcinoma had the highest mammaglobin expression.

We did not present a correlation between hormonal receptor status (ER and PR) and HER2 and protein expression. There are different data in the studies related to this. O'Brien. et al. [42] cite that the presence of mammaglobin in patients with ER+ and PR+ is a good prognostic indicator. Guan et al. [43] show that the presence of mammaglobin protein and gene expression correlates with ER positivity.

We did not show age dependence in mammaglobin expression since the results were like those of other studies [38]. We defined specific values of mammaglobin concentration in carcinoma tissue and peritumoral tissue, and we showed that there were patients who were potentially at risk of disease development. They were suggested an adjuvant cancer treatment. Protein expression value was 0.6704221 ng/mL in peritumoral tissue, and 0.5784426 ng/mL in carcinoma tissue (ELISA test).

We also dealt with mammaglobin gene expression in carcinoma tissue and peritumoral tissue, and the results were quantitatively different as described in other studies [42]. Nevertheless, Chen et al. [44] showed that only 21% of results of gene examination correlated with protein expression (adenocarcinoma lung).

Possible reasons are well-known phenomena of post-transcriptional and post-translational regulation and modification; in some cases, it is not certain that protein would be functional and detectable [45, 46].

As for the gene expression in carcinoma tissue and peritumoral tissue, our study showed a gradual increase of mammaglobin gene expression depending on the tumor size, though it was less shown in peritumoral tissue. Mammaglobin gene expression values of concentration in carcinoma tissue were 1.5 at T1 stage up to 7.4 at T3 stage, being lower in peritumoral tissue: 0.9 at T1 stage up to 1.7 at T3 stage.

Mammaglobin gene expression was in correlation with lymph nodes status. Results showed significantly lower values in peritumoral tissue: 0.1 at N0 up to 0.4 at N2 stage; in carcinoma tissue, these values were 1.9 at N0 up to 5.6 at the N2 stage. These data are like the ones from the other studies [36]. Marchetti et al. [47] consider mammaglobin one of the most sensitive and most specific markers for lymph nodes micrometastasis detection.

Gene expression of mammaglobin was slightly increased in carcinoma tissue of patients with metastasis. In peritumoral tissue, it had values of 0.3 at M0 up to 0.5 at M1, and in carcinoma tissue 2.0 at M0 up to 5.5 at M1. In 12% of cases, M status was not defined. Therefore, these patients were excluded from the study.

The ROC curve showed that the value of mammaglobin gene expression in peritumoral tissue could not be used as a prognostic factor of distant metastasis (AUC = 0.553, $p = 0.546$).

The ROC curve showed that the value of mammaglobin gene expression in carcinoma tissue could be used as a prognostic factor of distant metastasis (AUC = 0.838, $p < 0.01$).

The study by Span et al. [15] showed that increased gene expression was independently associated with a longer non-relapse period. This was particularly evident in patients taking tamoxifen, indicating a relation to the hormonal status of the tumor. Therefore, mammaglobin gene expression is considered to be a good prognostic marker. Nevertheless, some authors stated that there was no statistically significant correlation between gene expression level and hormonal status [48, 49].

Our results match previously described data. A Korean group [50] found a statistically significant correlation between mammaglobin level of peripheral blood and ER, PR status of patients and their hormonal status. There was no connection related to HER2 status.

Our study indicated that gene expression was significantly higher in carcinoma tissue than in peritumoral tissue and statistically more significant in ductal than in lobular breast cancer. These data correspond to those of other studies [51].

It can be concluded that mammaglobin gene expression in carcinoma tissue (not the one in peritumoral tissue) could be used as a prognostic marker of hematogenous dissemination of breast carcinoma. We determined specific values of gene expression for carcinoma tissue. Thus, it is possible to identify high-risk patients of metastatic disease; these patients are suggested adjuvant cancer treatment. Boundary value of mammaglobin gene expression is considered to be 1.003 for the sensitivity of 0.73 and specificity of 0.76 in carcinoma tissue.

Conclusion

To sum up, the basic results of this study are the following: protein expression of mammaglobin in peritumoral and carcinoma tissue can be used as a prognostic marker for dissemination of breast carcinoma; specific values of mammaglobin concentration in peritumoral and carcinoma tissue have been defined above, from which can be assumed that metastatic dissemination of disease could occur; in peritumoral tissue, mammaglobin concentration determined with ELISA test was 0.6704221 ng/mL, and in carcinoma tissue, this value was 0.5784426 ng/mL; mammaglobin gene expression can be a prognostic marker for hematogenous dissemination of breast carcinoma concerning carcinoma tissue; the determined value of mammaglobin gene expression in carcinoma tissue was 1.003; the analysis of mammaglobin in peritumoral and carcinoma tissue makes it possible to define high-risk patients of disease development, and we suggest adjuvant cancer treatment in order to prevent disease development.

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Conflict of interest

The authors declare that they have no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Dental health status in children with type 1 diabetes mellitus in Montenegro

Dentalno zdravlje kod dece sa dijabetesom melitusom tipa I u Crnoj Gori

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Abstract

Background/Aim. The number of children with diabetes is constantly increasing. The aim of this study was to evaluate oral health in children with type 1 diabetes mellitus (T1DM) compared to healthy children. **Methods.** The study included 177 patients aged 10–15 years, divided into two groups. The group T1DM included children with type I diabetes mellitus, and healthy children were in the control group. Dental health was assessed using the decayed, missing, filled teeth (DMFT) index. The Plaque Index (PI), according to Silness-Löe, and the Calculus Index (CI) by Green, were used to determine oral hygiene. Salivary status involved determining the amount of secreted stimulated saliva and buffer capacity of the same patient. **Results.** In terms of average values of the DMFT index of permanent teeth, there were no statistically significant differences between the groups. A significant difference ($p = 0.001$) was found in the PI value between the T1DM group (1.29 ± 0.56) and the control group (1.01 ± 0.50). Also, a significant difference was found in the CI value between the T1DM group (0.09 ± 0.23) and the control group (0.03 ± 0.14) ($p = 0.047$). The average amount of secreted stimulated saliva for diabetic children was significantly lower (0.99 ± 0.14 mL/min) compared to healthy children (1.06 ± 0.20 mL/min) ($p = 0.020$). **Conclusion.** Children with T1DM do not have more diseased teeth; however, they have more dental plaque, dental calculus, and lower salivation than the children in the control group. Also, our results point to the absence of preventive measures and programs in maintaining dental health in Montenegro.

Key words:
child; dental caries; dmf index; diabetes mellitus, type 1; oral health; oral hygiene; saliva.

Apstrakt

Uvod/Cilj. Broj dece sa dijabetes melitusom u stalnom je porastu. Cilj istraživanja je bio da se utvrdi stanje dentalnog zdravlja kod dece obolele od dijabetes melitusa tip I (T1DM) u odnosu na zdravu decu. **Metode**. Studijom je bilo obuhvaćeno 177 pacijenata uzrasta 10–15 godina, podeljenih u dve grupe. Grupu T1DM činila su deca obolela od T1DM. Zdrava deca činila su kontrolnu grupu. Stanje zdravlja zuba ocenjeno je pomoću indeksa karioznih, ekstrahovanih i plombiranih (KEP) zuba. Za utvrđivanje oralne higijene primenjivan je Plak indeks (PI) po Silness-Löe i Indeks zubnog kamenca po Green-u (CI). Salivarni status je podrazumevao određivanje količine izlučene stimulisane pljuvačke i puferski kapacitet istog pacijenta. **Rezultati.** Nije bilo statistički značajnih razlika između obolele i zdrave dece u prosečnim vrednostima indeksa KEP stalnih zuba. Uočena je značajna razlika u vrednostima PI između grupe T1DM (1,29 ± 0,56) i kontrolne grupe (1,01 ± 0,50), ($p = 0,001$), kao i značajna razlika u vrednostima CI između grupe T1DM (0,09 ± 0,23) i kontrolne grupe (0,03 ± 0,14), ($p = 0,047$). Prosečna količina izlučene stimulisane pljuvačke kod obolele dece bila je značajno niža i iznosila je 0,99 ± 0,14 mL/min, u odnosu na zdravu decu, kod koje bila 1,06 ± 0,20 mL/min ($p = 0,02$). **Zaključak.** Deca obolela od T1DM u Crnoj Gori nemaju više obolelih zuba, ali imaju više dentalnog plaka, zubnog kamenca i manji protok pljuvačke u odnosu na decu iz kontrolne grupe. Takođe, naši rezultati ukazuju na nepostojanje preventivnih mera i programa u očuvanju zdravlja zuba u Crnoj Gori.

Ključne reči:
deca; zub, karijes; dmf indeks; dijabetes melitus, tip 1; usta, zdravlje; usta, higijena; pljuvačka.

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Introduction

Diabetes mellitus (DM) is a metabolic disorder caused by an absolute or functional insulin deficiency. Type 1 DM (T1DM) is a common metabolic disease in childhood [1, 2]. The main characteristic of childhood diabetes is instability, variability, sudden changes in blood glucose levels from day to day, and changes that occur during growth and development.

The increase in the incidence of diabetes in childhood reaches a worrying stage; therefore, the 21st-century epidemic can be rightfully discussed. The occurrence of the incidence is greatest in the population up to 14 years of age, equally among ill boys and girls. The incidence of T1DM decreases going from North to South Europe and is highest in children aged 0–14 years in the Scandinavian countries. Compared to other former Yugoslavian countries, Montenegro has the highest incidence of T1DM in the 0–14 years age group, and it is 18.6 in 100,000 children [1, 2].

This disease has a significant influence on oral health, as shown by numerous studies [3–6]. The most common complications in oral health in DM are the changes in the tissues of the periodontium (periodontitis and gingivitis) salivary dysfunction, xerostomia, changes in salivary composition, oral mucosal diseases, taste dysfunction, oral lichen planus, skin hyperpigmentation, infection by *Candida albicans*, dental caries, odontogenic abscesses, and tooth loss. In 1993, periodontal diseases were identified as the sixth complication of diabetes, and four years later, it has been listed as one of the pathologic states diagnosed in these patients (Expert Commitment on the Diagnosis and Classification of Diabetes Mellitus) [7].

The relationship between DM and dental caries has not yet been clarified. Dental caries can be defined as an infectious and easily transmissible disease caused by a specific bacterial infection. Saliva, with its numerous exogenous and endogenous factors, is considered an important factor in the etiology of dental caries. Reduced salivary flow rate and consequently reduced salivary clearance can lead to dental caries development as well as various other disorders of the mucosal lining of the oral cavity. Reducing the flow rate modifies the buffer's salivary effect resulting in reduced resistance to dental plaque microorganisms and can present favorable conditions for dental caries development in these patients. On the other hand, restricted sugar intake can slow the development of dental caries in diabetics [4–6]. Plaque control and fluoride use are quite important in preventing dental caries and periodontal disease. Brushing teeth with fluoride paste at least twice a day is a professional recommendation. However, children do not usually follow these recommendations.

The aim of this study was to evaluate the dental health status in children with T1DM compared to healthy subjects in Montenegro.

Methods

The study was designed as a cross-sectional study and was carried out in the period from June 2014 to December 2015. The study was conducted in accordance with the Helsinki Declaration and principles of Good Clinical Practice. The study protocol was approved by the Ethics Committee of the Clinical Center of Montenegro in Podgorica (No. 03-5/23).

One dental team was qualified and calibrated for clinical measurements, regardless of experience. The reliability of inspection criteria was measured by a pretest performed on a group of 20 randomly selected schoolchildren (10 children with T1DM and 10 healthy children) aged 10–15 years. Inter-rater agreement was measured by the Cohen kappa index. Kappa values evaluated after the study for inter-rater agreement amounted to 0.94.

Subjects

The study was conducted on 177 children, including 87 children with T1DM (48 boys and 39 girls) aged 10–15 years, treated at the Institute of Child Diseases of the Clinical Center of Montenegro. All diabetic children were treated with insulin. Insulin was dosed according to individual patient needs (0.7–1.3 U/kg/24 h). These children constituted one group of the respondents [8, 9]. The parents of the affected children were fully acquainted with the research protocol. Only children whose parents have given their signed consent were included in the study.

Children with T1DM were examined in the dental offices of the Faculty of Medicine, study program Dentistry in Podgorica, after regular control examination of endocrinologists in the presence of parents. Data on the value of glycosylated hemoglobin (HbA1c) not older than six days were obtained from the patients' medical documentation. The exclusion criteria for this group with T1DM were the presence of other systemic disorders unrelated to the complications of DM.

The control group, aged 10–15 years, included 90 healthy children (47 boys and 43 girls) with the absence of active diseases and no history of drug therapy, selected from elementary schools in the municipality of Podgorica by random sample method. The school principal, school staff, and the parents were informed of the research protocol. The realization of the study started after receiving the written consent of the principal and parents' signatures. These children were examined in the school dental offices, in the presence of their parents, in a period that did not interfere with their regular teaching.

General exclusion criteria for all participants were the need for antibiotic prophylaxis and the children who refused to cooperate. The patients and their families lived in the same geographic area.

Questionnaire

The parental questionnaire consisted of two parts. Part 1 included questions on socioeconomic data (municipality, school, grade, gender, date of birth, parents' education and employment, parents' marital status, number of children at home, family income). The second part was focused on children, including questions on their oral hygiene habits (the frequency of tooth-brushing and the use of fluoride toothpaste). The questionnaire is given in Addendum.

Furthermore, the socioeconomic status estimate was implemented. Socioeconomic status was classified into low, moderate, or high, according to the household income, with nationally defined cutoffs according to Eurostat [10]. After examining, every child was trained to properly brush his/her teeth.

Dental examination

The dental caries status of permanent teeth was registered using the decayed, missing, filled teeth (DMFT) system according to the World Health Organization (WHO) standards [11]. Caries was diagnosed with an inspection with the use of standard dental diagnostic instruments. Clearly visible lesions with the cavity formed on the tooth surface were registered as dental caries, while changes in transparency or initial demineralization of the eyes with an intact surface and without cavitation were registered as healthy teeth. The oral hygiene was assessed using the Plaque Index (PI) according to Silness-Löe [12], which determines the absence or the presence, the quantity and distribution of dental plaque and other soft deposits on teeth, as well as the Calculus Index (CI) by Green, which determines the absence or the presence of dental calculus on the teeth [13].

Saliva collection

The children involved in the study were healthy and did not undergo antibiotic therapy for at least 15 days until saliva sampling or taking any other therapy (other than anti-diabetic for patients suffering from the disease) and were not in the process of tooth repairing during saliva sampling. Investigations were carried out on samples of total stimulated saliva taken at least two hours after the last meal (between 10 and 11 a.m.).

The Dentobuff Strip System (Orion Diagnostics, Espoo, Finland) was used to determine the amount of stimulated saliva and buffer capacity. Samples of saliva were collected by giving medical paraffin tablets to children, who chewed them to induce stimulation and spontaneously spit in a graduated plastic bowl for a period of 5 min. The measurement did not include the foam formed during shrinkage. After that, the readout value was divided by number five, and thus the amount of stimulated secretion of saliva in one minute (mL/min) is obtained. Then one drop of saliva was applied to the test strip. After five minutes, the test strip changed color, and the buffer capacity of saliva was recorded. The results of the saliva buffer capacity of diabetic children were compared with those of the control group. The scoring, or the results of the buffer capacity of the saliva, was carried out as follows: 0 – blue color; the test strip is currently turned into this color; indicates a very high buffering capacity (pH > 6); 1 – blue color; the test strip changed colors within 5 min; indicates a high buffering capacity (pH = 6); 2 – green color; represents a mean buffer capacity (pH = 4.5–5.5); 3 – yellow color; represents a low buffering capacity (pH = 4, or less than 4). The blue color of the test strip, that is, the values 0 and 1, indicate a high buffering capacity [14].

Statistical analysis

Statistical data processing was done in SPSS v.11.5 for Windows (SPSS Inc., Chicago, IL, USA). Methods of descriptive and analytical statistics were used to describe the results. The descriptive statistical methods used were the mean value, standard deviation, and percentages. Student's *t*-test and χ^2-test were used to test statistical significance in the average values between two independent samples. *P*-values of less than 0.05 were considered statistically significant.

Results

Subjects characteristics

A total of 177 children (95 boys and 85 girls), aged 10–15 years, participated in this research. The average age of children suffering from DM was 12.7 ± 1.6 years, while the average age of children from the control group was 12.8 ± 1.6 years. The disease lasted for 4.44 ± 2.02 years from the moment of diagnosis. The mean hemoglobin A1c (HbA1c) value in the diabetics was 9.9 ± 1.7%, reflecting an inadequate control of their diabetes. Of those, 75% had HbA1c higher than 8%.

All the examined children brushed their teeth with fluoridated toothpaste. Only 33.3% of patients with T1DM and 31.1% of respondents from the control group brushed their teeth twice a day or more frequently (χ^2-test, $p > 0.05$). Socioeconomic family statuses were similar for both groups (χ^2-test, $p > 0.05$). Oral hygiene habits and socioeconomic status of the study groups are shown in Table 1.

Table 1
Oral hygiene habits and socioeconomic status of the examined patients

Parameter	T1DM group (%)	Control group (%)	*p* (χ^2-test)
Daily brushing			
1 daily	66.7	68.9	n. s.
≥ 2 daily	33.3	31.1	
Using fluoridated toothpaste	100.0	100.0	
Socioeconomic status			
low	22.9	21.1	n. s.
medium/high	77.1	78.9	

***Note*: All values are expressed in percentages of the examined patients. T1DM – a group of children with type I diabetes mellitus; Control group – a group of healthy children; n.s. – non-significant.**

Dental examination

The percentage of children with all healthy teeth in the T1DM group was 4.6%, while the number of children with permanent teeth affected by the illness was 83 (95.4%). The percentage of children with diseased permanent teeth was slightly lower in the control group compared to the group of children with diabetes mellitus (92.2%), and the number of children with all healthy teeth was 7 (7.8%). Testing the results did not show statistically significant differences in the values of this index (Student's *t*-test, $p > 0.05$).

The average value of DMFT index was almost identical for both groups and it was 4.3. The average values of carious, extracted, and filled teeth were very similar in both groups ($p > 0.05$, Student's *t*-test).

The distribution of the DMFT index in the examined patients is shown in Table 2.

Oral hygiene

Children with DM had significantly higher average PI ($p = 0.001$, Student's *t*-test) and CI values ($p = 0.047$, Student's *t*-test) compared to healthy children. These values are shown in Table 4.

Saliva

The mean value of the stimulated salivary flow for the children with DM was significantly lower (0.99 ± 0.14 mL/min) compared to the children in the control group (1.06 ± 0.2 mL/min), ($p = 0.020$, Student's *t*-test). The average buffer capacity of the saliva value for diabetic children was somewhat higher than in the control group, but there was no statistically significant difference between groups ($p = 0.652$, Student's *t*-test) (Table 4).

In the group of patients with T1DM, the highest percentage (44.8%) of children examined had a high buffering capacity of saliva (grade 1), and only 5.7% of subjects had a low buffering capacity of saliva. The highest percentage (44.4%) of children in the control group had a high buffering capacity of the saliva. The

Table 2

The values of DMFT components and DMFT index in the observed groups

Index	T1DM group* mean ± SD (range)	Control group* mean ± SD (range)	*p* (*t*-test)
D	1.66 ± 1.58 (0–6)	1.96 ± 1.47 (0–6)	0.190
M	0.20 ± 0.48 (0–2)	0.26 ± 0.49 (0–2	0.427
F	2.45 ± 1.59 (0–7)	2.11 ± 1.52 (0–8)	0.148
DMFT	4.30 ± 1.79 (0–8)	4.33 ± 1.99 (0–9)	0.866

DMFT – decayed (D), missing (M), filled (F) teeth (T); SD – standard deviation.
***For explanations see under Table 1**.

The filled teeth dominated in the structure of DMFT in both groups. The percentage of carious and extracted teeth was slightly higher in the control group (Table 3). There were no significant differences found in the values of the DMFT components (χ^2-test, $p > 0.05$).

Table 3

Components of the DMFT index in the examined patients

Components of DMFT*	T1DM group* (%)	Control group* (%)	*p*-value
D	38.5	45.4	n. s.
M	4.8	5.9	n. s.
F	56.7	48.7	n. s.
Total	100.0	100.0	

***Note*: All values are expressed in percentages of the examined patients.**
***For explanations see under Tables 1 and 2.**

Table 4

The values of Plaque Index (PI), Calculus Index (CI), stimulated salivary flow and buffer capacity of the saliva in the examined patients

Parameter	T1DM group* mean ± SD (range)	Control group* mean ± SD (range)	*p* (*t*-test)
PI	1.29 ± 0.56 (0.12–2.63)	1.01 ± 0,50 (0.11–2.12)	0.001
CI	0.09 ± 0.23 (0.00–1.16)	0.03 ± 0,14 (0.00–1.16)	0.047
Salivary flow (mL/min)	0.99 ± 0.14 (0.70–1.60)	1.06 ± 0.20 (0.70–1.70)	0.020
Buffer capacity of saliva	1.35 ± 0.77 (0–3)	1.29 ± 0.85 (0–3)	0.652

***For explanations see under Table 1.**
SD – standard deviation.

difference between the observed groups was not statistically significant (χ^2-test, $p > 0.05$). Values of the saliva buffering capacity are shown in Table 5.

Table 5

Buffer capacity of the saliva in the examined patients

Buffer capacity	T1DM group* n (%)	Control group* n (%)
0	11 (12.7)	15 (16.7)
1	39 (44.8)	40 (44.4)
2	32 (36.8)	28 (31.1)
3	5 (5.7)	7 (7.8)
Total	87 (100.0)	90 (100.0)

***Note*: All values are expressed in number (percentage) of the examined patients.**

***For explanations see under Table 1.**

Buffer capacity: 0 – very high (pH > 6); 1 – high (pH = 6); 2 – mean (pH = 4.5–5.5); 3 – low (pH = 4, or less than 4).

Discussion

This study examined the relationship between T1DM in schoolchildren and their oral health condition. We believe that this is the first study dedicated to this topic in Montenegro.

Dental caries is the ultimate result of a complex, dynamic multifactorial effect. Some factors may increase the risk of developing dental caries in diabetes, and others may reduce it. Reduced salivation, accelerated accumulation of dental plaque, and increased frequency of meals in diabetics are factors that could increase the risk of developing dental caries [4]. On the other hand, reduced consumption of fermentable carbohydrates and a well-balanced diet are factors that could slow the development of dental caries. Bearing these facts in mind, it is logical that numerous studies devoted to the impact of diabetes on the appearance of dental caries have shown contradictory results. Findings of individual studies indicate an increased prevalence of dental caries, especially in patients with poorly controlled disease [15–18], while others find no differences between people suffering from DM and the healthy population [3, 19–23]. Studies conducted in Portugal [19], Brazil [21], Egypt [22], and Iran [23] did not find the correlation between these two diseases. In contrast to their results, a higher incidence of dental caries in children with DM has been determined by studies carried out in Kuwait [24], Bosnia [25], and India [16, 17].

The results of this study indicate a high percentage of children with diseased permanent teeth in the control group (92.2%). Following the WHO criteria, the average value of the DMFT control group is considered high. These results point to the absence of preventive measures and programs in Montenegro; therefore, it is necessary to form a strategy for controlling dental caries.

In our study, the percentage of children in Montenegro with DM and with all healthy teeth was slightly lower than in the control group, which was expected. Namely, we established a significantly worse oral hygiene condition and significantly lower average values of the stimulated salivary secreted in these children. The lower buffer capacity of saliva was observed in diseased children. The occurrence of salivary glycoside, the increase in its viscosity, the salivary gland dysfunction, and dry mouth in this disease favor a faster accumulation of dental plaque and the formation of calculus [19, 20, 23]. Accordingly, our respondents with DM had significantly more calculus deposits on their teeth compared to the subjects from the control group. The results of our research are generally in agreement with the results of studies conducted by Orbak et al. [25] in Turkey, Daković et al. [26] in Serbia [27], and López del Vall and Ocasio-López [28] in Puerto Rico. Hyposalivation was confirmed in patients with insulin-dependent diabetes [17, 29, 30]. Reduced saliva secretion reduces its bactericidal role; this creates a disposition for oral infection. Increased saliva density increases the concentration of enzymes; this enhances fermentation and leads to acidosis. Increased acidity in the mouth causes changes in the biofilm structure and makes it easier to colonize *streptococci* and *lactobacilli*, thereby increasing the risk of dental caries [31, 32]. In contrast, the results of our study did not find an association between the amount of salivation and dental caries prevalence. Similar observations were found earlier [20, 33]. Namely, the average value of the DMFT index of 4.3 was almost identical for both of the investigated groups. The explanation of this statement lies in the etiology of dental caries that arises as a result of the interaction of three primary factors: host (tooth), causative agent (microorganism), and environment (nutrition, substrate) in the function of time. Additionally, fluoride use, nutrition, social factors, habits, and patients' behavior are of great importance. Furthermore, the results of our research show that most children from both groups brush their teeth with fluoride paste at least once a day, and they had very similar oral hygiene habits. Similar findings have also been found by Lai et al. [33]. Moreover, the participants of our research were very similar according to socioeconomic status, which could explain the same values of the DMFT index.

After analyzing the individual components of DMFT, no significant differences were found between the comparing groups. The filled teeth dominated both of the observed groups, but the ratio of dental caries and tooth fillings was a bit better in the group of diabetics. A slightly better structure of DMFT in diabetic children in Montenegro can be explained by the relatively good cooperation between pediatricians and dentists.

Similar oral hygiene habits in both study groups indicate the necessity of implementing oral health education in children and their parents. Effective removal of dental plaque is quite significant for good oral health. It is, therefore, necessary to apply this knowledge to promoting oral health on the individual and social level [34, 35].

The relationship between DM and oral disease is not only at the level of clinical signs of illness but at the level of inflammatory mediators, especially those occurring in

autoimmune processes [3, 4, 27]. Regular dental appointments for guidance, dental plaque control, fluor prophylaxis, fissure sealing, a well-balanced diet, and glycemic control will certainly contribute to improving both oral and overall health [35].

In order to relate this variable to the patient's oral health, our study provides significant data on the oral health condition of children with T1DM in Montenegro even though there were some limiting factors, such as a small number of subjects and a small number of diabetics with good glycemic control. Moreover, the study points to the importance of additional salivary analysis in assessing the state of oral health.

Conclusion

The results of this research indicate that children with T1DM do not have more diseased teeth; however, they have more dental plaque, dental calculus, and a lower saliva rate than the children in the control group. The high average value of DMFT for both groups points to the absence of preventive measures and curative-oriented dental policy in Montenegro. It is crucial to propose a plan of preventive activities aimed at educating diseased children and at applying prophylactic measures at an individual level in dental offices.

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Addendum

Questionnaire

Number _____

Answer the questions by filling out the number of answers or enter the 'X' .

General data

Name and surname of the child _____

Day, month, year, and place of birth _____

Gender _____

School _____

Grade _____

Address _____

Municipality _____

Part 1

1. Paternal education
   a) No education
   b) Elementary school
   c) Secondary school
   d) College
   e) University
2. Maternal education
   a) No education
   b) Elementary school
   c) Secondary school
   d) College
   e) University
3. Paternal employment
   a) Employed
   b) Unemployed
4. Maternal employed
   a) Employed
   b) Unemployed
5. Family income in the last month
6. Number of siblings
7. Number of family members
8. Child living with
   a) Both parents
   b) Mother
   c) Father
   d) Custody

Part II

9. The child brushes the teeth
   a) Only in the morning
   b) Only in the evening
   c) In the morning and in the evening
   d) After each meal
   e) Not brushing every day
10. The child brushes his/her teeth with fluoride paste
    a) Yes
    b) No

ORIGINAL ARTICLE

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Frequency and effects of seasonal flu vaccines on exacerbations of chronic obstructive pulmonary disease in Serbia

Učestalost i efekti vakcinacije protiv sezonskog gripa na pojavu egzacerbacija hronične opstruktivne bolesti pluća u Srbiji

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Abstract

Background/Aim. The influenza virus is often the cause of exacerbations among chronic obstructive pulmonary disease (COPD) patients, especially during the winter season. However, vaccination rates are still below recommended even in developed countries. The aim of the study was to determine the rates and examine the effectiveness of immunization against seasonal influenza in preventing exacerbations among COPD patients in Serbia. **Methods.** The prospective cohort study of stable COPD outpatients was conducted over three years (between October 1, 2014, and September 30, 2017) at the Polyclinic Department of Institute for Pulmonary Diseases of Vojvodina, Sremska Kamenica, Serbia. The rates and effects of seasonal flu vaccination on COPD exacerbation rates were evaluated using univariate and multivariate logistic regression analysis, taking into account comorbidity, age, and body mass index (BMI). **Results.** The study included 840 patients. The flu vaccination rate was 37.1%. Exacerbations occurred more frequently in unvaccinated patients [176 (31.9%) vs. 375 (68.1%), $p = 0.043$]. In elderly vaccinated patients (≥ 65 years) there were fewer exacerbations compared to unvaccinated patients [89 (56.0%) vs. 188 (69.4%), $p = 0.005$]. Lower frequency of exacerbations was also observed in vaccinated patients with comorbidities [165 (61.1%) vs. 327 (69.4%), $p = 0.021$] and low BMI [27 (64.3%) vs. 78 (83.9%), $p = 0.011$]. Multivariate logistic regression analysis identified BMI < 21 kg/m^2 [relative risk (RR): 0.490; 95% confidence interval (CI): 0.318–0.758; $p = 0.001$] and heart failure (RR: 2.734; 95% CI: 1.121–6.669; $p = 0.027$) as independent predictors of COPD exacerbations. **Conclusion.** Immunization for seasonal influenza in Serbia is below recommended rates. Flu vaccination was associated with a significant reduction in COPD exacerbation rates, particularly in elderly patients and patients with heart failure and low BMI.

Key words:
influenza, human; influenza, vaccines; pulmonary disease, chronic obstructive; recurrence; risk assessment; serbia; vaccination.

Apstrakt

Uvod/Cilj. Egzacerbacije hronične opstruktivne bolesti pluća (HOBP) često nastaju usled infekcija virusima influence, posebno u zimskim mesecima. Međutim, i u razvijenim zemljama je stepen imunizacije i dalje niži od preporučenog. Cilj rada je bio da se utvrdi učestalost i efektivnost imunizacije protiv sezonskog gripa u cilju prevencije egzacerbacija kod bolesnika sa hroničnom opstruktivnom bolešću pluća (HOBP) u Srbiji. **Metode.** Prospektivna kohortna studija sprovedena je kod ambulantnih bolesnika sa HOBP, lečenih u periodu od 1.10.2014. do 30.09.2017. u Polikliničkoj službi Instituta za plućne bolesti Vojvodine, Sremska Kamenica. Povezanost vakcinacije i egzacerbacija HOBP, ali i komorbiditeta, starosti i indeksa telesna mase (*body mass index* - BMI), ispitivana je pomoću univarijantne i multivarijantne logističke regresione analize. **Rezultati.** Studijom su bila obuhvaćena 840 bolesnika. Protiv sezonskog gripa vakcinisano je 37,1% bolesnika. Egzacerbacije HOBP bile su češće kod nevakcinisanih bolesnika [176 (31,9%) vs. 375 (68,1%), $p = 0,043$]. Stariji, vakcinisani bolesnici (≥ 65 godina), imali su manje egzacerbacija u odnosu na nevakcinisane [89 (56,0%) vs. 188 (69,4%), $p = 0,005$]. Manje egzacerbacija je potvrđeno u grupi vakcinisanih bolesnika sa komorbiditetima [165 (61,1%) vs. 327 (69,4%), $p = 0,021$] i niskim BMI [27 (64,3) vs. 78 (83,9%), $p = 0,011$]. U multivarijantnoj analizi nezavisni prediktori pojave egzacerbacije su bili BMI > 21 kg/m^2 [relativni rizik (RR): 0,490; 95% interval poverenja (CI): 0,318–0,758; $p = 0.001$] i srčana slabost (RR: 2,734; 95% CI: 1,121–6,669; $p = 0.027$). **Zaključak.** Imunizacija protiv sezonskog gripa u Srbiji je i dalje ispod preporučenog nivoa.

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Vakcinacija protiv sezonskog gripa značajno redukuje egzacerbacije među HOBP pacijentima, posebno kod starijih sa srčanom slabošću i niskim BMI.

Ključne reči:
grip; grip, vakcina; pluća, opstruktivne bolesti, hornične; recidiv; rizik, procena; srbija; vakcinacija.

Introduction

Chronic obstructive pulmonary disease (COPD) is a significant sociomedical burden for both the patient and the society. It is estimated that 11–12% of the world population suffers from COPD [1, 2]. The more frequent exacerbations, the quicker the progression of the disease. The exacerbations are followed by a decrease in the quality of life, an increase in the number of hospitalizations, and mortality [3].

Viruses account for up to 50% of COPD exacerbations and the influenza virus for up to 28% of seasonal viruses (winter months) [4–7]. The World Health Organization (WHO) and European Center for Disease Prevention and Control (ECDC), Centers for Disease Control and Prevention (CDC) of the United States (US), as well as many national guides, recommend that patients with COPD undergo immunization for influenza (Evidence level A, Recommendation degree 1) [8, 9]. Vaccination against influenza leads to a reduction in viral infections by 60%, hospitalizations up to 40%, and a reduction in the occurrence of pneumonia [10–12]. Thus, the flu vaccination is recommended for all patients over 65 years of age and for the chronically ill [13, 14]. The aim of the European Union (EU) is to vaccinate 70% of patients with chronic diseases. From 2012–2016, the number of those vaccinated averaged 45%, and in the US, around 40% [15, 16].

The effects of seasonal flu vaccination among COPD patients have been demonstrated in most of the studies coming from highly-developed countries as opposed to low or middle-income countries. The vaccination rates and effects of vaccination in these settings remain largely unknown even though these countries face a high COPD burden due to high smoking prevalence and the fact that simple and inexpensive interventions such as vaccination may have a large impact on patients' health.

The aim of this study was to examine the frequency and effectiveness of vaccination against seasonal flu among COPD patients in Serbia. More specifically, we aimed to determine whether the flu vaccine reduces COPD exacerbation rates. Additionally, we examined the influence of patient-related factors such as age, comorbidities, and body mass index (BMI) in relation to the vaccination status on reducing COPD exacerbations.

Methods

Study design and population

This prospective cohort study was conducted over three years, and it included consecutive ambulatory patients with COPD (October 1, 2014 – September 30, 2017) at the Polyclinic for Pulmonary Diseases, Institute for Pulmonary Diseases of Vojvodina (IPDV) in Sremska Kamenica, Serbia. The Institute is a tertiary institution in Vojvodina (a northern province in Serbia) and covers a population of approximately 1.9 million (21.56% of the total population of the Republic of Serbia). The Polyclinic Department of the IPDV is a major outpatient clinic for the municipality of Novi Sad with surrounding settlements, covering almost 400,000 inhabitants.

The criteria for being included in the study were the following: patients over 40 years of age, established COPD diagnosis (based on a post-bronchodilator forced expiratory volume in the first second/forced vital capacity – FEV1/FVC ratio of < 0.70) [1] for at least one year, and being in the stable phase of the diseases (no systemic use of corticosteroid therapy or respiratory infection within 6 weeks prior to the study and no change in current medications). Patients who were suffering from cancer within the 5-year period prior to the study were excluded from it. Basic demographics data, comorbidities, and data regarding COPD exacerbations in the previous year were collected from the patients' medical files. The demographic data included gender, age, smoking habits (packs per year), and BMI.

At the beginning of the study, the patients were divided into two groups according to the immunization status against seasonal flu and followed for a one-year study period. Vaccination status and comorbidities were obtained from patients' files and medical history at the IPDV but were also given by the patients. The patients' vaccination status for the current year was collected. All the patients were vaccinated during the standard vaccination season (October-January) [15, 16]. Vaccinations were performed by the patients' general practitioners in their local health centers. The major outcomes were moderate/severe exacerbations during the one-year follow-up.

A patient was considered to have a moderate COPD exacerbation if treated with systemic corticosteroids or antibiotics [17]. Severe COPD exacerbations were defined as the need for hospitalization or evaluation in the emergency department [17]. We also analyzed whether vaccination influenced the stability of COPD groups according to the Global Initiative for Chronic Obstructive Lung Disease (GOLD) ABCD classification of COPD [1] over a one-year period.

Ethics committee approval

All research procedures were in accordance with the ethical standards of the Institute where the research took place, following good clinical practices and the the Helsinki Committee Declaration and its later amendments or

comparable ethical standards. The research was approved by the IPDV Ethics Committee.

Statistical analysis

Descriptive statistics were generated for all study variables, including mean and standard deviation (SD) for continuous variables and relative frequencies for categorical variables. The χ^2 test was used to determine whether there was a significant difference between the expected frequencies and the observed frequencies in one or more categories. The predictive values of evaluated variables for COPD exacerbations were evaluated with univariate and multivariate logistic regression analysis. All statistically significant predictors in univariate analysis were included in multivariate logistic regression analysis. All probability values were calculated by assuming a 2-tailed α value of 0.05 with confidence intervals at the 95% level. All statistical analyses were performed with SPSS for Windows version 17 (SPSS Inc., Chicago, IL).

Results

The study included 840 patients (468 males and 372 females); a total of 312 patients (37.1%) were vaccinated against seasonal flu. There were 741 patients with comorbidities, the most frequent being arterial hypertension (n = 597; 71.1%). During the previous year (prior to entering the study), COPD exacerbations were present in 663 (78.9%) patients. The basic demographic characteristics of the study population, comorbidities, and exacerbations are shown in Tables 1 and 2. During the one-year follow-up, 551 (65.6%) patients experienced COPD exacerbations.

Table 1

Characteristics of the study population and comorbidities

Characteristics	Values
Demography, n (%)	
males	468 (55.7)
females	372 (44.3)
Age (years), mean ± SD	65.39 ±8.6
Patients aged ≥ 65 years, n (%)	430 (51.2)
Duration of COPD (years) , mean ± SD	7.64 ± 5.44
Smoking (pack/year), mean ± SD	45.67 ± 25.09
Smoker, n (%)	348 (41.4)
Ex-smoker, n (%)	448 (53.3)
Nonsmoker, n (%)	44 (5.3)
BMI (kg/m2), mean ± SD	26.39 ± 4.77
Patients with < 21 kg/m^2, n (%)	135 (16.1)
Comorbidity, n (%)	741 (88.2)
arterial hypertension	597 (71.1)
hyperlipoproteinemia	255 (30.3)
ischemic heart disease	198 (23.6)
DM	180 (21.4)
arrhythmia	177 (21)
tuberculosis	56 (6.6)
depression	52 (6.2)
heart failure (NYHA ≥ 2)	36 (4.3)
osteoporosis	31 (3.7)
hyperplasio prostate	14 (1.6)
hypothyroidism	9 (1.1)
breast cancer	5 (0.6)
lung cancer	4 (0.5)
Number of CMB, n (%)	
1	290 (34.5)
2	233 (27.7)
≥3 CMB	185 (22.0)
CVD+DM+depression	19 (2.4)

COPD – chronic obstructive pulmonary disease; BMI – body mass index; DM – diabetes mellitus; CVD – cardiovascular disease; NYHA – New York Heart Association; CMB – comorbidity; SD – standard deviation.

Table 2

Exacerbation characteristics of the chronic obstructive pulmonary diseases patients (n = 840)

Exacerbations	Previous year	Study year
Yes	663 (78.9)	551 (65.6)
None	147 (17.5)	271 (32.3)
Pneumonia	30 (3.6)	18 (2.1)
Total number of exacerbations		
moderate	1,219	892
severe	206	217
ABCD classification		
A	240 (28.6)	351 (41.7)
B	189 (22.5)	146 (17.4)
C	96 (11.4)	62 (7.5)
D	315 (37,5)	281 (33,4)

***Note*: Values are expressed as number (%) of the patients, except total number of exacerbation.**
A – low symptom severity, low exacerbation risk; B – high symptom severity, low exacerbation risk; C – low symptom severity, high exacerbation risk; D – high symptom severity, high exacerbation risk.

Table 3

Characteristics of COPD patients with exacerbations according to the vaccination status

Characteristics	Vaccination		*p*
	yes 312 (37.1)	no 528 (62.9)	
With exacerbation (moderate/severe)	176 (31.9)	375 (68.1)	0.043
Age ≥ 65 years	89 (56.0)	188 (69.4)	0.005
Age < 65 years	103 (67.3)	171 (66.9)	0.935
With CMB	165 (61.1)	327 (69.4)	0.021
Without CMB	27 (64.3)	33 (57.9)	0.520
With heart failure (NYHA ≥ 2)	6 (66.7)	24 (88.9)	0.043
Without heart failure	186 (61.4)	335 (67.1)	0.102
Without CVD + DM + Depression	192 (61.5)	340 (66.9)	0.116
BMI ≥ 21 kg/m^2	168 (61.1)	278 (64.6)	0.353
BMI < 21 kg/m^2	27 (64.3)	78 (83.9)	0.011

***Note*: Values are expressed as number (%) of the patients.**
COPD – chronic obstructive pulmonary disease; CMB – comorbidity; NYHA – New York Heart Association; CVD – cardiovascular disease; DM – diabetes mellitus; BMI – body mass index.

Patients who were vaccinated had less frequent severe and moderate COPD exacerbations than unvaccinated patients [176 (31.9%) vs. 375 (68.1%); $p = 0.043$]. Frequencies of COPD exacerbations according to vaccinal status are presented in Table 3. Among patients ≥ 65 years, more COPD exacerbations occurred in unvaccinated compared to vaccinated patients [188 (69.4%) vs. 89 (56.0%), $p = 0.005$]. A lower frequency of exacerbations was observed in vaccinated patients with at least one comorbidity compared to unvaccinated patients [165 (61.1%) vs. 327 (69.4%), $p = 0.021$]. In patients without comorbidities, there was no statistically significant difference in the frequency of exacerbations according to the vaccination status [33 (57.9%) vs. 27 (64.3%), $p = 0.520$]. Vaccinated patients who suffered from heart failure (NYHA ≥ 2) had a lower frequency of exacerbations compared to unvaccinated patients [6 (66.7%) vs. 24 (88.9%), $p = 0.043$]. Among patients with multiple comorbidities [cardiovascular diseases (CVD), diabetes mellitus (DM), and depression], nobody was vaccinated. In this subgroup, everybody had COPD exacerbation during the follow-up (one of them passed away from exacerbation and pneumonia in the hospital). In vaccinated patients with BMI < 21 kg/m^2, there was a lower frequency of exacerbation [27 (64.3%) vs. 78 (83.9%), $p = 0.011$].

In univariate logistic regression analysis, significant predictors of COPD exacerbation were vaccination,

COPD duration, BMI < 21 kg/m^2, and heart failure, while age ≥ 65 and comorbidities were not (Table 4). In multivariate logistic regression analysis, only BMI < 21 kg/m^2 and heart failure were independent predictors of COPD exacerbations.

Table 4

Logistic regression analyses of predictors of COPD exacerbations

Analysis	RR	95% CI	*p*
Univariate			
age ≥ 65 years	0.889	0.668–1.182	0.418
CMB	1.284	0.835–1.976	0.255
vaccination	0.747	0.557–0.898	0.048
COPD duration	1.032	1.004–1.061	0.025
BMI < 21 kg/m^2	0.492	0.319–0.759	0.001
heart failure	2.701	1.111–6.567	0.028
Multivariate analysis			
BMI < 21 kg/m^2	0.490	0.318–0.758	0.001
heart failure	2.734	1.121–6.669	0.027

COPD – chronic obstructive pulmonary disease; CMB – comorbidity; BMI – body mass index; RR – relative risk; CI – confidence interval.

During the one-year follow-up, vaccinated patients were more likely to improve and move to a milder category according to GOLD ABCD stages, as shown in Figure 1. Unvaccinated patients were more likely to remain in the same stage at the end of the study.

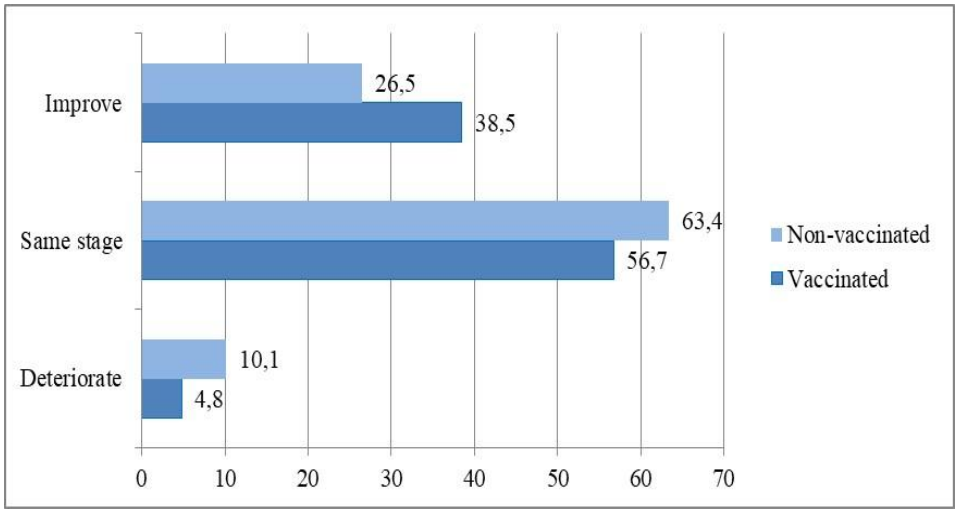

Fig. 1 – Tendency to different category of disease severity regarding vaccination status during one-year follow-up in chronic obstructive pulmonary disease patients.

Discussion

The results of the study demonstrate that only one-third of COPD patients received the seasonal flu vaccine. Those who were vaccinated experienced a significant reduction in COPD exacerbations compared to unvaccinated patients during a one-year follow-up. The observed effects were more pronounced in patients with comorbidities, low BMI, and the elderly.

The study by Aka Aktürk et al. [18] showed a similar number of vaccinated patients, 36.5%. The effects of flu vaccination on COPD exacerbations are well known. A Cochrane meta-analysis by Poole et al. [19] has shown the results of 11 studies regarding the efficacy of the influenza vaccine in COPD. There was a significant reduction in the number of exacerbations, but because of the small number of patients, there was no observed effect on mortality. In our study, COPD exacerbations were significantly reduced in vaccinated patients. A lower number of ER visits in vaccinated patients was also observed in other studies [20]. Montserrat-Capdevila et al. [21] analyzed 1,323 patients over a three-year period and reported fewer hospitalizations among the vaccinated patients.

Our observation on the lower frequency of exacerbations among vaccinated patients older than 65 is similar to a South Korean study in which 828 (54.2% vaccinated) patients were analyzed [22]. Nichol et al. [20] noted that persons older than 65 outside the flu season are often hospitalized for the flu and pneumonia (55 of 1,000), and during the season, that number doubles. In our study, BMI < 21 kg/m^2 was an independent predictor of exacerbations, and among the vaccinated patients, those with BMI < 21 kg/m^2 experienced fewer exacerbations, which is in line with the study by Gajanan et al. [23]. Compared to our study, the Montserrat-Capdevila et al. [21] study from 2014 demonstrated similar results in the number and type of comorbidities, the most frequent being arterial hypertension and diabetes. We observed that vaccinated patients with comorbidities had a lower frequency of exacerbations compared to unvaccinated patients, which had been previously reported by Søgaard et al. [24]. Vardeny et al. [25], in 2016, reported that there was less worsening of heart failure in patients vaccinated against seasonal flu. In our study, among patients with multiple comorbidities (CVD, DM, and depression) none had been vaccinated, and all had experienced COPD exacerbations. These findings indicate that the effects of vaccination are of paramount importance in the subpopulation of COPD patients who are older, underweight, and have comorbidities.

The reason for a lower level of immunization (37.1%) could be due to the distrust of the effectiveness of immunization against seasonal flu worldwide [26, 27]. Zimmerman et al. [28] reported that 38% of unvaccinated persons thought that they could get the flu after vaccination. That study also showed that one-third of the unvaccinated persons were not informed by their doctors regarding vaccination, and similar findings were also observed by other authors [18]. The impact of health care workers is very important for promoting immunization but only 8.7% of the health staff in Serbia were vaccinated in the 2016–2017 season [29]. Interestingly, a recent study found that even health care workers who suffer from COPD are often unaware of their disease which could lead to a lower level of immunization [30].

There were two important limitations to this work. First, there was no laboratory confirmation of vaccination against seasonal flu. Immunization information was obtained from patients' files and medical histories but was also given by the patients. Second, there were probably varying criteria for hospitalization or observation in COPD exacerbation rates among health institutions. Despite these limitations, to our knowledge, this is the first longitudinal study investigating the effects of the flu vaccination in COPD patients in this region (Southeast Europe - West Balkan). The study showed that vaccination rates were far below the recommended, especially in high-risk patients. We believe our study is important as it underlines that in resource-limited settings there is a great area for improvement in COPD care using low-cost interventions such as seasonal flu vaccination.

Conclusion

There is a clear reduction in the number of exacerbations among vaccinated patients with COPD. Multivariate logistic regression analysis confirmed that patients with a low BMI and heart failure (NYHA ≥ 2) were independent predictors of COPD exacerbations. In these subgroups, vaccination led to a significant decrease in exacerbations. From the aforementioned, the study demonstrates that there is a great need for consistent information and education for all COPD patients with an emphasis on non-pharmacological prevention of exacerbation and progression of the disease.

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Correlation between suboptimal vitamin D concentration and secondary hyperparathyroidism in women with low-energy fractures

Korelacija nedovoljne koncentracije vitamina D i sekundarnog hiperparatireoidizma kod žena sa prelomima na malu traumu

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Abstract

Background/Aim. Osteoporosis is the most common metabolic bone disorder worldwide characterized by decreased bone strength that predisposes to an increased fracture risk, especially in postmenopausal women. Today, over 25 million people, mainly women, suffer from this metabolic disorder. In addition to genetic predispositions, hormonal disorders, lifestyle, and insufficient vitamin D levels in the blood are significant risk factors for the occurrence of osteoporosis and low-energy fractures. The aim of our study was to analyze the incidence of osteoporosis and the correlation between vitamin D deficiency and secondary hyperparathyroidism in women of different ages with low-energy fractures. **Methods.** This cross-sectional study included 559 women who were not previously treated for osteoporosis. All women were clinically examined and their anamnesis of chronic illnesses, fractures, and therapies was taken. Height and weight were measured, and body mass index (BMI) was calculated. Risk factors for osteoporosis, including the concentration of 25-hydroxyvitamin D [25(OH)D] and parathyroid hormone (PTH), were measured. Vitamin D deficiency was defined as serum level of 25(OH)D less than 30 ng/mL (75 nmol/L). **Results.** The study included a total of 559 women, of which low-energy fractures were identified in 102 women. Women with fractures were older (63.69 ± 13.88 years) compared to women without fractures (54.39 ± 14.10 years) ($p < 0.0005$). Furthermore, BMI was also higher (27.75 kg/m^2) in women with fractures compared to the other group (26.49 kg/m^2) ($p < 0.025$). Out of 102 women with fractures, 88 were postmenopausal. The most frequent fractures were the humerus and radius fractures, 11.62% (65/559), followed by femoral neck fractures, 7.15% (40/559), and body *vertebrae* fractures, 3.04% (17/559). A significant decrease of the bone mineral density (BMD) in the spinal and the femoral neck sites was observed in women with femoral neck and body *vertebrae* fractures, but not in women with humerus and radius fractures. Vitamin D deficiency did not have a significant impact on fracture incidence. The increased concentration of PTH was statistically significant in women with femoral neck and body *vertebrae* fractures. **Conclusion.** In women with all three types of low-energy fractures, compared to women without fractures, significant risk factors for osteoporosis were age, BMD, and the strength of mechanical force during fall (estimated through BMI).

Key words:
osteoporosis; risk factors; vitamin d; fractures, bone; hyperparathyroidism, secondary; women.

Apstrakt

Uvod/Cilj. Osteoporoza je metabolička bolest kostiju koju karakteriše smanjenje koštane čvrstine, posebno kod žena nakon menopauze. Danas, više od 25 miliona ljudi pati od ovog metaboličkog poremećaja. Pored genetske predispozicije, hormonski poremećaj, način života i nedovoljna koncentracija vitamina D u krvi su značajan faktor rizika za pojavu osteoporoze i preloma na malu traumu. Cilj naše studije bio je da ispitamo učestalost osteoporoze, kao i vezu između deficita vitamina D u krvi i hiperparatireoidizma kod žena različite životne dobi koje su imale prelom na malu traumu. **Metode.** U studiju preseka bilo je uključeno 559 žena koje prethodno nisu bile lečene od osteoporoze. Sve žene bile su klinički pregledane i od svih je uzeta anamneza o hroničnim bolestima, prelomima i prethodno uzimanoj terapiji. Svakoj ispitanici izmerene su masa i visina, izračunat je indeks telesne mase (ITM) i uzeta je krv za laboratorijske analize. Analizirani su faktori rizika za osteoporozu, uključujući koncentraciju 25-hidroksi vitamina D [25(OH)D] i paratireoidnog hormona. Snižena koncentracija vitamina D definisana je kao serumska koncentracija 25(OH)D manja od 30 ng/mL (75 nmol/L). **Rezultati.** Studija je

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obuhvatila 559 žena, od kojih su 102 imale prelome na malu traumu. Žene koje su imale prelom bile su starije (63,69 ± 13,88 godina) u poređenju sa ženama bez preloma (54,39 ± 14,10 godina) ($p < 0,0005$). Indeks telesne mase bio je veći kod žena sa prelomima (26,49 kg/m^2) u poređenju sa drugom grupom (24,79 kg/m^2) ($p < 0,025$). Od 102 žene sa prelomom, 88 je bilo u menopauzi. Ispitanice su najčešće imale prelom distalne podlaktice i proksimalne nadlaktice, 11,62% (65/559), zatim prelom vrata butne kosti, 7,15% (40/559) i prelom tela pršljena, 3,04% (17/559). Značajno niže vrednosti mineralne koštane gustine na kičmi i vratu butne kosti zapažene su kod žena sa prelomom vrata butne kosti i tela pršljena, ali ne i kod žena sa prelomom nadlaktice i podlaktice. Snižene koncentracije vitamina D nisu pokazale statistički podržanu značajnost kod žena sa prelomom. Povišena koncentracija paratireoidnog hormona (> 65 pg/mL) pokazala se statistički značajnom kod žena sa prelomom vrata butne kosti i tela pršljena. **Zaključak.** Kod žena koje su imale prelom kosti na malu traumu, na sve tri lokalizacije, u poređenju sa ženama bez preloma, značajni faktori rizika za osteoporozu su godine života, mineralna koštana gustina i jačina mehaničke snage tokom pada (procenjena preko indeksa telesne mase).

Ključne reči:
osteoporoza; faktori rizika; vitamin d; prelomi; hiperparatireoidizam, sekundarni; žene.

Introduction

Osteoporosis is the most common metabolic bone disorder worldwide characterized by decreased bone strength that predisposes especially postmenopausal women to an increased risk of fracture [1]. In addition to genetic predispositions, hormonal disorders, lifestyle, and insufficient vitamin D levels in the blood are significant risk factors for the occurrence of osteoporosis and low-energy fractures. Bone strength is defined by bone mineral density (BMD), which makes 60–80% of bone strength and bone quality. BMD is a measurable category, expressed as grams of mineral *per* area or volume. Bone quality refers to architecture, turnover, and mineralization. Osteoporotic bones are characterized by a reduced trabecular thickness, broken horizontal bonds, and a total reduced bone mass. Osteoporosis is characterized by the imbalance between resorption and deposition in favor of resorption [2].

The association between falls and osteoporosis have been analyzed in several population studies showing that a reduced vitamin D concentration (< 30 ng/mL) is present in 63.9% of people in the general population and the elderly patients older than 60 years of age with a hip fracture in about 97.8% of cases [2].

Today, over 25 million people, mainly women, suffer from this metabolic disorder. Osteoporosis is the cause of 1.5 million fractures annually, including 500,000 body *vertebrae* fractures, more than 250,000 hip fractures, and about 200,000 radius fractures [2, 3]. The highest prevalence of osteoporosis, about 21% of women aged between 50 and 84 years (over 12 million women), has been noted in the European Union (EU) countries (Germany, France, Italy, Spain, and the United Kingdom) [2–8]. It is estimated that about 77% of the population is without diagnosis and therapy, 14% with diagnosis but without treatment, and only 9% with diagnosis and therapy. In the elderly people, 90% of the hip fracture is associated with a fall, while vertebral fractures are most commonly spontaneous and are not associated with a fall. Rarely, a fracture caused by osteoporosis can occur on the pelvis, ribs, and distal part of the tibia and femur. The significance of hip fracture is very important because of the devastating impact on patients: 50% of patients have functional incapacity, 15–25% require long-term care and assistance, while 10–20% die during the first year of fracture [6]. In 2010, there were over 43,000 deaths related to osteoporosis in the EU, of which 50% were caused by hip fractures, 28% by spine fractures, and 22% were caused by other fractures [7]. Less than 20% of patients with low-energy fractures receive therapy in the first year of the fracture [8]. A fall is a significant cause of morbidity and mortality in the older population. About 30% of people over 65 years of age experience falls each year. Aging is one of the factors that increase the incidence of falls, especially in hospital conditions [9, 10]. Based on the previously published data, the total costs of treatment for fractures caused by osteoporosis in the EU were about 39 billion euros in 2010. Moreover, 26 billion was spent on direct costs of fracture treatments, 11 billion on long-term treatments, and 2 billion euros on fracture prevention [7]. A fall is the cause of 90–95% cases of hip fractures, 95% of radius fractures, 75% of proximal humerus fractures, and 25% of *vertebrae* fractures. Vertebral fracture is by far the most prevalent osteoporotic fracture (50%), especially in the spine region of Th 11 to L1, followed by Th 8 [2, 11]. In approximately 2/3 of cases, osteoporotic fractures of body *vertebrae* are asymptomatic. The femoral neck, located near the top of the femur bone, is also susceptible to fracture due to osteoporosis, and it is the next most common complication caused by osteoporosis. The risk of suffering a new femoral neck fracture increases multiple times after the first fracture. Thus, a third of patients with hip fractures have experienced a fracture of the other hip [12]. Moreover, localized fractures caused by osteoporosis can occur in the distal limb.

Among the most commonly used techniques, dual-energy X-ray absorptiometry (DXA) is considered the current “gold standard” for diagnosing osteoporosis. The hip is the most relevant measurement site since this site suffers the most severe fracture. In particular, the World Health Organization (WHO) classifies BMD on the basis of the T-score as normal (≥ -1.0), osteopenia (< -1.0 but > -2.5), osteoporosis (≤ -2.5), and severe osteoporosis (≤ -2.5 with a fragility fracture). The T-score represents the standard deviation (SD) of measured BMD, compared to an average BMD for a female person aged 25 years [13–16]. Osteoporotic fracture of the body *vertebrae* is defined as a reduction in the front of the craniocaudal diameter of the *vertebrae* on lateral

radiography by more than 20% (at least 4 mm), which is a radiological and rather approximate definition of fracture.

The aim of our study was to analyze the incidence of osteoporosis and the correlation between vitamin D deficiency and secondary hyperparathyroidism in women of different ages with low-energy fractures.

Methods

This cross-sectional study included 559 women not previously treated for osteoporosis. All women were presented to the Military Medical Academy in Belgrade. The study was approved by the Ethics Committee of the Military Medical Academy and informed consents were signed by the patients. All women were clinically examined, and their history of chronic illnesses, fractures, and therapies was taken. Height and weight were measured, and body mass index (BMI) was calculated (kg/m^2). Blood samples after 8-hour fasting (8 mL) were collected from all patients for laboratory analyses. Risk factors for osteoporosis, including concentration of 25(OH)D and parathyroid hormone (PTH), were measured. Serum 25(OH)D levels were measured by the competitive binding chemiluminescence immunoassay, IDS Ltd, Boldon, England (reference range 9.3–151.2 nmol/L; intra-assay precision 5.3% at 39 nmol/L, 5.6% at 67.1 nmol/L, and 6.7% at 165 nmol/L, inter-assay precision 4.6% at 40.3 nmol/L, 6.4% at 72.0 nmol/L, and 8.7% at 132 nmol/L, lower limit of sensitivity 5 nmol/L). Serum 25(OH)D concentrations were presented as nmol/L. Serum intact PTH was measured using the commercially available competitive immunoradiometric assay from DiaSorin, Stillwater, Minnesota, USA with detection limit < 0.1 pmol/L. The intra-assay coefficient of variation (CVs) ranged from 2.4% to 3.6%, and the interassay CVs ranged from 3.4% to 4.9%. Vitamin D deficiency was defined as a serum 25(OH)D level less than 30 ng/mL (75 nmol/L); a level of 25(OH)D of 21 to 29 ng *per* milliliter (52 to 72 nmol *per* liter) can be considered to indicate a relative insufficiency of vitamin D.

DXA scans were performed to measure BMD at the spine and hip with the osteodensitometer GE Lunar Prodigy Advance (GE Healthcare Lunar Co, Madison, USA), and the results were expressed as T-scores, Z-scores, and BMD (mg/cm^2). The diagnosis of osteoporosis by DXA was made following the WHO definition.

The obtained data were analyzed by appropriate statistical tests, univariate analysis of variance (ANOVA) followed by the Bonferroni test, linear regression tests and correlation. Statistical data analysis was done using the Origin Pro 8.5 program. The *p*-value < 0.05 was considered significant.

Results

The study engaged a total of 559 women, age range from 18–88 years. Low-energy fractures were identified in 18.24% (102/559) of women included in the study. Women with fractures were older (aged 63.69 ± 13.88 years) compared to those without fractures (aged 54.39 ± 14.10 years) ($p < 0.0005$). Furthermore, BMI was also higher (27.75 kg/m^2) in women with fractures compared to the other group (26.49 kg/m^2) ($p < 0.025$). Out of 102 women with fractures, 88 were postmenopausal. All subjects included in the study were divided into four groups: three groups of women based on the type of fracture and one group of women without fractures. The first group consisted of women with humerus and radius fractures, followed by the group of women with femoral neck and body *vertebrae* fractures.

In the first studied group of women with humerus and radius fractures, the fractures were identified in 11.62% (65/559) of women, of which 84.61% (55/65) were postmenopausal. Women with fractures were significantly older and had a higher BMI compared to the women without fractures (Table 1). BMD of femoral neck and spine were not statistically lower in women with fractures. We did not find a significant association of 25(OH)D deficiency and secondary hyperparathyroidism in humerus and radius fractures. Osteoporosis was diagnosed in 53.84% (35/65) of women with fractures compared to 39.16% (179/457) of women without fractures ($p < 0.034$). Osteopenia was diagnosed in 38.46% (25/65) of women with fractures compared to 19.3% (87/457) of women without fractures ($p = 0.000$).

The second group consisted of women with femoral neck fractures. The fractures were detected in 7.15% (40/559) of women, of which 90% (36/40) were postmenopausal women. Women with fractures were significantly older and had a higher BMI compared to the

Table 1

Characteristics of women with humerus and radius fractures and without fractures

Risk factors	Without fractures (457/559, 81.75%)	Humerus and radius fractures (65/559, 11.62%)	*p*-value
Age (years), mean ± SD	54.39 ± 14.10	64.42 ± 12.91	0.008
BMI (kg/m^2), mean ± SD	26.49 ± 5.12	30.54 ± 4.20	0.003
Spine BMD (mg/cm^2), mean ± SD	966 ± 177	926 ± 158	
Femoral neck BMD (mg/cm^2), mean ± SD	833 ± 150	790 ± 166	
25(OH)D < 75 nmol/L, n (%)	424 (92.77)	65/65 (100)	0.049
Increased concentration of PTH (> 65 pg/mL), n (%)	89 (19.47)	14 (21.53)	
T-score of spine, mean ± SD	-1.6 ± 1.4	-1.9 ± 1.3	
T-score of femoral neck, mean ± SD	-1.2 ± 1.2	-1.5 ± 1.4	

BMI – body mass index; BMD – bone mineral density; 25(OH)D – 25-hydroxyvitamin D; **PTH – parathyroid hormone; SD – standard deviation.**

women without fractures (Table 2). Femoral neck and spine BMD were statistically associated with the incidence of hip fracture. There was no significant difference in 25(OH)D deficiency in these two groups, while the secondary hyperparathyroidism was significantly more frequent in women with fractures. Osteoporosis was diagnosed in 55% (22/40) of women with fractures compared to 39.19% (179/457) of women without fractures (p = 0.074). Osteopenia was diagnosed in 30% (12/40) of women with fractures compared to 19.3% (87/457) of women without fractures (p = 0.145).

In the third studied group, body *vertebrae* fractures were identified in 3.04% (17/559) of women, of which 88.23% (15/17) were postmenopausal. Women with fractures were significantly older, while the BMI was not statistically associated with the incidence of body *vertebrae* fractures (Table 3). BMD of the femoral neck and lumbar spine were statistically lower when fractures are present. There was no significant difference in 25(OH)D deficiency, while the secondary hyperparathyroidism was significantly more frequent in women with fractures. An average vitamin D concentration was statistically lower in the group of women with fractures (p < 0.005). Osteoporosis was diagnosed in 88.23% (15/17) of women with fractures compared to 39.16% (179/457) of women without fractures (p = 0.0007). Osteopenia was diagnosed in 11.76% (2/17) of women with fractures compared to 19.03% (87/457) of women without fractures (p = 0.002).

energy fractures. Women with all three types of fractures were significantly older. We found that women with humerus and radius and hip fractures had statistically higher BMI compared to those without fractures. Furthermore, a significant decrease of the BMD in the spinal and the femoral neck sites was observed in women with femoral neck and body *vertebrae* fractures, but not in women with humerus and radius fractures. However, the suboptimal 25(OH)D concentrations are not the only risk factor for increased incidence of fractures. The increased concentration of PTH was statistically significant in women with femoral neck and body *vertebrae* fractures.

Six studies were included, with a total of 105,129 participants followed from 3 to 19 years. The pooled relative risk (RR) with 95% confidence interval (CI) for vertebral fracture per each standard deviation increase in BMI was 0.94 (95% CI = 0.80–1.10). Across various studies of women not adjusting for bone mineral density (BMD), there was no significant association between BMI and risk of vertebral fracture (RR = 0.91, 95% CI = 0.80–1.04; p = 0.18; n = 72,755 participants) [17]. A prospective, multinational, observational cohort study of 52,629 postmenopausal women participating in the Global Longitudinal Study of Osteoporosis in Women (GLOW) investigated the relationship between BMI, weight and height and fracture risk at multiple fracture sites. It showed that the relationship between BMI and fracture risk is site-specific. The different

Table 2

Characteristics of women with hip fractures and women without fractures

Risk factors	Without fractures (457/559, 81.75%)	Femoral neck fractures (40/559, 7.15%)	*p*-value
Age (years), mean ± SD	54.39 ± 14.10	63.70 ± 14.04	0.008
BMI (kg/m^2), mean ± SD	26.49 ± 5.12	27.98 ± 4.77	0.01
Spine BMD (mg/cm^2),	966 ± 177	902 ± 162	0.002
Femoral neck BMD (mg/cm^2), mean ± SD	833 ± 150	756 ± 149	0.00003
25(OH)D < 75 nmol/L, n (%)	424 (92.77)	39 (97.5)	
Increased concentration of PTH (> 65 pg/mL), n (%)	89 (19.47)	14 (35.00)	0.034
T-score of spine, mean ± SD	-1.6 ± 1.4	-2.3 ± 1.3	0.0002
T-score of femoral neck, mean ± SD	-1.2 ± 1.2	-1.8 ± 1.1	0.00005

For abbreviations see under Table 1.

Table 3

Characteristics of women with body *vertebrae* fractures and women without fractures

Risk factors	Without fractures (457/559, 81.75%)	Body *vertebrae* fractures (17/559, 3.04%)	*p*-value
Age (years),	54.39 ± 14.10	63.64 ± 13.48	0.008
BMI (kg/m^2),	26.49 ± 5.12	26.54 ± 4.85	ns
Spine BMD (mg/cm^2),	966 ± 177	861 ± 126	0.016
Femoral neck BMD (mg/cm^2),	833 ± 150	722 ± 138	0.003
25(OH)D < 75nmol/L, n (%)	424 (92.8)	17 (100)	0.507
Increased concentration of PTH (> 65 pg/mL), n (%)	89/457 (19.47)	11/17 (64.70)	0
T-score of spine, mean ± SD	-1.6 ± 1.4	-2.6 ± 0.7	0.009
T-score of femoral neck, mean ± SD	-1.2 ± 1.2	-2.6 ± 1.0	0.001

For abbreviations see under Table 1.

Discussion

The present study showed an association between specific risk factors, such as age, BMD, BMI, the concentration of 25(OH)D and PTH, and different low-

associations may be mediated by their effects on BMD, bone structure and geometry, and patterns of falling [18].

In the previous study that included 88 postmenopausal women with osteoporosis, 21 (23.86%) women with fractures had significantly lower 25(OH)D concentration (p < 0.01).

The obtained results implied that vitamin D deficiency in women with osteoporosis is a significant risk factor for fractures [19]. Vitamin D deficiency was also studied in 415 older women, and results showed that it was a contributing factor to different types of fractures of body *vertebrae*. This result highlights the importance of vitamin D insufficiency as a possible risk factor for older women [20]. The results of a meta-analysis of randomized controlled trials showed that vitamin D supplementation, at doses of 700 to 1000 IU per day, reduces the risk of falling in the 65-year-olds by 19%. Doses less than 700 IU in the serum do not reach the serum level of vitamin D of 60 nmol/L and cannot reduce the risk of falling in the elderly women [21, 22]. The association between hypovitaminosis D and the fracture was also studied in postmenopausal women in Tunisia. The first group consisted of 102 women with fractures and the second group of 32 women without fractures. Obtained results showed that all fractures in postmenopausal women were related to BMD and vitamin D deficiency [23]. In the study aimed to analyze the association between concomitant upper limb fractures and both vitamin D status and hip BMD in 549 women with fall-related hip fracture, the concentration of vitamin D was significantly lower in the women with concomitant fractures of both hip and upper limbs compared to the women with hip fractures ($p < 0.001$). However, hip BMD levels were not significantly different between the observed groups [24].

In a case-control study, 105 postmenopausal women with recent distal radial fracture were prospectively engaged. It showed that the mean 25(OH)D levels were similar in the fracture and control groups (44.4 ± 14.6 ng/mL *vs* 41.3 ± ng/mL; $p = 0.08$). The 25(OH)D levels were not associated with distal radial fracture and did not appear to affect the risk assessment for distal radial fracture in the postmenopausal women [25].

In a study that included 1,775 postmenopausal women with osteoporosis, the association between the femoral and total proximal BMD and the incidence of spine and hip fractures was examined. The changes in the femoral neck and total proximal BMD were statistically correlated with the incidence of hip and fragility fractures after 3 years ($p < 0.001$). However, changes in BMD on the spine did not affect the occurrence of *vertebrae* fractures [26].

A recent study has reported that serum levels of PTH > 65 pg/mL and severe vitamin D deficiency were associated with trochanteric fractures and recurrent falls as well. On the other hand, patients without the PTH response to low vitamin D levels were not repeated fallers and suffered mostly from subcapital fractures [27].

Association between fall and fracture of the femur neck was studied in more than 34,000 people who experienced a fall. Obtained results suggested that data on the previous fracture and fall should be taken into consideration during the assessment of risk for osteoporosis [28].

In order to study the relative significance of dietary calcium intake and vitamin D concentration regarding the hip BMD, 4,958 women and 5,003 men ≥ 20 years of age were examined in the United States of America. Among both sexes, BMD increased stepwise and significantly with higher vitamin D concentrations (< 50, 50–74, 75 + nM; women: $p < 0.0001$; men: $p = 0.0001$) [29].

Conclusion

We concluded that aging and low bone mineral density are important risk factors for all three types of low-energy fractures. Body mass index is an important risk factor for non-vertebral fractures, probably because of the strength of the mechanical force during the fall. The suboptimal concentration of vitamin D did not have a significant impact on the incidence of fractures. The increased concentration of PTH was statistically significant in women with femoral neck fractures and body *vertebrae* fractures.

Conflict of interest

Authors declare that there is no conflict of interest.

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Association of bone fracture type and degree of callus formation with leptin concentration in children with long bone fractures

Povezanost tipa preloma kosti i stepena formiranja kalusa sa koncentracijom leptina kod dece sa prelomima dugih kostiju

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Abstract

Background/Aim. Recent studies indicate that adipokines have an important role in bone physiology and pathology. Recent data indicate that adipokine leptin functions as a regulator of bone growth at multiple levels, systemically and locally. So far, it has been shown that leptin influences bone volume and bone mineral density in a population with metabolic and/or hormonal abnormality. Data concerning leptin values in non-obese children with fractures are scarce. **Methods.** This study included 93 non-obese children with long bone fractures (LBF), 14 children with short bone fractures (SBF), and 19 healthy children. Leptin concentration was determined in two blood samples (day 0 and day 21) and analyzed according to gender, fracture type, anatomical localization of the fracture, fracture topography, callus formation, and the healing outcome. **Results.** Children with LBF demonstrated significantly increased leptin levels compared to the control group (both day 0/day 21). In the control group, girls had significantly more leptin than boys. Leptin value was significantly influenced by anatomical localization since boys and girls with humerus fracture and girls with femur fracture had the highest average leptin concentration in the initial sample. Boys with incomplete callus formation had the highest leptin concentration (both day 0 /day 21), significantly elevated compared to boys' samples in the control group, boys' samples with an intermediary and well-formed callus, and also increased compared to the initial samples of girls with incomplete callus. Better callus formation in girls was associated with an increment of leptin concentrations in the second over the initial sample. Girls with partially and satisfactorily formed callus had significantly increased leptin concentration in the second sample (day 21) compared to the boys' group. **Conclusion.** Leptin concentration was significantly increased (both samples) in children with LBF compared to children with SBF and corresponding controls. Leptin concentration was highly influenced by gender. High blood leptin concentrations in boys or low leptin concentrations in girls immediately upon fracture could be used to identify groups of children with incomplete callus formation.

Key words:
fractures, bone; humeral fractures; radius fractures; tibial fractures; ulna fractures; child; leptin; bony callus; gender; prognosis.

Apstrakt

Uvod/Cilj. Novije studije pokazuju da adipokini imaju važnu ulogu u fiziologiji i patologiji kostiju. Takođe, najnoviji podaci pokazuju da adipokin leptin funkcioniše kao regulator rasta kostiju sistemski i lokalno. Pokazano je da leptin utiče na volumen kostiju i mineralnu gustinu kostiju u populaciji sa metaboličkom i/ili hormonskom

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abnormalnošću. Podaci o vrednostima leptina kod negojazne dece sa frakturama su oskudni. **Metode**. U ovu studiju bila su uključena 93 negojazna deteta sa prelomima dugih kostiju (LBF), 14 dece sa prelomima malih kostiju (SBF) i 19 zdrave dece. Koncentracija leptina određena je u 2 uzorka krvi (0. dana i 21. dana) i analizirana prema polu, tipu frakture, lokalizaciji anatomske frakture, topografiji frakture, formiranju kalusa i ishodu zarastanja. **Rezultati.** Deca sa LBF imala su značajno povećane nivoe leptina u poređenju sa kontrolnom grupom u oba uzorka krvi (0. dana/21. dana). U kontrolnoj grupi devojčice su imale značajno više nivoe leptina od dečaka. Na vrednost leptina značajno je uticala anatomska lokalizacija, jer su dečaci i devojčice sa prelomom humerusa i devojčice sa prelomom femura imali najveću prosečnu koncentraciju leptina u početnom uzorku. Dečaci sa nepotpuno formiranim kalusom imali su najveću koncentraciju leptina (u oba uzorka, 0. dana/21. dana), značajno višu u odnosu na kontrolne uzorke dečaka, uzorke dečaka s intermedijarnim i dobro formiranim kalusom, a takođe višu u odnosu na koncentracije leptina u početnim uzorcima djevojčica s nepotpunim kalusom. Bolje formiranje kalusa kod devojčica je bilo povezano sa povećanjem koncentracije leptina u drugom (21. dan) u odnosu na početni uzorak (0. dan). Devojčice sa delimično i zadovoljavajuće formiranim kalusom imale su značajno višu koncentraciju leptina u drugom uzorku (21. dan) u odnosu na grupu dečaka. **Zaključak**. Koncentracija leptina je značajno povećana (u oba uzorka krvi) kod dece sa LBF u poređenju sa decom sa SBF i odgovarajućim kontrolama. Koncentracija leptina je zavisna od pola. Visok nivo leptina u krvi kod dečaka ili niska koncentracija leptina kod devojčica odmah nakon preloma može se koristiti za identifikaciju grupa dece sa nepotpunim formiranjem kalusa.

Ključne reči:
prelomi; humerus, prelomi; radijus, prelomi; tibija, prelomi; ulna, prelomi; deca; leptin; kalus; pol; prognoza.

Introduction

The recovery of fractured bone represents unique biological phenomena in which the healing bone repairs itself through a complex interaction with immune cells, blood, bone marrow cells, and soft tissue cells. Finally, the healing bone again gains previous functional mechanical stability. According to classic understanding, there are three phases of this process, each represented with specific mediators. The initial, inflammatory, or the early remodeling phase, which starts immediately after bone injury, is characterized by increased secretion of IL-1, IL-6, and TNF-alpha [1, 2]. The reparative phase, which starts after a few hours upon fracture, is conducted by local and systemic production of numerous growth and differentiation factors, such as transforming growth factors (TGF), bone morphogenic proteins (BMP), platelet-derived growth factors (PDGF), fibroblast growth factor (FGF), and others [3, 4]. The third, remodeling phase, which starts with endochondral ossification, is mediated with metalloproteinases and angiogenic factors, like vascular endothelial growth factors (VEGF) and angiopoietins [5].

Data from a growing number of studies indicate that adipokines, mediators that are discovered primarily as fat and energy regulators, are also involved in the remodeling and development processes in bone physiology. Adiponectin, leptin, resistin, visfatin, and others are recognized as important regulators of bone metabolism [6]. Beyond its effects on fat tissue, leptin modulates immune response and inflammation [7]. Arguments demonstrating leptin's influence on bone tissue came both from experimental and clinical data. Mesenchymal stem cells and osteoblasts express functional leptin receptors [8, 9], and leptin ligation of specific receptor-induced cell proliferation [10] comparably intensive as seen in response to insulin live growth factor 1 (IFG-1), major anabolic bone stimulator [11]. Furthermore, it has been shown that leptin induces differentiation of mesenchymal cells to osteoblasts, increasing both messenger ribonucleic acid (mRNA) and protein levels of osteocalcin, type I collagen, alkaline phosphatase (ALP), and mineralization [12, 13]. Experimental leptin administration *in vivo* increased bone mineral density and bone length in the genetically manipulated ob/ob mice [14], significantly reduced bone fragility in male mice [11], and prevented bone loss in estrogen-deficient ovariectomized rats [15]. It seems that leptin acts directly on the bone because a study in which ovariectomized rats were treated with a virus vector expressing leptin directly in the hypothalamus showed no changes in bone parameters compared to peripheral administration of leptin [16].

Data connecting leptin with bone physiology in humans came mainly from studies of osteoporotic women. These results are still controversial; some of them indicate no leptin influence on bone metabolism [17], but others demonstrate association between leptin and bone mineral density [18, 19]. Newer results have indicated that leptin is valuable marker in osteoporosis [20], and that the increase in serum leptin level correlates with bone mineral density increase in postmenopausal women with primary knee osteoarthritis [21].

Studies in which leptin concentration was determined in children were mainly focused on the obese population, and they demonstrated a direct correlation between serum leptin values, body mass index (BMI), and obesity [22, 23]. Besides data from control children in some studies [24], we did not find any studies that investigated adipokines' influence on bone recovery after a fracture in the population of healthy children.

Methods

Participants

All children were admitted, diagnosed, and treated at the Department of Orthopedics and Joints/Bone Trauma, at

the Institute for Health Protection of Mother and Child, "Dr. Vukan Čupić", Belgrade, Serbia. This study was approved by the Ethics Committee of this Institute (No 8/26, 13/10/2015). Parents of all investigated children were informed by the attending medical doctor and gave written consent for their participation in the study.

Inclusion criteria

Nonobese children (BMI 15.0–24.0 kg/m^2), both boys and girls, aged 4 to 18 years, were included in the study. The investigated groups consisted of children with long bone fractures (LBF, n = 93), children with finger fractures – short bone fractures (SBF, n = 14), and a control group of children (control, n = 19) (Tables 1 and 2). The control group consisted of children admitted to the Clinic due to trauma of extremities but without any evidence of bone fracture.

Exclusion criteria

Obese children (BMI over 24.0 kg/m^2), children with other injuries beyond long bone fracture, children with malignant diseases, systemic connective tissue diseases, metabolic diseases/disorders, and children with congenital anomalies of the joint/bone were not included in the study.

For each patient, radiological records were made as standard two X-ray projections [anteroposterior (AP) and profile], a total of 5 records at different time intervals (immediately before the orthopedic procedure, immediately after the intervention, follow-up after 7 days, follow-up after 21 days, and after removing the cast immobilization). All fractures were classified according to type (open, closed fracture), anatomical localization (humerus, radius, radius + ulna, femur, tibia, tibia + fibula), the topography of the affected bone (fracture of the proximal or distal segment,

Table 1

Demographic characteristics of investigated children

Groups of patients	All	Boys	Girls
LBF (n = 93)			
age (years)	9.3 ± 3.4	9.3 ± 3.7	9.3 ± 3.0
weight (kg)	32.5 ± 11.8	32.7 ± 12.6	31.3 ± 11.4
height (cm)	136.7 ± 18.4	139.2 ± 20.2	135.2 ± 17.2
BMI (kg/m^2)	16.6 ± 1.9	16.3 ± 1.6	16.9 ± 2.1
SBF (n = 14)			
age (years)	9.7 ± 5.9	9.8 ± 6.6	9.6 ± 5.1
weight (kg)	32.5 ± 10.8	31.7 ± 18.6	31.3 ± 12.2
height (cm)	134.1 ± 20.0	137.1 ± 26.5	131.1 ± 14.2
BMI (kg/m^2)	16.4 ± 1.6	16.1 ± 1.7	16.8 ± 1.5
Controls (n = 19)			
age (years)	9.1 ± 5.0	9.1 ± 5.4	9.0 ± 4.6
weight (kg)	31.4 ± 10.7	31.5 ± 10.5	31.1 ± 10.9
height (cm)	134.1 ± 13.8	135.2 ± 14.5	133.0 ± 13.1
BMI (kg/m^2)	16.4 ± 1.8	16.1 ± 2.0	16.7 ± 1.5

***Note*: Results are given as mean ± standard deviation.**
LBF – long bone fractures; BMI – body mass index;
SBF – small bone fractures.

Table 2

Number (%) of LBF patients according to fracture properties

Fracture properties	All	Boys	Girls
Anatomical localization			
humerus	25 (27)	16 (30)	9 (23)
radius	7 (7)	4 (8)	3 (7)
radius + ulna	33 (35)	18 (34)	15 (37)
femur	10 (11)	5 (9)	5 (13)
tibia	11 (12)	6 (11)	5 (13)
tibia + fibula	7 (7)	4 (8)	3 (7)
Total	93 (100)	53 (100)	40 (100)
Type			
oblique	21 (23)	14 (27)	7 (18)
transverse	58 (62)	33 (62)	25 (62)
spiral	14 (15)	6 (11)	8 (20)
Total	93 (100)	53 (100)	40 (100)
Callus formation (%)			
< 25	17 (18)	9 (17)	8 (20)
< 50	43 (46)	24 (45)	19 (48)
> 75	33 (36)	20 (38)	13 (32)
Total	93 (100)	53 (100)	40 (100)

LBF – long bone fracture.

diaphysis fractures), the severity of bone injury (easy, heavy, complicated), callus formation (based on radiological analysis as < 25% – incomplete, < 50% – partial, > 75% – satisfactory) and the healing outcome (unsatisfactory, incomplete, complete). According to the data obtained from parents, children were analyzed for previous bone fractures, propensity, and history of upper respiratory tract infections, allergies, previous nursery or school residence, and feeding habits.

Study design

The study was designed as a cross-sectional investigation. From all investigated children, the first initial blood sample (on the day 0 – 0d), 2 mL of venous blood from the cubital vein, was taken within the 1st hour upon admission. Where possible, a second blood sample (on the day 21 – 21d), 2 mL of venous blood from the cubital vein, was taken after 21 days of bone fracture. After separation of the serum, samples were frozen at –70ºC until testing.

Leptin determination

The concentration of leptin was determined with a commercial flow cytometric test (LEGENDplex 13-plex Human Adipokine Panel) on a flow cytometer Beckman Coulter FC500.

Statistical analysis

Parameters of descriptive statistics were used to estimate the central tendency of data (mean, median), and to analyze group variability (standard deviation, standard error, range, 95% interval of confidentiality). The analysis between more than two groups, groups according to fracture type (oblique, transverse, spiral), anatomical localization of fracture (humerus, radius, radius + ulna, femur, tibia, tibia + fibula), and between groups according to the degree of callus formation (< 25%, < 50%, > 75% of formed callus) was performed using the one-way analysis of variance (ANOVA), with Bonferroni post-testing. Mann-Whitney test was used for all other comparisons between two independent groups. The sensitivity and specificity of leptin determination were analyzed with receiver-operating characteristic (ROC) curves constructed on the basis of values detected in the control boys' and girls' groups. All statistical analyses were done using the statistical package GraphPad Prism 5.01 (GraphPad Prism Software Inc. California, USA).

Results

Leptin concentration in children with long bone fractures vs. children with small bone fractures and control healthy children

Average leptin concentration in samples of all investigated children with long bone fractures was insignificantly increased compared to the control group in both time intervals (Tables 3 and 4). Same data were demonstrated in groups of boys and girls; both groups had an increased leptin value compared to their adequate peers. Both groups showed a slight leptin increase in the second sample. Boys with long bone fractures had insignificantly more leptin compared to boys with fractures of short bones (SBF). Leptin was significantly increased in the samples of girls compared to boys, both from the control group (Table 5).

Table 3

Average leptin concentration (ng/mL) in initial blood samples (on the day 0) of children with LBF, SBF and control children

Parameters	All	Boys	Girls
LBF	2.46 ± 1.29	2.27 ± 1.22	2.59 ± 1.16
long bone type			
oblique	2.85 ± 0.17	2.73 ± 0.19	3.01 ± 0.58
transverse	2.31 ± 0.11	2.24 ± 0.13	2.46 ± 0.84
spiral	2.14 ± 0.71	2.12 ± 0.76	2.17 ± 0.80
long bone localization			
humerus	2.72 ± 1.61	2.72 ± 1.74	2.73 ± 0.71 [b]
radius	1.61 ± 1.12	2.10 ± 0.97	1.12 ± 0.14 [b]
radius + ulna	2.27 ± 1.10	2.34 ± 1.46	2.19 ± 0.75
femur	2.76 ± 1.41	1.98 ± 0.72	3.54 ± 1.78 [b]
tibia + fibula	1.70 ± 0.34	1.88 ± 0.51	1.52 ± 0.15
Callus (%)			
< 25	3.23 ± 2.05	4.47 ± 2.05 [c, d, e, f]	1.73 ± 0.32 [f]
< 50	2.42 ± 1.53	2.27 ± 1.29 [c]	2.60 ± 1.06
> 75	2.31 ± 0.75	2.20 ± 0.77 [d]	2.56 ± 0.68
SBF	2.16 ± 0.41	1.91 ± 0.50	2.47 ± 0.29
Control	2.02 ± 0.22	1.67 ± 0.49 [a, e, h]	2.38 ± 0.41 [a]

Note: **Results are given as mean ± standard deviation.**
LBF – long bone fractures; SBF – small bone fractures.
Superscript letters mark pairs of groups that differ significantly (see Table 5).

Table 4

Average leptin concentration (ng/mL) in blood samples of children with LBF, SBF, and control children on the day 21

Groups of patients	All	Boys	Girls
LBF	2.59 ± 1.43	2.40 ± 1.26	2.85 ± 1.57
long bone type			
oblique	3.15 ± 1.81	3.63 ± 2.28	2.51 ± 0.98
transverse	2.39 ± 1.27	2.17 ± 0.95	2.99 ± 1.85
spiral	2.21 ± 0.89	1.82 ± 0.22	2.78 ± 1.41
long bone localization			
humerus	2.16 ± 0.71	2.20 ± 0.87	2.14 ± 0.53
radius	1.96 ± 0.52	2.10 ± 0.65	1.82 ± 0.38
radius + ulna	2.99 ± 1.30	2.65 ± 2.01	3.32 ± 0.59
femur	2.06 ± 0.32	2.14 ± 0.11	2.00 ± 0.54
tibia + fibula	2.23 ± 1.25	2.04 ± 0.61	2.42 ± 1.61
Callus (%)			
< 25	2.87 ± 2.01	3.92 ± 2.24 [g, h]	1.61 ± 0.33 [i]
< 50	2.27 ± 1.34	1.86 ± 0.78 [g, j]	3.13 ± 1.89 [j]
> 75	2.64 ± 0.83	2.36 ± 0.72 [k]	3.13 ± 0.83 [i, k]
SBF	2.16 ± 0.41	1.91 ± 0.50	2.47 ± 0.29
Control	2.02 ± 0.22	1.67 ± 0.49 [a, e, h]	2.38 ± 0.41 [a]

LBF – long bone fractures; SBF – small bone fractures.
Superscript letters mark pairs of groups that differ significantly (see Table 5).

Table 5

Statistical analysis of differences in leptin concentrations according to level of callus formation inside groups of boys and girls, and between groups of boys and girls

Marker	Group	Sample	Callus (%)		Group	Sample	Callus (%)	Significance
a	Boys	Control	none	*vs*	Girls	Control	none	**
c	Boys	Day 0	< 25	*vs*	Boys	Day 0	< 50	**
d	Boys	Day 0	< 25	*vs*	Boys	Day 0	> 75	**
e	Boys	Day 0	< 25	*vs*	Boys	Control	none	***
f	Boys	Day 0	< 25	*vs*	Girls	Day 0	< 25	*
g	Boys	Day 21	< 25	*vs*	Boys	Day 21	< 50	**
h	Boys	Day 21	< 25	*vs*	Boys	Control	none	**
i	Girls	Day 21	< 25	*vs*	Girls	Day 21	> 75	*
j	Boys	Day 21	< 50	*vs*	Girls	Day 21	< 50	*
k	Boys	Day 21	> 75	*vs*	Girls	Day 21	> 75	*

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

Leptin concentration in children with long bone fractures according to the type of fracture

The analysis of all investigated children showed no difference between groups with oblique, transverse, or spiral long bone fractures (Tables 3 and 4). There was a slight increase of leptin concentration in the samples after 3 weeks, irrespective of the fracture type. Girls with transverse and spiral types of fracture had an increased leptin concentration compared to boys, while boys with oblique fractures demonstrated an increased average concentration of leptin after 3 weeks.

Anatomical localization of bone fracture and leptin concentration in children with long bone fractures

Different localization of long bone fractures was associated with various leptin concentrations in investigated children (Tables 3 and 4). Initially, the highest average leptin concentration was detected in samples of girls with a femur fracture and boys and girls with a humerus fracture. After 21 days from fracture, the highest average leptin concentration was detected in children, both girls and boys, with fractures of radius and ulna. The analysis of boys' samples only demonstrated smaller variations in leptin concentration, both between different anatomical localization and between the samples and controls. On the contrary, girls' samples showed significant variations between those that suffered femur and humerus fracture as opposed to girls with radius fracture in the initial samples (Table 3).

Callus formation and leptin concentration in children with long bone fractures

Stratification of children according to the level of callus formation demonstrated an increased average leptin concentration in all investigated samples compared to the controls. Children with incompletely formed callus had the highest leptin value, both in the initial and second sample, insignificantly elevated compared to all others and controls (Tables 3 and 4).

A more detailed diversification of examinants in groups of boys and girls with long bone fractures according to the quality of formed callus showed significant differences, both inside and between the investigated groups (Tables 5). Boys with incomplete callus formation (< 25%) had the highest leptin concentration both at the time of trauma (initial sample) and after 3 weeks. The average concentration of leptin in their initial samples was significantly elevated compared to boys' control samples, samples of boys with an intermediary and well-formed callus and also increased compared to the initial samples of girls with incomplete callus. The average concentration of leptin in boys with incomplete callus formation was still significantly increased after 3 weeks compared to the second sample of boys with intermediary formed callus and the control values in boys' samples. According to callus quality, later incomplete callus formation was associated with the highest leptin values in boys' samples and with the lowest leptin concentration in girls' samples at the time of bone trauma. Leptin in the initial samples of boys and girls with an intermediary and well-formed callus did not differ significantly (Tables 3 and 4, Figure 1).

After 3 weeks, leptin concentration in boys with incompletely formed callus decreased significantly but was still higher than the level measured in other groups. Intermediary and well-formed callus was associated with insignificant leptin change. In girls' samples after 3 weeks, incomplete callus formation was followed with a further decrement of leptin concentration. On the contrary, better callus formation in girls was associated with the increment of leptin concentrations in the second over initial samples.

Girls with partially (< 50%) and satisfactory (> 75%) formed callus had a significantly increased leptin concentration in the second sample (21d) compared to the boys' group (Table 4, Figure 1).

Finally, leptin concentration was significantly increased in girls' control samples compared to boys' control samples. Leptin was increased in all samples of boys with long bone fractures compared to their control values, both at the time of trauma and 3 weeks after. Girls with incomplete callus formation had lower leptin compared to their control values, while better callus formation was associated with an increased leptin concentration (Tables 3 and 4, Figure 1).

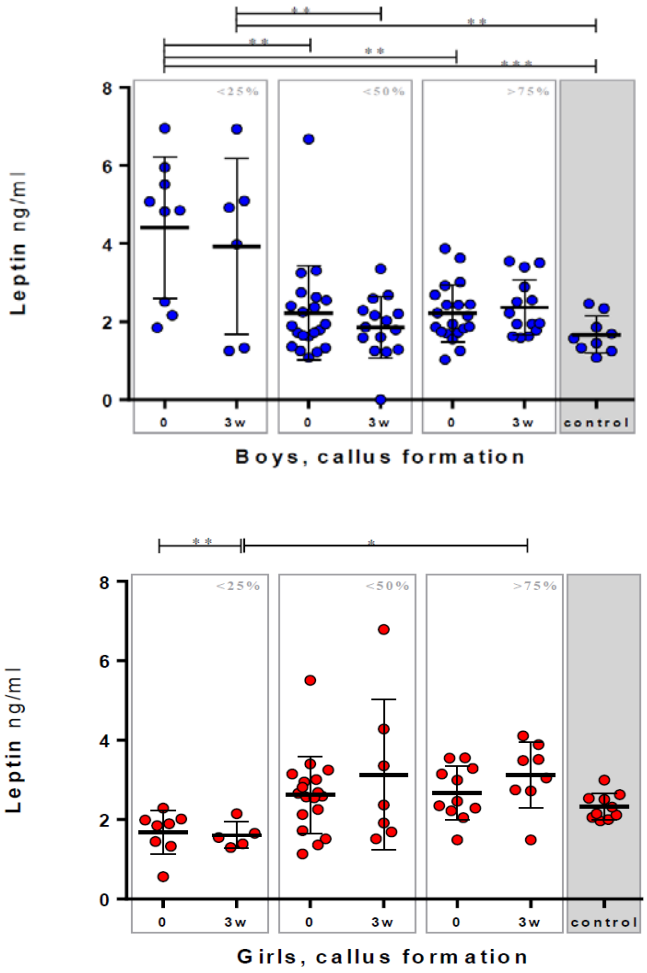

Fig. 1 – Serum concentration of leptin in children with long bone fractures according to the callus formation (*$p < 0.05$; **$p < 0.01$; ***$p < 0.001$, Mann-Whitney test). 0 – initial values (before bone fracture); 3 w – values, three weeks after bone fracture.

Leptin ROC curve analysis in children with long bone fractures

ROC curve analysis comparing the diagnostic power of serum leptin concentration in children with long bone fractures demonstrated further differences between boys and girls (Figure 2). A more uniform distribution of blood leptin levels in boys resulted in a better area under the curve (AUC = 0.777) compared to the girls' group (AUC = 0.600). Cutoff leptin concentration for 100% specificity in boys was above 2.49 ng/mL, compared to 3.00 ng/mL in girls. Leptin concentration above the cutoff value in the boys' group was significantly associated with fractures localized exclusively in the arm and those with incomplete callus formation. Interestingly, the analysis showed that high leptin concentrations were detected in the majority of girls (67%) that were later subjected to a certain type of orthopedic therapy, namely open reposition and osteosynthesis.

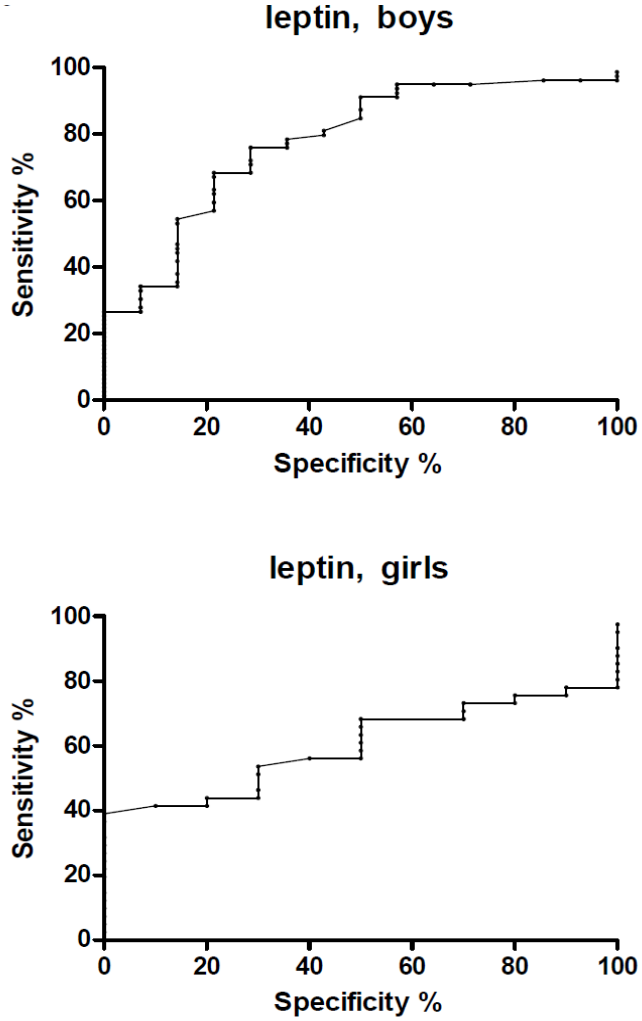

Fig. 2 – Receiver operating characteristic (ROC) analysis of serum leptin concentration in children with long bone fractures.

Discussion

Leptin is a representative adipokine produced by differentiated adipocytes, crucial in regulating energy balance and fat storage. Newer data demonstrated that leptin has almost the same relevance in numerous physiological processes, such as regulating both female and male reproduction, hematopoiesis and angiogenesis, glucoregulation, wound healing, inflammation, and osteogenesis [25]. Furthermore, leptin has a major role in chronic inflammatory mechanism characteristic for obesity and atherosclerosis [26], sepsis [27], chronic viral infection [28], and cancer [29]. Leptin exerts its action by binding specific membrane receptor (Ob-R), expressed on different cell types, including immune cells [30]. On the other hand, microbial products as lypopolysaccharide (LPS) together with inflammatory cytokines stimulate intense leptin production, indicating complex mutuality between this hormone and inflammatory processes. Mesenchymal stem cells and osteoblasts, same as immune cells, express both types of leptin receptors on their membrane [8, 9]. Leptin functions as a regulator of bone growth at multiple levels, both systemically and locally. After penetrating the blood-brain barrier and binding to specific receptors, leptin indirectly

regulates bone metabolism through the sympathetic nervous system [31–33]. There are still controversies about leptin and bone mineral density, at least regarding conditions without bone fractures. Several studies pointed out that there is no significant correlation between leptin levels and bone density [4, 34–40]. It must be emphasized that the most frequently studied population was among postmenopausal women, although from different ethnicities. Contrary to these data, Rhie et al. [41] reported a positive correlation of serum leptin level with bone density (measured as the spine and femoral bone mineral density) in a group of pre-pubertal obese girls compared to an age-adjusted group, indicating a positive role of leptin in bone metabolism.

In an experimental model where rats were subjected both to traumatic brain injury and femoral fracture, two studies demonstrated leptin significance in the process of healing bone [42, 43]. Callus formation was significantly associated both with increased serum leptin values and an abundant presence of leptin in various cells in fracture sites, osteoblasts, fibroblasts, and mesenchymal cells. The serum concentration of leptin and local expression of leptin documented by immunohistochemical analysis reached their peak in the 4th week after trauma.

There are also clinical data supporting leptin's importance in bone homeostasis. Women with hypothalamic amenorrhea demonstrate low serum leptin concentration together with decreased estrogen, growth hormones, and thyroid hormones. This hormonal disorder is associated with insufficient bone mass density, which is often the cause of bone fractures despite age [44, 45].

Interestingly, supportive therapy with synthetic leptin preparation not only improves hormonal abnormalities but also significantly mends bone density [44–47].

Increased serum leptin concentration immediately after bone trauma could be explained from several aspects. Bone fracture is accompanied by the hypermetabolic response, characterized by mobilization of free fatty acids, which cause leptin rise by the neuroendocrine mechanism [33, 48–50]. An early inflammatory response to acute bone trauma is mediated with inflammatory cytokine production, which also results in stimulating further leptin secretion [51, 52]. Another important source of posttraumatic leptin rise is the bone marrow, its adipose part (“medulla flava”), which abundantly releases leptin at the edges of a fractured bone [53]. Besides these, complex neuroendocrine response after bone trauma, represented in the release of multiple cytokines and hormones, influences the production and levels of leptin.

Several studies reported contradictory findings of improved healing in patients with long bone fractures and concomitant traumatic brain injuries, both at experimental and/or clinical levels [54–60]. Although far from clear understanding, data from these studies indicate that both serum and cerebrospinal fluid of patients with concomitant brain trauma and long bone fractures have stimulatory effects on bone healing. It is assumed that these osteoinductive factors are secreted and/or released from the injured brain. Based on these results, leptin is one of these factors.

Interesting data came from a study by Wang et al. [61]. They investigated the association between serum leptin concentration, bone density, and healing of long bone fractures in two groups of men, with fractures combined with spinal cord injury and one group with fractures only. Four and eight weeks after trauma, patients with spinal cord injury had significantly less formed callus, significantly reduced bone density, and significantly increased leptin concentration compared to the fracture-only group. Although our investigated population consisted of children with no other trauma (as one of the inclusion criteria), some children demonstrated an association of very high leptin concentration with unsatisfactory bone recovery. Namely, the group of boys with insufficiently formed callus had the highest leptin concentration, both initially after bone fracture and in the samples after 3 weeks. Gender conditionality of high leptin concentrations seems to be important because the leptin concentration was significantly decreased in girls with insufficient callus formation in both their samples.

Studies from the late 2000s demonstrated a strong association between leptin and gender, presented with increased serum leptin concentration in girls compared to boys of the same age [60–66]. This gender-based leptin domination was explained with a higher rate of leptin secretion from adipose tissue in girls, genetically determined higher subcutaneous/visceral fat ratio, and higher estrogen levels throughout puberty. Our data demonstrated a clear difference in leptin concentration between boys and girls, as well as in the control group of healthy children compared to the long bone fracture group. Girls generally have higher leptin serum concentration than boys, but this is not an absolute rule, at least not in our study. Average leptin concentration was higher in boys with an oblique type of fracture (21d), boys that had a fracture of the humerus (21d), radius (0d, 21d), combined radius and ulna (0d), and most strikingly, in boys with incomplete callus formation (0d, 21d).

Theoretically, fracture of larger bones should be associated with a more intensive systemic reaction, and a larger inner surface of the fractured bone should induce increased liberation of leptin from frontiers of lesion [53]. This is in compliance with our finding that boys and girls with humerus fracture and girls with femur fracture had the highest average leptin concentration in the initial sample. The association of high serum leptin with incomplete callus formation is even more complex to explain and leaves space for speculation. In the initial sample, the association of high serum leptin with unsatisfactory bone recovery in boys could indicate its compensatory increase as a result of the ongoing inflammatory process in incompletely formed callus. On the contrary, leptin could be considered as an adipokine necessary for bone recovery in a group of girls with insufficiently formed callus and extremely low leptin. Another possibility is that these boys and girls with incompletely formed callus had leptin abnormality before the resulting fracture. This issue should be investigated in the population of children with poor recovery after fractures of long bones. The determination of leptin concentration after 21 days from fracture demonstrated a greater variation. At that time, beyond the processes in a fractured bone, leptin concentration could be influenced by numerous factors, such as immobilization quality, the discipline of the patient, sleep, feeding, or infection.

Conclusion

Children with long bone fractures demonstrated a significant increase of leptin concentration in samples taken immediately upon bone trauma and three weeks after, compared to children with short bone fractures and corresponding controls. Leptin concentration is highly influenced by gender. High leptin concentrations in boys or low leptin concentrations in girls immediately upon fracture could be used to identify groups of children with incomplete callus formation.

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Iron status among blood donors deferred due to low haemoglobin level

Ispitivanje statusa gvožđa kod davalaca krvi vraćenih zbog niskog nivoa hemoglobina

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Abstract

Background/Aim. Haemoglobin (Hb) determination is a routine part of the blood donor selection process. Previously reported studies have revealed that iron deficiency is common in frequent donors. This prospective investigation was aimed at examining iron status among blood donors with low circulating Hb and evaluating correlation between Hb values determined by capillary methods and those obtained by reference method from venous blood count (BC), as well as ferritin level. **Methods.** Between February 2017 and December 2018, 200 consecutively recruited regular blood donors with low Hb, aged 19 to 64 years (median 39), were included. Hb level was determined using the copper sulphate method, the HemoCue capillary method, and also from venous blood within the complete blood count (CBC) test. Plasma ferritin was determined turbidimetrically. **Results.** In 42.7% of men and 57.3% of women, ferritin concentration was low (p = 0.008). The relative numbers of males and females, with levels < 12 μg/L (p = 0.023) or > 50 μg/L (p = 0.022), differed. Comparison of the values obtained with the capillary methods with reference Hb levels obtained from the CBC test showed that the copper sulphate procedure gave false fails in 10.5% of cases (p < 0.001). Hb values from HemoCue were significantly correlated with Hb values from the CBC test, but no correlation was observed between ferritin levels and Hb levels determined by both capillary method. **Conclusion.** Low ferritin was observed in 51.5% of Serbian blood donors deferred due to low Hb. Based on our results, the determination of the algorithm in the iron deficiency detection is necessary, while the capillary method (HemoCue) represents a more convenient method for Hb testing prior to blood donation.

Key words:
blood donors; hemoglobins; iron; ferritin; clinical laboratory techniques.

Apstrakt

Uvod/Cilj. Određivanje nivoa hemoglobin (Hb) je rutinski deo selekcije dobrovoljnih davalaca krvi. Prethodno publikovane studije pokazale su da se nedostatak gvožđa javlja kod redovnih davalaca krvi. Cilj ove prospektivne studije je bio da se utvrdi status gvožđa kod davalaca kod kojih je pre davanja utvrđen nizak nivo Hb i da se proceni stepen korelacije kapilarnih metoda, sa referentnom metodom određivanja Hb iz venske krvi, kao i sa i nivoom feritina. **Metode.** U periodu od februara 2017. do decembra 2018. godine, bilo je uključeno 200 dobrovoljnih davalaca sa niskim nivoima Hb, starosne dobi 19–64 godine (medijana 39 godina). Hb je određivan primenom metode bakar sulfat, kapilarnom metodom „HemoCue“, i iz venske krvi u sklopu određivanja kompletne krvne slike (KKS). Nivo feritina određivan je primenom turbodimetrijske metode. **Rezultati.** Nizak nivo feritina utvrđen je kod 42,7% muškaraca i 57,3% žena (p = 0,008). U odnosu na nivo feritina < 12 μg/L, odnosno > 50 μg/L, zabeležena je značajna razlika između polova (p = 0,023, odnosno p = 0,022). Poređenje vrednosti Hb dobijenih kapilarnim metodama u odnosu na referentne vrednosti Hb, određene iz KKS, pokazalo je da metoda s bakar sulfatom daje lažno niske vrednosti Hb kod 10,5% slučajeva (p < 0,001). Vrednosti Hb dobijene metodom „HemoCue-a“ značajno su korelirale sa vrednostima Hb iz KKS, dok korelacija između nivoa feritina i Hb, određenog pomoću obe kapilarne metode, nije uočena. **Zaključak.** Kod 51,5% naših davalaca krvi koji su vraćeni zbog niskih vrednosti Hb utvrđen je snižen nivo feritina. Na osnovu ovih rezultata neophodno je odrediti algoritam za detekciju nedostatka gvožđa, dok je kapilarna metoda (HemoCue) pogodnija metoda za testiranje Hb pre davanja krvi.

Ključne reči:
krv, davaoci; hemoglobini; gvožđe; feritin; laboratorijske tehnike i procedure.

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Introduction

Blood donor selection is one of the most important measures used in blood transfusion centres in order to ensure blood safety [1–5]. Determining haemoglobin (Hb) level is a routine part of the donor selection process in order to ensure high quality of the red cell concentrates collected and, at the same time, protect the health of a potential donor [1, 6]. However, iron deficiency has been found to be common in frequent blood donors, particularly women, while the Hb level measured may not accurately reflect iron stores [7–10]. Determining Hb levels during the donor selection process using capillary methods distinctly saves time and expenditure without endangering blood donors [11]. Data from the forum investigation [12] state that the capillary copper sulphate procedure is used to determine Hb level before donation in three European countries (the United Kingdom, Spain, and Croatia); in one country, both capillary methods are employed, and in the remaining European countries, the capillary photometric method is preferred, most often HemoCue®. In Serbian transfusion centres, we use the capillary copper sulphate method, considering the minimum acceptable Hb level to be > 135 g/L for male and > 125 g/L for female donors.

Low circulating Hb is globally the most common reason for deferral of prospective blood donors [13–16]. During 2017, the total rate of all deferrals in our centre was 14.2%, among which 30.5% were due to low Hb level. Considering the relatively high proportion of deferrals due to low Hb among our voluntary blood donors, we performed a prospective study aimed to determine iron status among such blood donors. In order to indicate the most appropriate procedures in our blood transfusion centre, the second aim was to evaluate the agreement between values for Hb obtained using capillary methods with reference hemoglobin values obtained from the complete blood count (CBC) test and their association with ferritin level.

Methods

Between February 2017 and December 2018, this prospective study included 200 consecutively recruited regular blood donors (102 male and 98 female) with low Hb, aged 19 to 64 years (median 39). The term regular blood donor was defined as someone who had routinely donated blood in the same centre within the previous 2 years, in accordance with minimum time intervals [17]. The total number of previous blood donations in the study group was 3,340, and the median time interval between them was 5.3 months. All study participants were recruited from the Blood Transfusion Institute of Serbia, Belgrade. In the Serbian transfusion service, copper sulphate is used as the standard method for Hb determination in a finger prick sample. This method was applied during the recruitment of the study participants. Another capillary method for a finger prick sample was applied, and Hb was measured photometrically using the HemoCue® Hb 201+ System (Mission Viejo, Ca, USA). In addition, Hb was determined in a venous blood sample taken into EDTA tubes, using a haematology analyzer (Horiba Medical ABX Micros ES 60 blood counter, France). Ferritin concentration was determined turbidimetrically in a second venous blood sample collected in plastic tubes for biochemical analysis, using test reagents from Linear Chemicals, Spain. The reference range designated as normal by the manufacturer was 20–250 μg/L for males and 20–200 μg/L for females. In particular, a plasma ferritin level < 12 μg/L is defined as absent iron stores (AIS), and ferritin concentration < 29 μg/L is defined as depletion [18]. These were used in statistical analyses in order to point to the iron status of blood donors deferred due to low Hb.

All study participants were approached with the standard questionnaires for voluntary blood donors. Data related to age, gender, number of previous donations, date of the last donation, dietary regime, and health problems since the last donation, such as haemorrhage, menstrual bleeding, fever, respiratory infection, and stomach problems, were analyzed.

Institutional approval for the study was granted by the Local Research Ethics Committee (EK-number 7767/2016) in accordance with the internationally accepted ethical standards. Each participant signed the informed consent form.

Statistical methods

The Statistical Package for Social Sciences 20.0 for Windows (SPSS Inc., Chicago, Illinois, USA) was used for statistical analysis. The Mann-Whitney U-test, Fisher's exact test, and Pearson Chi-squared (χ^2) test were employed to evaluate differences in the demographic and haemoglobin/ferritin test characteristics among the study participants. The probability $p < 0.05$ was considered statistically significant.

Spearman's tests were used for correlation analysis, and $p < 0.01$ was taken as statistically significant.

Results

Referring to their state of health, 25% of our subjects reported fatigue, 6.5% nutrition changes (diets, fasts), 7% bleeding episodes, and 61.5% good health. Considering the lower limit of the reference range to be 20 μg/L, a decreased ferritin level was found in 103/200 (51.5%) subjects (Table 1).

Table 1

Demographic characteristics of the patients

Characteristics	Value
Total number of patients, n (%)	200 (100)
Age (years), median (range)	39 (19–64)
Gender (M/F), n	102/98
Total number of previous donations, n	3,340
Interval from last donation (months), median (range)	5.3 (3.0–9.5)
Condition status, n (%)	
fatigue	50 (25)
diet	13 (6.5)
recently bleeding	14 (7)
good condition	123 (61.5)
Patients with low ferritin level, n (%)	103 (51.5)

M – male; F – female.

Equal frequencies of reduced Hb levels for the genders were indicated with all three methods ($p = 0.323$, $p = 0.796$, and $p = 0.422$, respectively). Statistically significant differences were found between male and female donors regarding age, the total number of donations, and the time interval following the previous donation ($p < 0.001$). Concerning ferritin levels, 44 (42.7%) males and 59 (57.3%) females had low ferritin concentrations, the difference being statistically significant ($p = 0.008$) (Table 2). Moreover, the median plasma ferritin for females (15.0 μg/L) was lower than that for males (22.5 μg/L; $p = 0.002$). For ferritin levels defined as < 12 μg/L or > 50 μg/L, significant differences between male and female donors were also observed ($p = 0.023$ and $p = 0.022$, respectively) (Table 3).

Comparison of the results from the capillary methods with the reference Hb values obtained from BC showed that the copper sulphate method pointed to false fails in 21 (10.5%) donors, 13 male and 8 female ($p < 0.001$). Values for Hb obtained with the capillary HemoCue method were positively correlated with those from BC ($p < 0.001$) (Figure 1) but not with plasma ferritin level ($p = 0.393$) (Figure 2). However, in relation to ferritin level defined as AIS (< 12 μg/L), a significant correlation was observed in the case of the capillary HemoCue method and ferritin level in female donors ($p < 0.001$), but not in male donors ($p = 0.148$). There were no significant correlations when results for Hb levels using the copper sulphate method were compared with those from BC ($p = 0.209$) or ferritin level ($p = 0.855$).

Table 2

Haemoglobin (Hb) and ferritin levels in relation to gender

Parameters	Male n = 102	Female n = 98	*p*
Age (years), median (range)	43 (22–64)	30 (19–64)	< 0.001
Total number of previous donations, median (range)	16 (2–120)	5 (2–48)	< 0.001
Interval from last donation (months), median (range)	4 (3–9.5)	5 (4–17)	0.001
Number with low Hb level by copper sulphate, n <135 g/L M, < 125 g/L F	102 (100)	98 (100)	0.323*
Number of patients with low Hb level by HemoCue, n	90 (88.2)	89 (90.8)	0.796
Number of patients with low Hb from BC, n	89 (87.2)	90 (91.8)	0.422
Hb level (g/L), median (range)			
by HemoCue	128 (92–145)	118 (84–136)	< 0.001
from BC	127 (93–149)	114 (83–136)	< 0.001
Number of patients with low ferritin, n (%)	44 (42.7)	59 (57.3)	0.008
Ferritin level (μg/L), median (range)	22.5 (4–209)	15 (2–349)	0.002

M – male; F – female; BC – blood count; *p* (Mann-Whitney test; *Fisher test). Difference with regard to number with low Hb – copper sulphate vs. BC ($p < 0.001$). The reference ranges for ferritin designated as normal by the manufacturer were 20–250 μg/L for males and 20–200 μg/L for females.

Table 3

Iron status in relation to the different ferritin levels and gender

Ferritin level (μg/L)	Male (n = 102) n (%)	Female (n = 98) n (%)	*p* (Pearson χ^2 test)
<12	30 (29.4)	44 (45)	0.023
< 29	32 (31)	28 (28.5)	0.659
30–50	16 (15.6)	15 (15)	0.942
> 50	24 (23.5)	11 (11.5)	0.022

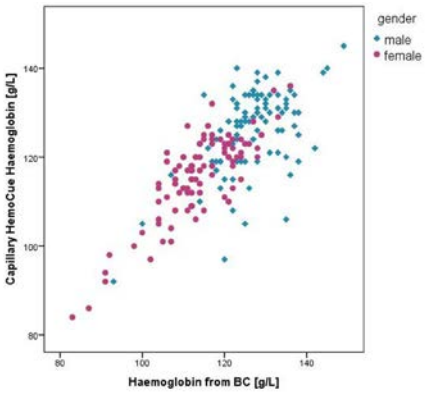

Fig. 1 – Correlation of haemoglobin (Hb) level measured by the HemoCue capillary method with reference Hb from BC (for correlation analysis, Spearman's tests were used, $p < 0.001$).

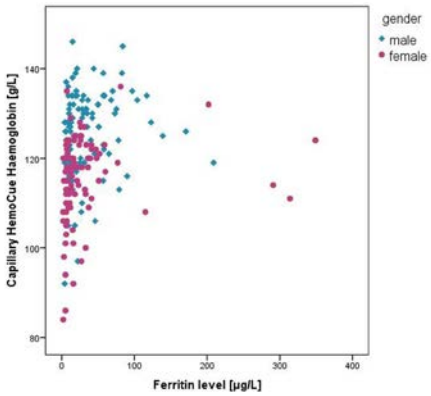

Fig. 2 – Correlation of haemoglobin (Hb) level measured by the HemoCue capillary method with ferritin level (for correlation analysis, Spearman's tests were used, $p = 0.393$).

Discussion

Our evaluation of iron status among blood donors deferred due to low Hb level showed that half of them had decreased plasma ferritin concentrations, while a quarter of them reported fatigue prior to donation. The frequency of low ferritin levels was significantly higher in females, and a greater proportion of women had ferritin levels lower than 12 μg/L, defined as AIS.

A negative correlation between blood donation and the total iron reserve was demonstrated over three decades ago [19, 20] and has been confirmed in recently published studies [7–10]. Our results indicated the same. Based on all these data, additional measures are needed to improve the health of blood donors in order to prevent iron deficiency anemia. Several studies have aimed to determine the best approach for preventing iron deficiency in blood donors [21–27]. Their findings point to a need to re-evaluate current criteria in blood donor selection concerning the interval between donations, optimal testing strategy for measuring iron stores, and the necessity of iron supplementation [28, 29]. A plasma ferritin level below 12 μg/L, defined as AIS, or less than 29 μg/L, defined as depletion, requires deferral of blood donation and confirmation [28]. In Italy, donors with iron deficiency are invited to lengthen the interval between whole blood donations. In Denmark, if plasma ferritin is less than 15 μg/L, the donor is sent 100 iron tablets by post. When the ferritin level is 15–40 μg/L, the donor is given 60 iron tablets for supplementation [12].

In our study, 60% of male and 73.5% of female donors had ferritin values that required deferral of blood donation for three to six months. We should point out that almost half of our low Hb female donors (45%) were observed in the group with ferritin < 12 μg/L, while only 11.5% of them had a ferritin concentration that indicated optimal iron status.

It should be emphasized that donor selection criteria have generally been adopted through health system regulations, but practice according to the current approach in vary among different transfusion centers [30, 31]. Although the demand for blood supplies is decreasing in many countries due to the implementation of Patient Blood Management, in Serbia, this demand is constantly increasing because of the aging of the population and a relatively high incidence of malignant diseases. Another problem of the Serbian society is the emigration of young people during the last few decades for economic reasons. Therefore, defining a rational, evidence-based donor selection process in our transfusion services is crucial in order to minimize unnecessary rejection of voluntary blood donors and to prepare them for future donation so that continuous blood supply is maintained.

Determining Hb levels before donation with two capillary methods and comparing them with the value obtained from BC showed that the copper sulphate method gave false fails in 10.5% of donors. On the other hand, the capillary HemoCue method provided Hb values comparable with those obtained from BC. Moreover, in female donors with ferritin below 12 μg/L, there was a significant association with Hb level obtained with the capillary HemoCue method. Regarding that, every effort should be made to improve the accuracy of Hb screening in our centres. This implies a recommendation that the capillary photometric method should be introduced to replace the copper sulphate procedure for Hb determination. In addition, taking our results for iron status into consideration, determining the algorithm for detecting iron deficiency together with iron supplementation should be included in the Serbian transfusion services.

Our study has limitations that should be considered. Namely, the number of participants was relatively small. Concerning the study design, no selection was made during recruitment; thus, all donors with identified low Hb were included. Therefore, differences among donors regarding age and number of previous donations could have an implication that resulted in recall bias. However, this is the first study conducted among Serbian voluntary donors, and the results obtained need to be confirmed in further investigations involving a larger number of participants.

Conclusion

For determining hemoglobin prior to donation, the HemoCue capillary method is more suitable than the copper sulphate method. A low plasma ferritin concentration was observed in 51.5% of Serbian blood donors deferred due to low hemoglobin. Our findings indicated that determining the algorithm for detecting iron deficiency is necessary. However, in addition to the optimal testing strategy for measuring iron stores, the necessity of iron supplementation and treatment of iron deficiency anemia among blood donors are extremely important.

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Early childhood caries predictors

Prediktori nastanka karijesa u ranom detinjstvu

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Abstract

Background/Aim. Dental caries represents a health problem of all ages, but it is especially significant in the earliest age of children. Early childhood caries can develop immediately after the eruption of deciduous teeth, resulting in many complications, such as tooth extraction, which is reflected in the quality of life and health. Early childhood caries is influenced by a large number of predictors. Considering that the data on predictors for the development of deciduous teeth caries are scarce, the aim of this paper was to determine the prevalence of deciduous teeth caries and detect the predictors for the development of early childhood caries. **Methods.** Our survey included 117 children of both genders, aged 3 to 6 years, from the territory of Kosovska Mitrovica and Zvečan. All respondents, including children and parents, were surveyed, and thus general data was obtained. A dental examination was performed on all children. The current state of dental health was inserted into special research cards that were in line with the recommendations of the World Health Organization. Those predictors of dental health level that were statistically significant in models of univariate ordinal logistic regression at the significance level of 0.05 were included in the model of multivariate ordinal logistic regression. **Results.** According to the full structure, there were 49.6% of boys and 50.4% of girls. Healthy teeth were present in 25 (21.4%) children, 19 had reversible changes (16.2%), while 73 (62.4%) children had irreversible carious changes on teeth. The most important detected predictors were sweets consummation, sleeping with a baby bottle, 6 and more meals per day, frequent usage of medical syrups, child's daily tooth-brushing frequency, child's independent tooth-brushing frequency, usage of non-fluoride toothpaste, a lack of professional fluoride application, and mouth breathing. **Conclusion.** This research contributed to the detection of a large number of predictors that are most important for the development of early childhood caries. In order to reduce the risk of caries development, it is necessary to act in more spheres. This certainly requires planning and implementing preventive dental programs.

Key words:
child, preschool; dental caries; food and beverages; habits; oral hygiene; preventive dentistry; risk factors.

Apstrakt

Uvod/Cilj. Karijes zuba predstavlja problem u svim uzrastima, ali je posebno veliki kod dece u najmlađem dobu. Karijes mlečnih zuba se može javiti nakon nicanja zuba, dovodeći do nastanka komplikacija i ekstrakcije zuba, što se odražava na kvalitet života i zdravlje. Na nastanak karijesa u ranom detinjstvu utiče veliki broj prediktora. S obzirom na to da su podaci o prediktorima nastanka karijesa mlečnih zuba oskudni, cilj rada bio je da se utvrdi rasprostranjenost karijesa na mlečnim zubima i otkriju prediktori nastanka karijesa u ranom detinjstvu. **Metode.** Istraživanjem je bilo obuhvaćeno 117 dece starosti od 3 do 6 godina, oba pola, sa teritorije Kosovske Mitrovice i Zvečana. Svi ispitanici su bili anketirani, uključujući i decu i roditelje, čime su dobijeni njihovi opšti podaci. Kod sve dece je izvršen stomatološki pregled, a zatečeno stanje zdravlja zuba uneseno je u posebne istraživačke kartone koji su bili u skladu sa preporukama Svetske zdravstvene organizacije. U model multivarijantne ordinalne logističke regresije bili su uključeni oni prediktori stepena zdravlja zuba koji su u modelima univarijantne ordinalne logističke regresije bili statistički značajni na nivou značajnosti od 0,05. **Rezultati.** Prema polnoj strukturi bilo je 49,6% dečaka i 50,4% devojčica. Zdrave zube imalo je 25 (21,4%), reverzibilne promene imalo je 19 (16,2%), dok je ireverzibilne promene

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na zubima imalo 73 (62,4%) dece. Kao najznačajniji prediktori pokazali su se konzumacija slatkiša, spavanje sa flašicom, 6 i više obroka dnevno, česta upotreba medicinskih sirupa, učestalost dnevnog pranja zuba, učestalost samostalnog pranja zuba, upotreba pasti bez fluora, izostanak profesionalne fluorizacije i disanje na usta. **Zaključak.** Istraživanje je doprinelo otkrivanju velikog broja prediktora nastanka karijesa. Kako bi se smanjio rizik od nastanka karijesa, neophodno je delovati u više sfera, što svakako zahteva planiranje i sprovođenje preventivnih stomatoloških programa.

Ključne reči:
deca, predškolska; zub, karijes; hrana i piće; navike; usta, higijena; stomatologija, preventivna; faktori rizika.

Introduction

Oral health has a high level of impact on general health and thus has a significant impact on the quality of life [1]. Dental caries represents a chronic infective disease of extremely high prevalence in modern society and is created by the interaction of a large number of predisposing factors (predictors) over time. The most important predictors are the presence of acidogenic microorganisms capable of metabolizing free carbohydrates, frequent ingestion of carbohydrates, the quality of dental tissue, the quality and composition of saliva, behavioral characteristics of the individual, as well as numerous psychological, biological, ecological, and socioeconomic factors. All these factors are predictors for the occurrence of caries and its development [2].

Dental caries represents an extremely large problem at all ages, but it is especially prominent in children in the earliest years of life [3]. Deciduous teeth compared to permanent teeth are completely different in many aspects including morphology, histology, mineralization, and chemical composition; they are also different in the physiological aspect. The main differences exist due to the smaller percentage of mineral matter and lesser thickness of the deciduous teeth enamel. Studies have shown that deciduous teeth are significantly rich in organic matter, while the percentage of calcium and phosphorus is much lower than in permanent teeth [4]. Dental caries can occur very early on deciduous teeth, even 3–6 months after eruption [5].

The occurrence of dental caries in young children is influenced by a large number of predictors. In the foreground, breastfeeding, bottle feeding, too frequent intake of saccharose-rich food (sweet and salty snacks, hidden carbohydrates), as well as the lack of adequate oral hygiene by parents or caregivers are on the forefront [6]. Other risks include dietary habits, the transmission of cariogenic pathological microorganisms, especially from parents or caregivers, hygienic habits, family values, tradition, and lifestyle [7]. The carious lesion does not occur suddenly; it passes through numerous development stages, starting from non-cavitated carious lesions in the form of white or brown spots to the presence of cavitation of varying degrees. Carious lesions are also followed by many complications such as the loss of vitality of dental pulp, presence of periapical pathological processes, abscesses, and teeth extraction, which are the worst outcomes [8].

Because of the complex dental caries etiology and the lack of data relating to the earliest age, it is considered that any additional research contributes to the detection of the risk factors for caries occurrence in the youngest age and, therefore, the possibility of prevention [9]. Moreover, one of the conclusions of an internal seminar of the European Academy of Paediatric Dentistry, held in 2016 in Brussels, whose main topic was dealing with the treatment of early carious lesions of deciduous and young permanent teeth, was that more studies which include deciduous dentition are necessary for more precise data [10].

Since the data on predictors for caries development of the deciduous teeth are scarce, the aim of this study was to determine caries distribution on deciduous teeth and assess possible predictors for dental caries development in very young children.

Methods

The sample for this study consisted of 117 children of preschool age. The children were between 3 and 6 years old, of both genders, attending preschools on the territory of Kosovska Mitrovica and Zvečan, regardless of where they live. After informing the management of preschool institutions about the public health significance of this research and obtaining their permission for its implementation, children's parents, who also participated in the study, were informed in written form. The study included only children of parents who agreed to participate in the research. All respondents were interviewed, and the state of the health of their deciduous teeth was determined by an objective clinical examination.

The study was implemented according to the principles of good clinical practice, following domestic and international legal and ethical norms as well as the Helsinki Declaration of Human Rights, and with the approval of the Ethics Committee of the Faculty of Medicine of the University of Priština/Kosovska Mitrovica, Serbia.

The survey provided general data on respondents, including examined children and parents. Data were obtained regarding pregnancy, nutrition during pregnancy, fluoride use, and regular visits to the dentist, as well as data on the state of parents' oral health, including their habits and level of awareness of oral health. The survey included data related to the history of infants and young children, with special emphasis on nutrition, bad habits,

oral hygiene, tooth eruption, use of fluoride preparations, visits to the dentist, and early medical history.

Dental examination was performed using a dental mirror and a graduated Community Periodontal Index (CPI) probe, with artificial light sources. These methods are fully in line with the recommendations of the World Health Organization (WHO) for examining oral tissues. Accordingly, the WHO advises using a plane dental mirror, artificial light sources (in blue-white colour spectrum) for better detection of structural changes on oral tissues, and using the CPI probes on white or chalky spots on the teeth, which are not soft to touch [11]. Sterile cotton pads and portable air-dryers were used to remove soft deposits from the teeth and dry them.

In terms of obtaining data on caries prevalence, a decay-missing-filled (DMF) index (DMF for deciduous dentition) was also used.

Dental caries prevalence data were recorded in a research card that was specially designed for this study. The research card was in line with the WHO recommendations [12]. The following nosological units were diagnosed and numerically recorded: a healthy tooth (0), a tooth with a present reversible carious lesion, i.e. a white or dark spot (1), and a tooth with clinically determined cavitation or irreversible carious lesion (2).

Statistical analysis of data

Statistical data analysis was performed using IBM SPSS Statistics 22 (IBM Corporation, Armonk, NY, USA). The results were presented as a frequency (numerical and percentage). The χ^2 test and the Fisher test were used to examine the differences between the nominal data. A Kruskal-Wallis test was used to process ordinal data. Numerical data with normal distribution were analyzed by variance analysis. Predictors that influence the level of tooth health were analyzed by a univariate and multivariate ordinal logistic regression. All *p*-values less than 0.05 ($p < 0.05$) were considered statistically significant.

Results

The survey included 117 children. The children were 3 to 6 years old. According to the gender structure, there were 49.6% of boys and 50.4% of girls. The sociodemographic characteristics of the examinees are shown in Table 1. Healthy teeth were present in 25 (21.4%) children, 19 (16.2%) had reversible changes (white and brown spots), while 73 (62.4%) children had irreversible carious lesions on their teeth cavitated lesions.

The peculiarities of the pregnancy that were considered concerning the normal course of pregnancy, illnesses and the use of drugs in pregnancy, the type of mother's nutrition, and regular visits to the dentists, did not affect the level of oral health of children.

Based on the obtained results, the characteristics of nutrition, such as the length and frequency of breastfeeding, or the duration of baby bottle usage, showed that they did not affect the level of oral health. Children with irreversible teeth lesions had significantly 6 and more meals per day ($p = 0.001$). The degree of teeth lesions was associated with more frequent consumption of sweets ($p < 0.001$). Children with irreversible carious lesions were more often breathing on their mouth during sleep ($p < 0.001$), which can be seen in Table 2.

Table 1

Sociodemographic characteristics of children and their parents

Variables	Total	Healthy teeth	Carious lesions		*p*
			reversible	irreversible	
Gender					
male	58 (49.6)	11 (44.0)	9 (47.4)	38 (52.1)	
female	59 (50.4)	14 (56.0)	10 (52.6)	35 (47.9)	0.768
Age of the child (years)	4.7 ± 0.5	4.6 ± 0.5	4.7 ± 0.5	4.8 ± 0.5	0.241
Professional qualifications (mother)					
secondary education	61 (52.1)	14 (56.0)	10 (52.6)	37 (50.7)	
highly qualified	56 (47.9)	11 (44.0)	9 (47.4)	36 (49.3)	0.899
Professional qualifications (father)					
secondary education	66 (64.1)	13 (59.1)	10 (55.6)	43 (68.3)	
highly qualified	37 (35.9)	9 (40.9)	8 (44.4)	20 (31.7)	0.527

All values are given as number (percentage) of children or mean ± standard deviation.

Table 2

Dietetic regime and habits of children

Variables	Total	Healthy teeth	Carious lesions		*p*
			reversible	irreversible	
Nutrition of infants					
natural	95 (81.2)	22 (88.0)	17 (89.5)	56 (76.7)	0.364
artificial	22 (18.8)	3 (12.0)	2 (10.5)	17 (23.3)	
Breastfeeding duration					
to 20 min	92 (83.6)	21 (87.5)	15 (83.3)	56 (82.4)	0.933
longer than 20 min	18 (16.4)	3 (12.5)	3 (16.7)	12 (17.6)	
Daily breastfeeding intakes					
4–8	88 (79.3)	23 (95.8)	14 (77.8)	51 (73.9)	0.055
over 8	23 (20.7)	1 (4.2)	4 (22.2)	18 (26.1)	
Children slept on breast	23(20.5)	4 (16.7)	2 (11.1)	17 (24.3)	0.474
Baby bottle usage					
until 6 months	8 (9.4)	3 (15.8)	0 (0.0)	5 (9.1)	
until 12 months	51 (60.0)	13 (68.4)	10 (90.9)	28 (50.9)	0.093
longer	26 (30.6)	3 (15.8)	1 (9.1)	22 (40.0)	
Six and more meals a day	38(32.5)	3(12.0)	2(10.5)	33(45.2)	0.001
Sweets					
no	10 (8.5)	6 (24.0)	2 (10.5)	2 (2.7)	
rarely	80 (68.4)	19 (76.0)	16 (84.2)	45 (61.6)	< 0.001
often	27 (23.1)	0 (0.0)	1 (5.3)	26 (35.6)	
Sleeping with baby bottle	56 (47.9)	3 (12.0)	5 (26.3)	48 (65.8)	< 0.001
Mouth breathing	39(33.3)	4(16.0)	1(5.3)	34(46.6)	< 0.001

All results are shown as number of children (percentage).

Fluoride tablets were given to children in only 5.1% of cases and did not show any effects on the oral health of the children ($p = 0.261$). Children with healthy teeth and reversible lesions were significantly more frequently subjected to professional fluoridation by dentists (24.0% and 21.1%) compared to children with irreversible tooth changes (8.2%) ($p < 0.001$). Children with irreversible carious lesions were significantly more commonly using a toothpaste without fluoride ($p < 0.001$). Four percent of children with healthy teeth brushed their teeth once a day; reversible carious lesions were present in 15.8% of children and irreversible carious lesions in 43.8% of children, which was a statistically significant difference ($p < 0.001$). Sixty percent of children with irreversible carious lesions brushed their teeth independently, without parental control ($p < 0.001$) (Table 3). Eight percent of children with healthy teeth used medical syrups more than 5 times a year; 5.3% of children were with reversible tooth changes, and 31.5% of children were with irreversible tooth changes, which is a statistically significant difference ($p = 0.007$). Twelve percent of children with healthy teeth were sleeping with a baby bottle; 26% of children were with reversible carious lesions changes, and 66% of children were with irreversible carious lesions, which was a statistically significant difference ($p < 0.001$).

Table 3

Fluoride usage and hygienic regime of children

Variables	Total	Healthy teeth	Carious lesions		*p*
			reversible	irreversible	
Use of fluoride tablets in children	6 (5.1)	0 (0.0)	0 (0.0)	6 (8.2)	0.261
Professional fluoride application	16 (13.7)	6 (24.0)	4 (21.1)	6 (8.2)	< 0.001
Use of non-fluoride toothpaste while brushing	36 (30.8)	3 (12.0)	1 (5.3)	32 (43.8)	< 0.001
Frequency of tooth brushing - daily					
once a day	35 (29.9)	1(4.0)	3 (15.8)	31 (42.5)	
twice a day	73 (62.4)	20 (80.0)	15 (78.9)	28 (52.1)	0.001
three and more	8 (6.8)	4 (16.0)	1 (5.3)	3 (4.1)	
Child brushes teeth independently					
no	19 (16.2)	6 (24.0)	8 (42.1)	5 (6.8)	
sometimes	53 (45.3)	18 (72.0)	11 (57.9)	24 (32.9)	< 0.001
yes	45 (38.5)	1 (4.0)	0 (0.0)	44 (60.3)	
Use of medical syrups during a year					
never	18 (15.4)	6 (24.0)	4 (21.1)	8 (11.0)	
rarely	73 (62.4)	17 (68.0)	14 (73.7)	42 (57.5)	0.007
more than 5 times	26 (22.2)	2 (8.0)	1 (5.3)	23 (31.5)	
Teeth eruption in proper time	103 (88.0)	24 (96.0)	16 (84.2)	63 (86.3)	0.393

All results are shown as number (percentage) of children.

The model of multivariate ordinal logistic regression included tooth health level predictors in children who were statistically significant at a significance level of 0.05 in models of a univariate ordinal logistic regression. The model contains 9 predictors listed in Table 4. The entire model (with all predictors) was statistically significant ($p < 0.001$) (Table 4).

In the model of multivariate ordinal logistic regression, statistically significant predictors of the tooth health level in children were a higher incidence of sweets consumption ($p = 0.023$), a lesser frequency of tooth-brushing per day ($p = 0.049$), and a higher frequency of autonomous tooth-brushing in children ($p = 0.049$).

Table 4

Multivariate ordinal logistic regression with the level of dental health as the dependent variable

Variables	Multivariate ordinal logistic regression	
	OR (95%CI)	*p*
Sweets	4.4 (1.2–15.6)	0.023
Sleeping with baby bottle	2.3 (0.8–7.1)	0.142
6 and more meals per day	3.2 (0.8–12.7)	0.106
Medical syrups usage frequency	1.8 (0.8–4.0)	0.176
Daily frequency of child's tooth-brushing	0.3 (0.1–1.0)	0.049
Frequency of child's independent tooth-brushing	3.1 (1.4–7.0)	0.006
Tooth-brushing with non-fluoride toothpaste	3.6 (0.9–13.8)	0.064
Professional fluoride application lack	2.9 (0.8–10.9)	0.117
Mouth breathing	2.6 (0.7–9.0)	0.135

OR – odds ratio; CI – confidence interval.

Discussion

Deciduous teeth caries, known as early childhood caries (ECC) [13], represents a serious health problem worldwide, especially in developing countries [14]. This is supported by the data from Tušek et al. [15, 16], who showed that the prevalence of caries in Vojvodina was 46.64%, while it was 62.4% in children involved in this study. Their data show that the distribution of caries between sexes is different, which in our sample was not the case. This study analyzed the prevalence of ECC and its relationship with behavioural, nutritional, and hygienic factors as the risk factors for disease presence. ECC prevalence of 62.4% in northern Kosovo was on a higher level of moderate values of prevalence compared to Sweden (low prevalence - 11.4%) [17] and the recorded lower prevalence in children from Southwest China [18], (85%), and in Canada (high prevalence - 98%) [19]. Studies from the neighboring countries stated that 40.29% of the children had ECC and our data values were on a higher level, which is troubling [15]. Reversible carious lesions were found in 16.2% of mostly younger children, which is connected to the fact that the level of ECC is directly dependent on age [16]. If treatment is not approached in a timely manner and in the right way, not only can ECC negatively affect all aspects of the child's health, but it can also significantly increase the possibility of caries in permanent dentition [17, 18]. ECC is a disease for which the treatment is not just restorative. Prevention of caries based on predictor detection and risk management are important concepts in epidemiological studies of ECC [19]. According to the classic understanding of the etiology of early childhood caries, it is considered a multifactorial disease that includes bacterial, dietary, and host-related determinants, intertwined with numerous sociological and environmental factors [20, 21]. Studies that have dealt with detecting predictors for the development of deciduous teeth caries have yielded very diverse results connected with breastfeeding, feeding on the bottle [22], and inadequate dietary habits in very young children [23]. The presence of cariogenic microorganisms [24], the frequency of consumption of beverages and food [25, 26], the level of oral hygiene [27, 28], the level of parental education, but also the involvement of parents in the maintenance of oral hygiene of children, as well as the use of fluoride toothpaste [29] play an extremely important role. Each additional examination and detection of the predictors for the occurrence of ECC contributes to more effective prevention of this disease, which was the main idea of this study.

The oral health of preschoolers is often a neglected aspect of general health in children. These children are a unique part of the population that is extremely receptive to caries, primarily because of their dependence on parents, limited manual abilities, and lack of understanding the oral health importance. There are data from previous studies that clearly show that parents play a central role in preserving the oral health of children, starting from developing awareness of how important oral health is, including brushing children's teeth, forming quality nutrition, and eliminating bad habits [29, 30].

It can be said that at the right age, habits related to tooth -brushing and proper nutrition can be easily transferred from parents or caregivers to children with everyday usage. Additionally, in order to improve oral hygiene and quality of life, education for both parents and children is necessary [31].

Observing the individual effects of predictors, it can be concluded that a large number of examined variables show significance, which certainly supports the notion that caries is a disease of extremely complex etiology.

The results show that the parental level of education is not related to the health of children's teeth, which corresponds to data obtained in our previous research [32]. Our results related to the impact of maternal education are completely identical to the results of the study [33], which has

shown that there is no correlation between the level of mother's education and the state of children's oral health. Certain data obtained by this study are in contradiction with the results of some other authors. This is primarily related to the frequency of caries in girls and boys. The obtained results showed that the incidence of reversible and irreversible carious lesions cannot be related to the child's gender, which is contrary to the results obtained in Canada [34] and India [35]. Moreover, according to the data obtained, the time of birth, pregnancy, and the use of drugs during pregnancy showed no effects on caries incidence in children. The data obtained regarding the use of fluorinated water, which is related to lower incidence of caries, appear in full accordance with the results obtained in the United States [36].

The results of this research, related to the child's nutritional habits, showed that caries incidence, whether the lesion was reversible or irreversible, was on a high level in those children who used a baby bottle in their nutrition, had more than six meals a day, were sleeping with a baby bottle, and who consumed extra sugar or beverages, which is in full accordance with the data obtained in other studies [37–39]. For comparison, similar data were obtained in the research by Tušek et al. [15] for a region of the province of Vojvodina. Although all of these factors are extremely important and work together, special importance is given to the effect of sleeping with a baby bottle. The results showed that the presence of caries in children who practiced this habit was at a high level, which can be explained by decreased salivation during sleep, which reduces the self-cleaning mechanism, and by a constant inflow of nutrients into the oral cavity, which results in a constant decrease of pH value of the oral cavity. Therefore, it is considered that children should only use water during the night with a baby bottle [40].

A review of the results obtained in this study related to oral hygiene maintenance in children provides a clear picture that adequate oral hygiene significantly determines the possibility of carious lesion occurrence in preschool children. First of all, this refers to the beginning of tooth-brushing, parental involvement in the maintenance of oral hygiene, the use of fluoride toothpaste, and the frequency of tooth washing, which coincides with the results obtained in other studies [41]. However, although all of these factors are significant and affect one another, special importance is given to the fact that the higher frequency of caries is visible in those children who lack the help of parents in maintaining oral hygiene, which is closely related to the underdeveloped manual abilities in preschool age, but also underdeveloped awareness of the importance of oral health. It is a fact that parents play a central role in the lives of children and in acquiring habits; however, it is also considered that teachers and babysitters should be educated about the importance of oral health, proper nutrition, and oral hygiene maintenance. This is because they are considered to have an extremely good effect in creating awareness of healthy nutrition and hygiene of mouth and teeth [32]. It should also be noted that changing children’s and parents' habits is an extremely challenging task, especially for pediatric and preventive dentists [32]. The obtained data show that professional fluoridation significantly contributes to the preservation of teeth health, which is in line with the results of similar research [42, 43].

Mouth breathing, which is one of the bad habits, is also one of the caries predictors [44]. The exact mechanism is not completely clarified, but this is considered a risk factor due to the dehydration of the teeth surface and the absence of the protective effect of the saliva [45].

Studies carried out in Turkey also indicate an increased caries risk in children who have often used medical syrups. Higher caries prevalence in children who use medical syrups more often is connected to industrial sugar presence in syrups [46]. The connection between higher ECC prevalence and the frequent usage of medical syrups is also shown in data collected in Vojvodina [15, 16].

With every degree of more frequent sweets consumption, children had a 4.4 times higher chance of worsening every teeth health level, with the control of all other factors in the model. With every minor degree of tooth-brushing, children had a 70% higher chance of worsening any teeth health level, with the control of all other factors in the model. With every degree of more frequent autonomous tooth-brushing, children had a 3.1 times more chance for worsening every stage of teeth health, with the control of all other factors in the model.

Conclusion

The results of this research, as well as the results of many other studies worldwide, undoubtedly show that caries is quite prevalent in preschool children, and its occurrence is influenced by a large number of interconnected predictors. However, the most important predictors were sweets consumption, daily frequency of tooth-brushing, frequency of independent tooth-brushing in children, frequency of medical syrups use, breathing on the mouth, sleeping with a baby bottle, tooth-brushing with fluoride-free toothpaste, six or more meals per day, as well as the absence of professional fluoridation of teeth.

Predictors identified as the most important, i.e. nutritional and factors related to oral hygiene maintenance and bad habits, are readily subject to correction with adequate access and education. This can, therefore, significantly affect the oral health of this sensitive population, which would result in reducing early childhood caries incidence and providing a better quality of life.

In order to reduce the risk of caries development, it is necessary to act in more spheres, starting by educating parents, children, and preschool institutions staff, which certainly requires planning and implementing preventive dental programs.

Conflict of interest

The authors have no actual or potential conflicts of interest to disclose, related to this manuscript.

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Forensic characteristics of chest injuries among subjects who died in road traffic accidents

Forenzičke karakteristike povreda grudnog koša kod osoba poginulih u saobraćajnim nesrećama

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Abstract

Background/Aim. In road traffic accidents, chest injuries are a critical factor since death usually occurs as a result of injuries to the heart and lungs, flail chest, pneumothorax, exsanguination, etc. The aim of this study was to analyze the most vulnerable subjects in road accidents, as well as the most frequent and most significant types of chest injuries sustained by different categories of subjects, and to examine the relevance of certain injuries or factors to outliving the injuries. **Methods.** The autopsy, the retrospective, and the cross-sectional study were performed at the Clinical Centre of Kragujevac, Department of Forensic Medicine and Toxicology. The study included all participants in road traffic accidents on the territory of Kragujevac and the surrounding area who died from the injuries sustained from the accidents or due to complications of the injuries during the period from 2001 to 2016. The subjects were divided into the following groups: pedestrians, motor vehicle drivers, front-seat passengers, back-seat passengers, bicyclists, motorcyclists, and tractor drivers. The occurrence of injuries in three regions of the body (chest, head, and abdomen) was analyzed in all the subjects. **Results.** The study included 525 subjects who died due to the injuries sustained in traffic accidents, which makes up to 38.4% of the total number of 1,366 autopsy cases covered by the study period. The average age of the subjects was 52 ± 19 years. The study sample consisted of 391 (74.5%) men and 134 (25.5%) women. The most vulnerable subjects were pedestrians (220, i.e. 41.9%), followed by motor vehicle drivers (98, i.e. 18.7%), front-seat passengers (79, i.e. 15%), motorcyclists (39, i.e. 7.4%), bicyclists (38, i.e. 7.2%), back-seat passengers (29, i.e. 5.5%) and tractor drivers (22, i.e. 4.2%). Chest injuries were identified in 408 subjects (77.7%), while the most frequent type of injury was rib fracture, observed in two-thirds of the sample. Out of the total number, 291 (55.4%) subjects died at the scene of the accident or on their way to the hospital, while 234 (44.6%) of them outlived injuries for a certain period of time. Drivers exhibited the highest risk of dying at the scene, while bicyclists outlived their injuries more frequently. **Conclusion.** Chest injuries are very common in subjects who died at the scene of the road traffic accident.

Key words:
accidents, traffic; thoracic injuries; autopsy; cause of death.

Apstrakt

Uvod/Cilj. Povrede grudnog koša su značajne u saobraćajnom traumatizmu jer su čest uzrok smrti zbog povreda vitalnih organa – srca i pluća, torakalnog kapka, pneumotoraksa, povreda velikih krvnih sudova itd. Cilj ove studije bio je analiza vulnerabilnosti različitih učesnika u saobraćajnim nezgodama, utvrđivanje najučestalijih i najznačajnijih povreda grudnog koša kod različitih učesnika, kao i da se ispita značaj pojedinih povreda ili faktora za nadživljavanje povreda. **Metode.** Urađena je retrospektivna, autopsijska, studija preseka na materijalu Službe za sudsku medicinu i toksikologiju Kliničkog centra Kragujevac. U studiju su bili uključeni svi učesnici saobraćajnih nezgoda koji su umrli od zadobijenih povreda ili njihovih komplikacija na teritoriji grada Kragujevca sa okolinom, u periodu od 2001. do 2016. godine. Učesnici su bili podeljeni u sledeće grupe: pešaci, vozači motornih vozila, suvozači, putnici u vozilu, biciklisti, motociklisti i vozači traktora. Kod svih učesnika je analizirano prisustvo regionalnih povreda (grudnog koša, glave i abdomena). **Rezultati.** Studijom je bilo obuhvaćeno 525 učesnika čija je smrt bila posledica saobraćajne nezgode, što čini 38,4% od ukupnog broja obdukcija (1 366). Prosečna starost učesnika iznosila je 52 ± 19 godina. Muškaraca je bilo 391 (74,5%), a žena 134 (25,5%). Najvulnerabilniji učesnici su bili pešaci (220 ili 41,9%), vozači motornih vozila (98 ili 18,7%), suvozači (79 ili 15%), motociklisti (39 ili 7,4%), biciklisti (38 ili 7,2%), putnici (29 ili 5,5%) i vozači traktora (22 ili 4,2%). Povreda nekog dela grudnog koša je bila prisutna kod 408 (77,7%) učesnika, a najučestalija povreda je bila prelom nekog rebra, koja je evidentirana kod dve

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trećine učesnika. Od ukupnog broja, 291 (55,4%) učesnik je stradao na licu mesta ili na putu do odgovarajuće zdravstvene ustanove, dok je 234 (44,6%) učesnika nadživelo povrede neko vreme. Vozači su imali veću šansu da umru na licu mesta, dok su biciklisti češće nadživljavali povrede. **Zaključak.** Povrede grudnog koša su veoma česte u slučajevima smrtnog ishoda na licu mesta u saobraćajnim nezgodama.

Ključne reči:
udesi, saobraćajni; toraks, povrede; autopsija; smrt, uzrok.

Introduction

Out of all causes of death on the global scale, road traffic trauma is currently ranked ninth, albeit with a tendency to grow [1]. It is estimated that in 2030 it might come to be the fifth leading cause of death in the world, with 2.4 million deceased per year [1]. In road traffic accidents (RTAs), 1.25 million people die every year, which is more than 3,000 death instances per day [1]. The World Health Organization (WHO) has identified pedestrians, bicyclists, and motorcyclists as the most vulnerable categories of participants in RTAs, given the increased likelihood of sustaining a serious injury in accidents compared to drivers of motorized vehicles and passengers inside [1].

Blunt chest trauma (BCT) makes up 10–15% of all injuries and is identified as the main cause of death in approximately 25% of the subjects, while in approximately another 25% of instances, it is the contributing factor of deathly outcomes [2, 3]. In RTAs, chest injuries are the most numerous ones and are observed in approximately 60–80% of the instances [4–6], while in terms of causes of death, these injuries are found in approximately 15% of the casualties [3]. The most frequently injured are men in their 40s [5, 7].

RTA injuries to the chest remain a significant cause of morbidity and mortality. In the majority of RTAs, injuries to the chest are combined with the trauma of other regions of the body, such being the head or abdomen [5]. A rib fracture is one of the most common BCTs [5, 8]. Studies have reported that as the number of rib fractures increases, the number of complications increases, too, as well as mortality rates [8, 9], which is particularly pronounced in the elderly population [10]. Deceleration thoracic injuries are of great relevance (traumatic aortic transection, flail chest, myocardial contusion), most commonly occurring in motor vehicle collisions, and are classified as life-threatening injuries [11].

There are merely a few studies dealing with the frequency and the consequences of chest injuries in RTAs on the territory of the Republic of Serbia. A study conducted between 1973–1988 on the territory of Serbia concluded that chest injuries were observed in 62% of subjects involved in RTAs [12]. Thirty years later, RTAs remain the most common cause of chest injuries on the territory of Serbia (approximately 64%) [5]. Nikolić [13] and Nikolić et al. [14] demonstrated that chest organ injuries are most frequently concomitant RTAs and that there is a high probability that the fatally injured one is the motor vehicle driver, with a number of concomitant injuries of the thoracic aorta, heart, and pericardium, as well as with fractured thoracic cage bones.

Daskal et al. [15] proved that severe chest injuries were most common in front-seat passengers when compared to drivers and other passengers in the vehicle.

The aim of this study was to analyze the most vulnerable RTA participants, the frequency and types of chest injuries among different subjects, as well as to examine the relevance of certain specific chest injuries and factors in outliving the injuries.

Methods

The epidemiological (observational) analytical, retrospective and cross-sectional autopsy study was conducted at the Clinical Centre of Kragujevac, Department of Forensic Medicine and Toxicology, for sixteen years (between 2001 and 2016). The study included 525 RTA subjects (38.4% of 1,366) who died from sustained injuries or complications of the injuries on the territory of Kragujevac and the surrounding area. The subjects were included in the study according to the police reports, requests for forensic autopsy obtained from competent courts or prosecutions, or, in rare instances, based on retrospectively acquired hetero-anamnestic information. The data on the sustained injuries were obtained by analyzing the autopsy records, as well as the available medical documentation. The study did not include children under 14 years of age and subjects shorter than 150 cm.

Subjects were analyzed in terms of gender, age, and type of involvement in traffic accidents. The frequency of accidents was observed depending on the month of the year and the day of the week. According to their type of involvement in RTAs, all subjects were classified into the following groups: pedestrians, motor vehicle drivers, front-seat passengers, back-seat passengers, bicyclists, motorcyclists, and tractor drivers. Chest injuries were classified into the following groups: bone fractures of the chest (sternum, thoracic spine, unilateral or bilateral rib fractures), lung injuries (unilateral or bilateral: contusions, lacerations, disruptions), cardiac injuries (rupture of the pericardium, myocardial rupture, contusion, cardiac tamponade), aortic injuries (haematoma of adventitia of the aorta, aortic rupture), pneumothorax (unilateral or bilateral) and haemothorax. In addition, the analysis included the simultaneous occurrence of chest injuries and head or abdominal trauma. When considering the outliving period, the subjects were divided into two categories: subjects who

died at the scene of the accident and those who outlived their injuries for a certain period of time.

Statistical Package for Social Sciences – SPSS for Windows, Version 20 (SPSS Inc. Chicago, IL) was used for data processing. All numerical variables were tested with the Kolmogorov-Smirnov and Shapiro-Wilks tests for normal distribution as criteria for further implementation of parametric methods. According to the data distribution, appropriate descriptive statistics were employed (mean values with standard deviation or median with interquartile range – IQR). To estimate the differences between variables that exhibited parametric distribution, the Student's *t*-test was used. The Pearson's chi-squared test (with Yates correction) and Kruskal-Wallis test were applied in variables that showed a nonparametric distribution. The analysis of the connection of dichotomous dependent variables (immediate death *versus* death after a period of outliving) and observational independent variables (injuries of certain body parts) was carried out by binary logistic regression. The results were presented as crude odds ratio (OR) with a 95% confidence interval (CI). After applying corrections for the influence of other independent and confounding variables, the acquired data were expressed as adjusted OR with a 95% CI. The *p*-value of 0.05 has been considered significant.

This study was conducted with the approval of the Ethics Committee of the Clinical Centre of Kragujevac (18/10/2016, No 01/13221).

Results

The study included 525 subjects, whose average age was 52.4 ± 19.4 years (ranging from 16 to 92 years). There were 391 (74.5%) men, aged 51.3 ± 19.6 years (ranging from 16 to 92 years), and 134 (25.5%) women, aged 55.8 ± 18.5 years (ranging from 16 to 84 years). The ratio of male and female subjects was approximately 3:1 (χ^2 = 125.808; df = 1; p = 0.000). On average, women were older than men (t test = -2.370; p = 0.019), which has been shown in all age groups (χ^2 = 8.735; df = 3; p = 0.033). A detailed overview of the results is provided in Table 1.

Among all the different categories of subjects, tractor drivers were the oldest (Kruskal-Wallis test = 100.34; df 7; p = 0.000). In addition, tractor drivers were the subjects with the highest percentage of identified chest injuries. The distribution of subjects according to RTA participation, their age, and occurrence of chest injuries is provided in Table 2.

Depending on the month of the year, the largest number of fatal RTAs was recorded in September (63, i.e. 12%), followed by August and October (60, i.e. 11.4%), while the fewest accidents took place in March (20, i.e. 3.8%) and February (25, i.e. 4.8%) (χ^2 = 47.274; df = 11; p = 0.000). When observing the days of the week, the highest number of fatal RTAs was recorded on Wednesdays (92, i.e. 18%), followed by Fridays and Sundays (85, i.e. 16%), while the fewest such accidents took place on Mondays (47, i.e. 9%) (χ^2 = 20.853; df = 6; p = 0.002).

In the study sample, chest injuries were identified in 408 (77.7%) out of 525 subjects, followed by head injuries (skull fractures or intracranial bleeding), which were observed in 339 (64.6%) out of 525 subjects. Abdominal injuries were found in 201 (38.3%) out of 525 subjects. Almost half of the subjects (241 out of 525, i.e. 45.9%) suffered from concomitant chest and head injuries, while one-third of them (188 out of 525, i.e. 35.8%) sustained concomitant chest and abdomen injuries. Simultaneous occurrence of chest, head and abdominal injury was identified in 103 out of 525 (19.6%) instances (χ^2 = 193.830; df = 1; p = 0.000).

Table 1
The distribution of road traffic accidents (RTA) subjects by age ranges and sex

Age ranges (years)	Men n (%)	Women n (%)	Total n (%)
16–35	97 (24.8)	21 (15.7)	118 (22.5)
36–50	76 (19.4)	19 (14.2)	95 (18.1)
51–65	104 (26.6)	44 (32.8)	148 (28.2)
> 65	114 (19.2)	50 (37.3)	164 (31.2)
Total	391 (100)	134 (100)	525 (100)

Table 2
The distribution, age, and presence of chest injuries according to types of road traffic accidents (RTAs) participation

RTA participants	Total number (%)	Age (years), median (IQR)	Presence of chest injuries, n (%)
Pedestrians	220 (41.9)	62 (51–74.5)	172 (78.2)
Motor vehicle drivers	98 (18.7)	43.5 (32–55)	81 (82.7)
Front-seat passengers	79 (15)	47 (26–62)	63 (79.7)
Back-seat passengers	29 (5.5)	53 (26–64)	20 (69)
Bicyclists	38 (7.2)	58.5 (49–67)	26 (68.4)
Motorcyclists	33 (7.4)	34 (24–40)	23 (69.7)
Tractor drivers	22 (4.2)	63.5 (54–71)	20 (90.9)

IQR – interquartile range.

Subjects who died at the scene in RTAs were more likely to have sustained chest injuries, which proved to be statistically significant (χ^2 = 14.99; df = 1; p = 0.000). Regional types of injuries analyzed by using the binary logistic regression are presented in Table 3.

From the total number of 525 subjects, 291 (55.4%) died at the scene of the accident or on their way to the hospital (average age 48.8 ± 19.3 years), while 234 (44.6%) out of 525 subjects outlived their injuries (average age 56.9 ± 18.7 years) for a certain period of time. Subjects who outlived their injuries were of older age (t-test = 4.89; p = 0.000). In 46 (8.8%) out of 525 subjects, the main cause of death was a complication of injury (pneumonia, sepsis, thromboembolism, or fat embolism). Subjects who outlived the injuries differed in terms of the kind of their RTA involvement: pedestrians and bicyclists outlived their injuries more frequently, while motor vehicle drivers were more likely to die at the scene (Table 4).

The most frequent type of chest injury was the fracture of the ribs on both sides of the chest. The odds for subjects who died at the scene in RTAs to have sustained fracture of the ribs on both sides of the chest is approximately two times higher than for the other subjects who outlived the accidents for some time. The presence of different rib cage fractures analyzed by using binary logistic regression is presented in Table 5.

The association of lung parenchymal injuries with rib fractures was statistically significant (χ^2 = 109.563; df = 6; p = 0.000). The most common type of lung injury was bilateral lung contusion (122 out of 525 subjects, i.e. 23.2%), and odds that the subjects who died at the scene to have sustained this type of injury were twice as high as for the rest of the subjects (OR 1.816; 95% CI 1.189–2.773). The odds of occurrence of bilateral lung lacerations in those who died instantly were approximately four times higher than in the other subjects (OR 4.098; 95% CI 2.017–8.324).

Subjects who died at the scene of the accident had frequently sustained injuries of the heart and the aorta (Table 6).

Table 3

Regional types of injuries analyzed by using the binary logistic regression

Types of injuries	Subjects who died at the scene, n (%)	Subjects who outlived the injuries, n (%)	Crude OR (95% CI)	p-value
Chest injury	245 (60)	163 (40)	1.778 (1.076–2.940)	< 0.05
Chest and head injury	134 (55.6)	107 (44.4)	0.397 (0.231–0.684)	< 0.05
Chest and abdomen injury	128 (68.1)	60 (31.9)	2.320 (1.523–3.533)	< 0.05
Chest, abdomen, and head injury	73 (70.9)	30 (29.1)	3.208 (1.467–7.016)	< 0.05

OR – odds ratio; CI – confidence interval.

Table 4

The distribution of subjects according to the type of road traffic accidents (RTAs) participation and outliving period

RTA participants	Subjects who died at the scene, n (%)	Subjects who outlived the injuries, n (%)	p-value (χ^2 test)
Pedestrians	101 (45.9)	119 (54.1)	< 0.05
Motor vehicle drivers	73 (74.5)	25 (25.2)	< 0.05
Front-seat passengers	49 (62.0)	30 (38.0)	> 0.05
Back-seat passengers	17 (58.6)	12 (41.4)	> 0.05
Bicyclists	12 (31.6)	26 (68.4)	< 0.05
Motorcyclists	24 (58.3)	15 (41.7)	> 0.05
Tractor drivers	15 (68.2)	7 (31.8)	> 0.05

Table 5

Presence of different rib cage fractures analyzed using binary logistic regression

Place of fracture	**Subjects who died at the scene, n (%)**	**Subjects who outlived the injuries, n (%)**	**Crude OR (95% CI)**	***p*-value**
Sternum	1 (0.3)	3 (1.3)	0.266 (0.027–2.569)	> 0.05
Ribs on one side	36 (12.4)	43 (18.4)	0.627 (0.388–1.014)	> 0.05
Ribs on both sides	198 (68)	114 (48.7)	2.241 (1.571–3.198)	< 0.05
Ribs on both sides with sternum	119 (40.9)	57 (24.4)	2.148 (1.471–3.139)	< 0.05
Thoracic spine	48 (16.5)	14 (6)	3.104 (1.665–5.786)	< 0.05

OR – odds ratio; CI – confidence interval.

Table 6

The distribution of subjects according to the presence of heart and aortic injuries and outliving period

Heart and aortic injuries	Subjects who died at the scene, n (%)	Subjects who outlived the injuries, n (%)	p-value (χ^2 test)
Heart tamponade	6 (2.1)	0	< 0.05
Heart contusions	13 (4.5)	6 (2.6)	> 0.05
Rupture of pericardium	14 (4.8)	3 (1.3)	< 0.05
Heart destruction	39 (13.4)	0	< 0.05
Hematoma of adventitia aortae	90 (30.9)	12 (5.1)	< 0.05
Aortic rupture	68 (23.4)	8 (3.4)	< 0.05

The odds of occurrence of pericardial rupture in those who died at the scene were approximately five times higher than in the other subjects (OR 4.730; 95% CI 1.341–16.685), while the odds of occurrence of aortic rupture in those who died at the scene were approximately nine times higher than in the other subjects (OR 9.388; 95% CI 4.404–20.015). Aortic adventitial hematoma was significant, as well, hence the odds of occurrence of this type of injury were approximately six times higher for the subjects who died at the scene of the accident (OR 6.075; 95% CI 2.058–17.929).

Haemothorax was identified in 206 (39.2%) out of 525 subjects, while in 203 (98.5%) out of 525 of them, combinations of haemothorax and rib fractures occurred, and haemopneumothorax was identified in 85 (41%) out of 525 subjects. The connection between haemothorax and fractured ribs was statistically significant (χ^2 = 94.605; df = 1; p = 0.000). Unilateral and bilateral pneumothorax were often accompanied by rib fractures, which was statistically significant (χ^2 = 15.237; df = 1; p = 0.000 and χ^2 = 8.921; df = 1; p = 0.003). The occurrence of unilateral pneumothorax, bilateral pneumothorax, and haemothorax in subjects dying at the scene of the accident was statistically significant (Table 7).

Numerous risk factors (the existence of different chest injuries or demographic features) proved to be in significant connection with immediate deathly outcomes of RTAs in two groups of subjects. After adjusting the results for gender and occurrence of other chest injuries, only a few of the above-mentioned risk factors remained statistically significant to be associated with immediate deathly outcomes. The multivariate logistic regression resulted in strong associations (Cox & Snell R square 0.336, Nagelkerke R square 0.450, Hosmer-Lemeshow, χ^2 = 6.876, df = 8, p = 0.550, overall model accuracy of 77.1%). Details are presented in Table 8.

Table 7

Presence of pneumothorax/hemothorax, rib fractures and the outliving period

Pneumothorax/Hemothorax		Rib fractures		Subjects who died at the scene, n (%)	Subjects who outlived the injuries, n (%)	*p*-value (χ^2 test)
		Yes	No			
One–sided pneumothorax	Yes	53 (10.1)	1 (0.2)	46 (85.2)	8 (14.8)	< 0.05
	No	344 (65.5)	127 (24.2)			
Both–sided pneumothorax	Yes	36 (6.8)	1 (0.2)	30 (81.1)	7 (18.9)	< 0.05
	No	361 (68.8)	127 (24.2)			
Hemothorax	Yes	203 (38.7)	3 (0.6)	160 (77.7)	46 (22.3)	< 0.05
	No	194 (36.9)	125 (23.8)			

Table 8

Risk factors and time of death analyzed using multivariate logistic regression

Risk factors (independent variables)	Adjusted OR (95% CI)	*p*-value
Gender	1.588 (0.932–2.707)	> 0.05
Older than 51 years	0.334 (0.164–0.680)	< 0.05
Pedestrians	0.666 (0.321–1.383)	> 0.05
Motor vehicle drivers	2.703 (1.380–5.295)	< 0.05
Front-seat passengers	1.370 (0.511–3.668)	> 0.05
Back-seat passengers	0.571 (0.225–1.453)	> 0.05
Bicyclists	1.004 (0.362–2.786)	> 0.05
Motorcyclists	0.206 (0.024–1.779)	> 0.05
Tractor drivers	2.580 (0.777–8.567)	> 0.05
Fracture of sternum	0.162 (0.008–3.404)	> 0.05
Fractures of ribs on one side	0.229 (0.036–1.442)	> 0.05
Fractures of ribs on both sides	0.262 (0.042–1.619)	> 0.05
Fractures of ribs on both sides with Fracture of the sternum	0.283 (0.045–1.764)	> 0.05
Fracture of thoracic spine	4.173 (1.921–9.066)	< 0.05
One–sided lung contusions	1.183 (0.549–2.550)	> 0.05
Bilateral lung contusions	1.274 (0.640–2.539)	> 0.05
One–sided lung lacerations	0.889 (0.259–3.047)	> 0.05
Bilateral lung lacerations	1.885 (0.639–5.561)	> 0.05
One–sided lung disruption	1.097 (0.013–0.752)	< 0.05
Heart contusion	2.185 (0.472–10.113)	> 0.05
Rupture of pericardium	0.663 (0.175–2.517)	> 0.05
Hematoma of *adventitia aortae*	2.400 (0.576–10.002)	> 0.05
Aortic rupture	3.606 (1.157–11.240)	< 0.05
One–sided pneumothorax	5.619 (1.959–16.117)	< 0.05
Both–sided pneumothorax	1.864 (0.596–5.831)	> 0.05
Hemothorax	1.647 (0.556–4.877)	> 0.05
Chest injury	6.234 (0.899–43.254)	> 0.05
Chest and head injury	0.550 (0.278–1.089)	> 0.05
Chest and abdomen injury	0.361 (0.162–0.807)	< 0.05
Chest, abdomen, and head injury	3.606 (1.341–9.673)	< 0.05

OR – odds ratio; CI – confidence interval.

Discussion

The aim of this study was to estimate the frequency and characteristics of RTA chest injuries in the region of Kragujevac in order to better understand their significance and define preventive measures for the most vulnerable population categories. Demographic data revealed that males contributed to the total account of subjects in much higher numbers compared to females, and this was also applicable to the total number of the deceased and RTA participants pertaining to different categories. Similar results have been reported in studies conducted in Germany [16], Hungary [17], India [18], and Turkey [19], where men accounted for 70–85% of casualties in RTAs.

According to the results obtained in this study, tractor drivers were among the oldest of subjects, and comparable results have been reported in other studies, as well. In Portugal, for instance, most of the tractor drivers involved in RTAs are between 60 and 70 years old [20], while in Sweden, more than half of the casualties are aged 55 and above [21]. The most vulnerable subjects in this research were pedestrians, which is in accordance with conclusions of similar studies conducted in the world, where pedestrians made up to 45% of the deceased RTA participants in underdeveloped countries, 29% in developing countries, while in the developed countries, pedestrians approximately made up to 18% of the casualties [16, 22]. A high number of deceased pedestrians in the present research could be explained by a large number of decrepit and old vehicles, poor road infrastructure as well as irresponsible behavior of pedestrians. Pedestrians have been identified as the most numerous and the most heterogeneous category of traffic participants, and they are the least protected subjects. A great majority of the deceased pedestrians in RTAs were older than 65. These findings could be explained by the fact that elderly pedestrians have difficulties noticing potential dangers and do not respond to them on time. They frequently have dementia, hearing, or sight impairments, which also makes it more difficult for them to participate in traffic [17, 23, 24], while recovery from trauma is more demanding than in the younger population [10, 16]. The largest number of motorcyclists and passengers in motor vehicles was in the 35–45 age category, which included the working population. This also correlates to the results of the rest of the studies [25–28].

Seasonal differences were noticed regarding RTA deathly outcomes, and these could be explained by greater activity during summer and autumn as compared to winter, which is partially in accordance with the results from other studies [17, 19]. The smallest number of fatal RTAs took place on Mondays, the first workday of the week, i.e. when subjects were not tired, while the majority of fatal RTAs took place in the middle of the week and at weekends, when the working population was more exhausted and when the younger population was usually more active, due to restaurant visiting and alcohol abuse [17, 19].

In the present study, injuries of individual parts of the chest were identified in two-thirds of the subjects, head injuries came in second by incidence, and abdomen injuries were the least frequent, which largely coincides with results from other studies [16, 25]. Traffic injuries exhibit certain characteristics according to the type of injury and extension, which is explained by the presence of an enormous action force generated as a result of mass and acceleration multiplication that is absorbed by the body during an accident. The injury occurs due to the absorption of the external force upon impact, acceleration, or deceleration, whereas the body tends to maintain its original position and speed [29, 30]. According to this study, the majority of the deceased subjects were pedestrians, while due to the polyphase mechanism of injury, chest injuries were among the most common types of trauma. Primary injuries sustained by pedestrians were caused by the first impact of the vehicle (usually in the legs), secondary injuries were caused by contact with the vehicle, while tertiary ones were caused by subsequent contact with the terrain as a result of falling against the ground [29, 31]. Depending on the profile of the front end of the car, the pedestrian struck was either thrown forward in the direction of movement in cases of the bonnet front being high and blunt or scooped up onto the bonnet top, as with many slope-fronted modern vehicles. If the car speed is appreciable, the body can be thrown into the air or knocked down flat with a severe impact [11, 29–31]. Motor vehicle drivers sustained direct chest injuries due to the impact against the front part of the vehicle interior, i.e. the steering wheel, as well as hyperextension and deceleration injuries [32–34]. The large percentage of chest injuries among tractor drivers in this study sample could be explained by the characteristics of the vehicle and the mechanism by which the accident occurred. Namely, these are usually tractors without a cab from which drivers can be knocked out of rather easily. Furthermore, the tractors or implement machinery could turn over, or trailers could tip and end on top of the driver, and in those instances, chest injuries are inevitable.

Subjects who died at the scene of the accident are virtually twice as more likely to have sustained chest trauma than the other subjects, which is in correlation with El-Menyar et al. [3], who point out that such chances are approximately two times higher if chest injuries are sustained. The chances that subjects who have died at the scene sustained simultaneous chest and abdominal injuries are more than two times higher, while the chances for the occurrence of simultaneous injuries to the head, abdomen, and chest are more than three times higher for subjects who died at the scene. Similar results have been reported in other studies, according to which subjects with simultaneous chest, head, and abdominal injuries were most likely to die at the scene or during the first few hours [3, 35] following the accident.

In this study, more than half of the subjects died at the scene of the accident or on their way to a healthcare facility, while the remaining ones outlived their injuries for a certain period of time. This is in accordance with the data from other studies, in which approximately 50% of the subjects died at the scene or on their way to the hospital [17, 19]. In several studies, the published results deviate from those presented here, where the mortality rate at the scene of the accident is approximately 65% [16, 18], while Reddy et al. [25] stated that only 20% of subjects died at the scene. The organizational structure of the services for transporting injured persons, technical equipment of vehicles, and availability of vehicles could explain the differences in the aforementioned results. Subjects who died at the scene were younger compared to those who died in the hospital because

these are mainly motor vehicle drivers and motorcyclists who are prone to risky behavior, and they suffer from serious injuries, which correlates to the results of other studies [17]. The results of this study accentuate that a large number of subjects who died at the scene of the accident pertained to the group of motor vehicle drivers and motorcyclists, who were classified in the group of younger RTA subjects. Bicyclists were the category of subjects who outlived their injuries most frequently. This could be explained by the distribution of injuries, localized mostly in the pelvic region and lower limbs, and least often in the chest area. It is important to mention that one-quarter of the bicyclists experienced pneumonia complications during hospitalization. A similar distribution of bicyclist injuries is evidenced by other certain studies [17, 36, 37].

Rib fractures are among the most frequent injuries in RTAs, with a share of 60–70% [5, 25]. The authors established that rib fracture was followed by damaged blood vessels and haemothorax, as this was largely present in all subjects. Subjects with bilateral fractures of the ribs and bilateral fractures of the ribs accompanied by sternal fracture were approximately twice as likely to die at the scene. In their study, Kent et al. [38] found that the odds ratio for death of younger subjects (aged 18–45) with rib fractures was smaller than for older subjects (over 64 years). Regardless of the presence or absence of concomitant trauma, subjects with rib fractures are at a significantly increased risk of in-hospital mortality [38].

Lung contusions were either caused by the effects of direct force originating from the fractured ribs, or via the effects of indirect force on lung tissue, in cases without rib fractures. Bilateral lung contusions were the most frequent type of injury of intrathoracic organs of the following RTA subjects: pedestrians, passengers, bicyclists, motorcyclists, and tractor drivers. Similar results have been reported in other studies [3, 25].

Subjects with myocardial rupture and tamponade died at the scene of the accident. In cases of myocardial rupture, death occurs within a few moments, and the diagnosis is most frequently made during autopsy [39]. According to the results of this study, the aortic rupture was the most frequent injury of intrathoracic organs in motor vehicle drivers and front-seat passengers. Aortic rupture is classified in the group of deceleration thoracic injuries, most commonly occurring in motor vehicle collisions, with front-seat occupants being the most vulnerable category [11, 13].

By analyzing the occurrence of different types of chest injuries in subjects, the authors obtained results that accentuated the relevance of certain types of injuries and the characteristics of deathly outcomes at the scene of the accident. Motor vehicle drivers were nearly three times more likely to die at the scene when compared to other subjects. This could be explained by the fact that motor vehicle drivers frequently sustain aortic rupture, pneumothorax, or haemothorax, which are life-threatening injuries that dramatically increase the chances for deathly outcomes at the scene of the accident. According to the obtained results, subjects who died at the scene of the accident are highly likely to have sustained aortic rupture, fracture of the thoracic spine, and pneumothorax.

Unlike other studies that employ data from all RTAs [3, 15, 22, 40], the authors only used the data on RTAs with fatal outcomes, i.e. autopsy reports. This could explain the partial difference between the results obtained herein and certain results reported by other global studies. The present results should be interpreted considering several limitations: the absence of traffic police reports from the scene of the accident, limited geographical area, exclusion of children under 14 years of age and of subjects shorter than 150 cm. These would provide data on the exact time of an RTA, road conditions at the time of the accident, traffic accident expertise (the type of vehicle, speed of impact), application of protective gear (seatbelts, helmets), as well as the extent of driving experience of RTA participants.

Conclusion

The majority of fatalities in RTAs were male pedestrians, with the average age being 51, who died after outliving their injuries for a certain period of time. Chest injuries were the most frequent in such accidents, and the most common type of injury was rib fracture combined with haemothorax. Chest injuries were often life-threatening or result in serious health consequences.

Performing autopsy in cases of fatal RTAs is extremely important because it is the only way to identify all of the injuries and explain the mechanisms of their occurrence; hence the data obtained in such procedures are irreplaceable.

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Salivary alpha-amylase and tooth pulp evoked potentials in paroxysmal trigeminal neuralgia patients

Salivarna alfa amilaza i evocirani potencijali zubne pulpe kod bolesnika sa paroksizmalnom trigeminalnom neuralgijom

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Abstract

Background/Aim. The sudden and excruciating pain that characterizes paroxysmal trigeminal neuralgia (PTN) has a negative effect on the wellbeing of the affected individuals, causing psychological distress. Salivary alpha-amylase (sAA) level represents an objective assessment of physical, physiological, and psychological stress. Evoked potentials (EPs) reflect nerve function and evaluate a functional aspect of the trigeminal nerve conduction. The aim of this study was to analyze possible modifications in painful impulses conduction related to sAA level by registering tooth pulp EPs in PTN patients. **Methods.** The study included ten PTN patients and twelve healthy subjects. The activity of sAA was measured using the Nipro Salivary Amylase Monitor. In order to record EPs response, the dental pulp of vital teeth was electrically stimulated through intact enamel. For stimulation and impulse registration, we used Xltek Protektor 32 system, software EPWorks, version 5.0. **Results.** The results obtained in PTN patients showed a higher number of waves and significantly shorter latencies and lower amplitudes N2-P2 and N3-P3 at the neuralgic side compared to the healthy side of the same patient, as well as to the controls ($p < 0.05$). Moreover, latencies were significantly shorter in patients with higher sAA levels than in those with normal sAA levels ($p < 0.05$). Late latencies (N2 and P2) at the healthy side in patients with higher sAA levels were significantly shorter compared to patients whose sAA levels were normal ($p < 0.05$). **Conclusion.** This study showed that psychical stress associated with PTN probably further increased hyperexcitability and conduction velocity of the affected nerves. Moreover, it seems that anticipation of stressful pain even increases the conduction velocity of unaffected nerves at the thalamocortical level in PTN patients. However, in healthy individuals, stress itself had no influence on painful impulses conduction.

Key words:
alpha-amylases; evoked potentials; neuralgia; pain; stress, psychological; trigeminal neuralgia.

Apstrakt

Uvod/Cilj. Paroksizmalna trigeminalna neuralgija (PTN) se karakteriše iznenadnim i intenzivnim bolom koji nepovoljno utiče na stanje obolelog i može prouzrokovati psihičku uznemirenost. Nivo salivarne alfa amilaze (sAA) predstavlja objektivnu procenu fizičkog, fiziološkog i psihološkog stresa. Evocirani potencijali (EP) odražavaju sprovodnu funkciju neurona, zbog čega se mogu primeniti za procenu neurotransmisije duž puta trigeminalnog nerva. Cilj rada bio je da se kod bolesnika sa PTN registrovanjem EP zubne pulpe ispitaju promene u prenošenju bolnih impulsa u odnosu na nivo sAA. **Metode**. Studijom je obuhvaćeno 10 bolesnika sa PTN i 12 zdravih ispitanika. Aktivnost sAA određivana je upotrebom aparata „Nipro Salivary Amylase Monitor". U cilju dobijanja odgovora EP, zubna pulpa je stimulisana električnom strujom preko intaktne gleđi. Za stimulaciju i registraciju korišćen je apparat „Xltek Protektor 32 sistem", softver „EPWorks", verzija 5.0. **Rezultati**. Rezultati dobijeni od bolesnika sa PTN pokazuju veći broj talasa i značajno kraće latence na neuralgičnoj strani u poređenju sa zdravom stranom i sa kontrolnom grupom ispitanika ($p < 0,05$). Na neuralgičnoj strani su sve latence bile značajno kraće, a amplitude N2-P2 i N3-P3 značajno niže kod bolesnika sa povišenim nivoom sAA u odnosu na one koji su imali normalan nivo sAA ($p < 0,05$). Kasne latence (N2 i P2) na zdravoj strani kod bolesnika sa povišenim nivoom sAA bile su značajno kraće u poređenju sa bolesnicima čiji je nivo sAA bio normalan ($p < 0,05$). **Zaključak.** Naše istraživanje je pokazalo da psihički stres udružen sa

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PTN dodatno povećava hiperekscitabilnost i brzinu sprovođenja neurona. Osim toga, anticipirani stres povećava brzinu sprovođenja na talamokortikalnom nivou čak i na nepogođenoj strani kod pacijenata sa PTN. Međutim, kod zdravih ispitanika, stres sam po sebi nema uticaja na brzinu prenosa bolnih impulsa.

Ključne reči:
alfa amilaze; evocirani potencijali; neuralgija; bol; stres, psihički; neuralgija, trigeminalna.

Introduction

Paroxysmal trigeminal neuralgia – (PTN) is a neuropathic pain condition characterized by paroxysmal, lancinating pain attacks along the somatosensory distribution of one or more divisions of the trigeminal nerve [1–3]. Its pathogenesis is not fully understood. It is believed to be related to microvascular compression of the trigeminal nerve root by aberrant blood vessels [1–4]. However, it seems that additional neurophysiological mechanisms are also involved [2]. Ultrastructural and biochemical changes in axon and myelin are not only seen in the trigeminal root but also in Gasserian ganglion [1, 5]. Chronic nerve compression results in demyelination, with progressive axonal degeneration in small unmyelinated and thin myelinated fibers. Demyelination may give an increase to electrical hyperexcitability, spontaneous and triggered ectopic impulses, and cross excitation among neighboring afferents [1, 3, 5]. Atrophy of the trigeminal nerve [6, 7] and reduced volume of gray matter [1, 8] are also seen.

The sudden and excruciating nature of the pain in PTN has a negative effect on the wellbeing of the affected individuals, leading to psychological distress [9]. Depression and anxiety are the most frequent psychiatric comorbidities associated with trigeminal neuralgia (TN) [7, 9]. Stress is implicated in the etiology of depressive and anxiety disorders [10, 11]. Chronic exposure to stress leads to morphological damage [11, 12], as well as functional disorders like neuronal hypersensitivity [13, 14].

Salivary alpha-amylase (sAA) level is shown to be an objective assessment of physical, physiological, and psychological stress [15, 16]. Evoked potentials (EP) can reflect the function of the trigeminal nerve conduction; therefore, a functional level of the trigeminal nerve conduction pathway may be evaluated by the EP [17]. To our knowledge, studies about the effects of sAA level on the tooth EP in PTN patients have not been previously reported. Thus, the aim of this study was to analyze modifications in painful impulse conduction (registered by the tooth pulp EP) related to sAA level in PTN patients. Although this is a preliminary report, we have studied the tooth pulp EP in stress and pain states and their modulation by analgesics and anxiolytics.

Methods

Ten PTN patients (eight females and two males, mean age 51.40 ± 20.07) were recruited from the Clinic for Oral and Maxillofacial Surgery at the Institute of the Faculty of Stomatology in Pančevo. Additionally, twelve healthy subjects participated in the study. The study was approved by the local Ethics Committee and was in accordance with the Declaration of Helsinki [18]. All subjects gave their written informed consent after a full explanation of the study, focusing on the purpose of the study and precise procedures. Exclusion criteria were as follows: avital central incisors of the upper jaw, prosthetics and fillings on the same teeth, oral mucosal changes, fractures, trauma or surgery in the maxillofacial region, as well as the use of any drug that could contribute to the action of the sympathetic nervous system, such as alpha-blockers, beta-blockers [15, 19], alpha-methyldopa, etilefrine hydrochloride, amezinium metilsulfate, midodrine hydrochloride, L-threo-3,4-dihidroxyphenylserine, and antidepressants [20]. The participants were asked not to smoke, eat or drink anything 30 min prior to testing. All participants were examined under the same conditions, from 8 am to 12 pm.

The activity of sAA was measured using a hand-held sAA monitor manufactured by Nipro (Osaka, Japan). This analyzer enables automatic measurement of sAA activity within one minute from collection to completion of the measurement, using a dry-chemical system [16].

To record cortical somatosensory-evoked response, we stimulated the dental pulp electrically through the intact enamel by a pair of specialized Ag-AgCl electrodes on the vestibular and palatal surface of the intact maxillary incisor under condensation silicone dental impression Zetaplus (Zhermack Clinical, Italy). Single square pulses of 1 ms and 1 mA intensity were delivered at a rate of 1 Hz (machine-delivered stimulation). Brain potentials were collected with surface cup electrodes placed on the Vertex and kept in place by collodion (Aquasonic 100 Ultrasound Transmission Gel, Parker Laboratories Inc., Fairfield, New Jersey, USA), the reference being 2 cm above the Inion. The analysis time was 300 ms. Signals were amplified (10 μV), filtered (0.5–70 Hz), and averaged (2 sweeps). For stimulation and registration, we used Xltek Protektor 32 system, software EPWorks, version 5.0 (Natus Medical Inc., Canada) that contained a complete data acquisition system with built-in amplifiers, A/D converters, digital signal processors, central processing units, and storage devices.

Five recordings were performed for each participant. Normal EP values were collected from all volunteers (six females and six males, mean age 21.59 ± 1.08). In PTN patients, the recording was performed on both upper central incisors. The peak latencies and the peak-to-peak amplitude of all components were measured.

Data were statistically analyzed with the SAS System for Windows, release 9.3 [21]. We used the Kruskal-Wallis test to determine statistical significance. Values of $p < 0.05$ were considered significant. Results are expressed as mean ± the standard error of the mean.

Results

The tooth pulp EP obtained from PTN patients demonstrated a higher number of waves with significantly shorter latencies at the neuralgic side compared to the healthy side and the controls ($p < 0.05$). The amplitudes decreased at the neuralgic side vs. the healthy side and controls but without statistical significance (Figure 1, Tables 1 and 2). There were no significant differences in the tooth pulp EP components (latencies and amplitudes) between the healthy side and the controls (data not shown).

Higher sAA levels were found in 2 (20%) patients and 5 (41.67%) controls.

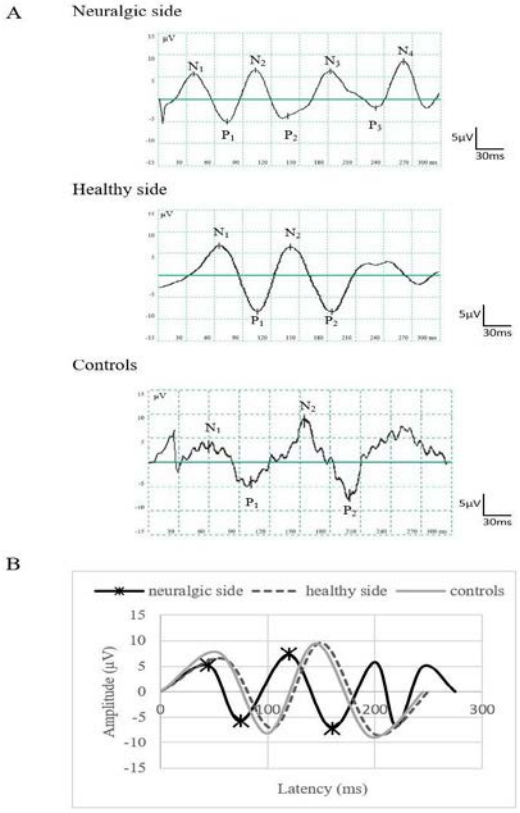

Fig. 1 – A) Original waveform recordings from Vertex after stimulation of the tooth at the neuralgic side, healthy side, and controls: An upward deflection of the evoked potential (EP) waveform was defined as N (negative) and downward deflection as P (positive); B) The pattern of the mean values of EP from the neuralgic side, healthy side, and controls: There were higher numbers of waves and the latencies significantly shorter at the neuralgic side compared to the other two groups [* - statistically significant difference ($p < 0.05$)].

Table 1
Comparison of evoked potential (EP) parameters in paroxysmal trigeminal neuralgia patients between neuralgic and healthy side

EP parameters	Side		*p*
	neuralgic	healthy	
Latency (ms)			
N1	44.91 ± 2.48	58.63 ± 1.93	0.0008*
P1	77.21 ± 1.57	102.80 ± 3.80	0.0001*
N2	118.50 ± 4.13	151.98 ± 6.67	0.0018*
P2	157.70 ± 7.31	202.20 ± 5.76	0.0014*
N3	199.40 ± 11.22		
P3	217.62 ± 11.09		
N4	243.00 ± 12.30		
Amplitude (μV)			
N1-P1	10.96 ± 1.33	13.62 ± 2.04	0.1720
N2-P2	14.54 ± 1.64	18.04 ± 2.16	0.0585
N3-P3	12.95 ± 2.35		

All values are expressed as mean ± standard error; *$p < 0.05$ (Kruskal-Wallis test).

Table 2

Comparison of evoked potential (EP) parameters parameters in paroxysmal trigeminal neuralgia patients between neuralgic side and controls

EP parameters	Neuralgic side	Controls	*p*
Latency (ms)			
N1	44.91 ± 2.48	62.21 ± 2.54	0.0001*
P1	77.21 ± 1.57	102.46 ± 2.35	< 0.0001*
N2	118.50 ± 4.13	153.42 ± 3.99	0.0002*
P2	157.70 ± 7.31	201.71 ± 4.59	0.0012*
N3	199.40 ± 11.22		
P3	217.62 ± 11.09		
N4	243.00 ± 12.30		
Amplitude (μV)			
N1-P1	10.96 ± 1.33	12.55 ± 0.69	0.1129
N2-P2	14.54 ± 1.64	15.33 ± 0.75	0.0985
N3-P3	12.95 ± 2.35		

All values are expressed as mean ± standard error; *$p < 0.05$ (Kruskal-Wallis test).

At the neuralgic side, all latencies were significantly shorter, and amplitudes N2-P2 and N3-P3 were significantly lower in patients with higher sAA levels ($p < 0.05$) than in those with normal sAA levels (Figure 2, Table 3).

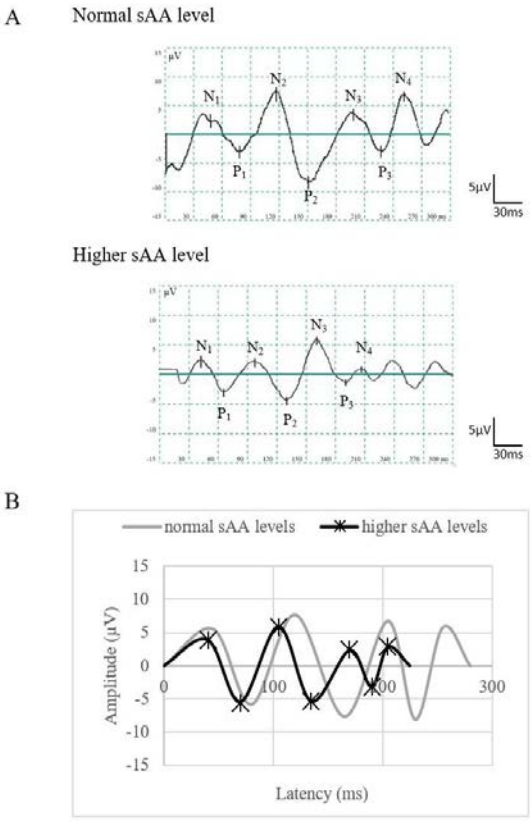

Fig. 2 – A) Original waveform recordings from patients with normal and higher salivary alpha-amylase (sAA) levels after stimulation of the tooth at the neuralgic side; B) The pattern of the mean values of evoked potential (EP) from the neuralgic side related to normal and higher sAA levels: All latencies were significantly shorter, and amplitudes N2-P2 and N3-P3 were significantly lower in patients with higher sAA levels than in those with normal sAA levels [* - statistically significant difference ($p < 0.05$)].

Table 3

Comparison of evoked potential (EP) parameters in paroxysmal trigeminal neuralgia patient at the neuralgic side related to salivary alpha-amylase (sAA) levels

EP parameters	Normal sAA levels	Higher sAA levels	*p*
Latency (ms)			
N1	46.00 ± 3.00	39.85 ± 0.45	0.0293*
P1	79.13 ± 1.15	69.53 ± 1.07	0.0345*
N2	122.13 ± 4.17	104.58 ± 1.53	0.0345*
P2	163.25 ± 7.93	135.27 ± 2.10	0.0345*
N3	207.00 ± 12.64	168.09 ± 4.04	0.0345*
P3	227.33 ± 12.31	188.15 ± 8.36	0.0417*
N4	255.00 ± 12.81	206.45 ± 6.95	0.0417*
Amplitude (μV)			
N1-P1	11.35 ± 1.57	9.42 ± 0.11	1.0000
N2-P2	15.35 ± 1.15	11.53 ± 0.09	0.0339*
N3-P3	15.44 ± 1.22	5.75 ± 0.25	0.0417*

All values are expressed as mean ± standard error; *$p < 0.05$ (Kruskal-Wallis test).

Late latencies (N2 and P2) at the healthy side in patients with higher sAA levels were significantly shorter compared to patients whose sAA levels were normal, whereas amplitudes showed no significant changes (Figure 3, Table 4). In the control group (healthy patients), tooth pulp EP components showed no significant difference in relation to sAA levels (data not shown).

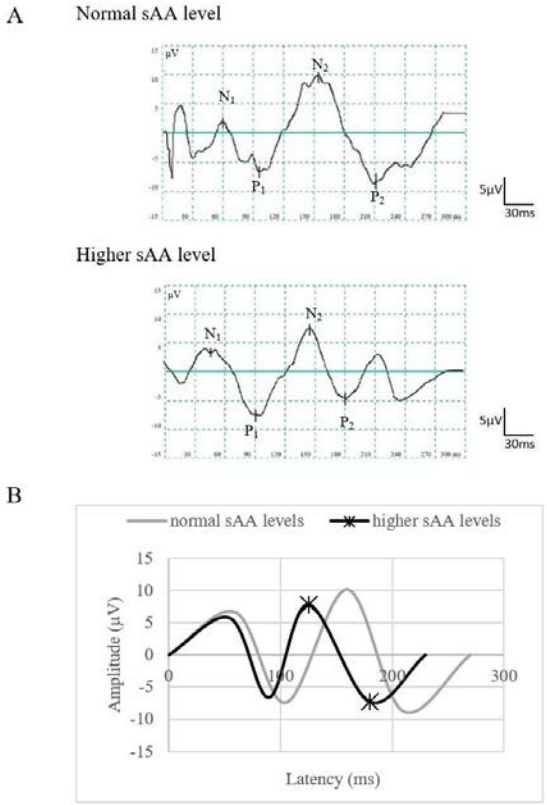

Fig. 3 – A) Original waveform recordings from patients after tooth stimulation at the healthy side related to normal and higher salivary alpha-amylase (sAA) levels; B) The pattern of the mean values of EP from the healthy side related to normal and higher sAA levels: Late latencies (N2 and P2) at the healthy side in patients with higher sAA levels were significantly shorter compared to patients whose sAA levels were normal [* - statistically significant difference ($p < 0.05$)].

Table 4

Comparison of evoked potential (EP) parameters in paroxysmal trigeminal neuralgia patients at the healthy side related to salivary alpha-amylase (sAA) levels

EP parameters	Normal sAA levels	Higher sAA levels	*p*
Latency (ms)			
N1	59.78 ± 2.24	54.38 ± 0.27	0.2888
P1	105.75 ± 4.02	89.60 ± 0.15	0.2765
N2	158.10 ± 6.58	127.12 ± 1.49	0.0339*
P2	207.88 ± 5.39	179.78 ± 1.53	0.0339*
Amplitude (μV)			
N1-P1	13.95 ± 2.37	12.78 ± 2.51	1.0000
N2-P2	12.30 ± 0.05	15.11 ± 0.10	1.0000

All values are expressed as mean ± standard error; $^{*}p < 0.05$ (Kruskal-Wallis test).

Discussion

To our knowledge, this is the first study investigating sAA activity in relation to painful impulses registered by tooth pulp EP in PTN patients.

The present findings support previous results that PTN patients show a higher number of waves at the neuralgic side [4, 22–24]. However, the results of EP latencies are inconsistent. Some publications describe prolonged EP latencies [4, 22, 24], while others found no changes in the EP latencies length [25, 26]. We could presume that prolonged EP latencies are due to the compression of Gasser ganglion, which is mentioned as a cause of neuralgia in previous studies. It is well established that compression of the peripheral nerve produces both conduction block and decreased conduction velocity [4, 24]. Our results showed shortened mean latencies of all EP components at the neuralgic side compared to both the healthy side and healthy controls. This is in agreement with the findings of Lekić and Cenić [23] and suggests an increased conduction velocity at the second-order neurons and a greater number of synaptic discharges in the ventral posteromedial nucleus of the thalamus, which is reflected in twice the number of waves in the thalamocortical pathway after a latency period of 150 ms. According to the ignition hypothesis, due to microvascular compression near the root, focal demyelination of the trigeminal sensory root generates spontaneous ectopic impulses responsible for the short-lasting attacks.

Psychological stress can produce several effects in a variety of physiological systems, similar to those produced by physical challenges due to activation of two stress response systems: the sympathoadrenal medullary (SAM) system and the hypothalamic-pituitary-adrenal axis [27]. Stress requires heightened excitability or arousal, which can be operationally measured using electroencephalography, behavioral (motor) activity, or neurochemical (adrenaline, glucocorticoid) levels [14]. There have been many attempts to quantify stress by various methods, including psychological tests and measurements of hormonal, cardiovascular responses, and other physiological parameters. However, sAA is increasingly used as an indicator of stress because sAA activity is a reliable stress marker of the sympathoadrenal system, not being influenced by the salivary flow [28]. In contrast to other parameters, sAA seems to have the advantages of being a noninvasive, painless, and fast method, allowing easy and stress-free quantification and multiple sampling [16]. The role of biological stress markers as mediators between stress and pain is confirmed [27–29].

The pain was identified as psychological stress [30] and, *vice versa*, stress is a factor that causes and maintains the pain intensity [29]. Several studies have shown morphological as well as functional changes in both neuralgic [1, 3, 5–8] and stress states [11–14]. We noticed that latencies at the neuralgic side were shorter as a result of the hyperexcitability of dysfunctional nerves. Findings in which the latency period was additionally reduced when the activity of sAA was increased suggest that psychical stress associated with PTN further increases hyper-excitability and conduction velocity. In patients with higher sAA levels, at the neuralgic side, late amplitudes were significantly lower than in those with normal sAA levels. The progressive reduction in late amplitude could be induced by cortical plastic modification in the pain matrix structures or in the brainstem monoaminergic nuclei. These structures are central effectors of the endogenous pain control system and play a crucial role in the central processing of sensory stimuli [31]. A recent study [32] also showed that reduced EP amplitude significantly predicted higher questionnaire scores of anxiety/depression, reports of increased life dysfunction, greater comorbidity, and clinician ratings of heightened severity and poorer prognosis. Moreover, EP amplitudes decreased in post-traumatic stress disorders [33], attention-deficit/hyperactivity disorders, and schizophrenia [34].

Late latencies at the healthy side in PTN patients with higher sAA levels were significantly shorter compared to the patients whose sAA levels were normal. Although these results relate to the nerves unaffected with TN, they reflect stress possibly due to anticipating a painful procedure. This is because TN patients live in fear of receiving the next pain attack. According to Capranica et al. [35], increases in sAA indicate psychological arousal due to anticipating the upcoming event. Pain modulatory networks in the brain play an active role in controlling nociceptive responses so that pain perception is influenced by our state of arousal, attention, and expectation [36]. Endogenous factors, such as the state of the subject, vigilance, psychological meaning, and demands of the stimulus, determine the EP [37, 38]. Stimuli associated with emotions, such as surprise, shock, uncertainty, obtrusiveness, or pain, also influence late EP components. Late EP components are not specific to the modality of the eliciting stimulus; they reflect the emotional-motivational aspects of pain, relating to factors such as degree of discomfort or unpleasantness associated with pain [37]. In contrast, early EP components are determined by the characteristics of the afferent input and, hence, the eliciting stimulus. In reality, the two phenomena cannot always be clearly separated [38].

This study confirms the involvement of stress in the PTN-affected nerve hyperexcitability. Furthermore, the results might support a functional rather than a structural

alteration in the trigeminal sensory pathway. As for the results, the following limitations should be considered: this is a preliminary study, limited by its sample size; however, the design, findings, and inclusion of physiological measures present a contributory role in the essential line of research. Those results, nevertheless, require further observation in a larger number of participants.

PTN is a relatively rare condition and can be sometimes confused with other painful conditions affecting the orofacial region [1]. These patients usually seek many health care providers before receiving a proper diagnosis and management. Dentists and physicians tend to consider, first of all, more common conditions that are likely to occur in the facial region (like a toothache) rather than PTN [39]. The initial misdiagnosis may lead to unnecessary interventions in many patients, especially unneeded dental restorative and surgical procedures [40]. In addition, the mean age of the TN patients is in the sixth decade [1, 9]. At that age, many persons have already lost their teeth for various reasons and have prosthetic replacements. Moreover, many PTN patients are in fear of an upcoming pain attack. It is not surprising to note that any sensation can be perceived as pain and may cause retreatment. It is also difficult to establish age-matched controls since persons after their fifties usually take medications for some other health condition. However, these criteria are not necessary because EP waveforms tend to be remarkably stable within and across the subject age in both amplitude and latency [41].

Conclusion

We found that psychical stress associated with PTN further increased hyperexcitability and conduction velocity. Besides, stress anticipation increased conduction velocity at the thalamocortical level even of unaffected nerves in PTN patients. However, in healthy individuals, stress itself had no influence on painful impulses conduction.

Acknowledgement

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Medical care of patients in the emergency department of the Military Medical Academy in Belgrade during the epidemic of COVID-19

Zbrinjavanje pacijenata u Centru hitne pomoći Vojnomedicinske akademije u Beogradu tokom epidemije COVID-19

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Key words:
covid-19; diagnosis; emergency service, hospital; hospitals, military; serbia; triage.

Ključne reči:
covid-19; dijagnoza; hitna služba, bolnica; bolnice, vojne; srbija; trijaža.

Introduction

A coronavirus is a group of single-stranded RNA viruses that cause respiratory tract infections [1, 2]. The first case of the coronavirus disease 2019 (COVID-19), caused by a severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), was recognized in December 2019 in Wuhan (Hubei Province, China) [3, 4].

The first case of the infection caused by SARS-CoV-2 recorded in Serbia was on March 6, 2020 [5]. Therefore, the COVID-19 Infection Disease Crisis Response Team was formed on March 13. The team was formed in order to ensure timely and coordinated treatment and undertaking of activities of the competent authorities, services, and organizations, as well as other entities related to the control of COVID-19. All activities at the Military Medical Academy (MMA) in Belgrade were in accordance with it since then.

After that, the number of infected and sick patients began to grow rapidly. Hence, on March 15, the Government of the Republic of Serbia imposed a state of emergency in the entire country [6]. Most hospitals were dedicated to treating patients with COVID-19, while certain hospitals were oriented to taking care of the remaining patients with other acute and chronic diseases.

After introducing the state of emergency, due to the central ventilation system, the MMA was assigned to provide all specialist healthcare services during the COVID-19 outbreak, except for infectious diseases patients [7, 8].

This work organization also required an urgent transfer of the work regime to the daily twenty-four-hour duty. In order to protect our patients admitted to the hospital from the danger of potential entry of COVID-19 patients, the field hospital (admission-triage tent) has been set up in front of the entrance of the MMA Emergency Center. The field hospital is like a triage center where all patients must first pass through this center before entering the MMA (Figures 1 and 2). The medical triage is performed by the on-duty medical staff headed by a doctor based on information obtained from the patients' initial interview and examination. If they suspect a possible COVID-19 case patient, based on the clinical picture (fever, shortness of breath, chest pain, dry cough, changes in senses of taste and smell, stomach problems), a rapid serological test for COVID-19 is required, as well as an X-ray of the lungs and heart and laboratory

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Fig. 1 – Admission-triage tent in front of the entrance to the Military Medical Academy Emergency Center during the fight against the coronavirus disease 2019 (COVID-19).

Fig. 2 – Inside the admission-triage tent in front of the entrance to the Military Medical Academy Emergency Center during the fight against the coronavirus disease 2019 (COVID-19).

analysis [complete blood count, sedimentation, lactate dehydrogenase (LDH), C-reactive protein (CRP), ferritin, D-dimer] [9–11]. After that, based on the results, these patients were referred further to the Emergency Center of the MMA for examination, or if the patients were febrile and had at least one of the other signs in favor of COVID-19, then they were further referred to the Infectious Diseases Clinic of the MMA where they were treated until the polymerase chain reaction (PCR) test for SARS CoV-2 result arrived. In the case of a positive PCR test, a COVID-19 patient was transferred to the Clinical Center of Serbia, the main triage clinic designated exclusively for managing COVID-19 patients. A negative test did not rule out a subsequent development of COVID-19, and, therefore, infection control precautions were continued during the incubation period, despite the negative result.

This current topic presented the provision of healthcare at the MMA in Belgrade starting from the introduction of the state of emergency onwards.

From March 16 to July 31, 2020, a total number of 39,249 patients passed through the admission-triage tent, set up at the entrance to the Emergency Center of the MMA, i.e.

Table 1

Number of patients treated at the Military Medical Academy Emergency Center during the period of the coronavirus disease 2019 (COVID-19) spread (16/03/2020–31/07/2020) and during the same period in previous year (16/03/2019–31/07/2019)

Parameter	From 16th March to 31th July	
	2019	2020
Number of patients in the admission – triage tent	–	39,249
Number of patients treated		
civil insured	13,275	24,158
military insured	6,497	4,411
Total number of patients treated	19,772	28,569
Number of hospital admissions		
civil insured	1,952	3,429
military insured	1,081	853
Total number of hospital admissions	3,033	4,282

285 patients on average per day (Table 1). On the other hand, a total number of 28,569 patients was treated at the Emergency Center of the MMA in the same period (from March 16 to July 31, 2020). Compared with the same period in 2019, during which a total of 19,772 patients were treated in the Emergency Center of the MMA, an increase of 44.49% was seen. Prior to the period of the state of emergency, the MMA was open for emergencies on the territory of Belgrade only on Wednesdays, while on other days, only first aid was provided to military insured persons. Since the introduction of the state of emergency, military insured persons have come to the MMA Emergency Center less often compared to the same period in 2019 (decrease by 32.11%) because they were mostly taken care of at the military medical centers. In contrast, the number of civilians who got first aid increased significantly. Table 1 shows that the number of civilians, who got first aid, almost doubled in that period (an increase of 81.98%). These data covered a period of 138 days, therefore, during this period an average of 207 patients per day were taken care of, while during the same period last year, 143 patients per day were taken care of. Otherwise, the MMA Emergency Center is designed to receive and examine about 100–130 patients per day. On the other hand, out of the total number of examined patients in the Emergency Center of the MMA during the analyzed period in 2019, 15.34% of patients were hospitalized, while that percentage was 14.99% in 2020. However, the number of hospitalized patients among civilian insured persons increased by 75.66%, while among the military insured persons, the number decreased by 21.09%.

According to the testing recommendations of the COVID-19 Infection Disease Crisis Response Team, we used a COVID-19 rapid serology antibody test SARS-CoV-2 coupled with a reverse transcription PCR (RT-PCR) swab test to provide a more complete picture of the COVID-19 in our patients. In these circumstances, with the growing of COVID-19 pandemic and shortages of laboratory-based molecular testing capacity and reagents, the rapid point-of-care serological assay, cheap and simple enough to be afforded by the MMA, if used correctly (with the knowledge on their limited sensitivity at an early stage, when the host has not yet developed specific antibodies), was allowed, thus enlarging the spectrum of the tested population. We also took into consideration that the World Health Organization (WHO) did not recommend the use of antibody-detecting rapid diagnostic tests for patient care purposes but encourages their continuous usage in order to establish their usefulness in disease surveillance and epidemiologic research [12].

During the observed period, we took specimens (whole blood) for rapid serologic tests from 5,202 patients treated at the Emergency Center of the MMA (Figure 3). IgM was detected in

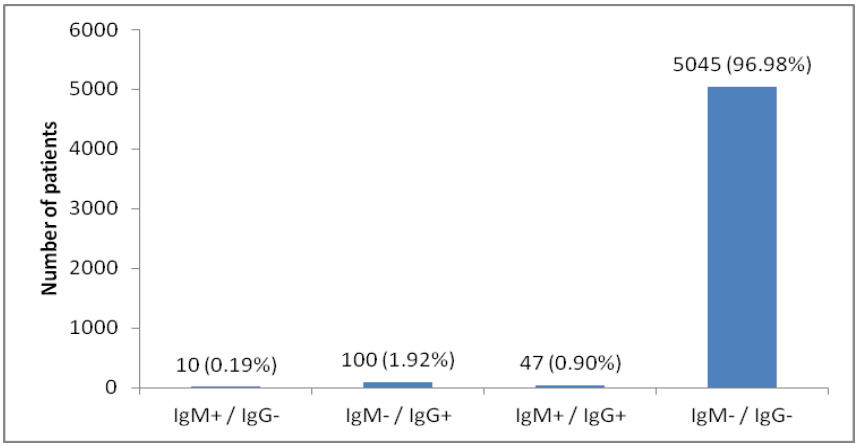

Fig. 3 – Number of patients tested with rapid serology antibody test for severe acute respiratory syndrome corona virus 2 (SARS-CoV-2) at the Military Medical Academy during the period from 16/03/2020 to 31/07/2020.

10 (0.2%) patients and IgG was detected in 100 (1.9%) patients. IgM and IgG were positive in 47 (0.9%) patients.

The presence of SARS-CoV-2 using the RT-PCR method was confirmed in 95 of 1,096 hospitalized patients at the National Referent Laboratory "Torlak Institute of Virology, Vaccines, and Serums" in Belgrade. The largest number of tested and confirmed COVID patients, 32 (16.0%) PCR positive patients of 200 tested, were hospitalized at the Clinic for Infectious and Tropical Diseases of the MMA.

The coronavirus infection is a great challenge for the whole society and every health institution. The care of such patients requires large financial allocations for institutions that deal with COVID-19 patients. However, the facilities that treat all other patients are under great pressure, as they have to take care of all other patients, treated on all levels of healthcare with a wide variety of comorbidities. One of the additional pressures that the healthcare workers had was the constant wearing of protective equipment (goggles, masks, gloves, hats, tights, face shields, hazmat suits, coveralls), increased disinfection, and maintaining social distance during the rest period [13–15]. All this further complicates the work of the medical staff, because the patient's health condition, protection from COVID-19 infection, economical but correct use of the protective equipment, and the possibility of making omissions must be considered the entire time.

Considering COVID-19 and the previous knowledge about the modes of transmission of SARS-CoV-2, healthcare facilities need to provide care for all patients and healthcare personnel in the safest way possible and at the appropriate level, regardless of the fact whether the patients need outpatient care, emergency center care, inpatient care, or intensive care. At the MMA, preparations for preventing and controlling COVID-19 began immediately after the WHO Director-General declared that the outbreak constitutes a Public Health Emergency of International Concern, in early February 2020. In the preparations, we used the recommendations of the WHO [16], European Center for Disease Control and Prevention (ECDC) [17], and Centers for Disease and Control Prevention (CDC) [18]. The experiences of the expert team from China were also implemented [19]. At all times, we followed the additions and the new announcements of the aforementioned bodies. A massive load was on the trainee specialist physicians who worked all the time on the triage of patients. Five basic principles were used in the prevention and control of COVID-19 at the MMA:

1. Screening and triage for early recognition of patients with suspected COVID-19 and rapid implementation of source control measures were done in all departments of the MMA.
2. Applying standard precautions in the care of all patients treated at the MMA. These measures were used when providing care to all patients, whether or not they appear infectious or symptomatic: hand hygiene before and after all patient contacts; consistent use of personal protective equipment, which included gloves, gowns, masks (medical or FFP2 depending on the type of activities), face shields or eye protection, respiratory hygiene/cough etiquette, needlestick and sharps injury prevention, cleaning and disinfection, reprocessing of instruments and equipment, regular waste disposal and safe injection practices.
3. Implementing additional precautions (Contact Precautions, Droplet Precautions, and Airborne Precautions) which were used in addition to the standard precautions. Special prevention measures were applied during the performance of aerosol generating procedures.
4. Implementing administrative measures which were included: the education of healthcare workers upon early recognition and diagnosis of COVID-19, education on the proper use of personal protective equipment (PPE), providing sufficient quantities of quality PPE, ensuring access to laboratory testing for COVID-19 detection, prevention of overcrowding, especially in the emergency center, etc.
5. Implementing environmental and engineering measures which were included: adequate ventilation according to specific areas at the MMA, spatial separation, adapted structural design, as well as adequate environmental cleaning and disinfection.

Conclusion

Data presented can serve as a good basis for further healthcare planning, considering that the number of patients infected with SARS-CoV 2 is again increasing, both in the world and in Serbia. Additionally, based on these data, new measures can be planned, which implies that the MMA, together with the Clinical Center of Serbia, are marked as hospitals that provide all specialist healthcare services, except for COVID-19 patients on the territory of Belgrade, while all other hospitals in Belgrade are dedicated to treating patients with COVID-19.

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Immunization in inflammatory bowel diseases: recommendations on vaccines administration

Imunizacija kod inflamatornih bolesti creva: preporuke za primenu vakcina

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Ključne reči:
creva, zapaljenske bolesti; imunizacija; vakcinacija; imunosupresija.

Introduction

The planning and implementation of immunization in patients with inflammatory bowel diseases (IBD) is just a necessary step in treating and monitoring the underlying disease, especially in preparing a patient for the introduction and application of immunosuppressive therapy.

This opinion is the result of the following findings: the majority of patients with IBD will be treated with immunosuppressive therapy during the illness; a large number of IBD patients are immunocompromised due to the nature and the course of the underlying disease; vaccination is the only form of prevention in certain infectious diseases that can complicate the condition of patients with IBD. The possible, more favourable relationship between the cost of the treatment of the infectious complications, the resulting hospitalization, and the costs of vaccination of these patients should not be neglected either.

The immunization of patients with IBD has certain specificities since the immune system and the immune response of the patients have been altered under the influence of etiopathogenesis, clinical course characteristics, and the treatment of the underlying disease. Due to all this, adequate vaccination planning is needed – the time of vaccine use, the type of vaccine, as well as the knowledge of safe and effective application, possible interactions with immunosuppressive drugs, and the effect of immunization on the immune response.

In routine clinical practice, special attention is rarely given to immunization data and its implementation in patients with IBD. Namely, the implication of compulsory vaccinations, which have been conducted in most patients and prior to the diagnosis of IBD, separates the clinician from the idea of the necessity of using vaccines during the treatment of these patients. It is, therefore, very important to define methods of vaccination of IBD patients, establish recommendations based on which the immunization of IBD patients will become an integral part of their treatment and an important segment in preventing infectious complications of these patients.

Predisposition for developing complications from infectious diseases in IBD patients

Infections that complicate IBD are on the rise. They are the basis of the highest percentage of complications and the need for hospital treatment of these patients. Among them, opportunistic infections, but also infectious diseases that can be prevented by immunization, are particularly important.

Opportunistic infections are defined as serious, usually progressive, infections caused by microorganisms that have limited pathogenic or even non-pathogenic capacity in people with uncompromised immune system. The same microorganisms can cause severe illnesses in the conditions of the predisposing effect of another associated disease or its treatment [1].

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Table 1

Some causes of the state of deficiency of the immune system (according to the Center for Disease Control, The Unites States Advisory Committee on Immunization Practices – US ACIP)

People who are in severe immunodeficiency which is not the result of human immunodeficiency virus (HIV) infection (congenital immunodeficiency, leukaemia, lymphoma, malignancy or application of therapy – alkylating agents, antimetabolites, radiation, high doses of corticosteroids – 2 mg/kg/bw or more than 20 mg of prednisone daily);
People with HIV infection;
People with conditions that cause a limited deficit of the immune system (e.g. hyposplenism, renal insufficiency, etc.).

Patients who are immunocompromised have altered cellular and/or humoral immunity, which increases the risk of opportunistic infections and infections in general. In patients with IBD, gene mutations for molecules that play an important role in innate immunity have been described, such as receptors for molecular patterns of pathogens and damaged cells (NOD2) or cytokine receptors (IL23R). These mutations are responsible for the changed functional capacity of innate immunity [2, 3]. Moreover, the activity of the acquired (adaptive) immunity cells in patients with IBD is also changed. In Crohn's disease, for instance, there is a description of increased activity of Th1 subpopulation of helper lymphocytes (CD4+ T lymphocytes) manifested by increased production of interferon gamma (INF-γ), as well as increased production of cytokines IL-12 and IL-18 by macrophages of the mucosa. In contrast to Crohn's disease, in ulcerative colitis, there is primarily an over-response of Th2 subpopulation of helper lymphocytes characterized by increased production of cytokines IL-13 and IL-5. Furthermore, T lymphocytes of patients with ulcerative colitis have a slower cell cycle and are more susceptible to programmed cell death (apoptosis) compared to control cells [4]. Bearing in mind all the above findings, it is clear that the intestinal lesion in IBD patients is completely under the influence of different "branches" of the immune system.

Despite the deficiencies described in the function of innate immunity and the changed immune function of the cells of the acquired immunity, not all patients with IBD can be considered immunocompromised [5]. Although there is no clear definition of the immunocompromised condition in the IBD, IBD patients are considered immunocompromised if treated with the following: corticosteroids for two weeks and longer or repeated within three months, with azathioprine (AZA) or 6-mercaptopurine (6MP), methotrexate (MTX), anti-tumor necrosis factor (TNF) agents, as well as patients with high protein-caloric malnutrition (Table 1).

Corticosteroids reduce the synthesis of cytokines with pro-inflammatory activity by inhibiting the transcription of the gene for these cytokines. This results in a decrease in the activity of various immune system cells, including inhibition of leukocyte migration, inhibition of the function of phagocytes (neutrophils and monocytes), and the function of T lymphocytes. AZA and 6MP, in the form of nucleotides, have been shown to lead to apoptosis of T lymphocytes. MTX, as a folic acid antagonist, inhibits the synthesis of purine, DNA and RNA structures, consequently inhibiting the S phase of the cell cycle. Cyclosporin, as the most commonly used calcineurin inhibitor, reduces the production of cytokine (IL-2, IL-3, IL-4, IL-5, TNF-α, TNF-β, INF-γ) by T helper lymphocytes. One of the main activators of the inflammatory process in IBD is TNF. The biological agents, antibodies that bind TNF (anti-TNF antibodies), except that they can inactivate the effect of TNF, induce monocyte apoptosis, and thus suppress the inflammatory process in IBD.

Applying the combination of immunosuppressants in therapy thus modulates the immune system at multiple levels.

Other risk factors for the development of infections, which also intensify the degree of immunosuppression, are malnutrition, surgical interventions, old age, co-morbidities, and leukopenia within the immunosuppressant application [6].

The occurrence of opportunistic infections is a problem for clinicians. These infections are often more difficult to recognize and diagnose, and are associated with high morbidity and mortality because they are potentially serious and less responsive to effective treatment (Table 2). Within a number of clinical studies, an increased incidence of opportunistic infections in IBD patients has been observed, including the occurrence of opportunistic infections associated with the use of immunosuppressive therapy in the treatment of patients with IBD [5–9].

Table 2

Opportunistic infections associated with the use of immunosuppressive therapy in the treatment of inflammatory bowel diseases (IBD) [7]

Causes of opportunistic infections
Viral infections
Varicella zoster virus
Herpes simplex virus
Cytomegalovirus
Epstein Barr virus
Human papillomavirus
Bacterial infections
Escherichia coli
Salmonella spp.
Streptococcus pneumoniae
Clostridium difficile
Staphylococcus spp.
Mycobacterium tuberculosis
Legionella pneumophila
Listeria monocytogenes
Mycobacterium avium spp.
Nocardia spp.
Fungal and parasitic infections
Candida spp.
Pneumocystis carinii
Aspergillus spp.
Histoplasmosis
Cryptococcus spp.
Coccidioides immitis
Blastomycosis

Vaccination

Vaccines are used to achieve a qualitatively and quantitatively appropriate (adequate) immune response from a recipient that ensures the usefulness of the applied protection. The time of immunization should be such as to provide a balance between the desire to achieve an optimal immune response and the practical need to achieve protection against an illness. The principles of childhood immunization are based on the above.

Since most IBD patients were vaccinated prior to the diagnosis of IBD, when their immune response was not changed due to the illness, and according to the mandatory vaccination plan (Table 3), the specific effect of administered vaccines in patients with IBD does not differ significantly from their effect in the non-IBD population. Therefore, although no precise data are available, it is considered that the incidence of the disease against which early immunization was carried out (diphtheria, pertussis, polio, measles, rubeola, tetanus) in the IBD population is negligible. Recommendations for immunizing patients with IBD (immediately after the diagnosis of IBD) are based on the assumption that compulsory vaccines have been previously used, which requires adequate evidence.

Planning and implementation of vaccination in patients with IBD is part of the infection prevention process (Table 4). Infections can complicate IBD during the application of immunosuppressive therapy, like in immunocompromised patients in general. This plan and its implementation depend on the assessment of the immune response status of each patient individually at a given moment.

Table 3

Calendar of compulsory vaccinations in Serbia, according to age (Institute of Public Health of Serbia "Dr Milan Jovanović Batut")

Age	BCG	HB	DTP	OPV	MMR	Hib
1st month (birth)	Vaccination	I dosage				
2nd month		II dosage				
3rd month			I dosage	I dosage		I dosage
3 and a half months			II dosage	II dosage		II dosage
By 6th month		III dosage	III dosage	III dosage		III dosage
From 12th to 15th month					Vaccination	
From 17th to 24th month			DTP Revaccination I	Revaccination I		
7 years of age (before going to school)			DT Revaccination II	Revaccination II	Revaccination	
12 years		3 doses*				
14 years of age			DT Revaccination III	Revaccination III		

HB – hepatitis B vaccine, contains purified hepatitis B surface antigen (HbsAg); DTP – diphtheria, pertussis, and tetanus vaccine, contains diphtheria toxoid, tetanus, and inactivated corpuscle *B. pertussis*; DT – adult vaccine, contains diphtheria toxoid and tetanus; MMR – vaccine against measles, mumps, and rubella, contains live, attenuated viruses; OPV – oral poliomyelitis vaccine, contains live, attenuated, 2 types of poliovirus; Hib – a conjugated vaccine against *Haemophilus influenzae* type B.

***children who have not been vaccinated by 12 years of age with three doses of vaccine by the scheme of 0, 1, 6 months.**

The calendar also includes the use of a tetanus vaccine (toxoid) that is applied after 30 years of age, every ten years, as well as the use of hepatitis B immunoglobulins (applied in newborn babies of mothers who are HBsAg positive).

Table 4

General recommendations for immunization of patients with inflammatory bowel diseases (IBD) [9]

1. Standard recommended immunization schedules for children and adults should be generally adhered to.
2. Upon diagnosis, children and adults should have a complete review of immunization history for completeness. All patients with incomplete series should commence catch-up vaccination.
3. Adults who cannot provide a clear history of chickenpox should have serologic testing for varicella. Nonimmune individuals should receive the varicella vaccine. Children who are not immune by vaccination or have not acquired immunity through infection should receive the varicella vaccine.
4. Live bacterial or viral vaccines should be avoided in immunocompromised children and adults with IBD. This includes the following: i) Treatment with glucocorticoids (prednisone 20 mg/d equivalent, or 2 mg/kg/d if less than 10 kg, for 2 weeks or more, and within 3 months upon stopping); ii) Treatment with effective doses of 6-mercaptopurine/azathioprine (effect on safety not established) and within 3 months upon stopping; iii) Treatment with methotrexate (effect on safety not established) and within 3 months upon stopping; iv) Treatment with infliximab (effect on safety not established) and within 3 months upon stopping; v) Significant protein-calorie malnutrition.
5. Whenever possible, adequate immune response (as reflected by serologic response) should be ascertained for individuals who have required immunization while immunosuppressed. Repeat dosing may be considered when the immune response to immunization is insufficient.

The effect of vaccination depends on the quality of the immune response and the compromising effect of immunomodulatory therapy on this response. There is still insufficient data based on which it is possible to assess the clinical response to the immunological changes caused by vaccination in IBD patients. The results of studies investigating the effects of immunization of immunocompromised patients or the immunization of patients suffering from immunosuppressive therapy [systemic lupus erythematosus (SLE), reumatoid arthritis (RA)] have shown that these patients create an adequate humoral immune response (specific antibodies have been detected). Moreover, these patients did not exhibit an increase in the activity of the underlying disease as a result of the response of their immune system to the use of vaccines [10, 15–18].

Vaccines can be classified into several categories, depending on the characteristics (forms) of the antigens used for their making. They may contain live (attenuated, avirulent) infectious agents (live vaccines), or they may be dead vaccines containing inactivated infectious agents of preserved immunogenicity. The immune system recognizes and responds to antigens by activating B lymphocytes (antibody production) and activating T lymphocytes. From activated T and B lymphocytes, in the process of developing the immune response to the pathogen, memory lymphocytes are created with mechanisms of even faster response in each future exposure to a given pathogen. By measuring the level of production of a specific antibody after immunization, as well as by comparing the antibody level before and after immunization, it is possible to estimate the degree of the immune response or the immunogenicity of the applied vaccine.

The immunogenicity of the vaccine (potential to produce an adequate immune response) can be determined in several ways and is usually estimated based on the titer of the antibodies produced. The titer of antibodies is determined before immunization and at a certain time interval after vaccination (usually four weeks after immunization). The process of seroconversion involves the formation of a specific antibody titer in seronegative persons (who did not have a measurable antibody titer prior to vaccination), which makes them seropositive. In the seroconversion process, a minimum titer of an anti-infection antibody (wild type) is required, and the achievement of said level or greater titer defines the so-called seroprotection – the expected protection [4].

Time of vaccine administration

In conditions of immunosuppression and, therefore, in patients with IBD, the use of live, attenuated vaccines is contraindicated since the entry of a causative agent in the condition of a compromised immune system can lead to the occurrence of an infectious disease (Table 5).

IBD patients need adequate access to the administration of vaccines with the knowledge of all of the aforementioned characteristics that relate to the changed immune system of the recipient and the application of immunosuppressive therapy. Bearing in mind the specificity of the recipient's immune response caused by the disease and/or the applied therapy that could affect the immunogenicity of the vaccine and the process of seroconversion, it is necessary to assess with great care the safety and efficacy of the administered vaccine.

Table 5

Live vaccines, generally contraindicated in patients receiving immunosuppressive therapy [9]

Anthrax vaccine
Intranasal influenza
Measles-mumps-rubella (MMR)
Oral polio live vaccine (OPV)
Smallpox vaccine
Tuberculosis BCG vaccine
Typhoid live oral vaccine
Varicella
Yellow fever

As part of the above, it is recommended that the appropriate vaccines be administered within 3 weeks upon starting the immunosuppressive therapy. Furthermore, if the treatment with immunosuppressants is in progress, the possible application is recommended 3 months after discontinued use of these drugs.

Vaccinating newborns of mothers treated with anti-TNF agents is a specific situation. According to the recent recommendations, the first vaccination should be administered from the 6th month of life. This is due to the fact that after this period, the anti-TNF antibodies, which the mother received as therapy and which were thus transferred to the child, have withdrawn from the child's bloodstream.

Specificity of vaccination of IBD patients

The most important infectious diseases of adult patients with IBD that can be prevented (mitigated) by vaccines and in which prevention is the most effective are influenza, varicella, and pneumococcal infections. Additionally, the recommendations for vaccination include specific situations that include vaccination against viral infections such as hepatitis B virus, human papillomavirus (HPV) infection in women, and the need for vaccination in case of traveling to certain parts of the world.

Influenza virus (flu)

Infection with the influenza virus has an annual epidemic character. Vaccines are formed each year according to the frequency of antigen properties of virus strains (type A, with H1N1 and H3N2 subtypes having global distribution and type B). In 2009, the World Health Organization (WHO) defined H1N1 as a pandemic strain, and since 2010, this strain is compulsorily contained in all vaccines produced for a given year.

Morbidity and mortality in influenza virus infections are increasing in immunocompromised patients. Thus, the current recommendations for vaccination are directed towards this population.

There are still insufficient results based on which it would be possible to evaluate clinical protection against influenza in patients with IBD after the vaccine administration. In several studies, where patients with IBD and patients with other immunologically mediated diseases were examined, vaccination efficacy was assessed based on the change in antibody titer after the vaccine administration. Data on the safety and tolerance of influenza vaccines are also limited, but they generally show that this vaccine is well tolerated and safe to use [19, 20].

As noted earlier, the application of a live, attenuated influenza vaccine is contraindicated in patients on immunosuppressive therapy. In this group of IBD patients, the use of trivalent influenza vaccine (TIV), a type of inactivated vaccine, is recommended. It is administered once a year (usually before the onset of influenza and in accordance with the recommended vaccine administration time). It has been shown that administration of the vaccine has no effect on IBD activity. It has also been confirmed that seroconversion has not been reduced and altered in patients on steroids, MTX, and anti-TNF agents, or dual therapy with these drugs. At the same time, the administration of thiopurine and cyclosporine affects the reduction in the percentage of seroconversion [21]. A routine check of a serological response in these cases is not necessary, given the above-mentioned existing knowledge [5].

Varicella–zoster virus (VZV)

VZV causes varicella and herpes zoster (after reactivating a latent VZV infection from the dorsal ganglia). Studies have shown that this is the most common herpes-viral infection in immunocompromised patients with IBD. Immunosuppression increases the incidence of herpes zoster (mainly in patients older than 50 years of age) and the risk of disseminated and complicated forms of illness (pneumonia, meningoencephalitis, and haemostasis disorders).

Primary prevention of varicella by vaccination is routinely recommended according to the calendar of vaccinations in childhood, in immunocompetent children (after the first year of life and in the period from 4 to 6 years of life-booster dose). This vaccination is not mandatory in our country. Given that these vaccines (against varicella and zoster) belong to the category of live, attenuated vaccines, the question arises as to the justification and risk of their use in immunocompromised IBD patients.

If the previous medical history of varicella and/or herpes zoster treatment is negative and if a patient is not vaccinated in childhood, the VZV vaccine should be administered immediately after the diagnosis of IBD or at least three weeks before initiating immunosuppressive therapy. If there is no vaccination or infection information, serological analyses – IgG VZV antibody titers should be done. Vaccination is performed in all seronegative patients. Two doses of live vaccine are administered, at a minimum interval of one month. If immunosuppressive therapy is discontinued, the vaccine should be administered no earlier than 3 months after the discontinuation of the therapy. The use of a vaccine is considered safe in patients with lower doses of immunosuppressive therapy (less than 20 mg of prednisone daily) or higher doses for less than two weeks, or AZA less than 3 mg/kg a day [9–11].

The levels of immunosupression are given in Table 6.

Previously treated VZV infection is not a contraindication for the use of immunosuppressive therapy but should not be initiated in the event of an acute infection. In the case of the VZV infection in the course of immunosuppressive therapy, antiviral drugs (acyclovir) should be used, and the immunosuppression should be stopped, especially in more complicated cases [13], and immunosuppressive therapy should be reintroduced after febrile and vesicle regression [14].

The need for the VZV vaccine, as well as all other vaccines, should be assessed individually in each IBD patient, depending on the application of immunosuppressive therapy, dose, and duration of treatment, as well as the assessment of the risk-benefit ratio of the above.

Table 6

Levels of immunosuppression based upon strength of immunosuppressive medication [10]

High-level immunosuppression
Treatment with glucocorticoids (prednisone >20 mg/day for ≥2 weeks and within 3 months of stopping therapy);
Treatment with effective doses of 6-mercaptopurine, azathioprine, or methotrexate compared with those with low-level immunosuppression (described below) or discontinuation within 3 months;
Treatment with adalimumab, certolizumab pegol, golimumab, infliximab, natalizumab, or vedolizumab, or recent discontinuation within 3 months.
Low-level immunosuppression
Treatment with effective doses of 6-mercaptopurine, azathioprine, or methotrexate compared with those with low-level immunosuppression (described below) or discontinuation within 3 months;
Treatment with lower total daily doses of corticosteroids compared with those with high-level immunosuppression for more than 14 days;
Patients receiving methotrexate (<0.4 mg/kg/week), azathioprine (<3.0 mg/kg/day), or mercaptopurine (<1.5 mg/kg/day).

Pneumococcal infections

Streptococcus pneumoniae-induced infections cause more deaths than other vaccine-preventable bacterial infections. Risk factors for the emergence of these infections are chronic immunosuppressive therapy (we see patients with IBD within this), chronic illness, and old age. Severe, invasive forms of pneumococcal infections – pneumonia and meningitis (with or without bacteraemia) are followed by higher mortality. In patients with IBD who are on immunomodulatory therapy, bacterial pneumonia caused by *pneumococcus* is one of the most common opportunistic infections.

Vaccination against *pneumococcus* should be performed in all patients with risk factors (old age, associated chronic illness, immunosuppression, splenectomized patients, and immunosuppressive therapy patients). Three types of vaccines are available, and they are the following: 23-valent polysaccharide (PPV23) that provides protection against the action of 80–90% of strains responsible for severe infections; 13-valent pneumococcal conjugate (PCV13), and 7-valent pneumococcal conjugate vaccine (PCV7). A patient with IBD should be vaccinated with pneumococcal vaccine according to recommendations.

IBD patients should be administered with pneumococcal vaccine prior to the introduction of immunosuppressive therapy (at least two weeks before). Combined immunosuppressive therapy has been shown to significantly reduce the immunogenic response to this vaccine (in particular, the combination of immunomodulators and anti-TNF agents), while monotherapy with immunomodulators (AZA) has no effect on the reduction of immunogenicity.

In the case of an active pneumococcal infection, the use of immunosuppressive therapy should be suspended until the infection is resolved. Any pneumonia in IBD patients should be treated with antibiotics acting on *pneumococcus* (penicillin, cephalosporins II and III generations) [9, 10].

Human papillomavirus

In all patients with IBD, regular gynaecological examinations and screening for cervical cancer should be performed. In particular, this refers to patients receiving immunosuppressive therapy and implies a compulsory prerequisite for the decision to include this therapy.

HPV is the most common sexually transmitted infection. Approximately 40 types of this virus are divided into those with low risk – skin and anogenital warts (condyloma) and those with high risk (high-grade dysplasia) – causing carcinoma of the cervix or anus (types 16 and 18).

The use of immunomodulatory therapy can cause the reactivation of HPV infection. Study data indicate an increased percentage of abnormal Papanicolaou (PAP) smear test findings in patients receiving immunosuppression (Table 7), an increased risk of cervical dysplasia, and a higher number of patients with persistent HPV infection [22–24].

Table 7

Comparison of abnormality of Papanicolaou (PAP) test findings in patients with inflammatory bowel diseases (IBD) and control group

Author	% of abnormal PAP test findings	
	Patients with IBD	Control group
Kane et al, 2008 [22]	42.5	7
Tamas et al, 2002 [23]	47	15
Bhatia et al, 2006 [24]	18	5

When it comes to HPV infection, especially infection with high-risk viral types, the best preventive measure is vaccination. The use of 4-valent (containing types 6, 11, 16, and 18) Gardasil vaccine, belonging to the type of inactivated vaccines, is recommended. It is effective, safe, and provides long-lasting immunity. Vaccination is recommended in young women with IBD (up to 26 years of age), as well as in men of the same age (especially those who practice homosexual relationships). Routine administration in the general population is recommended at the age before starting sexual intercourse (for the female sex, from 11 to 14 years of age).

The use of immunomodulatory therapy has no effect on the administration and effectiveness of this vaccine. In the case of a clinically high infection (extensive skin changes or genital warts), discontinuation of immunosuppressive therapy should be considered until the changes are cured.

Hepatitis B and C

The prevalence of infection with hepatitis B and C viruses (HBV and HCV, respectively) in patients with IBD is no different from that in the general population [21]. No direct connection was established between the application of immunosuppressive therapy in treating IBD and the course and outcome of chronic viral infection of the liver.

In each patient with IBD, screening for the presence of hepatitis B and C viruses should be done immediately after diagnosis. It has been shown that, in patients with IBD, impairment of liver function is significantly higher in patients with chronic hepatitis B virus infection than hepatitis C virus infection. In the case of hepatitis C infection, immunosuppressive therapy may worsen liver function, especially in the case of associated infection with another virus or hepatotoxic effect of drugs. Namely, this infection has increased prevalence of existence and associated, hidden, hepatitis B infection. Immunosuppressive therapy in chronic hepatitis C infection in IBD patients should be used with caution, depending on the severity of the IBD and the degree of damage to the liver. The application of immunosuppressive therapy does not affect the course of HCV infection, and progression to cirrhosis of the liver is the same as in the general population. The use of interferon in the treatment of HCV infection is contraindicated in Crohn's disease. There is no vaccine for preventing HCV infection.

Vaccination against hepatitis B is carried out by mandatory immunization schedule (after birth, and after 1 and 6 months), and efficacy is checked serologically. Provided it has not been conducted at the specified age, it is applied at the age of 12 in three doses (0, 1, and 6 weeks).

In all IBD patients, serological analysis of HBV surface antigen (HbsAg) and anti-HBs and anti-HBc antibodies is performed. In HBsAg positive patients, the viral DNA concentration is checked using a polymerase chain reaction (PCR) method. In seronegative patients, vaccination is carried out. In most cases, the standard protocol for the vaccine administration (0, 1, 6 months) will not provide seroprotection. Therefore, an accelerated protocol that implies a double dose of the vaccine and a schedule application of 0, 1, 2 months, with a mandatory serological conversion check, is recommended. Possible causative factors of reduced response include longer duration of IBD, decreased serum albumin level at the start of the vaccine protocol, administration of corticosteroids in more than one vaccination term [25, 26]. If the "accelerated" regime is insufficient for protection as well, performing revaccination according to the same application scheme is recommended. Serological testing is performed 1–2 months after administrating the last dose of the vaccine. It is believed that the concentration of anti-HBs antibodies greater than 100 mIU/L provides high protection [9, 10].

In HBsAg-positive patients, the use of antiviral drugs is required if they are within the immunosuppressive therapy in IBD. Nucleotide/nucleoside analogues (ribavirin, tenofovir) are applied before, during, and 12 months after the interruption of the immunosuppressive therapy. If the use of antiviral drugs was not effective, the reactivation of HB infection was described in 50% of cases.

In patients who are HBsAg negative and HBcAb positive (occult infection), virus reactivation rarely occurs during immunosuppressive therapy [5]. In these patients, virus activity should be monitored for 2–3 months, with DNA virus detection, using a PCR method, and in case of positivity (HBV-DNA detection), antiviral drugs should be applied according to the above protocol.

Recommendations for the use of inactiveated vaccines in patients with IBD are given in Table 8.

Bacillus Calmette–Guérin (BSG) vaccine

BCG vaccine is still one of the most commonly used childhood vaccines worldwide, with more than 1 billion recipients. The WHO recommends vaccinating babies, who are more likely to come in contact with someone with tuberculosis, as soon as possible after birth.

The BCG vaccine contains a live, attenuated form of *Mycobacterium bovis*, whose antigenic profile is akin to *Mycobacterium tuberculosis*.

In a child with a normal immune system, a granulomatous skin reaction develops only at the site of BCG vaccination. If an individual has an underlying immunodeficiency, this can lead to dissemination of the bacteria followed by widespread granulomatous inflammation. Disseminated BCG infection has an incidence of 1–20 per 10 million doses of the vaccine given, with mortality of 50–80%. The incubation period varies from 1 to 5 months, and children are usually reported as healthy prior to vaccination.

The majority of cases of disseminated BCG have been reported in immunocompromised hosts, particularly those infected with HIV.

There are no previous case reports of disseminated BCG following vaccination of individuals or infants born to mothers taking anti-TNF therapies. However, it is well

Table 8
Inactivated vaccines for patients with inflammatory bowel diseases (IBD) [10]

Influenza: All patients with IBD should be vaccinated seasonally with the intramuscular/intradermal inactivated influenza vaccine prior to starting immunosuppressive therapy.
Pneumococcal pneumonia: All patients with IBD should be vaccinated once with the PCV13 followed by the PPSV23 (first dose after 8 weeks if immunocompromised, or after ≥1 year if immunocompetent; second dose after 5 years; and third dose after 65 years of age). If previously vaccinated with the PPSV23, then the PCV13 should be administered at least 1 year after the PPSV23 in both immunocompromised and immunocompetent adults.
Hepatitis A: Check hepatitis A immune status at the patient's initial visit. If nonimmune to hepatitis A, vaccinate the patient with a 2-dose series (0 months and 6-12 months).
Hepatitis B: Check hepatitis B immune status at the patient's initial visit. If nonimmune to hepatitis B, vaccinate the patient with a 3-dose series (0 months, and 1 and 6 months after first dose) and recheck titers 1 to 2 months after last vaccination. If the patient remains nonimmune, offer booster with a double dose of hepatitis B vaccine or offer combined hepatitis A/B vaccination.
Human papilloma virus: All male and female IBD patients between the ages of 11 and 26 years should be vaccinated with the human papilloma virus vaccine.
Meningococcal disease: Patients with IBD should be vaccinated with the meningococcal vaccine according to standard ACIP recommendations for the general population.
Tetanus, diphtheria, and pertussis: All patients with IBD should be vaccinated with Td every 10 years. Tdap should be substituted once for the Td vaccine to provide additional coverage for pertussis.

PCV – preumococcal conjugate vaccine; PPSV – pneumococcal polysaccharide vaccine; ACIP – Advisory Committee on Immunization Practices; Td – tetanus, diphtheria; Tdap – tetanus, diphtheria, accelular pertussis

recognized that TNF-alpha is crucial to granuloma formation and anti-tuberculous immunity.

Infliximab is an IgG1 antibody that does not cross the placenta in the first trimester, thereby reducing exposure to the foetus during the period of organogenesis. The evidence suggests that the rates of miscarriage, prematurity, and congenital malformations in women exposed to infliximab are not different from non-exposed pregnancies. However, in the third trimester, it readily crosses the placenta, remaining detectable in the infant's serum for up to 7 months after birth.

When possible, infliximab should be stopped in the 3rd trimester. However, the decision must be made on a case-by-case basis when the active disease could have just as harmful consequence on the pregnancy outcome.

If BCG vaccination is accidentally given to an infant born to a mother on infliximab (avoid until 12th month of life), imperial mycobacterium prophylaxis may reduce the chances of dissemination infection [27–31].

Only a few studies have assessed the effects of vaccination with BCG on the subsequent risk of IBD [29–31]. A Danish prospective and population-based case-cohort study conducted on 47,622 participants showed that BCG vaccination does not have an effect on the later risk of developing Crohn's disease and ulcerative colitis [28].

Vaccination in case of travel

Patients with IBD do not have special restrictions on travel to developing countries or countries with endemic diseases. The specificities of these trips are reflected in the possibility of a relapse of the basic disease and the disease from the infectious endemic disease. In these cases, consultations with a doctor prior to travel are required, especially for those patients on immunosuppressive therapy. Vaccination before traveling to the mentioned areas is carried out according to the same recommendations as in the general population. The hepatitis A vaccine is administered in one or two doses before traveling to the endemic areas. Yellow fever vaccination against yellow fever is recommended for travelers to endemic regions in Africa and South America. However, the live, attenuated yellow fever vaccine may potentially lead to severe and possibly lethal symptoms in immunosuppressed patients and is thus contraindicated. Immunosuppressed individuals are advised to avoid traveling to endemic regions; if travel is unavoidable, travelers should be educated regarding the risks of such travels and instructed regarding the prevention of mosquito transmission. Vaccine use is only permitted in patients who have been treated with low doses of steroids (20 mg prednisone, shorter than two weeks) for a short period of time. Other vaccines that can be safely administered are the vaccine against Japanese encephalitis and rabies. In case of traveling to the countries with endemic diseases that are prevented by live vaccines, risk assessment of the onset of infection for each patient individually and good information about preventive measures are necessary [9, 10].

Preparation of patients with IBD for the application of immunosuppressive therapy

The majority of patients with IBD will be treated with immunosuppressive therapy (80% corticosteroids, 40% immunomodulators, and 20% biological therapy) during the course of the disease. Infectious complications are the most common complications of IBD and are responsible for increased mortality in IBD and increased hospitalization, and immunosuppression is the most important factor that suits the above. Measures to prevent opportunistic infections and infections in general in these patients involve the adequate preparation of patients for the introduction of immunosuppressive therapy, which reduces the risk of infection and allows, if necessary, appropriate treatment before the introduction of therapy. Immunization as a preventive measure of infection, which is prevented in this way, is only one part of the patient's preparation. The status of immunization that was previously performed is mandatory checked when diagnosing IBD, and then vaccination is planned regarding the specificity of the application of immunosuppressive therapy: routine applied vaccines – cardboard vaccination (diphtheria, tetanus, pertussis, polio, HPV vaccine); when diagnosing – HB and VZV vaccines; before introducing immunosuppressive therapy – PCV13, PPSV23, and TIV.

In addition to the planning of vaccination, the preparation of patients for the application of immunosuppressive therapy also implies the following: anamnestic data on treated bacterial, viral, fungal, and parasitic infections, treatment of tuberculosis, environmental factors (contact with tuberculosis, conditions of life), travel to endemic areas; physical examination; screening for tuberculosis: lung Rtg, reaction to a purified protein derivative (PPD), Quantiferon (test for interferon-gamma production in response to *Mycobacterium tuberculosis* antigens); laboratory tests: detection of titer and antibody class against viruses – EBV, hepatitis A virus (HAV), HCV, HIV, VZV, HBV, HbsAg virus antigens in the serum, examination of urine and stools on *Clostridium difficile*; patient education – hygienic diet regime during travel, travel consultations, screening on *portio vaginalis uteri* (PVU) carcinoma.

Conclusion

The results of testing the modality of administration and effect of vaccines in patients with IBD are still not at the highest level. Gastroenterologists who treat patients with IBD know best the current status of each patient. Therefore, they can and should be responsible for deciding when to use the most appropriate immunosuppressive therapy. For the same reasons, they can also assess the response capacity of IBD patients to applied vaccines or to define clear recommendations for vaccinating these patients. All this is necessary in order to improve and treat patients with IBD, as this could reduce the prevalence and incidence of infectious complications in these patients, in particular, complications that could be prevented by vaccination.

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Significance of diagnostic laparoscopy and determination of free cancer cells in peritoneal lavage fluid in patients with gastric carcinoma

Značaj dijagnostičke laparoskopije i određivanja slobodnih karcinomskih ćelija u tečnosti za peritonealnu lavažu kod pacijenata sa karcinomom želuca

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Ključne reči:
želudac, neoplazme; laparoskopija; lavaža, peritonealna; ćelije; dijagnostičke tehnike i procedure.

Introduction

Common features of gastric cancer (GC) are a late diagnosis, unsatisfactory results of surgical treatment, and poor effects of the oncological treatment [1]. Radical surgery is the only option for treating gastric cancer patients. According to the latest epidemiological data, GC ranks fourth in cancer incidence and mortality worldwide, preceded by lung cancer, liver cancer, and colon cancer [2].

The incidence of GC increases with age, the highest one being among the individuals aged between 50 and 70 years. Five-year survival in Western European countries was 14.3% in 1975, and 31.0% between 2008 and 2014. According to the World Health Organization (WHO) data, 754,000 people around the world died from GC in 2015 [3].

In the same year, in the Republic of Serbia, 1,100 patients (732 males and 368 females) were registered with GC, and 903 patients (587 males and 316 females) died, as indicated by the Cancer Registry data of the Public Health Institute "Dr. Milan Jovanovic Batut" [4].

Two-thirds of patients with GC in the United States present with advanced disease, and the majority have shown no significant findings on physical examinations [5, 6]. These patients have a high risk of metastatic disease in the abdomen at the time of diagnosis. Despite numerous endoscopic and radiological methods used in the preoperative evaluation of GC, metastatic disease was first diagnosed during laparotomy in a significant number of patients (6.7 %) [7].

Concerning the anatomical location, GC is divided into proximal (cardiac cancer) and distal ("non-cardiac" cancer). In Western Europe, for the past 30 years, the incidence of distal cancers has been declining, and the incidence of cardiac cancer has been increasing (8.9%). Cardiac cancers have a generally worse prognosis, a lower five-year survival rate, and higher operative mortality compared to antropyloric gastric cancer [3].

Characteristics of gastric cancer metastases

It is impossible to accurately determine the biological onset of GC. The two main histological subtypes of the disease, the intestinal and the diffuse type, as classified by Lauren, define two distinct entities that have different epidemiology, etiology, pathogenesis, and behavior [8].

Evolutionary changes in the gastric mucosa, going from normal through atrophic, metaplastic, dysplastic to neoplastic lesions for the intestinal type of cancer, take 15 to 20 years.

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Tsukuma et al. [9] have followed 56 patients with early GC that were not operated on for various reasons. They have shown that the average time for the transition from early to advanced GC was 37 months.

The diffuse subtype of GC is more aggressive than the intestinal type. It is often diagnosed in younger patients, more frequently associated with the loss of expression of E-cadherin, and the precancerous lesions are not clearly defined [10–12].

Invasion and metastasis are the most dangerous properties of malignant tumors and are the final phase of the multi-stage carcinogenesis. The outcome of the metastatic process is the result of the interaction between the metastatic cell and various host factors, above all, the immune system. This process implies the isolation of individual or groups of tumor cells from the primary tumor, their entry into the lymph and/or blood vessels, and the retention of these cells in small blood vessels of the target organs [13, 14].

Moreover, as in other human cancers, gastric tumorigenesis can also be profoundly influenced by epigenetic abnormalities, such as aberrant gene methylation, histone modification, and microRNAs. GC is a complex and molecularly heterogeneous disease involving dysregulation of canonical oncogenic pathways, such as p53, wnt/β-catenin, nuclear factor (NF)-κB, and PI3K/Akt pathways [15, 16]. GC is a disease with an early intra-abdominal spreading and an increase in the incidence of distant metastases during the follow-up. At the time of diagnosis, about 50% of patients have metastatic disease [11].

Metastases spread like other digestive tract cancers, including direct spreading to surrounding tissues and organs (liver, pancreas, diaphragm, spleen, transverse colon, bile ducts), lymphatic pathways (local and remote), hematogenous (liver, lungs, bone, brain) and peritoneal dissemination (surface visceral and parietal metastases, Kruckenberg's tumor). Tumor spreading often occurs simultaneously in different ways. The structure of hematogenous, peritoneal, lymph node metastases, and local recurrences depends on the biological properties and behavior of tumor cells [13, 17, 18].

Vascular invasion and metastases in lymph nodes in patients with advanced cancer are an independent risk factor for the development of synchronous and metachronous metastases in the liver [13]. Clinical-pathological studies have shown that the total incidence of metastases of GC in the abdominal lymph nodes is between 60% and 80%, on the peritoneal surface some 30% to 50%, and in the liver 25% to 40% [16–18]. The incidence of lymph node metastases is independent of the pathohistological type of tumor and is significantly associated with the degree of tumor invasion of the wall of the stomach [17].

Liver metastases are more common in patients with intestinal tumor type (50% to 70% vs. 3% to 30% for diffuse type), while peritoneal dissemination is most common in patients with the diffuse type of gastric cancer (45% to 75% vs. 10% to 30% for intestinal type) [16–18].

Peritoneal dissemination excludes surgical treatment of GC and is the most common cause of death in patients with GC. Peritoneal dissemination will occur despite curative resection in about 50% of patients with serosal invasion [17–19].

Diagnostic laparoscopy

The preoperative staging of gastric cancer makes use of chest X-ray, upper endoscopy, barium upper gastrointestinal examination, ultrasound of the upper abdomen (US), endoscopic ultrasonography, computerized tomography (CT) of the chest, upper abdomen, and small pelvis, laparoscopy, magnetic resonance (MR) and computer positron emission tomography (PET CT).

Despite all this, there is still no clear definition of what has to be done in the preoperative staging of GC. In recent years, the treatment of gastrointestinal tumors has become more complex and involves different treatment modalities such as neoadjuvant chemotherapy (HT), adjuvant HT, palliative systemic HT, or symptomatic treatment. In order to determine the optimal type of therapy, it is necessary to establish the stage of the disease more precisely at the time of diagnosis.

In spite of the significant technological advances in the development of highly sophisticated radiological equipment, peritoneal dissemination and lymph node metastasis are quite common in most patients diagnosed during laparotomy [20]. Laparoscopic exploration allows us to visualize the primary tumor, detect metastatic superficial metastases that cannot be diagnosed by other morphological methods (CT, MR, PET-CT), regional nodal metastases, peritoneal metastases, and free cancer cells in the peritoneal fluid [7, 21].

In the retrospective study of Tourani et al. [22], carried out in Australia with 199 GC patients included, diagnostic laparoscopy (DL) with peritoneal lavage in 19% of cases changed the treatment strategy of these patients.

DL significantly reduces unnecessary laparotomy in patients with an advanced stage of the disease [23–34]. In addition, it selects patients with advanced disease for various preoperative treatment modalities.

In the study of Stell et al. [32], the sensitivity of DL in detecting liver metastases was 96%, while the sensitivity of CT was 52% and US was 37%. In the diagnosis of peritoneal metastases, DL sensitivity was 69%, that of CT was 8% and for US it was 23%. The use of PET-CT for peritoneal metastases diagnosis in GC is also controversial, in view of the reported PET-CT poor sensitivity [33].

Absolute contraindications for laparoscopic exploration are severe coagulopathy and a high risk for surgery in general anesthesia. Relative contraindications include previous laparotomy, morbid obesity, and pregnancy. DL is a safe method in the preoperative staging of gastric cancer [31]. In the study by Muntean et al. [25], morbidity during DL was 2.2%, and mortality was 0%. During the monitoring period, no "port site" metastases were registered.

In a 1996 retrospective study by Adamek et al. [35], morbidity and mortality of DL in 747 patients were analyzed over a nine-year period. Eleven patients (1.5%) had serious complications, and one patient (0.13%) died after DL.

Table 1

Recommendations for staging laparoscopy from various professional societies

Society	Country of origin	Recommendation
SAGES [3]	USA	Patients with T3 or T4 gastric cancer without evidence of lymph node or distant metastases on high-quality preoperative imaging
ESMO [19]	Europe	All patients with resectable gastric cancer (III, Grade B)
S3 Guidelines [20]	Germany	Patients with advanced-stage gastric cancer (cT3-cT4); (II–III, Grade B)
GIRCG [21]	Italy	Cases deemed to be at risk of peritoneal carcinomatosis not visible or doubtful at CT examination
SEOM [24]	France	All patients with potentially resectable gastric cancer
JGCA [25]	Japan	Patients with clinical stage II-III prior to neo-adjuvant treatment

SAGES – Society of American Gastrointestinal and Endoscopic Surgeons; ESMO – European Society for Medical Oncology; GIRCG – The Italian Research Group for Gastric Cancer; SEOM – Spanish Society of Clinical Oncology; JGCA – Japanese Gastric Cancer Association; CT – computed tomography.

There is still no consensus when DL should be done. Table 1 lists DL indications according to different national associations.

The rate of DL doubled between 1998 and 2005. Despite the increased use of laparoscopy, occult metastases were identified in a similar proportion of patients [36, 37].

One of the key dilemmas is whether DL should be used as a special diagnostic procedure or immediately prior to the planned curative surgical resection (if there are no macroscopically visible metastases in the liver and peritoneum, and if the cytological examination of the peritoneal lavage fluid (PLF) excludes malignant cells). This mainly depends on the work organization in each hospital, as well as on how long it takes to obtain cytology results of a PLF. In our country, only a few hospitals have a cytology department. Therefore, DL is mostly based on a macroscopic examination (cytological examination of the peritoneal lavage is not a routine procedure). Further development of cytology as a science and an increase in the number of cytologists in our country would significantly improve diagnostic and therapeutic procedures, reduce morbidity (unnecessary laparotomy, unnecessary "curative" resections), and hospitalization costs in patients with advanced malignant digestive diseases.

The patients with metastatic disease (occult or otherwise) do not benefit from resection. Additionally, the minimal morbidity of DL argues strongly in favor of its widespread adoption in the management of patients with gastric cancer.

DL should be performed before chemotherapy for patients in whom a neoadjuvant approach is considered. Washing might increase the accuracy of DL [37].

DL is also used to evaluate the effects of systemic and neoadjuvant HT in patients with advanced GC [36–40].

The cost-effectiveness of DL for GC patients is highly dependent on the patient and the results of the diagnostic examination, and it is higher for locally advanced disease or in detecting peritoneal and superficial liver lesions [41].

Enhanced outreach and education of surgeons may help increase the use of DL in practice.

DL should be used in the following patients: patients with T3, T4 tumor of the stomach (determined by CT or EUS examination); patients with T2, N2 (certain CT or EUS examinations) with a diffuse type tumor greater than 5 cm in diameter; patients with gastric tumors, ascites, and negative cytological findings on malignant cells (sample taken percutaneously) regardless of T stage; patients treated with systemic or neo-adjuvant chemotherapy to evaluate the effects of the treatment [36–40].

DL should not be performed in the following cases: GC complicated by obstruction, bleeding or perforation; early GC; multiple previous laparotomies; in clearly diagnosed distant metastases (liver, lungs, bones, etc.) by other morphological methods [36–40].

Significance of free cancer cells detection in peritoneal lavage fluid (PLF) in patients with gastric carcinoma

Cytological analysis of PLF is an inexpensive and reliable method of testing the presence of free cancer cells (FCC) in the peritoneal cavity.

Laboratory methods for malignant cell detection in the aspirate include conventional cytology and reverse transcriptase-polymerase chain reaction (rt-PCR) [42, 43].

Cancer cells are found as single or groups of cells – epithelial type with all the morphological characteristics of malignant cells (enlarged nuclei of irregular shape, irregular chromatin structure, and prominent nucleolus) [42].

The first step in the development of peritoneal metastases is the detachment of cancer cells from the primary tumor invading serosa, followed by their peritoneal cavity spread.

The hypothesis that FCC play a significant role in the occurrence of peritoneal metastases is justified by the fact that postoperative metastases are present in almost all patients with free cancer cells proven during operative treatment of gastric cancer, even in those with potentially curative resection [42].

The possibility of finding FCC is increased with the degree of serosa involvement and the size of the surface of the affected serosa [42–45].

According to the multivariate analyses, the size of the tumor, the depth of the stomach wall of invasion, and the presence of metastases in lymph nodes are the most important prognostic factors in terms of patient survival [44–46].

Suzuki et al. [47] found that 50% of patients with GC greater than 14 cm had cancer cells in the PLF.

Kostić et al. [48] found that patients with tumor diameters less than or equal to 5 cm did not have FCC in PLF, while 30.95% of patients with cancer diameters greater than 5 cm had a positive cytological finding. This study has also shown

that tumor size is statistically highly significant for the frequency of a positive cytological finding. Positive cytological findings in patients with diffuse gastric cancer were 31.25% and 10.71% in patients with intestinal tumor type. The risk for the presence of FCC is 56 times higher in GC patients with serosal invasion (T3 and T4) than in those with T1 and T2 tumors and as much as 60 times higher in patients with tumor greater than 5 cm in relation to patients with tumors less than or equal to 5 cm.

Kaibara et al. [45] found FCC in 22% of patients with infiltration of serosa lesser than 10 cm^2, whereas the presence of FCC reached 72% in patients with infiltration of serosa greater than 20 cm^2.

A positive cytological finding is more often present in non-differentiated *versus* differentiated tumors [49–52].

The length of survival of patients with FCC does not differ significantly from patients with macroscopically visible peritoneal metastases (PM), even after curative resection of gastric cancer [46, 49].

The disease-free survival (DFS) of patients with a positive FCC without clearly seen peritoneal metastases is 13 months, whilst the DFS of patients with peritoneal metastases is about 10 months [47].

In the study by Bentrem et al. [51], the DFS of patients with R0 resection (a total of 371 patients), due to GC and a positive FCC in PLF, was 14.8 months, while patients with negative cytological findings had a DFS of 98.5 months.

A positive FCC in the PLF in the absence of visible peritoneal metastases is not uncommon in patients with gastric cancer and indicates a poor prognosis [49].

Sometimes, in patients with clear peritoneal dissemination, we get a negative finding for cancer cells in the peritoneal fluid.

Nakajima et al. [53] found that 32% of patients with macroscopic peritoneal dissemination did not demonstrate the presence of FCC in the peritoneal fluid. They concluded that such a high rate of false-negative findings is not a technical error but a consequence of the type of implantation of tumor cells into the peritoneum (often deeply implanted in the peritoneum).

The reliability of the cytological analysis of PLF in patients with advanced GC is about 91%, with a lower sensitivity of about 56% and a specificity of about 97% of the method [51].

Since the cytological examination of ascites on malignant cells has low sensitivity, new biomarkers are being examined to diagnose and predict the occurrence of gastric carcinoma peritoneal dissemination [54–57].

In a multicentre prospective study [57], miRNA expression of the genes encoding carcinoembryonic antigen (CEA) and cytokeratin 20 (CK-20), evaluated by RT-PCR, has proven to be useful for the prediction of overall survival and PM in GC. However, the disadvantage of mRNA-based diagnostic methods is the high degradability of mRNA in the course of surgical procedures.

In contrast, miRNAs enclosed in exosomes remain stable and can circulate in body fluids, such as serum, plasma, saliva, urine, breast milk, and tears, for long periods of time [56].

Cytology and molecular diagnostic assays are based on detecting the cancer cells, whereas profiling of miRNAs in PLF may be used for predicting the peritoneal premetastatic phenotype in GC, ensuring more effective preventive and curative measures [57].

The results of some randomized studies show that intraperitoneal chemotherapy is effective in preventing peritoneal recurrence in patients with FCC [58, 59]. Intraperitoneal chemotherapy statistically significantly reduces the incidence of peritoneal dissemination, though without affecting the incidence of liver or other metastases.

Cytological examination of PLF and PCR of PLF on FCC in patients with advanced GC is mandatory during a diagnostic laparoscopy. The presence of FCC in the PLF is a contraindication for curative surgical resection, and such patients are candidates for neoadjuvant chemotherapy [60].

Intraperitoneal FCC can also be found in earlier clinical stages of gastric cancer. In patients with low surgical and oncological risk (no serosa invasion, no lymph nodal spread, moderate or well-differentiated neoplasm), immediate surgery should be performed, and intraoperative peritoneal washing/lavage should be added [61].

The question remains whether it is necessary to do a PLF cytological examination on FCC (considering the pathogenesis of peritoneal metastases) in each patient during the surgical resection of GC, regardless of the stage of the disease. Further studies are necessary to better monitor and treat these patients.

Conclusion

Diagnostic laparoscopy is an important method in the preoperative staging of gastric cancer. Accurate preoperative disease staging is necessary for the optimal treatment of patients with gastric cancer. A cytological examination of the peritoneal lavage fluid is mandatory during the diagnostic laparoscopy in patients with advanced gastric carcinoma without macroscopically visible changes in the peritoneum. Further research on reliable biomarkers in peritoneal lavage fluid is needed to attain more reliable recruitment of patients with a phenotype for probable peritoneal dissemination, enabling a more aggressive therapeutic oncological approach and possibly a longer survival of patients with advanced gastric cancer.

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CASE REPORTS

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Myelodysplastic/myeloproliferative neoplasm with t(2;11)(P21;Q23)del(5) (Q22;Q33) but without mixed-lineage leukemia (MLL) rearrangement

Mijelodisplazna/mijeloproliferativna neoplazma sa t(2;11)(P21;Q23)del(5) (Q22;Q33) ali bez *mixed-lineage leukemia* (MLL) rearanžmana

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Abstract

Introduction. Myelodysplastic/myeloproliferative neoplasms represent a group of rare hematologic malignancies with concomitant characteristics of two different disorders. There are cytopenias and cytoses with dysplastic morphology in the circulating blood and hyperplastic bone marrow, respectively. Many cytogenetic and molecular features have been found in this rare entity, but t(2;11)(p21;q23)del(5) (q22;q33) has not been described so far. **Case report.** We present a patient with myelodysplastic syndrome, subtype refractory anemia without ringed sideroblasts, with unique translocation t(2;11)(p21;q23) associated with del(5)(q22;q33) in the karyotype. Fluorescence *in situ* hybridization analysis did not detect mixed-lineage leukemia (MLL) rearrangement, which can be found in other hematologic malignancies with this translocation. After a year on supportive treatment with packed red cells, thrombocytosis developed with a concurrent increase in white blood cells and the Janus kinase-2 gene mutation. This confirmed the presence of myelodysplastic/myeloproliferative neoplasms. Due to the high platelet count, the cerebrovascular insult has occurred. The patient was treated supportively and with lenalidomide. After introducing the lenalidomide steadily, the patient's condition improved, the peripheral blood count normalized, and he became transfusion independent. **Conclusion.** Patients with the cytogenetic finding of t(2;11)(p21;q23) associated with del(5)(q22;q33) but without MLL rearrangement and with Janus kinase-2 gene mutation presence, respond to lenalidomide therapy and have relatively longer overall survival.

Key words:
myelodysplastic syndrome; thrombocytosis; myeloproliferative disorders; janus kinase-2; mutation; antineoplastic agents; lenalidomide; treatment outcome.

Apstrakt

Uvod. Mijelodisplazne/mijeloproliferativne neoplazme predstavljaju grupu retkih hematoloških maligniteta sa istovremeno prisutnim osobinama dva različita oboljenja. U perifernoj krvi postoji citopenija jedne krvne loze uz citozu drugih krvnih elemenata sa displastičnom morfologijom, dok se u koštanoj srži nalazi hiperplazija. Mnoge citogenetske i molekularne osobine su nađene u ovom retkom entitetu, ali t(2;11)(p21;q23)del(5) (q22;q33) do sada nije opisana. **Prikaz bolesnika.** Prikazan je bolesnik sa mijelodisplaznim sindromom, podtip refraktorna anemija bez prstenastih sideroblasta, sa jedinstvenom translokacijom u kariotipu t(2;11)(p21;q23) udružena sa del(5)(q22;q33). Fluorescentna *in situ* hibridizacija nije utvrdila *mixed-lineage leukemia* (MLL) genski rearanžman, koji je inače osobina ove translokacije. Nakon godinu dana lečenja suportivnom terapijom sa koncentrovanim eritrocitima, razvila se trombocitoza praćena porastom belih krvnih zrnaca i prisustvom mutacije gena Janus kinaze-2. To je povrdilo evoluciju refraktorne anemije u mijelodisplaznu/ mijeloproliferativnu neoplazmu. Zbog visokog broja trombocita razvio se cerebrovaskularni insult. Bolesnik je u daljem toku lečen suportivno uz dodatak lenalidomida. Nekoliko nedelja nakon ove terapije, nalaz u perifernoj krvi se popravio i bolesnik je postao transfuziono nezavistan. **Zaključak. Bolesnici sa c**itogenetskim nalazom t(2;11)(p21;q23) udruženim sa del(5)(q22;q33), ali bez MLL rearanžmana, uz prisustvo mutacije gena Janus kinaze-2, povoljno odgovaraju na lečenje lenalidomidom i imaju relativno duže ukupno preživljavanje.

Ključne reči:
mijelodisplastički sindromi; trombocitoza; mijeloproliferativni poremećaji; janus kinaza 2; mutacije; antineoplastici; lenalidomid; lečenje, ishod.

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Introduction

Myelodysplastic syndrome (MDS) represents a heterogeneous group of diseases characterized by impaired haematopoiesis, dysplasia in one or more myeloid cell lines, and cytopenias in the peripheral blood. In 30% of patients, the disease progresses to acute myeloid leukemia (AML). MDS may be occasionally associated with thrombocytosis and most frequently myelodysplastic/myeloproliferative neoplasms (MDS/MPN). According to the 2016 World Health Organization (WHO) classification, MDS/MPN includes chronic myelomonocytic leukemia (CMML), atypical chronic myeloid leukemia (aCML), juvenile myelomonocytic leukemia (JMML), MDS/MPN with ring sideroblasts and thrombocytosis (MDS/MPN-RS-T)[1–3]. The incidence of MDS/MPN-RS-T is not well known, but it is estimated to be around 5% of all myeloid malignancies [4].

Cytogenetic abnormalities are found in around 50% of patients with MDS, which are significant for classification and prognostic stratification of the disease [5, 6]. Cytogenetic abnormalities in MDS are among the most valuable independent prognostic factors included in the International Prognostic Scoring System (IPSS), which assigns four risk categories of death or transformation to AML. This system is also based on a score that reflects the percentage of bone marrow blasts and the number of cytopenias [5]. However, due to the profound cytogenetic heterogeneity, the impact of many rare cytogenetic abnormalities in a substantial portion of patients with MDS is still unknown and can only be delineated on the basis of larger international studies [5].

In the literature, there are at least 26 cases with t(2;11)(p21;q23) described in MDS [7–9]. This translocation was most frequently associated with del(5q).

We describe a rare case of refractory anemia (RA) with t(2;11)(p21;q23) del(5)(q22;q33) in the karyotype. The erythroblasts did not display rings on Pearl's staining. The patient developed MDS/MPN-T with thrombocytosis and JAK2V617F mutation.

Case report

A 58-year-old male patient presented with malaise, fatigue, and headache starting at the end of 2015. MDS subtype of RA was diagnosed. The patient had an earlier history of hypertension. Upon examination, he was pale but without organomegaly and haemorrhagic syndrome. Complete blood count (CBC) at the time of diagnosis was the following: white blood cells (WBC) 5.57×10^9/L (55.3% segmented neutrophils, 8% monocytes, 1.3% basophils, 3.4% eosinophils, and 32% lymphocytes). Hemoglobin (Hb) was 69 g/L, red blood cells (RBC) 1.88×10^{12}/L, MCV121 fl, platelets 403×10^9/L. The C reactive protein (CRP) and other acute phase reactants were within normal ranges. The renal and hepatic function tests, lactate dehydrogenase (LDH) were normal. The concentration of vitamin B_{12} was 234 pmol/L, iron 17.9 μmol/L (normal range 11–30 μmol/L), and ferritin 409 μg/L (normal range 20–250 μg/L) with total iron-binding capacity – TIBC 42.5 μmol/L (normal range 44.8–80.6 μmol/L). The bone marrow aspirate showed increased cellularity, with slightly reduced megaloblastic erythropoiesis and normal granulopoiesis. Megakaryocytic lineage was profuse with some atypical mononuclear megakaryocytes and rare micromegakaryocytes. There were no manifestations of erythroid dysplasia, and ring sideroblasts were not found. The bone marrow trephine revealed 80% of hematopoietic cellularity with partly megaloblastic erythropoiesis. The ratio of myeloid to erythroid cells was normal with normal myelopoiesis. The megakaryocytes were increased in number showing hypolobulated nuclei. Their cytoplasm stained positively with periodic acid-Schiff (PAS). There were infrequent micromegakaryocytes. The cytogenetic analysis showed aberrant complex karyotype: 46,XY,t(2;11)(p21;q23),del(5)(q22;q33) in twenty mitoses (Figure 1). Fluorescence *in situ* hybridization did not detect mixed-lineage leukemia (MLL) rearrangement (Figure 2).

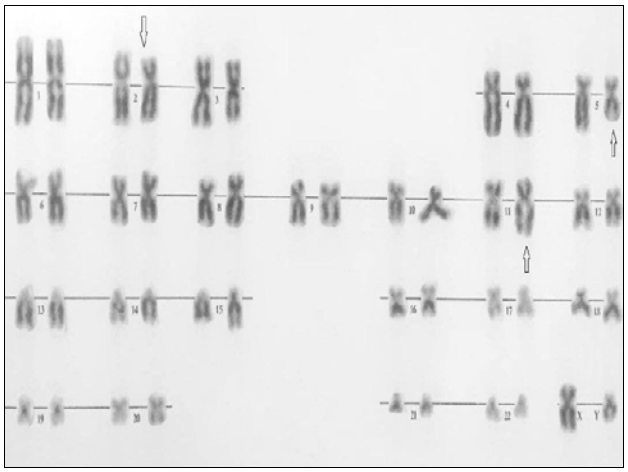

Fig. 1 – Karyotype showing 46,XY,t(2;11)(p21;q23),del(5)(q22;q33).

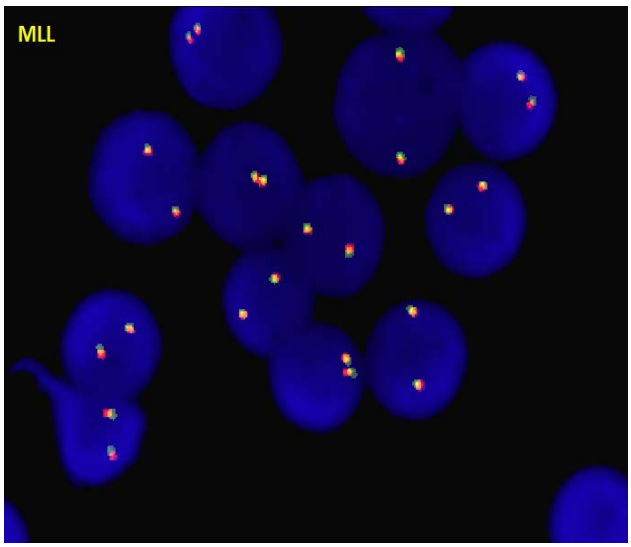

Fig. 2 – Fluorescence *in situ* hybridization – the mixed-lineage leukemia (MLL) gene rearrangement was negative.

The patient was transfusion-dependent and sporadically received twenty units of packed RBC by September 2016. The level of ferritin doubled 891 ug/L, and the erythropoietin level was 1,081 mlU/mL (normal range 3.70–31.50 mlU/mL). In November 2016, his CBC was the following: WBC 7.28×10^9/L (neutrophils 57%, lymphocytes 32%, monocytes 7%, eosinophils 3.1%, basophils 0.9%), Hb 75 g/L, MCV 120 fl and platelets 959×10^9/L. His ferritin increased to 2,710 μg/L. The treatment included folan, danazol, exjade, and prednisone 20 mg/day. The number of platelets and leukocytes increased steadily. Allogeneic bone marrow transplantation was planned, and the HLA typing was duly performed. At that time, no suitable unrelated donor was available. In January 2017, the CBC showed severe anemia, increase in WBC to 15.05×10^9/L with a normal differential. The platelet count was $1,729 \times 10^9$/L and ferritin 3,430 μg/L. The bone marrow histology was hypercellular (80%) with trilineage hematopoiesis with megakaryocytic hyperplasia with solitary or hypolobulated nuclei, some appearing in clusters (up to three cells) with few micromegakaryocytes. A focal paratrabecular dislocation of megakaryocytes existed. The number of blasts was normal (4% of CD34+/CD117+ cells). There was no reticulin fibrosis. On ultrasonography, the spleen was enlarged up to 170 mm in diameter. The treatment with hydroxycarbamide and aspirin was initiated. Unfortunately, the cerebrovascular insult (CVI) had developed. The molecular analysis, using a peripheral blood sample, detected the JAK2 gene mutation (V617F). The laboratory and molecular findings indicated that RA has evolved to MDS/MPN-T. Allogeneic stem cell transplantation has been planned, but a matching donor remained unavailable. Surreptitious supplementation with packed red cells was continued, but the treatment with exjade was irregular because it was financially unaffordable. Consequently, the level of ferritin remained high (4,130 μg/L). The lenalidomide, hydroxycarbamide, danazol, and folan remained his ongoing therapy. Several weeks after introducing lenalidomide, the patient's condition improved. He became transfusion independent, and his blood count in peripheral blood finally normalized.

Informed consent was obtained from the patient for the presentation of this case.

Discussion

The MDS are clonal stem cell disorders that are characterized by dysplasia in one or more myeloid lineages, ineffective hematopoiesis, and one or more cytopenias. In order to define categories within the MDS, the WHO Classification combined clinic, cytology, and cytogenetic analyses [3, 10, 11]. Rarely, certain MDS subtypes present with thrombocytosis rather than cytopenia [2, 3, 10, 12]. The incidence of thrombocytosis in MDS is 5% [9, 10, 13]. The new WHO Classification of the myeloid neoplasms introduced the category of MDS/MPN diseases. These include myeloid disorders, with both dysplastic and proliferative features at the time of initial presentation. This is due to the fact that it was difficult to assign distinctly either of the two features to the myelodysplastic or myeloproliferative group of diseases [10, 11]. Refractory anemia with ringed sideroblasts associated with marked thrombocytosis (RARS-T and MDS/MPN-RS-T), which is occasionally accompanied by increased platelet count, belongs to this category [2].

The presented patient had the clinical, laboratory, and morphologic characteristics of MDS subtype RA without ringed sideroblasts. Initially, the leukocyte and platelet number was normal. Cytogenetic finding showed 46,XY,t(2;11)(p21;q23),del(5)(q22;q33). The patient soon became transfusion-dependent, and after ten months, he was iron overloaded. After one year of supportive treatment, anemia progressed, the number of platelets increased with a concomitant increase in WBC count. The patient developed CVI as a complication. Bone marrow aspiration and biopsy showed hypercellularity, a marked proliferation of hypolobulated megakaryocytes with rare micromegakaryocytes, some focally dislocated to the paratrabecular region. Other signs of myelodysplasia were not in evidence. The spleen size increased to 170 mm, and the odds of essential thrombocythaemia (ET) were considered. However, the cytogenetic abnormality and the presence of micromegakaryocytes were not suggestive of ET. At all times, ringed sideroblasts were not present. The patient progressed from RA to MDS/MPN with thrombocytosis. Using the PCR method, the JAK2-V617 mutation was identified. Hydroxycarbamide was introduced, and the number of platelets dropped.

In the MDS, chromosomal abnormalities are found in about 50% of patients, most frequently as unbalanced structural aberrations and loss of material [5]. Rare cytogenetic abnormalities are observed in MDS with a frequency of less than 2% [5]. In the literature, the translocation of t(2;11)(p21;q23) was found in 26 patients with MDS [7–9]. In approximately half of the published cases, t(2;11)(p21;q23) was associated with deletion of the long arm of chromosome 5, (5q) [7–9]. The translocation breakpoint in 11q23 is near MLL gene. In most of these patients, the examination for the rearrangement of MLL had not been done. In a large cohort study of 1,185 patients with MDS, the presence of t(2;11)(p21;q23) was found in seven patients only. All of the seven patients were males with a median age of 52 years, cytological and histological signs of MDS, and marked dysplasia in megakaryocytopoiesis. Only two patients had a sole t(2;11)(p21;q23), 4 patients had associated 5q deletion, and in one patient, a subclone with deletion 5q was observed. They all lacked the MLL rearrangement. Their median survival was 72 months. It was concluded that t(2;11)(p21;q23) may have a good prognosis [9].

The JAK2 mutation was found in RARS-T in around 58% of cases and 20% of patients with MDS/MPN-RS-T [4, 14]. This mutation in myeloproliferative disorders is accompanied by thrombocytosis and erythrocytosis. In our patient, low numbers of RBC may have been a result of a defect in erythropoiesis. Consequently, an expected "protective effect" of the JAK2 mutation on the erythroid

cell line was suppressed [14, 15]. Similarly, JAK2 positive patients may also have leukocytosis as a result of a proliferative signal to leukocyte precursors [14, 16, 17].

Conclusion

This presentation suggests that specific chromosomal abnormality t(2;11)(p21;q23),del(5)(q22;q33) could be observed in patients with myelodysplastic/myeloproliferative neoplasms, most frequently without MLL gene rearrangement, and in addition with Janus kinase-2 gene mutation, they significantly respond to therapy with lenalidomide. A favorable response of our patient to lenalidomide indicates that the dominant cytogenetic finding in his karyotype was del(5)(q22;q33).

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CASE REPORT

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Recurring *myositis ossificans traumatica* of temporal muscle: A case report

Recidiv traumatskog osificirajućeg miozitisa temporalnog mišića

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Abstract

Introduction. *Myositis ossificans traumatica* (MOT) refers to a benign, localized ectopic bone formation within skeletal muscle bundles related to a traumatic injury. MOT rarely affects masticatory muscles, and it represents a major diagnostic and therapeutic problem for clinicians. Currently, the treatment of choice is complete excision of the calcified mass after bone maturation and resection of the affected bone. **Case report.** A 47-year-old male presented with a month-long severe restriction of mouth opening that was followed by extraction of the right lower third molar tooth under local anesthesia. A computed tomography (CT) scan revealed ectopic bone formation in the right temporal muscle extending to the right coronoid process. Surgical excision of the calcified mass was performed. Six years after the surgery, the patient reported the same symptoms. The CT scan revealed a calcified mass of the right temporal muscle extending to the medial pterygoid muscle. The patient was reoperated, and sent for the postoperative physical treatment. **Conclusion.** MOT represents a major diagnostic and therapeutic challenge for surgeons due to unclear etiology and frequent recurrences after surgical treatment. Further research is needed to clarify the mechanisms of ossification in MOT in order to develop conservative treatment approaches.

Key words:
myositis ossificans; temporal muscle; oral surgical procedures; recurrence; treatment outcome.

Apstrakt

Uvod. Traumatski osificirajući miozitis (*Myositis ossificans traumatica* – MOT) se odnosi na benigno, lokalizovano ektopično formiranje koštanog tkiva unutar skeletnih mišića nakon trauma. MOT retko zahvata mastikatorne mišiće i tada predstavlja ozbiljan dijagnostički i terapijski problem za hirurge. Trenutno je tretman izbora potpuna ekscizija kalcificifikovane mase i resekcija zahvaćene kosti. **Prikaz bolesnika**. Bolesnik, star 47 godina, javio se na pregled zbog otežanog otvaranja usta mesec dana unazad, nakon ekstrakcije desnog donjeg umnjaka pod lokalnom anestezijom. Nalaz kompjuterizovane tomografije (CT) glave ukazao je na stvaranje ektopične kosti u desnom temporalnom mišiću i koronoidnom nastavku donje vilice. Učinjena je hirurška ekscizija kalcificirane mase i resekcija zahvaćene kosti. Šest godina nakon operacije, bolesnik se žalio na iste simptome. Nalaz CT glave je pokazao ponovnu pojavu kalcifikovanog tkiva unutar desnog temporalnog mišića koje se protezalo na medijalni pterigoidni mišić. Bolesnik je reoperisan i upućen na postoperativni fizikalni tretman. **Zaključak.** Zbog nejasne etiologije i čestih recidiva nakon hirurškog lečenja MOT predstavlja dijagnostički i terapijski izazov za hirurge. Dalja istraživanja su potrebna kako bi se razjasnili mehanizmi osicifikacije kod MOT u cilju razvoja konzervativnog tretmana.

Ključne reči:
miozitis osifikans; temporalni mišić; hirurgija, oralna, procedure; recidiv; lečenje, ishod.

Introduction

Myositis ossificans is a rare disease in which ectopic benign bone formation occurs in muscle tissue. It has been divided into *myositis ossificans progressiva* (MOP) and *myositis ossificans traumatica* (MOT) [1]. MOP is an autosomal dominant disease characterized by systemic ossification of muscles and soft tissues with poor prognosis [1]. MOT refers to a benign, localized ectopic bone formation and ossification of fibrous connective

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tissue within skeletal muscle bundles related to traumatic completely understood, and proposed pathogenesis theories suggest inflammatory response in muscle tissue related to trauma followed by displacement of osteoprogenitor cells and overexpression of bone morphogenetic proteins, leading to ectopic bone formation [1–3].

MOT is most commonly seen in young patients due to bone metabolism, renewing periosteum, and richness in mesenchymal cells [4]. MOT rarely affects masticatory muscles, and, so far, a limited number of reports have been published. MOT of masticatory muscles represents a major clinical problem since there is no unified algorithm for diagnosis and treatment. The main clinical sign of MOT of masticatory muscles is progressive trismus followed by head and neck trauma. The only treatment modality widely accepted is complete excision of the calcified mass after bone maturation and resection of the affected bone.

In this case report, we presented a case of recurring MOT affecting the right temporal muscle after extraction of the right lower third molar under local anesthesia.

Case report

A 47-year-old male presented to the Department of Maxillofacial Surgery in 2011, complaining of a month-long jaw motion restriction. The patient's previous medical history was uneventful. The patient reported that he underwent extraction of the right lower third molar due to the pericoronal infection under local mandibular anesthesia in a private dental clinic approximately 2 months before the symptoms appeared. There was no history of other trauma to the head and neck.

Head and neck examinations revealed severe trismus, with a maximum incisal opening of 2 mm.

There was no tenderness of the right masseter and temporalis muscles. Intraoral examination was incomplete due to the trismus. Computed tomography (CT) scan of the facial bones depicted radiopaque entity attached to injury [1–3]. The pathological mechanism of MOT is not and extending superiorly to the right coronoid process. It also depicted the insertion of the right temporalis muscle. The lesion appeared as a central radiolucency surrounded by the circumscribed ossified periphery. Calcification in the right temporal muscle was approximately 43 × 15 mm in size when measured from the coronoid processus to the upper part of the lesion. It extended to the temporal fossa and was well-defined from the surrounding structures. There were no signs of bone destruction and infiltration of other masticatory muscles (Figure 1).

At this point, the differential diagnosis of extraskeletal bone formation included *myositis ossificans*, *fibrodysplasia ossificans*, osteochondroma, chondrosarcoma, osteosarcoma, osteoma, and vascular malformation with phlebolithis.

We decided to perform a right mandibular coronoidectomy and extirpation of the osseous tissue. By intraoral approach and elevation of the mucoperiosteal flap, a calcified mass extending to the temporalis muscle from the coronoid process, 4 × 3 cm in size, was visualized after striping the temporalis attachment from the coronoid process. Due to the size and localization of the lesion, an extraoral temporal approach was performed in order to gain access to the mass in the right temporal fossa, which was excised along with the coronoid processus. The resected coronoid process and the calcified mass were a normal-appearing bone with no evidence of a surrounding bony or soft tissue destruction or infiltration. After resection, there was a 2 cm gap between the mandibular ramus and the temporalis muscle. The immediate intraoperative maximal incisal opening, measured from the maxillary to the mandibular incisal edges, was 40 mm, compared to the 2 mm preoperatively.

A histological finding revealed a zonal pattern of the lesion. The innermost zone consisted of an immature vascularized fibroblastic zone with a mild degree of pleomorphism, sparse inflammatory cells, and rare multinucleated giant cells. The intermediate zone consisted of an irregular bone trabecula, and the

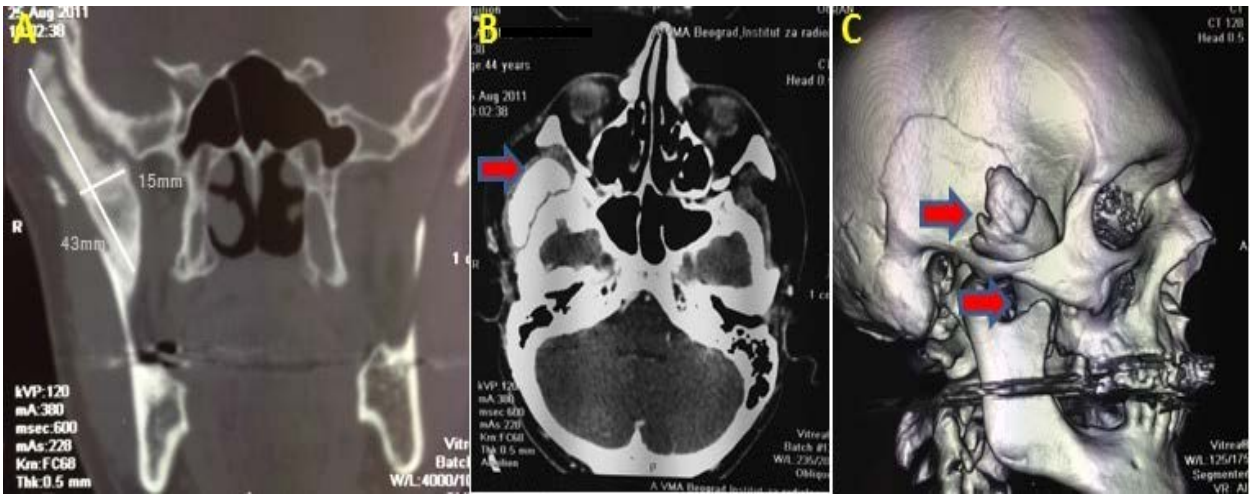

Fig. 1 – Preoperative computed tomography (CT) demonstrating heterotopic calcification in the right temporal muscle (red arrows): A) Sagittal view; B) Axial view; C) 3D reconstruction.

peripheral zone revealed ossification and mature lamellar bone (Figure 2). The histological finding was consistent with myositis ossificans.

Postoperatively, the patient was able to open his mouth passively up to 25 mm without assistance.

The patient presented to our Clinic for a follow-up evaluation two years after undergoing the right mandibular coronoidectomy, partial resection of the right temporal muscle tendon, and extirpation of the osseous tissue from the temporal fossa. He reported difficulties in mouth opening in the morning but no restriction or pain in mouth opening during the day. Extraoral and intraoral examinations were uneventful. The patient's maximum incisal opening was 30 mm without assistance or pain. However, the CT scan revealed an osseous lesion in the right temporal fossa, in the vicinity, but not attached to the resected coronoid processus, extending to the temporal muscle. The radiological features of the lesion were similar to the previous findings (Figure 3).

Since there were no functional problems, the patient was scheduled for a follow-up examination after 6 months. Two years later, the CT scan was repeated (2015), and the revealed condition was unchanged; however, the ossification extended towards the mandibular attachment of the right medial pterygoid muscle (Figure 4).

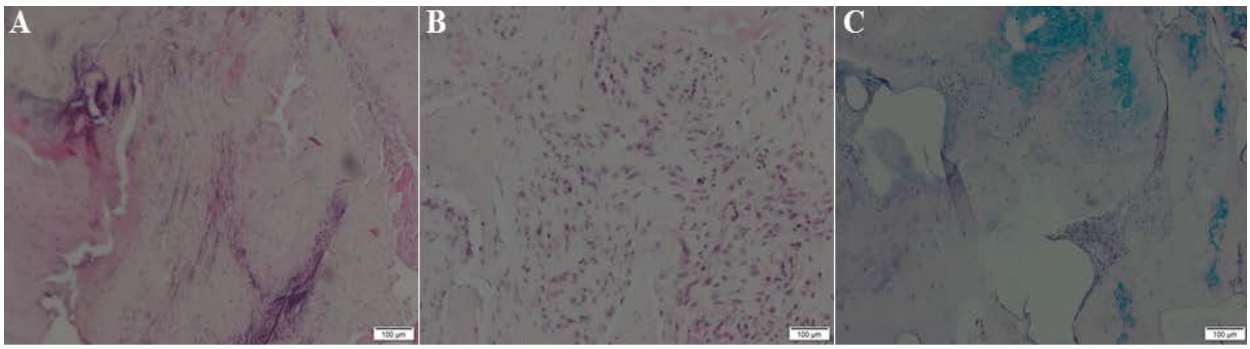

Fig. 2 – Photomicrograph of an excised extraosseous bony lesion: A) Section of specimen showing central loose connective tissue with immature bone containing osteocytes and mature bone at the periphery (hematoxylin and eosin – H&E stain, ×100); B) Photomicrograph showing central connective tissue zone surrounded by immature bone containing osteocytes in the lacunae (H&E stain, ×200); C) Periodic acid-Schiff (PAS) staining.

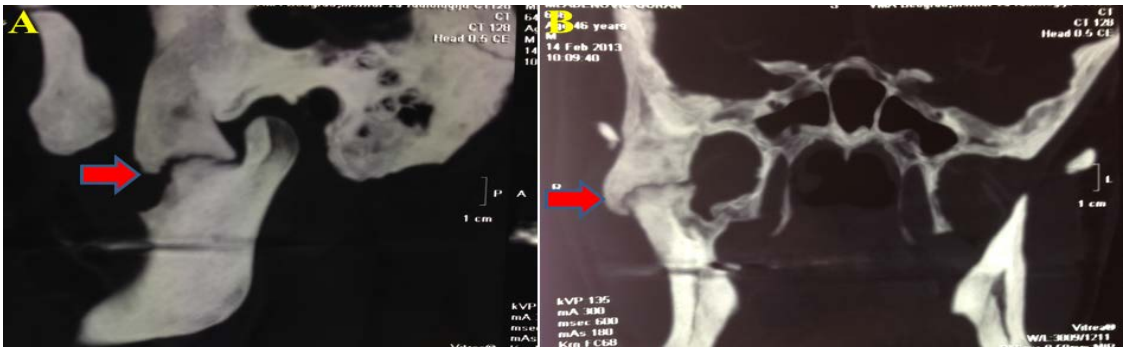

Fig. 3 – Postoperative computed tomography (CT) 2 years after surgical therapy demonstrating recurrence of heterotopic calcification in the right temporal muscle (red arrows). Coronoid processus is resected. A) Coronal view; B) Sagittal view.

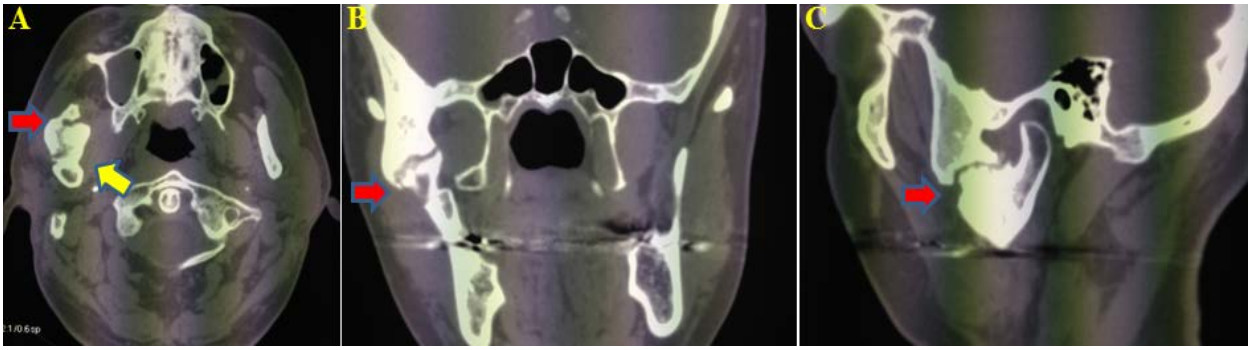

Fig. 4 – Postoperative computed tomography (CT) 4 years after surgical therapy demonstrating recurrence of heterotopic calcification in the right temporal muscle (red arrows) extending towards the right medial pterygoid muscle (yellow arrow). Note demarcated ectopic bone formation in the right temporal fossa.
A) Axial view; B) Sagittal view; C) Coronal view.

The patient was referred again to our Clinic in April 2017 because of a two-week-long inability to open his mouth. The CT scan revealed enlargement of the ossified mass in the right temporal muscle extending from the temporal fossa to the muscular space affecting the medial pterygoid muscle (Figure 5).

The decision on the surgical treatment was made. An intraoral incision was made along the external oblique ridge of the mandible, and a calcified mass extending from the resected mandibular coronoid to the temporal muscle was visualized and partially extirpated. Due to poor visibility and intraoperative bleeding, an extraoral submandibular incision was performed. With the preservation of major vascular and nerve structures, the right temporal fossa was approached and an osseous lesion was identified in the temporal muscle extending towards the medial pterygoid muscle tendon. The osseous mass was extirpated. The maximal incisal opening was 40 mm postoperatively. Macroscopically, the tissue resembled the previously extirpated tumor, and the histological finding was similar (Figure 6).

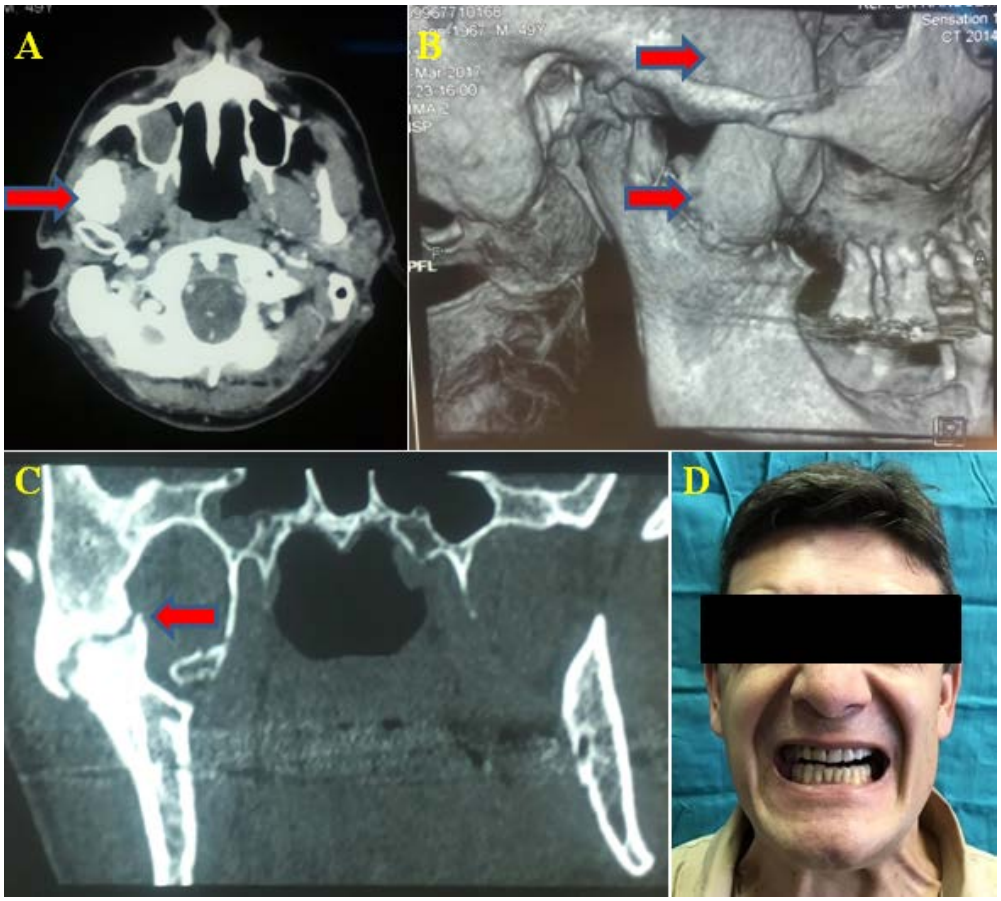

Fig. 5 – Postoperative computed tomography (CT) 6 years after surgical therapy demonstrating recurrence of heterotopic calcification in the right temporal muscle (arrow). Note fusion of calcification in temporal muscle to resected coronoid processus. A) Axial view; B) 3D reconstruction; C) Coronal view; C) Sagittal view; D) Preoperative photograph of the patient showing minimal mouth opening.

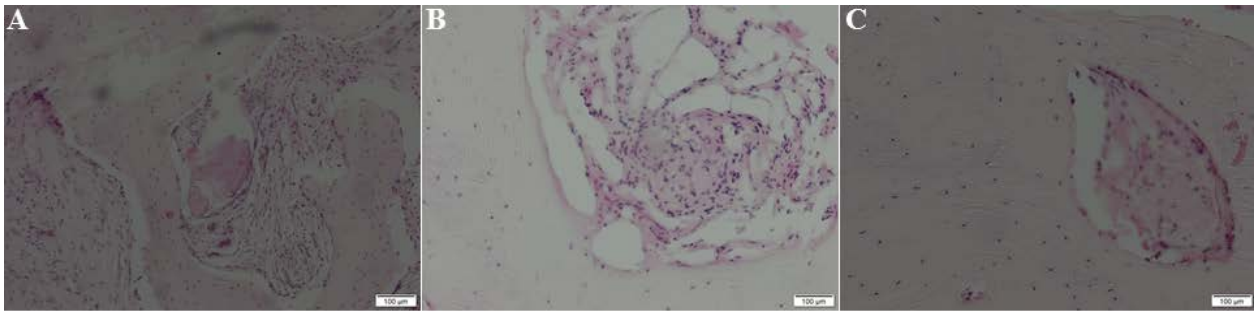

Fig. 6 – Photomicrograph of an excised extraosseous bony lesion: A) Section of specimen showing central loose connective tissue with immature bone containing osteocytes and mature bone at the periphery (hematoxylin and eosin – H&E stain, ×100); B) and C) Photomicrograph showing central connective tissue zone surrounded by immature bone containing osteocytes in the lacunae (H&E stain, ×200).

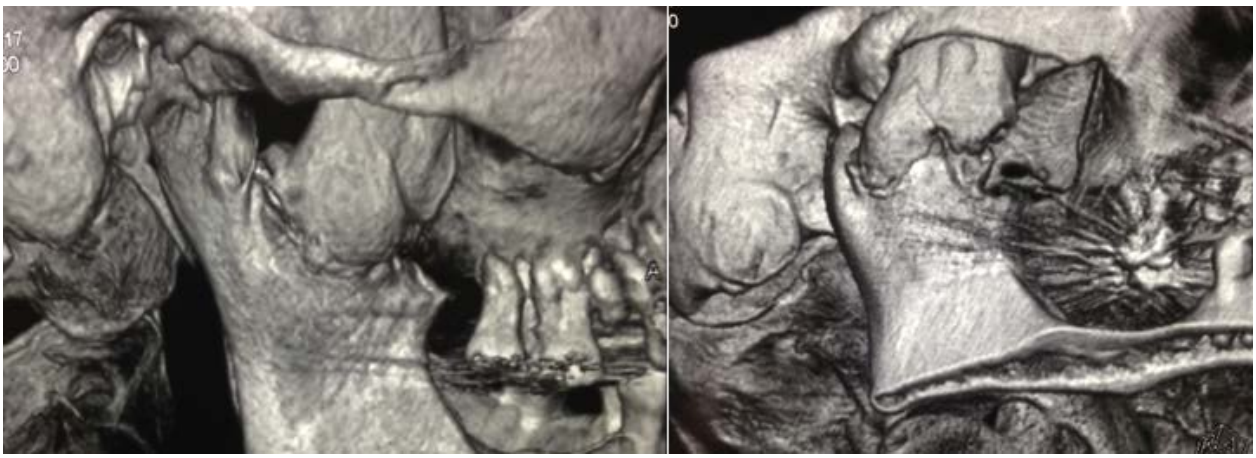

Fig. 7 – Postoperative computed tomography (CT) 3D reconstruction 6 months after second surgical treatment showing resected coronoid processus, and ectopic calcified mass in temporal and medial pterygoid muscle.

After the patient was released from the Clinic, he was able to open his mouth passively up to 10 mm without any assistance, and he was provided with physical treatment consisting of aggressive mandibular range-of-motion exercises. At the regular check-ups, the mouth opening was still reduced to 10 mm, and the patient was sent to perform the proposed physical therapy. On the CT scan follow-up 6 months after the surgery, the ectopic calcified mass affecting the right temporal and medial pterygoid muscles was still present (Figure 7).

Discussion

Although MOT of masticatory muscles presents a benign reparative ectopic bone formation in muscles, accompanying trismus is a major functional problem to the patients. Initial trauma causes an inflammatory response in the muscle and periosteum with subsequent displacement of bone fragments and osteoprogenitor cells into muscle bundles, which induce ectopic bone formation [3–6]. However, in about 25% of cases, a history of trauma is not found [1].

Previous reports found that the most commonly affected masticatory muscles were the masseter and medial pterygoid muscle, but in several reports, more than one muscle was affected [1–3]. Reports of affection of more than one muscle indicate that the inflammatory response following trauma is not localized on one individual muscle [7]. Most MOT lesions were caused by direct trauma to the masseter muscle or trauma to the medial pterygoid muscle after local anesthetic injection [1]. Temporal muscle is not commonly affected with MOT. In the present case, MOT could not be linked to any apparent trauma to the head except the mandibular anesthesia and extraction of the third molar, which occurred two months before the initial symptoms began. However, this type of surgical trauma would not cause direct trauma to the temporal muscle. There are indices that chronic subclinical infection, which often accompanies third molars due to the pericoronitis, could lead to the inflammation and periosteal reaction and subsequently cause bone formation [1–3]. In the present case, the trigger of ectopic ossification is still questionable. The most interesting clinical feature for discussion were difficulties in diagnosis and differential diagnosis. The diagnosis of MOT was based on the clinical picture, radiological examination, and histological findings. Bone formation was confined to the tendon of the temporal muscle and mandibular coronoid processus with no other areas of ectopic bone formation. Laboratory tests revealed no specific abnormalities of bone metabolism. However, even after mandibular coronoidectomy and resection of the temporal muscle tendon, the recurrence of the disease was observed after two years. Furthermore, seven years following the surgical treatment, the patient had limited mouth opening because of the enlargement of the ectopic bone and the affection of the nearby medial pterygoid muscle by the ossification process. The following surgical treatment included extirpation of the osseous lesion in the temporal muscle and the medial pterygoid muscle tendon. The uneventful postoperative course and relapse of the disease could be linked to the ongoing inflammatory process and activity of osteoprogenitor cells, leading to a continuous process of ectopic bone formation, as proposed in other trials [1–3].

MOT represents a significant diagnostic challenge for clinicians. The main criteria for diagnosing MOT include a history of local injury, clinical and radiological evidence of ossification within two months following the injury, and localization of the ossification in the muscle tissue [8]. The differential diagnosis for MOT includes other benign bone-forming lesions, such as fibro-osseous dysplasia progressive, calcified fibromatosis, phleboliths, osteoma, osteoblastoma, but also malignancies, such as osteosarcoma, chondrosarcoma, and rhabdomyosarcoma [9].

The radiographic appearance of MOT depends on the maturity of the lesion [10]. Radiologically, Shirkoda et al. [10] described 4 phases of MOT. The initial phase is characterized by inflammation and mesenchymal stem cell proliferation, without calcification. Initial bone formation is seen 1–2 weeks after trauma. The intermediate phase with peripheral ossification is seen after 4 weeks. The mature phase is seen after 6 weeks, and the lesion appears as a central radiolucency surrounded by peripheral mature bone.

During this phase, the lesion is well delineated from the surrounding tissue, and surgical treatment could be performed with minimal adverse events. CT scan is sensitive for identifying ossification. The radiological appearance of MOT is consistent with the zonal histological pattern of the lesion with a well-circumscribed ossified periphery and a low attenuating central portion. Early lesions appear as amorphous calcifications within the soft tissue, while mature lesions are well separated from the surrounding bone by a thin radiolucent area; however, older lesions can appear attached to the adjacent bone [11].

Histologically, the hallmark of MOT is the zonal pattern [12, 13]. The central or cellular zone represents the innermost region of the lesion, showing mitotic activity, undifferentiated cells, necrotic muscular tissue, giant multinucleated cells, and loose fibrovascular tissue. The middle or intermediate zone contains active osteoblasts and immature osteoid. The peripheral or outer zone of the lesion shows mature bone with active osteoclasts and collagenous fibrous stroma. The microscopic and radiographic zone pattern is strongly suggestive of a reactive lesion and helps rule out a diagnosis of sarcoma [11, 14].

Standard treatment of MOT is surgical excision of the ossification along with osteotomy. In the mature phase, the ectopic bone is well demarcated from the surrounding tissue, and it is easiest to excise [9]. Several authors proposed interposition of soft tissue graft between resected bone and muscle to prevent the bone and hematoma formation [3–6]. Although wide surgical excision of the lesion is performed, relapses are often reported. When several muscles are affected, surgical treatment may resolve significant functional impairment. Several trials reported the use of anti-inflammatory drugs, radiotherapy, and drugs affecting bone metabolism as means of controlling postoperative inflammation and the ossifying potential of the tissue [6]. Other treatment modalities include physical therapy, acetic acid iontophoresis, magnesium therapy, and bisphosphonate therapy [1]. However, these reports are confined to single case studies, and the development of new treatment methods is needed.

Conclusion

MOT represents a major diagnostic and therapeutic challenge for surgeons. It is fundamental that patients with an unspecific clinical history leading to trismus are referred to specialized centers for diagnosis. When MOT of masticatory muscles is suspected, a CT scan could be both a diagnostic and prognostic radiological tool. Surgical treatment remains the treatment of choice and should involve excision of osseous lesion and osteotomy of muscle attachment region of the bone. In cases when several muscles are affected, surgical treatment may resolve a major masticatory disfunction. Thus, further research is needed to clarify the mechanisms of ossification in order to develop conservative treatment approaches.

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Abdominal aortic aneurysm and horseshoe kidney – open surgical repair: A case report

Aneurizma abdominalne aorte i „potkovičasti bubreg“ – otvoreni hirurški tretman

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Abstract

Introduction. Horseshoe kidney (HSK) is a congenital anomaly of embryonic kidneys, which occurs in early gestation when both kidneys are in close proximity. This happens as a result of abnormal migration of nephrogenic cells. The presence of HSK may complicate an anterior approach to reconstructive surgery of the aorta and iliac vessels because the isthmus of the HSK lies across the aorta. HSK is often associated with anomalous renal vessels. **Case report**. A 71-year-old female patient was admitted with an abdominal aortic aneurysm, 50 mm in diameter, and HSK, and multiple aberrant renal vessels with subocluded upper left renal artery as seen on the multidetector computed tomography (MDCT). Open surgical treatment was applied. Endarterectomy of the left upper renal artery, perfusion of the right common bottom pole renal artery, and reimplantation of both bottom polar renal arteries were done. Isthmus was not divided. The coexistence of HSK and abdominal aortic aneurysm (AAA) is a rare condition. It presents a technical challenge to vascular surgeons because the surgical treatment of such an aneurysm is complicated due to the abnormal anatomy, difficulties in exposing the aneurysm, and a variable blood supply to the isthmus and lower poles of the HSK. **Conclusion.** Open surgical repair of AAA with HSK is a successful method and provides good exposure, the possibility of renal circulation preservation, and prevention of renal insufficiency.

Key words:
fused kidney; aortic aneurysm, abdominal; surgical procedures, operative; endarterectomy; reperfusion.

Apstrakt

Uvod. Potkovičasti bubreg (PB) je urođena anomalija u embrionalnom razvoju bubrega, javlja se rano u trudnoći kada se oba bubrega nalaze u neposrednoj blizini, a posledica je abnormalne migracije nefrogenih ćelija. Prisustvo PB može komplikovati anteriorni pristup rekonstruktivnoj hirurgiji aorte i ilijačnih arterija, jer „most“ PB leži preko aorte. PB je često povezan sa anomalijom bubrežnih sudova. **Prikaz bolesnika.** Žena, stara 71 godinu, primljena je sa aneurizmom abdominalne aorte (AAA) prečnika 50 mm i PB, i više aberantnih bubrežnih krvnih sudova i subokludiranom levom bubrežnom arterijom vidljivom na nalazu multislajsne kompjuterizovane tomografije (MSCT). Operisana je klasičnom hirurškom metodom gde je urađena resekcija aneurizme sa implantacijom Dakronskog grafta bez presecanja istmusa. Pored toga, urađena je endarterektomija leve gornje renalne arterije, perfuzija prave zajedničke donje polarne renalne arterije i reimplantacija obe donje polarne renalne arterije. Koegzistencija PB i AAA retko je stanje koje predstavlja tehnički izazov za vaskularnog hirurga zbog abnormalne anatomije, težeg prilaza aneurizmi i varijabilne vaskularizacije bubrega, a posebno istmusa. **Zaključak.** Otvorena hirurška metoda lečenja AAA sa PB predstvlja uspešan način rešavanja ovog problema jer pruža dobar pristup aneurizmi i renalnim krvnim sudovima uz mogućnost očuvanja bubrežne cirkulacije i, posledično, sprečavanja bubrežne insuficijencije.

Ključne reči:
bubreg, potkovičasti; aorta, abdominalna, aneurizma; hirurgija, operativne porocedure; endarterektomija; reperfuzija.

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Introduction

HSK is a congenital anomaly of embryonic kidneys, which occurs in early gestation when both kidneys are in close proximity. This happens as a result of abnormal migration of nephrogenic cells [1]. The HSK is found in approximately 0.1% of autopsy results and 0.1% to 0.6% of aortic operations [2]. The presence of HSK may complicate an anterior approach to the aorta because the isthmus of the HSK lies across the aorta and is often associated with anomalous renal vessels [3]. A medial fusion of the kidneys, mostly anteriorly to the aorta, is the main characteristic of this anomaly [4]. The HSK can be usually found preoperatively with multidetector computed tomography (MDCT) angiography of the abdominal aneurysm. Open surgical repair presents a challenge because of the possible complications including renal infarction, neuralgia, and collecting system disruption. Endovascular aortic repair (EVAR) is considered for this pathology, allowing aneurysm repair without isthmus dissection. However, whether to sacrifice commonly presenting aberrant renal arteries during EVAR is a point of controversy. Some authors recommended a hybrid treatment [5]. We report one case of open repair AAA with HSK, with aberrant renal arteries and its reattachment without dividing the renal isthmus.

Case report

A 71-year-old female patient was admitted to our hospital with an MDCT finding of 50 mm wide AAA and HSK. The isthmus of HSK was on the front side of the aneurysm (Figure 1).

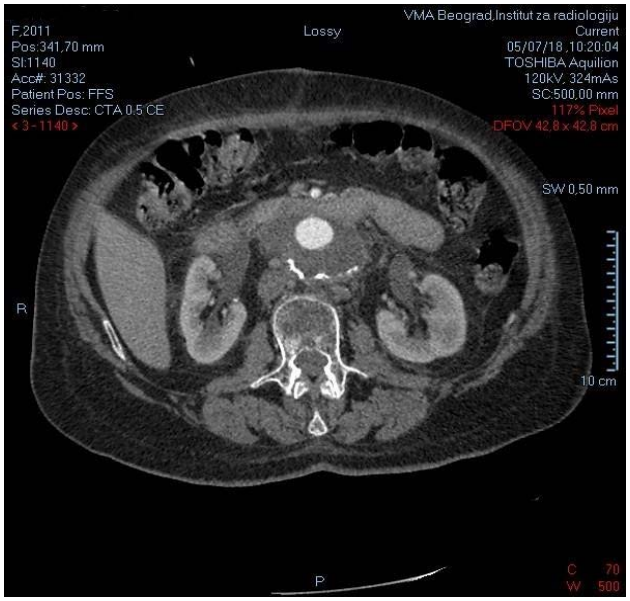

Fig. 1 – Abdominal aortic aneurysm (AAA) and horseshoe kidney (HSK).

The patient was asymptomatic for abdominal pain and had no urinary tract symptoms. Values of urea and creatinine were mildly elevated (Urea 13.5 IU/L and Creatinine 168 IU/L). On the MDCT analysis of renal vessels, it was seen that the left renal vein was in a preaortic position. The right and left upper polar renal artery originated from the healthy part of the abdominal aorta, but the left upper polar renal artery was subocluded at the exit of the aorta. The right and left lower polar renal artery originated from an aneurysm. The right lower polar renal artery had two branches: the right branch for the lower pole of the right kidney and the left branch for the lower pole of the left kidney (Figure 2).

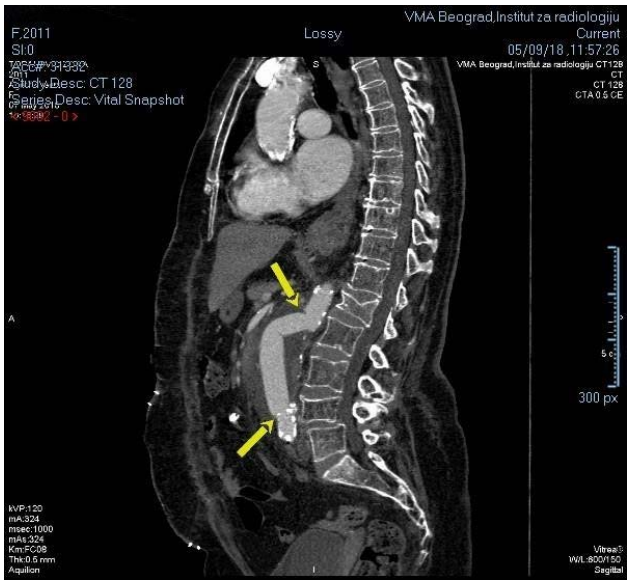

Fig. 2 – Aberrant renal arteries.

The operation was performed under general endotracheal anaesthesia with the monitoring of arterial tension, diuresis, gas analyses, electrocardiography, and pulse oxymetry. After medial laparotomy, all arteries were located. The arteries located are the following: artery for the upper pole of both kidneys, artery for the lower pole of the left kidney, 3 mm in diameter, and a common artery for the lower pole of both kidneys, 5 mm in diameter (Figure 3).

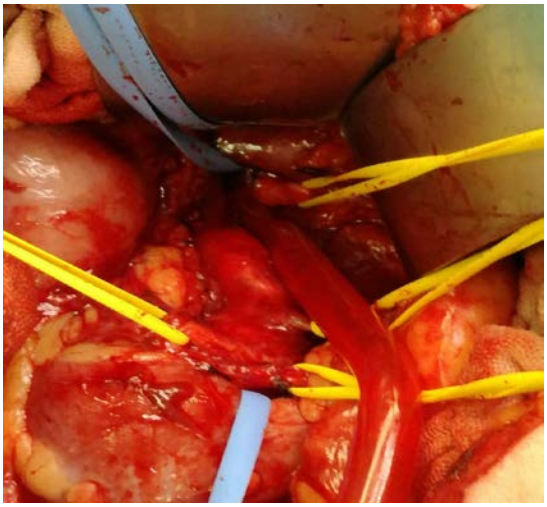

Fig. 3 – Left renal vein, left superior renal artery (subocluded), bottom pole renal arteries and isthmus of horseshoe kidney (blue tape).

The left ureter followed the isthmus of HSK. The artery for the right ureter originated from the right common iliac artery (Figure 4). Reconstructed aorta with graft pull-through beneath the kidney bridge is shown in Figure 5.

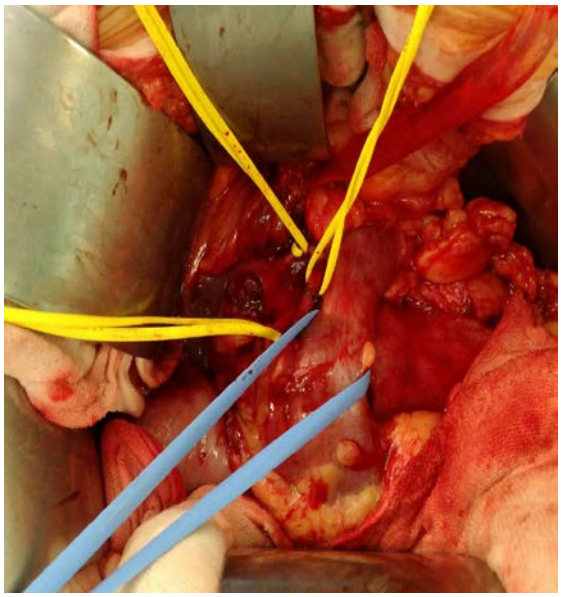

Fig. 4 – Abdominal aortic aneurysm (AAA) with horseshoe kidney (HSK). Bottom polar renal arteries, left ureter, isthmus of HSK (blue tape), and artery for right ureter.

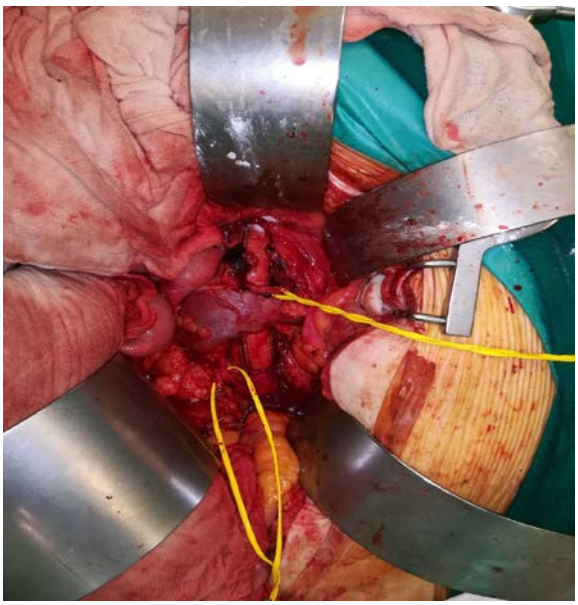

Fig. 5 – Reconstructed aorta with graft pull-through beneath the kidney bridge and reattached bottom polar renal arteries.

The aorta was clamped for 51 minutes, and the renal perfusion lasted for 46 min. Diuresis after declamping and reimplantation of renal arteries was 100 mL in the first hour. After control of hemostasis, we closed the retroperitoneum and the abdominal wall. In the Intensive care unit, the patient had a diuresis of 2600 mL postoperatively on the first day. Pulses were palpable and arterial pressure was between 100–140/70–100 mmHg. On the second postoperative day, urea was 13.5 IU/L and creatinine 168 IU/L. On the third postoperative day, creatinine was 180 IU/L. The patient was given intensive saline therapy and diuretics, and after one day, the values of urea and creatinine decreased (10.5 IU/L and 150 IU/L). Peristalsis was established on the second postoperative day and normalized in the next two days with a diet. The patient was discharged on the 8th postoperative day.

Discussion

The coexistence of the HSK and AAA is a rare condition that presents a technical challenge to vascular surgeons because the surgical treatment of such an aneurysm is complicated due to the abnormal anatomy, difficulties in exposing the aneurysm, and a variable blood supply to the isthmus and the lower poles of the HSK [6]. Abnormalities concerning the number and the origin of kidney vessels can be associated with ectopic kidney and HSK [7]. An appropriate preoperative evaluation of the HSK by computed tomography (CT)–angiography and the renal function is mandatory for optimal planning of the treatment strategy [8]. Horseshoe kidneys are frequently found in patients with other venous and particularly inferior vena cava anomalies, which should be evaluated using MDCT as a part of treatment planning [9]. The transperitoneal approach provides the best exposure to the aneurysm and kidney. However, the presence of the renal isthmus affects both surgical exposure and proximal aortic control. The left retroperitoneal approach has the advantage of avoiding interference with the renal isthmus and urinary tract; however, access to the right iliac artery is limited [10]. Division of the renal isthmus can be associated with an increased risk of retroperitoneal urinary leaks, bleeding, infection, and renal ischemia [6]. In the study by Davidović et al. [4], 25 patients with HSK underwent aortic surgery; in 18 cases, kidney tissue transection was successfully avoided by placing vascular grafts beneath the bridge of the HSK. In 12 cases, anomalous renal arteries were detected, and their reattachment into vascular graft has been performed. Defined guidelines for managing accessory renal arteries have yet to be established. Minimizing the risk of renal insufficiency and renal vascular hypertension is the ultimate goal. Individualizing the management of accessory renal arteries is necessary [11]. Kaplan et al. [12] noted that accessory vessels over 3 mm in diameter should be reattached in order to reduce the risk of postoperative renal insufficiency. The newest reports [5, 11] demonstrated cases in which hybrid surgical repair was performed for AAA in a patient with HSK and aberrant renal vasculature, including EVAR after debranching aberrant renal arteries. EVAR is regarded as a valuable alternative to open surgical therapy in the absence of renal failure, provided that accessory renal arteries are absent or small [8]. In our case, we had the initial level of renal failure and big accessory renal arteries that originated from an aneurysm. The superior left renal artery was subocluded, and endarterectomy was necessary. Customized endografts are a viable tool for preserving aberrant vessels and the renal mass in AAA and HSK. Customized endografts require an extensive work-up and are currently expensive to fabricate [13].

Conclusion

Open surgical repair of AAA with HSK is a successful method for an experienced team and provides good exposure of the aorta, kidneys, and vessels. Endarterectomy of renal arteries, reimplantation of accessory renal vessels, and preservation of isthmus of HSK represents a challenge, but it is also the best choice for preventing postoperative renal insufficiency.

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Acute kidney failure and extramedullary lung infiltration as the initial presentation of multiple myeloma: A case report

Akutna bubrežna slabost i ekstramedularna infiltracija pluća kao inicijalne prezentacije multiplog mijeloma

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Abstract

Introduction. Kidney failure in multiple myeloma is sometimes initial symptomatology and a very serious complication with an unfavorable effect on the course and prognosis of the disease. Multiple myeloma is a disease characterized by the proliferation of plasmocytes in the bone marrow, and in rare cases, it can be extramedullary in various organs and systems. Pulmonary plasmacytoma localization is a rare extramedullary localization, especially when it represents one of the initial manifestations of multiple myeloma. **Case report.** We present a patient with progressive acute kidney failure who has started hemodialysis treatment. On chest radiography, a homogeneous shadow was observed along the left chest wall, and the multislice computed tomography (MSCT) pointed to the tumor formation in the pulmonary parenchyma in the projection of the left upper pulmonary lobe with signs of pleural infiltration, intercostal muscles, and V rib destruction. Laboratory examination indicated the presence of Bence-Jones proteinuria in the urine sample in addition to anemia syndrome and azotemia with hyperuricemia. After bronchoscopy and needle biopsy, diffuse infiltration of mature plasma cells was demonstrated in the cytological and histopathological findings of the lungs. The histopathological finding of bone marrow biopsy indicated multiple myeloma of Lambda type with infiltration of plasma cells – about 70%. The hematologist determined a diagnosis of multiple myeloma BJ lambda III BCS, with extramedullary lung infiltration and acute kidney failure. Further treatment was continued according to the hematological protocol while performing intermittent hemodialysis. **Conclusion.** Sometimes, extremely rarely, and in completely asymptomatic patients with massive pulmonary infiltration observed initially, the differential diagnosis may also represent an extramedullary presentation of multiple myeloma, which should be considered.

Key words:
multiple myeloma; lung; acute kidney injury; diagnosis, differential.

Apstrakt

Uvod. Bubrežna slabost u multiplom mijelomu ponekad predstavlja inicijalnu simptomatologiju i veoma ozbiljnu komplikaciju sa nepovoljnim uticajem na tok bolesti. Multipli mijelom je bolest koju karakteriše proliferacija plazmocita u koštanoj srži, a u retkim slučajevima mogu biti zastupljeni i ekstramedularno u različitim organima i sistemima. Plućna lokalizacija plazmocitoma je retka ekstramedularna lokalizacija, a pogotovo kada predstavlja i jednu od inicijalnih manifestacija multiplog mijeloma. **Prikaz bolesnika.** Prikazali smo bolesnika sa akutnom bubrežnom insuficijencijom, progresivnog toka, kod koga je započeto lečenje hemodijalizama. Na radiografiji grudnog koša zapažena je homogena senka uz levi lateralni zid grudnog koša, a multislajsna kompjuterizovana tomografija (MSKT) je ukazala na tumorsku formaciju u plućnom parenhimu u projekciji levog gornjeg plućnog režnja sa znacima infiltracije pleure, interkostalnih mišića i destrukcijom V rebra. Laboratorijskim ispitivanjem, osim

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anemijskog sindroma i azotemije sa hiperurikemijom, u urinu je dokazana Bence-Jones-ova proteinurija. Nakon bronhoskopije i iglene biopsije, u citološkom i patohistološkom nalazu pluća dokazana je difuzna infiltracija zrelih plazma ćelija. Patohistološki nalaz biopsije koštane srži ukazao je na multipli mijelom lambda tipa sa infiltracijom plazma ćelijama – oko 70%. Od strane hematologa postavljena je dijagnoza multiplog mijeloma BJ lambda III BCS, sa ekstramedularnim zahvatom pluća i akutnom bubrežnom insuficijencijom. Dalje lečenje nastavljeno je po hematološkom protokolu uz obavljanje intermitentnih hemodijaliza. **Zaključak.** Ponekad, izuzetno retko i kod potpuno asimptomatskih bolesnika, masivne plućne infiltracije, zapažene inicijalno, diferencijalno dijagnostički mogu predstavljati i ekstramedularnu prezentaciju multiplog mijeloma, o čemu treba razmišljati.

Ključne reči:
multipli mijelom; pluća; bubreg, akutna insuficijencija; dijagnoza, diferencijalna.

Introduction

Multiple myeloma (MM) is a plasma proliferative disease that is more common in the older population (above the age of 60), with an incidence of about 5 cases per 100,000 persons. It represents about 1% of all malignancies, while it is the second most common hematological disease (10% of cases) [1–5].

The occurrence of acute kidney failure in MM sometimes also represents the initial symptomatology of this disease and further complicates this quite serious disease. It is believed that, precisely because of this presentation, 50% of cases with MM are initially nephrology patients [6]. That is why many authors suggest mandatory MM-related evaluation in acute kidney failure patients [7–9].

The extramedullary (EM) plasmacytoma is malignant plasma cell proliferation, not localized in the bone marrow. It is described in 7–17% of patients at the moment of diagnosis of MM and in 6–20% of cases during the course of treatment of the disease. However, the data of recent studies state the occurrence in about 34% of cases where it represents a very poor prognostic parameter [10–15].

The most common EM localizations (about 85% of cases) are in soft tissues around the skeletal system, and the remaining cases (15%) of EM localization occur in lymph nodes, liver, kidney, respiratory tract, spleen, skin, CNS, and others [13, 14]. EM localization in the lungs is described in less than 5% of cases [16]. Very rarely, as in the case of our patient, simultaneous infiltration of the lungs and acute kidney failure is observed as the initial manifestation of MM.

Case report

A 47-year-old male was admitted to the Clinic for Nephrology for acute kidney failure development, followed by arterial hypertension and anemic syndrome. The first complaints were reported one month before admission to the Clinic, in the form of fatigue, faster tiredness, and loss of appetite. At that time, elevated creatinine (683 µmol/L) and urine erythrocytes and proteinuria of 0.92 g/24 h were observed in laboratory analyses. The patient was sent to our institution for further assessment. At the time of admission, arterial hypertension (150/85 mmHg) was noted, along with paleness of skin and mucous membrane, while other results were within the normal range. The patient indicated that he was a long-term smoker who ceased smoking one month earlier. Laboratory analysis included the following: accelerated erythrocyte sedimentation rate (ESR 132 mm/h), anemia syndrome [hemoglobin (Hb) 97 g/L], azotemia (creatinine 876–1005 umol/L, urea 42,1 mmol/L), hyperuricemia (670 umol/L), total protein 72 g/L, albumin 50 g/L, and in the urine sample, hematuria with proteinuria of 0.656 g/24 h. Echotomographically, both kidneys were 12.8 cm in size with more echoing parenchyma and pronounced pyramids, and without hydronephrosis and calculus (Figure 1). Given the progressive course of kidney failure, a kidney biopsy was performed. In the meantime, oliguria with hypervolemia developed due to which a central venous catheter was placed in the right *vena jugularis*, and hemodialysis was started. Radiography of the chest revealed a homogeneous shadow of the polycyclic appearance on the

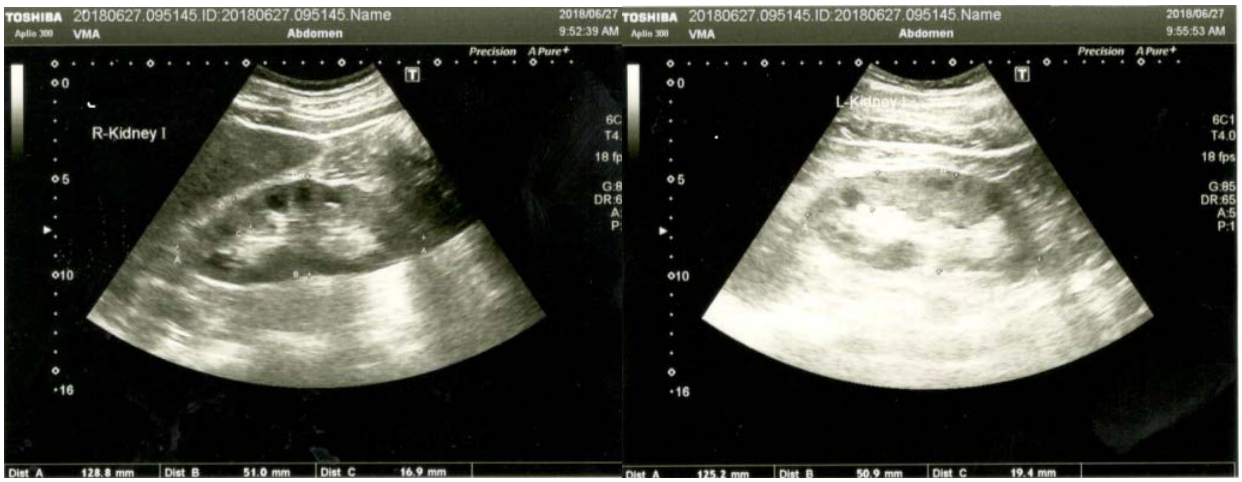

Fig. 1 – Kidney ultrasound: points to enlarged kidneys, echogenic parenchyma, and pronounced pyramids.

left side along the lateral wall of the thorax and multislice computed tomography (MSCT) was indicated (Figure 2). An infiltrative tumor formation was observed in the pulmonary parenchyma in the projection of the apical segment on the chest MSCT with dimensions 8 cm × 7 cm × 9 cm, with signs of pleural infiltration, intercostal muscles, and destruction of V rib (Figure 3).

Only then, did the patient recall that a year ago, he occasionally felt mild manifestations of unspecified disturbances on the left side of the chest, which he did not mention because he thought they were irrelevant. Discrete M-spike in the gamma fraction was found in the serum protein electrophoresis: 61.1% albumin, alpha-1 5.5%, alpha-2 13.6%, beta-1 5.3%, beta-2 4.8%, gamma 9.7%. The nephelometric finding indicated an extremely high concentration of Lambda F 5,820.0 mg/L (reference values 8.3–27.0 mg/L), which represents paraprotein seen as discrete M-spike in gamma region on serum electrophoresis. Levels of Kappa F 29.3 mg/L, IgL lambda light chain 1.62 g/L, and IgL kappa light chains 1.43 g/L, k/l ratio 0.88

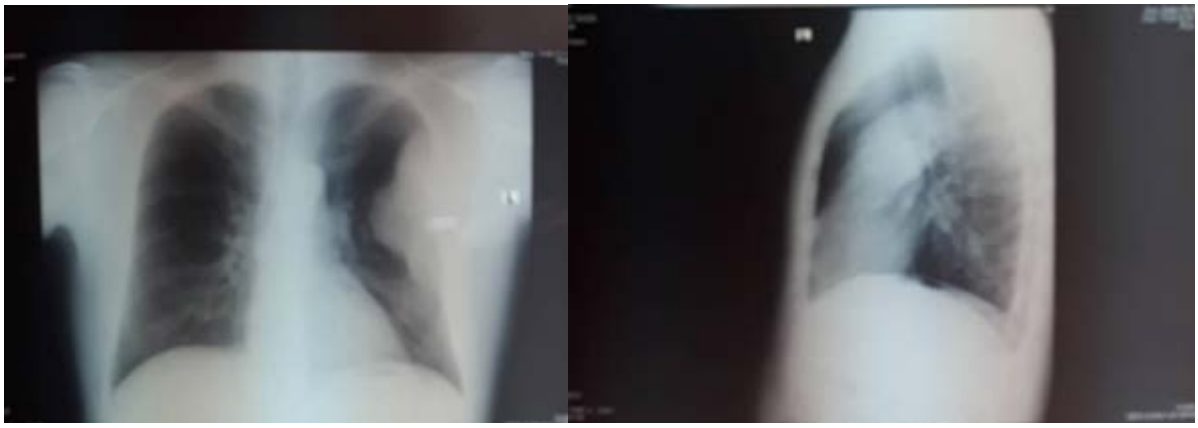

Fig. 2 – A radiographic record of the thorax on which a homogeneous shadow along the chest wall is seen on the left side in the middle lung field.

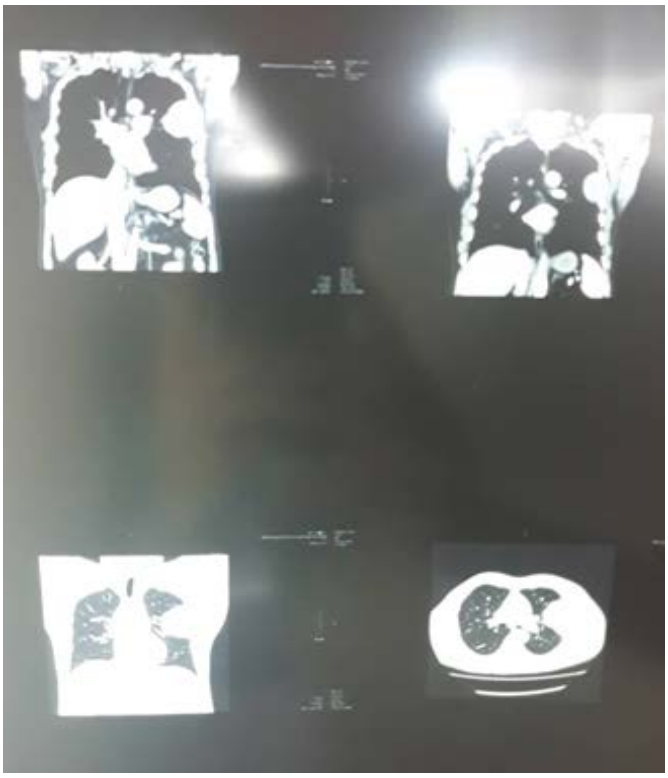

Fig. 3 – Chest multislice computed tomography (MSCT) with infiltrative tumor formation in the pulmonary parenchyma in the projection of the apical segment of the upper lung lobes, dimensions 8 cm × 7 cm × 9 cm, with signs of the infiltration of pleura, intercostal muscles, and destruction of V rib on the left.

(reference values 1.35–2.65 g/L), immunoglobulins IgG 7.2 g/L, IgA 0.68 g/l, IgM 0.18 g/L, and serum β2-microglobulin 29.7 mg/L (reference values 0.70–1.80 mg/L) were also determined.

With a diuresis of 700 mL and proteinuria of 0.656 g/24 h, protein electrophoresis in urine was performed: albumin 12.9%, alpha-1 5.9%, alpha-2 18.4%, beta 50.7%, gamma 12.1%, M-spike in the beta fraction. The nephelometric finding indicated: kappa F 29.3 mg/L, lambda F 5820.0 mg/L (reference values 8.3–27.0 mg/L), IgL lambda light chain 1.62 g/L, and IgL kappa light chains 1.43 g/L, k/l 0.88 (reference values 1.35–2.65), b2M 29.7 mg/L (reference values 0.70–1.80 mg/L). The presence of monoclonal light chain Lambda type (bound and free) and monoclonal free light chain lambda type was identified by immunofixation. The finding pointed to Bence-Jones proteinuria (Figure 4).

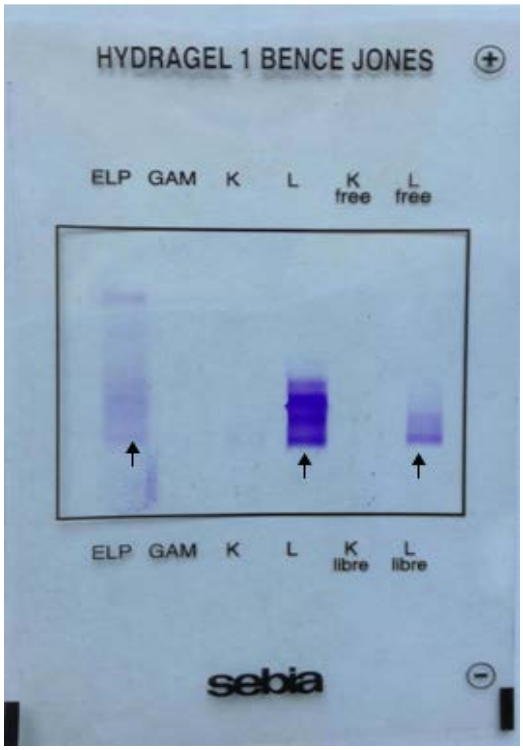

Fig. 4 – Bence-Jones immunofixation of the patient's urine: the presence of a monoclonal tape derived from the monoclonal free light chain λ type is observed in regions of ELP urine, L-lambda (free and bonded light chains), and L free (free light chains).

The pathohistological finding of the kidney biopsy indicated obstructive tubulopathy (in the lumen of the tubule crystalloid contents with the surrounding inflammatory reaction), and the characteristics of the "myeloma kidney" were present (Figure 5). In order to clarify the etiology of pulmonary changes, a video bronchoscopy with a needle biopsy of changes in the lungs was performed. The cytological smear of the needle biopsy already pointed to monomorphic cell populations, eccentrically arranged sails in the medium abundant, and basophilic cytoplasm suggesting an extramedullary plasmacytoma (Figure 6).

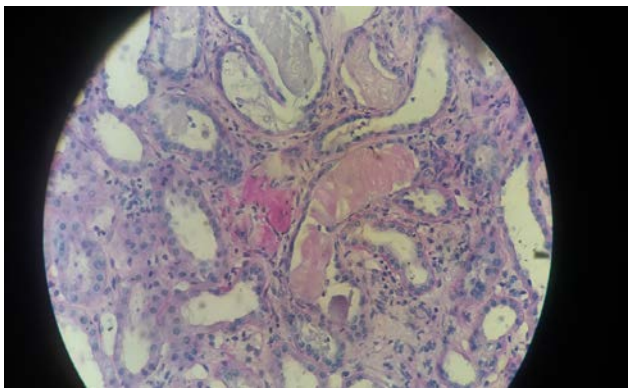

Fig. 5 – Histopathological findings of the kidney biopsy: obstructive tubulopathy – in the lumen of the tubule, crystalloid content with the surrounding inflammatory reaction, characteristic of the "myeloma" kidney is present [hematoxylin-eosin (HE) staining, ×40].

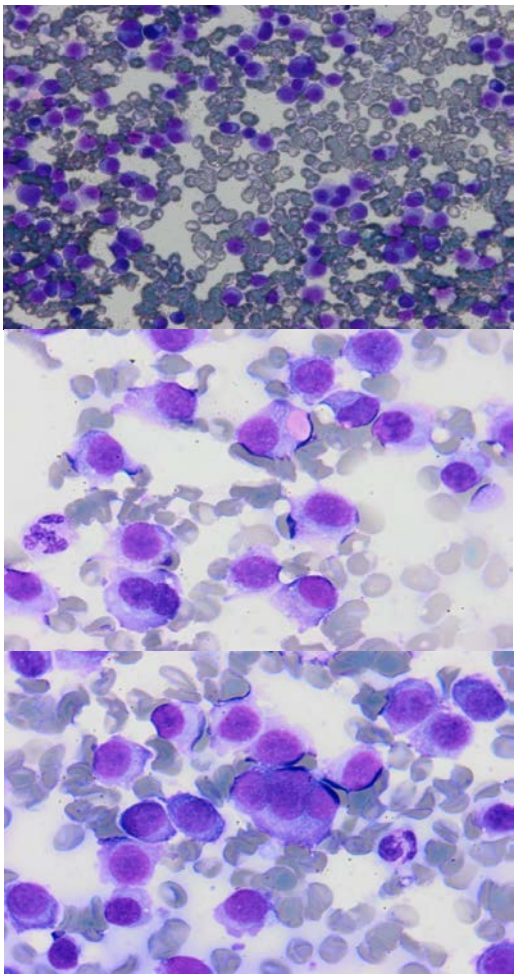

Fig. 6 – Cytological smears of needle lung biopsies: monomorphic population of cells, eccentrically arranged nuclei in medium abundant, basophilic cytoplasm. In the cytoplasm of certain cells, the focus of the eosinophilic substance corresponding to the deposited immunoglobulins is observed. Multinuclear forms are also present [May Grunwald-Geimsa (MGG) staining, × 200, × 1000, × 100].

The pathological findings of lung biopsy revealed diffuse infiltration of mature plasma cells. Immunohistochemical analysis in proliferating cells produced a diffuse membrane reaction for CD 138 and a focal membrane reaction for CD 56, while the cytoplasmic reaction for lambda light chains was apparent in a number of cells from the cytoplasmic reaction to kappa light chains (Figure 7). After consultation with a hematologist, a myelogram was performed, and infiltration of the plasma cells (25%) was observed in the cytological smear of the bone marrow aspiration (Figure 8). After that, a bone marrow biopsy was performed, which pointed to multiple myeloma–Lambda type (high infiltration of about 70%). Plasma cell immunophenotyped CD 138+/MUM-1+/Lambd +/Kapa+/CD20- (Figure 9). Flat bone radiography showed: on the bones of the skull, axial skeleton, and pelvis, no signs typical of the multiple myeloma were noticed. The diagnosis of multiple myeloma BJ lambda III BCS, with extramedullary lung infiltrations and acute kidney failure, was determined. Further treatment was continued according to the protocol by a hematologist while performing intermittent hemodialysis.

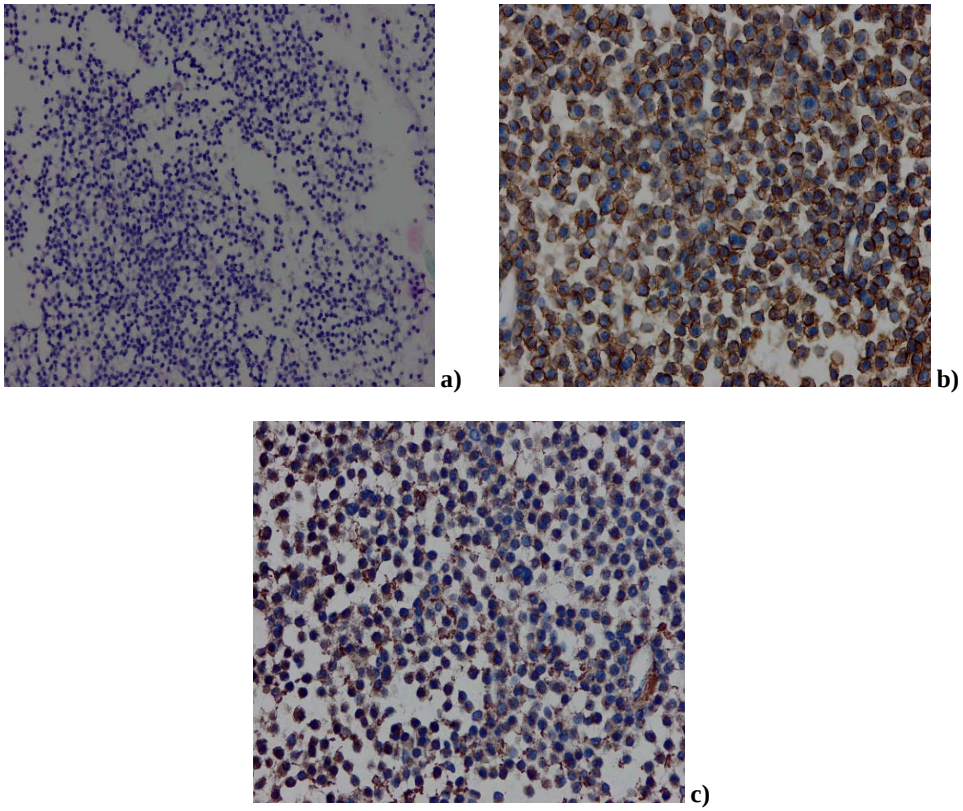

Fig. 7 – Histoathological findings of pulmonary biopsy: a) proliferation of monomorphic plasmacytoid cells [hematoxylin-eosin (HE) staining, × 200]; b), c) immunohistochemical analysis in proliferating cells resulted in a diffuse membrane reaction CD 138 and cytoplasmic reaction for lambda-light chains (CD 138, lambda, × 400).

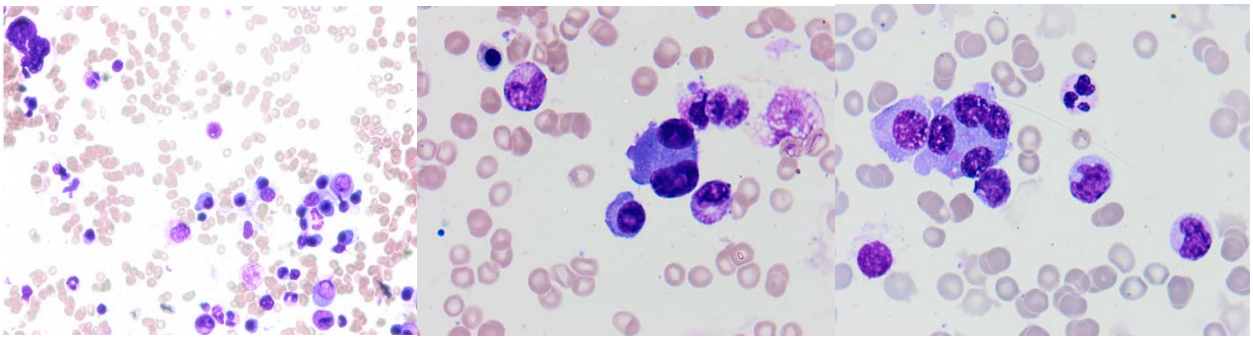

Fig. 8 – Cytological smear of bone marrow aspiration (MGG, ×10, ×100, ×100). In the lightly hypocellular bone marrow, 25% of plasma cells were found, including binucleate and multinucleate forms present [May Grunwald-Geimsa (MGG) staining, × 100, × 1000, × 1000]).

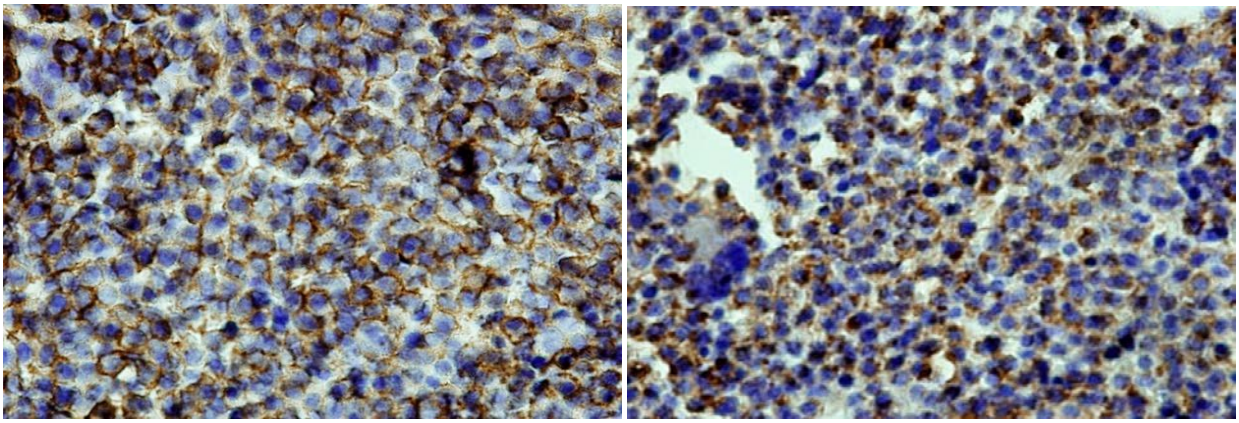

Fig. 9 – Pathohistological findings of bone marrow biopsy. Immunohistochemical analysis in proliferating cells resulted in a diffuse membrane reaction CD138 and cytoplasmic reaction for lambda-light chains (CD138, lambda, × 400).

Discussion

Kidney lesion in MM is a very serious condition with a significant impact on the survival of patients. Sometimes the occurrence of acute kidney failure is the initial manifestation of multiple myeloma, with kidney damage most often being the consequence of the so-called cast nephropathy or "myeloma kidney", which represents tubular damage caused by precipitation of light chain immunoglobulins, followed by deposits of light chains, amyloidosis, hypercalcaemia, drugs, etc. [17, 18]. At the moment of diagnostic MM, the existence of acute kidney failure is described in 20–40% of cases, and in severe cases, it is accompanied by oliguria, thus dialysis is necessary, as was the case with our patient [19].

In patients with MM and kidney failure, Dimopoulos et al. [19] describe median survival of 29–32 months mainly due to the new therapeutic modalities.

Extramedullary localization of MM is more common among males (about 3–4 times more frequent than in females), with only 1/3 of patients being younger than 50, as was the case with our patient [20–24].

In a study involving 1,027 patients with MM, Kyle et al. [21] described the extramedullary occurrence of MM before diagnosis in 4 patients (0.4%), and the median of the time interval was 18 months. According to anamnestic data, our patient experienced some unspecified difficulties on the left side of the chest a year earlier. Varettoni et al. [11] describe the occurrence of EM plasmacytomas in 13% of patients, with 7% having a pronounced EM plasmacytoma localization at the time of MM diagnosis, whereas in 6% of patients, there was a subsequent manifestation of EM plasmacytoma localization. Varga et al. [14] described that about 34% of patients treated for MM also express EM localization during treatment.

Data from other authors describe EM plasmacytoma in 20% of cases of plasma proliferative neoplasia, and the observation that the growth frequency is increasing is explained by the fact that the diagnostics are more sensitive, as well as the new treatment protocols that affect the survival of these patients [10–12, 23].

Five-year survival in patients with MM and EM plasmacytoma is described in 31% of cases, and in patients with MM but without EM plasmacytoma, in 59% of patients [24]. In MM light chains, the incidence of extramedullary localization is described initially in 2.1% of patients, and during the monitoring and treatment in 33.3% of cases, according to Zhang et al. [25]. EM plasmacytoma is sometimes described in patients who have previously been in remission, while in the course of the disease, relapse multiple organ involvement is observed – pancreas, kidney, adrenal glands, liver, lung skin, spleen, and lymph nodes [26]. The study of Oshima et al. [27] encompassed a period of twenty years and analyzed autopsy findings in 53 patients with multiple myeloma, and in 2/3 (63.5%), extraosseous localization was most represented in the spleen, kidney, and liver (about 30%), while pulmonary infiltration was observed in 15.4%.

Extramedullary localization in the respiratory tract is described in approximately 80% of patients, most often localized in the upper respiratory and nasopharyngeal tract (65–80%), while lung involvement is described less often, in 3–5% of cases [16, 20, 28, 29].

In a study published in 2004, in describing the cases of patients with pulmonary plasmacytoma (endobronchial localization), Edelstein et al. [30] state that only 22 proven cases of pulmonary infiltration have been described with immunohistochemical and other confirmatory assays. In a study published in 2011, in demonstrating a case of a patient with pulmonary plasmacytoma who had been experiencing difficulties in the form of shortness of breath, chest pain, cough, and loss of appetite, Prasad et al. [31]compare case studies of different authors with a review of pulmonary plasmacytoma, which is a rare occurrence (they describe 35 cases).

Extramedullary pulmonary plasmacytoma in our patient also represents the initial manifestation of multiple myeloma, which was clinically almost asymptomatic. The mild, nonspecific complaints of pain on the left side of the chest, which occasionally occurred, did not attract attention. At the moment of diagnosis, pulmonary changes were 9 cm in size, with pleural infiltration, intercostal muscles, and destruction

of the V rib. Only the occurrence of general signs of weakness and fatigue with laboratory confirmation of the patient's uremia brought the patient to a medical institution, where lung infiltration was observed during the examination of the etiology of kidney failure, and then multiple myeloma diagnosis was determined. EM pulmonary localization can clinically range from completely asymptomatic form to symptomatology in the form of cough, shortness of breath, and elevated temperature [32, 33].

Ravinet et al. [34] describe the case of a patient with a diagnosed MM who has experienced chronic respiratory insufficiency and emphysema, which had progressed to acute dyspnea and interstitial lung disease that was refractory to applied therapy. Postmortem autopsy analysis in the lungs verified nodal plasma cell infiltrates. Radiological investigations of pulmonary localization of plasmacytomas can present as infiltration in the form of a solitary mass of the nodular form, as in our patient, or as diffuse lung infiltration, which can sometimes occur on both sides [27, 35, 36].

Conclusion

The case of our patient confirms the opinion that the diagnosis of acute kidney failure should always be completed with screening for multiple myeloma. Extraosseus localization with pulmonary infiltration represents a rather rare presentation of multiple myeloma, indicating the aggressive course of this disease especially given the association with renal impairment and consecutive treatment limitations.

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Position of mentally ill persons in the 19th century Serbia – legal aspects

Položaj duševno obolelih lica u Srbiji 19. veka – normativni aspekti

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Key words:
history of medicine; hospitals, psychiatric; legislation, medical; mental disorders; serbia.

Ključne reči:
istorija medicine; bolnice, psihijatrijske, zakonodavstvo, medicinsko; psihički poremećaji; srbija.

Introduction

In medieval Serbia, mentally ill persons were treated in monasteries because it was believed that religious medicine had a crucial role in treating various neuropsychiatric diseases. Relics of saints were especially believed to have healing powers [1]. As of the 12th century, scientific medicine started developing under the influence of Byzantine and Western medicine [1]. It was also the time when the first Serbian hospitals were founded in the monasteries of Hilandar, Studenica, and Dečani [1]. Even after the Serbian Despotate fell under the rule of the Ottoman Empire, treatment and care for the mentally ill in monasteries were not abandoned [1]. It continued throughout Ottoman rule, but soon after the 1830 Hatt-i Sharif, by which Serbia was recognized as a vassal principality with its autonomous internal government, this practice started diminishing.

First attempt at solving the housing issue for the mentally ill

In the 19th century Serbia, sending mentally ill persons to monasteries, where they were treated by fasting, prayer, and "other known means," was common [2]. However, since monks, being busy doing other chores, could not take care of the patients, mentally ill people would sometimes physically hurt other people, even commit murders. On June 7, 1839, in the monastery of Vujan, a mentally ill person, Jovan Milovanović, from the village of Brđan, killed a servant of the monastery, Aksentije Stojanović, with an axe while he was sleeping [3]. This incident was a motive for imposing certain restrictions on the practice of placing mentally ill people in monasteries. With regard to this, on July 10, 1839, the Ministry of Justice and Education issued the "Circular on people who lack brains, and who come to monasteries and churches for healing, in order to prevent their harmful actions" [3]. In agreement with the Metropolitan of Belgrade, the Ministry decided that these persons, if acting aggressively, could not be placed in a monastery but should, as soon as the prayer was read to them, be sent home. The district court was advised that it should, on its behalf and in agreement with the district government, inform all the people through county officials that every family in which "by ill fate, there are crazy people or people affected by other dangerous diseases" should take all precautions [3]. Hence, every family was supposed to accompany ill persons to a monastery for treatment and prayer and to look after them throughout the whole time, so that they would not harm any member of the monastic fraternity nor the present faithful. The Ministry of Internal Affairs made the regulations of the Circular even more strict by demanding that every householder in whose house a person "is inflicted by insanity" should inform the local government of them and immediately take away any weapons or harmful tools from them [3]. The county and district prefects were obliged to take precautionary measures and instruct the householder as to how to take care of the patient's mental health in order to avoid unwanted consequences [3].

Considering the inconveniences that could be caused to monks by mentally ill people staying in monasteries, and constantly referring to the Circular of July 10, 1839, the Metropolitan allowed mentally ill patients to stay in a monastery only as a final measure and with numerous precautions [4]. In the second half of July in 1839, there was an

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issue of how to deal with the case of Petar Simić from the village of Ripanj in the Belgrade district, who "in the lack of common sense" cut his brother-in-law Ranko Nedeljković's throat with a knife [4]. The police authorities freed him from guilt and responsibility and sentenced him to two years in the monastery of Kalenić. Based on the testimony of Gavrilo Nedeljković, the abbot of the monastery, Petar had been healthy, sane, harmless, and diligent in doing monastery chores throughout the whole stay. On the way home, since he did not have his passport on him, he was arrested by the Smederevo district authorities and directed to the Belgrade court, where he was in custody at the time when his case was being discussed [4]. On August 12, fearing blood feud by relatives of the murdered man if Petar were sent home, the Ministry of Justice and Education appealed to the Metropolitan to direct the man either to the monastery of Kalenić or Studenica, where "prisons for wretched people can also be found," and where he would earn for food and clothing by doing monastery chores [4]. The Metropolitan thought that it would be most appropriate to send Petar as "a man in a dubious health condition" to a place where he could be constantly guarded, given the fact that it was not possible in a monastery setting. Nonetheless, if there was no other solution, he should be sent to Studenica, as it was quite remote from his place of residence, and "since abbot Gavrilo, today's Archimandrite, vouched for his harmlessness and calmness" [4]. In other words, if a patient had no brothers or any other male relatives who could look after him during his stay in a monastery, the Metropolitan agreed to issue an escort letter for taking the patient to a monastery for treatment only if he was verified to be harmless and calm.

No matter how unwillingly the Metropolitan sent mentally ill patients to monasteries, the problem of their housing was urgent. Namely, sometimes unguarded mental patients posed a risk of spreading contagious diseases. Such was the case of a man named Marko Taslak from Mokra Gora. As the head of the Mokra Gora quarantine informed the Ministry of Internal Affairs on August 11, 1839, Marko "had fantasies in the grip of insanity while wandering across hills and rocks, crossing the Turkish border, and leaving the Cordon" [5]. Since there was an epidemic of a contagious disease in Turkey at that time, upon returning to Serbia, he had to be taken to quarantine, but he could not be kept there "because he wanted to smash all doors and make a great noise". On August 31, after getting an opinion from the State Council, the corresponding ministry ordered the head of the quarantine that, when the quarantine period was over, Marko Taslak be put under arrest by the competent court, until a general statute on this very present issue of stationing mentally ill persons was enacted [5]. Two months later, the Belgrade police asked the same ministry for instructions on how to handle a man named Sima Nerandžić from the village of Ševarice in the district of Šabac, who was a craftsman in Belgrade [6]. He was "out of his mind" and was, therefore, sent to a hospital to see a district physician Florian Birg. However, since he was left in the hospital without supervision, it was necessary to put "the aforementioned delusional person" in the police station [6]. The police, nonetheless, did not have a place for him nor the money for his medication. Hence, on October 14, the Ministry of Internal Affairs decided to send the patient home, but at the same time appealed to Regency to issue a general instruction for acting in similar cases [6].

There was also a possibility of putting a mentally ill person under police supervision without his family knowing it. This happened on October 23, 1839, when the State Council ordered the district court of Jagodina to act upon the case of a former president of the Rudnik district court, Marko Rakić, who started showing signs of mental illness and suicidal tendencies while serving his sentence [7]. Namely, the State Council believed that the patient would recover sooner in the family environment and would not be aware of the supervision [7].

As a result of numerous incidents involving mentally ill people and persistent requests and endeavors to permanently solve the problem of their housing, an official action eventually came on November 24, 1839, in the form of the "Project for housing mentally ill and other unfortunate individuals with contagious diseases, within the monastery of Studenica," drafted by the Ministry of Justice and Education [4]. It consisted of five provisions. This document envisaged the construction of a building affiliated to the monastery, divided into four sections - three for ill people and one for two police officers who would guard them [4]. The cost of the food, clothing, and necessary furniture for the ill, as well as salaries for the police officers, would be paid from the state treasury [4]. The project recommended that, apart from monks who would care for "the wretched," a district physician should come as well in order to "examine the state of their health" and prescribe appropriate medical therapy [4]. All relevant state organs were unanimous in the opinion that placing mentally ill individuals on the property affiliated to the monastery of Studenica was the optimal solution at that time. Although the Ministry of Justice and Education had insisted upon establishing a separate institute at first, it was convinced that there was not enough money for that and accepted the opinion of the majority [4].

The project never came into force because it was strongly opposed by the Metropolitan. After receiving the text for appraisal, in his reply to the Ministry of Justice and Education on December 11, the Metropolitan presented his argument against the suggested way of housing the mentally ill [4]. Expressing his regret that executive authorities did not consult with him before making the draft, he firstly mentioned the practical advantages of building a dwelling for the mentally ill near a town, because then they would be able to easily reach physicians, who were very much needed [4]. The need to place mentally ill people near urban areas was recognized in all European countries since there was no example of these institutions being built on remote monastery properties. Of course, the Metropolitan pointed out that priests would continue to read prayers "to this group of people who suffer," but it did not necessarily mean that they would have to live in a monastery, where their clamor would disturb church service, and scare and offend the faithful [4]. The Metropolitan ended by indicating that

founding such an institute which would completely be under the jurisdiction of the executive authority and on the Studenica monastery property, would disturb the internal autonomy of the monastery. The Metropolitan suggested that, in the beginning, mentally ill persons should be taken to monasteries according to the provisions of the Circular of July 10, 1839 [4]. In case that staying in the monastery did not help them, and they proved to be dangerous, the head of the church would recommend that they be placed in an appropriate institution for care and treatment [4]. The Metropolitan's resolute refusal to accept the suggested project of placing persons with mental illnesses in Studenica indefinitely postponed the problem of housing mental patients.

Establishment of the first mental hospital

The problem of housing mentally ill individuals was revived in 1855 when a fund to build a department for the mentally ill within the general town hospital was set up in the state treasury [8]. The department was designed to admit 20 mentally ill persons, who would have their own backyard and a garden and would be isolated from other patients [8]. Until the building was over, the patients were supposed to be placed in barracks in Požarevac, which were adapted for that purpose. However, in the meantime, a garrison unit was moved to those premises, thus the initial plan was dismissed [9]. Competent authorities also considered the possibility of placing the patients in the administration building in Karanovac, but it turned out that the building was on the main street and, therefore, could not be enclosed and used for that purpose [9]. Eventually, at the end of 1860, it was decided to adapt the military warehouse on Vračar – the so-called "Doctor's Tower," and to use it for housing mentally ill patients [10].

On February 6, 1861, the Ministry of Internal Affairs brought a legal project, "The Establishment of Lunatic Asylum," to the State Council for consideration [11]. The State Council made only minor changes in the text. Thus, it can be said that the original form of the document was approved with insignificant changes. The paper was divided into sections in order to make it easier to read. The final version consisted of 37 paragraphs, as opposed to 35 in the original version; they were systematized in seven sections and marked with Cyrillic alphabet letters [11]. As early as February 11, the State Council brought the revised version to Prince Mihailo Obrenović, who authorized it on February 20 and ordered the State Council to print 100 originals and 450 copies of the text and have them brought back to him for signature and confirmation [12]. The State Council did it the next day, and "The Establishment of Lunatic Asylum" was published on March 3, 1861 [12].

"Lunatic Asylum" was situated in the "Doctor's Tower" and was managed by a principal, who was in direct subordination of the minister of internal affairs and took orders and instructions from him [11]. The personnel of this institution was comprised of one physician, one doctor of medicine, who was also the principal, one physician assistant, who had to be at least a "patron of surgery" [11], i.e. an educated physician of the lowest degree in the Habsburg Monarchy [13], one procurement clerk and the necessary number of servers [11]. All of them (except the servers) were appointed by a decree of the prince, according to the suggestion of the Ministry of Internal Affairs [11]. An Orthodox priest was chosen for performing religious duties and was obliged to visit the hospital three times a week, talk to patients, and comfort them, except when the physician estimated that it was counterproductive for the patient's recovery [11].

Paragraph 9 stated that this Asylum was for the treatment of all mentally ill persons, both male and female, adults and children [11]. Given the accommodation capacity and available resources, the corresponding ministry was supposed to decide on the number of mentally ill persons that could be admitted to the Asylum. Wealthier patients would pay for their own stay and treatment. The treatment of poorer patients would be paid for by the state [11]. Modifications of this paragraph from 1873 specified that poorer patients were to be financed through the fund of the general hospital from their hometown district or the district they had lived in lately [14]. According to patients' financial status, they were classified by the line ministry as "paying" and "not paying," based on the opinion of the police authorities of the patient's place of residence [11].

A person could not be placed in the Asylum unless they had been previously categorized as mentally ill by a decree of the competent court (the court of the city of Belgrade) and without the approval of the corresponding ministry [11]. Police authorities were obliged to notify the minister of internal affairs of every unaccountable, mentally ill person who had committed a crime. Police authorities were also obliged to inform the minister of the financial status of the offender and his family, with the purpose of deciding whether they should pay for the wrongdoer's hospital stay [11]. Then, the ministry would issue an order to put the delinquent in the hospital if no family member nor a friend wanted to take care of him. If, however, a family member or a friend offered to look after a mentally ill person, they would have to vow that the ill person would be guarded in such a manner that they "could not jeopardize neither other people's nor their own life, nor be an embarrassment to anyone" [11]. The same would happen if that person came to the Asylum while the patient was still in a "confusional state", except that in this case the approval of the Ministry of Internal Affairs was needed as well. The Ministry's approval was also needed for discharging the patients, who were cured, from the hospital [11].

The physician's main duty was "to take care of mentally ill patients according to laws of medical science, and cure them of confusion as well as any other illness which developed in this state," and he had no right to ask for a special reward for that [11]. As the principal, he was in charge of the internal management of the Asylum, and he was responsible for maintaining law and order, legal usage of medications, and supplying the necessary medical instruments. He had help from the physician assistant, who had to act upon the doctor's orders, and if necessary, be his substitute [11]. A procurement clerk was in charge of supplying

the necessary equipment for the Asylum, keeping business records in order, and taking inventory [11].

There was a plan to establish a hospital fund from donations, income from paying patients, money earned from selling deceased patients' possessions, and objects made by patients. The asylum would deposit the raised money to earn interest in the same way as other hospitals did. This fund was for financing "Lunatic Asylum" [11]. The document ended with a short section of only two paragraphs on the procurement of medicines. The provisions stated that the procurement of medications for the hospital should be done by the pharmacy from which the Ministry of Internal Affairs obtained medicines for poorer patients whose treatment was paid for by the state [11]. The asylum was opened soon after its establishment, and the first patient was admitted on August 26, 1861 [15]. All mentally ill people who had been in prisons until the establishment of the Asylum were sent to the hospital for treatment on the basis of the Decree issued on August 8, 1861, by the Ministry of Justice and Education [16].

The head of the Asylum was a physician, i.e. a doctor of medicine [17]. From 1861 to 1865, those physicians were the following: Florian Birg, the Master of Surgery and the second physician of the city of Belgrade, Dr. Vasa Teodorović, previously a quarantine doctor of Aleksinac, and Samuilo Pops, physician assistant (later Dr.) [17]. However, the first physician who was partly more skilled in psychiatry was Dr. Mladen Janković (1830–1885), who was appointed to this position in March 1865. He was soon sent to Vienna for three months in order to get acquainted with contemporary forms of the housing, procedure, and treatment of the mentally ill patients. Upon his return to Serbia, he was to propose amendments to the organization and procedure. With short breaks, he remained in that position, until his death [17].

"The Establishment of Lunatic Asylum" stopped applying on May 1, 1881, when "Law on the organization of the sanitary profession and public healthcare" came into force [18]. This law in 16 paragraphs regulated all important issues regarding mentally ill persons and their hospitalization. "Lunatic Asylum" was renamed "Hospital for Mental Diseases" and divided into two departments [18]. The first general department was designated for treating all mental patients, and the second one was created with the aim of "guarding and caring for incurable delusional patients until their death" [18]. The level of professional qualifications of the hospital principal was raised compared to the previous requirements so that only a psychiatrist, doctor of medicine, could be appointed to the position. Apart from the principal, the physician assistant, procurement clerks, an adequate number of servers, and the auxiliary staff, the hospital personnel also included a secondary doctor, who had to be at least a doctor of medicine and surgery [18]. The minister of internal affairs appointed a special priest for the "Hospital for Mental Diseases" to whom he assigned annual salary. The institute remained under the direct jurisdiction of the minister of internal affairs [18].

Persons who suffer from "all types of mental illnesses, from melancholia to insanity and dementia paralytica" were sent to the hospital [18]. Citizens of foreign countries were admitted to the hospital based on reciprocity with the country whose citizen the patient was [18]. This law also identified two categories of patients – the wealthier, who paid for their own treatment, and the poorer, whose treatment was paid for from the sanitary fund, which was decided by the minister of internal affairs upon receiving the opinions of affiliated municipality and police [18]. Subjected to his exclusive jurisdiction was the decision on hospital admission based on the professional opinion of three doctors who had been observing the patient. If the ill person in question or their family or friends filed a written objection to the hospitalization, the case was resolved by the court. The court also had jurisdiction to classify a person as delusional based on the results of the treatment and move them to another department [18]. The local police authorities were obligated to notify the line minister of all mentally ill people in their area because guarding delusional persons in private homes was not allowed unless their family swore to vouch for their actions and promised to treat them humanly [18]. Patients were discharged from the hospital only with the approval of a special committee formed by the corresponding minister. In order to be discharged from the hospital, a patient had to be completely cured, or, at least, their health had to be improved to the extent that they could not cause harm to anyone any more [18]. Since the hospital was under the direct jurisdiction of the minister of internal affairs, every year he decided which public pharmacy would handle the procurement of medicines for the hospital [18].

Legislation on mentally ill persons

In order to send people with mental disorders to the hospital, it was necessary to legally define the concept of a mentally ill person. It was done in paragraph 40 of the Civil Code of 1844, which stated that persons who completely or partially lacked sanity and free will were "insane, crazy, and delusional" [19]. They were under the special protection of the law because, due to the illness, they were not aware of the harmful consequences of their actions [19]. A commentator of the Civil Code, a notable professor of the Civil and State law at Belgrade Lyceum, Dimitrije Matić, was complimentary about the fact that the legislator had not gone into a more detailed classification of different types of mental disorders, given that it was a matter of disagreement among many doctors and psychologists [19]. Matić pointed out that even the behavior of mentally ill people in the so-called lucid intervals (*lucida intervalla*) had no legal importance because it would be very difficult to determine what a person incapable of reasoning did in the state of narrowed awareness or moments of full awareness of their actions [19].

Paragraph 53 of the Criminal Code of 1860 excluded criminal responsibility of mentally ill delinquents [20]. In the first edition, this paragraph stated that there was no criminal act if committed by a person who "is not in their right mind," and in the amendment of 1861, it was specified that "there is neither a criminal act nor wrongdoing if a person who committed the act did so while he was crazy" [20]. Such a vague definition, as our famous jurist Đorđe Cenić warned, left some room for the court's subjective judgement on which the offender was considered crazy and, therefore, both

unaccountable and criminally irresponsible. All the more so, "people don't call 'crazy' only those persons who are just silly, those who suffer from epilepsy are also put in the same category" [20]. It would thus be good, as Cenić thought, for judges to have at least some basic knowledge about mental disorders. Nevertheless, since these disorders were a matter of disagreement among doctors whose professional opinions, given in proceedings on offenders' mental states, were ultimately contradictory, it would be unrealistic to expect the judges to always be able to evaluate the mental state of a particular offender [20]. Cenić reasoned that it would be better if only "crazy" persons were considered criminally irresponsible than to go into the classification of mental disorders, as it had been done in paragraph 40 of the Civil Code, which only created more confusion [20].

Cenić's concern about courts' subjective judgments on the mental health of offenders proved to be justified, which was evident in two court case records sent to the minister of justice for consideration in 1869 by the Ministry of Internal Affairs [21]. In the first case, the accused of murder was declared crazy and sent to the Asylum, based on the doctor's opinion and hearing of the witnesses. After spending fifteen days in the Asylum, he was found perfectly healthy. Furthermore, it turned out that it was highly disputable whether he had been crazy at the time of committing the murder or whether his insanity was just "a result of his continuous drinking, which is why he got well when alcohol was forbidden for him" [21]. In the second case, a father reported that his son was crazy and socially dangerous, as confirmed by a doctor. The court handling the case declared him crazy without a further evidentiary procedure, but he could not be admitted to the hospital because he showed no symptoms of a mental illness [21]. Taking into consideration all the mentioned above, the minister of justice concluded that the courts had solved these cases very superficially, and in his 1869 Circular, he made an appeal to judicial organs not to easily trust every claim but to examine them with scrutiny [21]. He also addressed the Circular issued on May 14, 1863, by the minister of justice, which advised all courts that, before declaring a person mentally ill, they demand as evidence not only a medical report from a doctor, but also a statement from the local authorities of the municipality the defendant was from, as well as to hear the family and neighbors of the accused [21]. If they still had some doubts concerning the mental state of the offender, they were authorized to demand a second opinion from another doctor and bring the delinquent to court in order to personally discern his mental state [21].

Persons who, due to their mental illness, were not able to take care of themselves and their property were placed under guardianship [19]. A guardian was mostly chosen from the closest relatives and performed this duty, according to paragraph 180, until the reason for guardianship ceased to exist [19]. Guardianship over a mentally ill person was over, in the words of Dimitrije Matić, "when the one who is insane regains sanity" [19]. These provisions ceased to have an effect in 1872 when the "Law on Guardianship" was enacted [22]. The Law became a part of the Civil Code as a separate regulation [22]. The paragraphs concerning adult mentally ill persons were not substantially changed. Since 1872, decisions on guardianship were made by a guardianship judge, who would award guardianship to the spouse or a parent of the person being placed under guardianship [22]. According to paragraph 143, when the guardianship judge was assured that the person who had been placed under guardianship due to a mental illness was cured, based on the court-medical investigation, he would end the guardianship [22]. This meant that the person regained legal capacity at that moment and, thus, the ability to take care of themselves and their property.

Conclusion

With the establishment of the Lunatic Asylum in 1861, the treatment of mentally ill persons in the 19th century Serbia became significantly more humane. Until then, in the absence of an appropriate institution for their housing, ill people who were aggressive and showed suicidal tendencies, and did not have any relatives who would take care of them, were taken to prison. Staying in inhumane conditions without adequate care surely contributed to the deterioration in their mental health in periods when they would become aware of their surroundings and the place they were in. Therefore, the establishment of a special institute for the treatment of the mentally ill helped improve the mental state of those patients, and it was also beneficial for other mentally ill people who received professional medical help in the institution. A step forward was raising the level of professional qualifications of medical staff by the 1881 Law and dividing the hospital into two departments in an attempt to isolate very ill patients. The foundation of the hospital for the mentally ill was performed in compliance with the adequate legislature. Admittedly, it was terminologically imprecise, but it is completely understandable, given the fact that at the time, there were still numerous disagreements even among the experts concerning the diagnosis and classification of mental illnesses. Taking into consideration all the above said, it can be concluded that in the 19th century Serbia a lot was done for mentally ill persons, in terms of providing not only appropriate medical care, but also adequate legal protection for this particularly sensitive and vulnerable category of patients.

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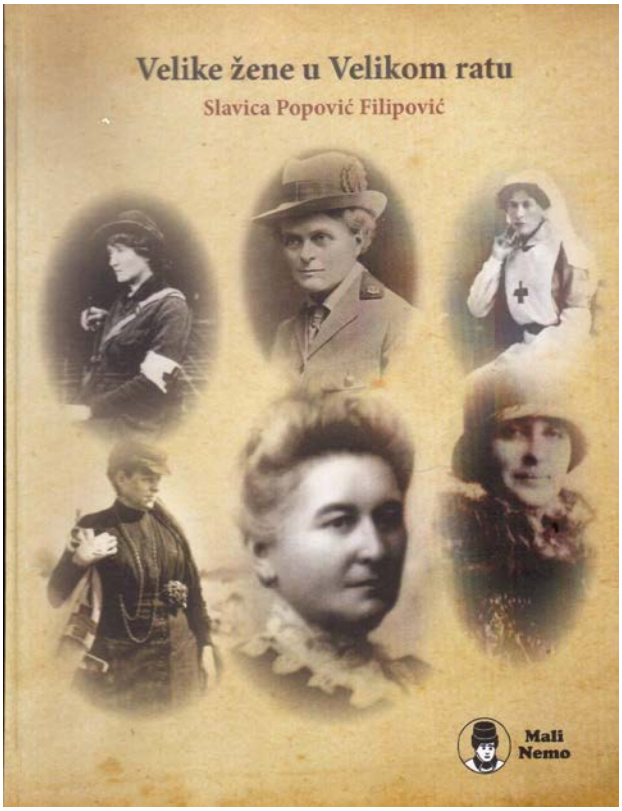
BOOK REVIEW

GREAT WOMEN IN THE GREAT WAR



Title: GREAT WOMEN IN THE GREAT WAR
Original title: Velike žene u Velikom ratu (Serbian)
Author: Slavica Popović Filipović
Publisher: Hrestomatija (Mali Nemo), Pančevo
Year: 2020
ISBN 978-86-7972-124-2
Circulation: 300

A new book by Slavica Popović Filipović entitled ***Great Women in the Great War***, reviewed by Dr. Veljko Todorović, PhD, was published in September 2020 by Mali Nemo in Pančevo. The Publisher has been promoting it as a capital work, an impressive opus created as a result of many years of research in the field of culture of remembrance, history of World War I (with special reference to the history of Serbian medicine and Serbian medical corps), cultural diplomacy, history of the suffrage movement in Europe and Serbia, and humanitarian and philanthropic activities. This book is the crowning achievement of the author's research work conducted over many years; she had already published a large number of her works on the history of Serbian medicine, foreign medical missions, and philanthropy during World War I.

In order to preserve the saga of humanity in the most difficult times of the war, and following her attitude towards the national history and the history of other peoples, the author collected and researched numerous archives, original documents, correspondence, manuscripts, and photographs preserved in archives on several continents, in libraries in different parts of the world, and public and private testimonies. The book *Great Women in the Great War* in a multi-layered view is dedicated primarily to women and their participation in the following activities: treating and healing war wounds in the great sufferings of World War I; treating a large number of patients with typhoid, relapsing, and spotted typhus in military surgical hospitals in Serbia; the operation of hospitals in occupied Serbia; the Serbian exodus through Albania; exile on Corfu, the Salonica Front, Corsica, North Africa, on the Russian Front, and in Dobrudja. The heroines are not shown only through their personal destinies, but they are also portrayed as the bearers of broad missions of the following organizations: the Serbian Women's Society (*Kolo*

srpskih sestara), the Serbian Red Cross Society, the Scottish Women's Hospitals, the Serbian Relief Fund, the Serbian Aid Fund, the St. Petersburg Slavic Charity Society, the First Serbian Surgical Hospital in Dragomanci, and other medical and humanitarian missions. The heroines were together with the Serbs in Serbia, with the Serbs in exile, and with the Serbs in rebuilding the destroyed and ravaged country.

Over the years, the author has collected valuable testimonies on women from all allied and friendly countries - bright and lesser-known examples of dedicated Serbian women, humanists and volunteer nurses, early Serbian and foreign doctors, Russian nobles, British suffragettes, Scottish women, French philanthropists, Canadian and Australian humanists. They were trained doctors, teachers, journalists, writers, painters, ladies, and heroines from all around the Mediterranean, across the Atlantic and the Pacific, all of them in a common mission for humanity, in the intertwined war drama of the world during World War I.

The chapters are dedicated to the following heroines: Ljubica Luković, the president of the Serbian Women's Society *Kolo srpskih sestara*, a volunteer nurse and war victim; Dr. Angelia Al. Yakchitch, a volunteer doctor in the Balkans and the First World War; Dr. Elsie Maud Inglis, a doctor and surgeon, the founder and manager of the Scottish Women's Hospitals; Dr. Isabel Emslie, Lady Hutton, a doctor of the Scottish Women's Hospitals in France, Gevgelija, Salonika, and Vranje; Scottish Baroness Honourable Evelina Haverfield, a member of the Scottish Women's Hospital in Serbia, on the Russian Front, and Dobrudja, after the War in Serbia; Nadežda Petrović, Serbian heroine, painter, and humanist, a volunteer nurse in the Balkans and World War I, fallen in the war; Delfa Ivanić, a teacher, co-founder of the Serbian Women's Society *Kolo srpskih sestara*, a volunteer nurse and humanist with a large and broad mission; Dr. Rosalie Morton, an American doctor at the French-Serbian Hospital in Sedes on the Salonica Front, a great philanthropist in war and peace; Margaret Leila Wemyss, Lady Paget, a volunteer nurse in Belgrade in 1912, and the head of the First Unit of the Serbian Relief Fund in the First World War, faithful to the Serbs to the end; Mrs. Gertrude Carrington Wilde, a longtime member of the Serbian Relief Fund, in the mission for the Serbian people and Serbian children; Mrs. Hannah Hankin Hardy, a volunteer nurse at the Second Reserve Hospital, founder of the National League of Serbian Women in Kragujevac; humanist Jelena Lozanić Frotingham, representative of the Serbian Red Cross, in a joint mission with Michael Pupin and John Frotingham in America and Canada; Dr. Agnes Elizabeth Lloyd Bennett, an Australian doctor, the head of the Scottish Women's Hospital in Ostrovo, and her compatriots on the Salonica Front; Lena Alexander Yovitchitch, a Serbian-Scottish writer and translator in the humanitarian mission for the Serbian people, author of great testimonies; Mrs. Mabel Annie St. Clair Stobart, a suffragette and volunteer nurse, the head of the Third Unit of the Serbian Relief Fund in Kragujevac and the founder of seven dispensaries throughout Šumadija; Miss Olive Kelso King, an Australian, a member of the Scottish Women's Hospitals, a sergeant in the Serbian army, and a great philanthropist; French Countess Marie de Shabannes la Palice, a volunteer nurse and philanthropist, who helped to establish the First Serbian Surgical Field Hospital on the Salonica Front; Madame Mabel Gordon-Dunlop Grouitch, an American humanist and Serbian daughter-in-law, promoter of the Serbian struggle in Europe and America; Russian noblewoman Mrs. Alexandra Pavlovna Hartwig, a humanist and volunteer nurse, who contributed to the formation of numerous Russian hospitals to help Serbia, personally delivered the medical mission Russian Pavilion in Niš; Dr. Slavka Mihailović-Klisić, a Serbian doctor, the only doctor at the Belgrade General Hospital after the Great Exodus, who left a diary of Belgrade's suffering in the Great War; Dr. Harriet Macmillan Cockburn, a Canadian humanist and doctor, a doctor in the Third Unit of the Serbian Relief Fund in Kragujevac and the head of the dispensary in Lapovo, but also her colleagues from the homeland of the maple tree, who treated Serbian soldiers in Serbia and on the Salonica Front.

This book impresses by its universal message, a wide ranging work, and the way it is presented. At the same time it is a work of special artistic design, written in 21 chapters on 761 pages, with the introduction in Serbian and English, each chapter having a summary in English, with extensive literature, and finally an index with thousands of names, a note on the author, and her gratitude to numerous associates, friends, and collaboration team, especially the reviewer Dr. Veljko Todorović, translator Bob Filipović, and publisher Milan Orlić, PhD.

According to Dr. Todorović, *"following the mentioned heroines, the author brings the mission of one of the hospitals or humanitarian organizations on different fronts of the Great War closer to each saga. It shows the strength and unity of intellectual elites, who stood on the right side in the first war of planetary proportions in history, who were not selfishly isolated but completely tied to ordinary people and small nations, guided by the principles of freedom, justice, and equality. "*

Prof. **Zoran Bojanić,** MD, PhD
Faculty of Medicine, University of Niš

E R R A T A

DOI: https://doi.org/10.2298/VSP210219013E

1. In the **Case report** titled "A fatal case of fulminant myocarditis caused by influenza A virus" by ***Mila Kovačević, Ilija Srdanović, Milana Jaraković, Dragana Bogdanović, Milenko Čanković,*** published in the ***Vojnosanitetski Pregled*** **2019; 76(12): 1290–6.** (https://doi.org/10.2298/VSP170928017K), there was an error in the byline. The correct byline is:

   Mila Kovačević*†, Ilija Srdanović*†, Milana Jaraković*, Dragana Bogdanović*, Golub Samardžija*†, Milenko Čanković*†
   *Institute of Cardiovascular Diserases of Vojvodina, Sremska Kamenica, Serbia; †University of Novi Sad, †Faculty of Medicine, Novi Sad, Serbia.

   The correction has been made to the online version of that issue of the Journal which is available at: http://www.vma.mod.gov.rs/vsp12-2019.pdf

2. In the **Case report** titled "Psychogenic diabetes insipidus – A case report of behavioral psychotherapy" by ***Miodrag M. Stanković, Jelena Stevanović, Aleksandra Stojanović, Sandra Stanković***, published in the ***Vojnosanitetski Pregled*** **2020; 77(12): 1332–5.** (https://doi.org/10.2298/VSP180527188S), there was an error in the byline. The correct byline is:

   Miodrag M. Stanković*†, Jelena Stevanović‡, Aleksandra Stojanović*, Jelena Kostić*†, Sandra Stanković§
   University of Niš, *Faculty of Medicine, Niš, Serbia; Clinical Centre Niš, †Center for Mental Health Protection, §Clinic for Children's Internal Diseases, Niš, Serbia; ‡General Hospital, Leskovac, Serbia

   The correction has been made to the online version of that issue of the Journal which is available at: http://www.vma.mod.gov.rs/vsp-12-2020.pdf

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Preparation of manuscript

Parts of the manuscript are: **Title page; Abstract with Key words; Text; Acknowledgements** (to the authors' desire), **References**, **Enclosures**.

1. ***Title page***

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Examples of references:

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

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

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

Abood S. Quality improvement initiative in nursing homes: the ANA acts in an advisory role. Am J Nurs [serial on the Internet]. 2002 Jun [cited 2002 Aug 12]; 102(6): [about 3 p.]. Available from: http://www.nursingworld.org/AJN/2002/june/Wawatch.htm

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Primeri referenci:

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

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

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

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

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Tabele

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

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