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# СРПСКИ АРХИВ
## ЗА ЦЕЛОКУПНО ЛЕКАРСТВО

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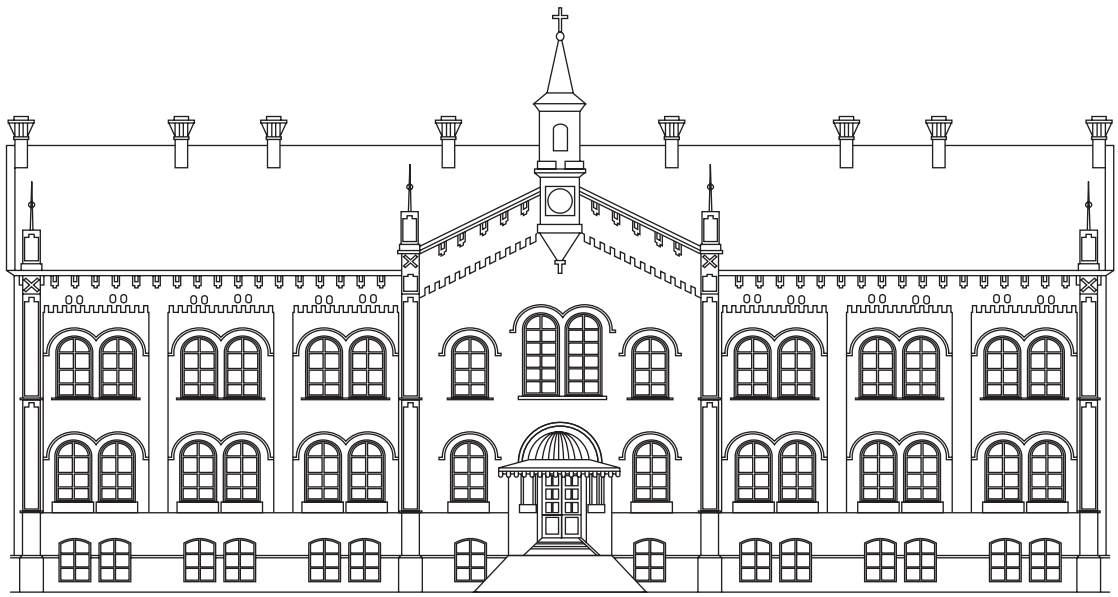

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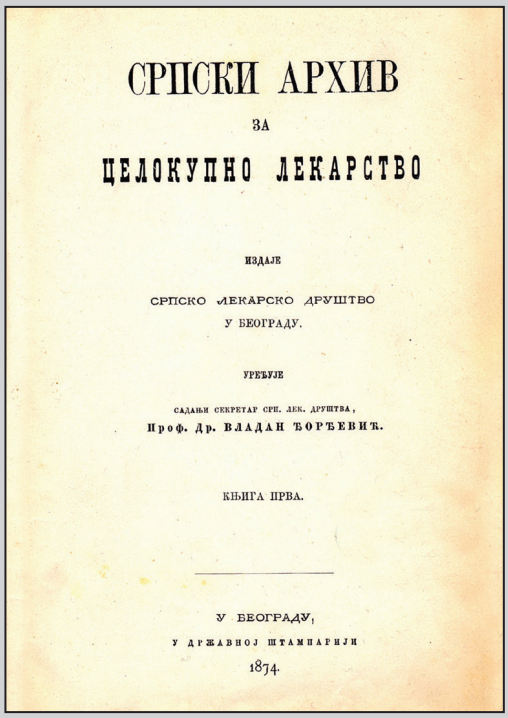

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ЗА

ЦЕЛОКУПНО ЛЕКАРСТВО

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1874.

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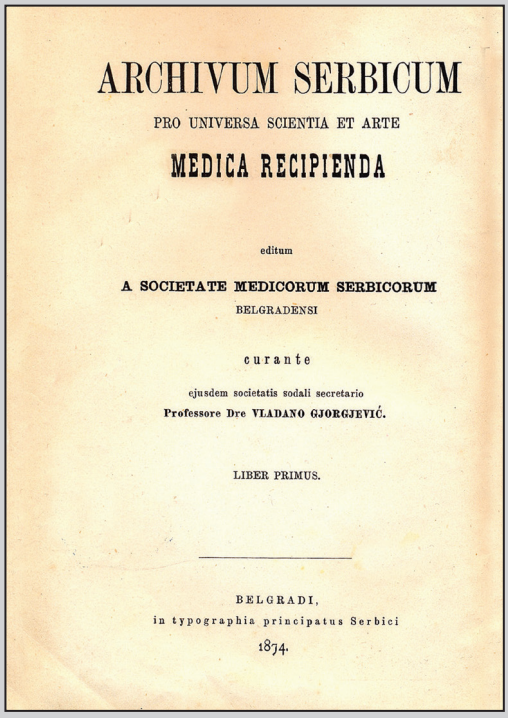

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1874.

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

# Expression of a fibronectin in the dental pulp of lead intoxicated rats with experimentally induced diabetes mellitus

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**SUMMARY**

**Introduction/Objective** Lead exposure represents one of the most important factors that affect the general health, including oral health and it is associated with enamel and dentin tooth defects. The aim of this paper was to determine expression of the fibronectin in the pulp of rats with experimentally induced diabetes mellitus (DM), after lead exposure, by using immunohistochemical analysis.

**Methods** The study was conducted on 42 rats of Wistar strain. Intoxication of rats with lead-acetate was done via drinking water *ad libitum*. The first group (Exp_14) consisted of 16 rats, which received lead in water for 14 days, the second group (Exp_30) consisted of 16 rats which received lead in water for 30 days at the same concentration (1500 ppm), while the control consisted of 10 healthy rats. Groups Exp_14 and Exp_30 were induced into DM, by using the Alloxan intraperitoneally. Pathohistological and immunohistochemical analysis determined fibronectin expression in pulp, odontoblasts, predentin and dentine of the teeth.

**Results** High diffuse positivity of fibronectin in group Exp_14 was noticed in 63.6% of rats, in group Exp_30 in 24% of rats, while in the control group it was noticed in 50% of rats. There was no statistically significant difference in the expression of fibronectin between the examined groups.

**Conclusion** Lead intoxication through drinking water, for a period of 14 and 30 days, had effect on the expression of fibronectin in the pulp, odontoblasts, predentin and dentin of the teeth of animals experimentally induced DM.

**Keywords:** fibronectin; odontogenesis; diabetes; lead

## INTRODUCTION

Exposure to lead represents one of the most important health problems because lead affects general health, development of bone and tooth defects and caries formation [1].

Although lead affects the pulp tissue, the number of studies concerning the influence of lead on the reparative dentinogenesis is very limited [1, 2].

Formation of dentin by odontoblasts is a genetically conditioned process involving the whole pulp-dentine complex, but it still represents an insufficiently explained biological phenomenon [2, 3]. During the long-term research the following conclusion has been made: the differentiation of new odontoblasts in the pulp is influenced by numerous factors such as: bone sialoproteins, calcium ions, extracellular matrix, growth factors, fibronectin, tenascin, nerve growth factor from the pulp or cytokines which, through the growth factors, affect the beginning of the formation of reparative dentine [4, 5].

Fibronectin belongs to the class of glycoproteins with high molecular weight and plays an important role in regulation of cell adhesion, migration and differentiation during developing and reparative processes. Fibronectin surrounds ectomesenchyme cells that polarize and secrete mantle predentin [6].

While the mechanisms of periodontal diseases associated with diabetes mellitus (DM) are largely clarified, cell and molecular mechanisms of dental pulp disorders associated with DM remain unclear. Many studies have shown that the formation of dentine in pathological conditions takes place in genetically predetermined order, depending on the influence of external factors that hinder or stimulate reparative dentinogenesis. It has also been confirmed that the structure of pathological dentine is very similar to the structure of mantle dentin and is most often dependent on the strength and length of irritation [2].

Thaweboon et al. [7] examined the effect of lead on the pulp tissue by observing the cells of the pulp-fibroblast, human teeth *in vitro*. Lead concentration significantly influenced the increased proliferation of the pulp cells and significantly lower protein production, procollagen and osteocalcin [7, 8].

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Tziafas et al. [9] in their research explained in more details the structure of pathological dentine i.e., its first layer (crystal zone), osteodentin tissue and tubular dentin, and indicated that in the dentin pulp surrounding the specific stimuli were found: TGF-β1 and fibronectin that initiate the process of the reparative dentinogenesis.

Wieser et al. [10] in his study also confirmed that fibronectin affects the pulp that differentiates in odontoblasts of a similar cell and that growth factors influence dentinogenesis due to the fibronectin that binds them.

Yoshiba et al. [11] examined the distribution of fibronectin in the dental pulp by using indirect immunofluorescence, a confocal laser scanning microscope, and observed the presence of fibronectin in the odontoblastic layer. Leite et al. [12] investigated tenascin-c and fibronectin expression in the pulp after direct calcium hydroxide capping and MTA in permanent teeth of pigs, and noticed that tenascin-C and fibronectin expression in both groups were similar.

DM leads to numerous changes in the human body, mainly in the blood vessels and the nervous system, including oral complications such as pulp microcirculation disorders, slowing down its metabolism, and thus the odontogenesis process [12, 13].

The aim of this paper was to use immunohistochemical analysis to determine the expression of fibronectin odontogenesis mediator in the dental pulp of rats with experimentally induced DM, after the exposure to the lead.

## METHODS

The research was conducted after the approval of the Ethics Committee of the University Clinical Center in Banja Luka no. 01-9-192.2 /15, Bosnia and Herzegovina. The sample consisted of 42 Wistar rats.

The animals were two months old with a body weight of 150–200 g and were kept under special conditions. Rats were kept in group plexiglass cages, at 12 hours of light regime (07.00–19:00) at an air temperature of 22°C (± 2) and humidity of 60% ± 10%, where they had free access to food and water during the experiment. At the beginning of the experiment, the individuals were separated into appropriate test and control groups. They were given a 15-day adaptation period, after which the treatment began. For immunohistochemical analysis, thin sections of dental pulp soft tissue were collected on positively charged Super Frost slides (Menzel-glaser), after which tissue sections were deparaffinized, rehydrated, and stained by blocking endogenous peroxidase activity. The reaction was performed by incubating the tissue in 3% hydrogen peroxide solution for 10 minutes at room temperature and then the hydrogen peroxide was washed with distilled water, and the procedure was continued by enzymatic unmasking of the target antigens. The enzyme trypsin (Thermo Fisher Scientific Fremont, Waltham, MA, USA) was used for unmasking. Enzyme digestion was being performed for 10 minutes at room temperature. Then tissue sections were washed with distilled water and TBS buffer (Tris buffered saline) pH 7,4 and treated with commercial solutions to block nonspecific reactions: Rodent Block R (Biocare Medical, Concord, CA, USA) for fibronectin.

On the tissues prepared in this way, immunohistochemical detection of the target antigen was performed by horseradish peroxidase reaction using rabbit polyclonal Anti-Fibronectin antibody (Rabbit polyclonal Anti-Fibronectin antibody, PA1-23693, Thermo Fisher Scientific, Waltham, MA, USA). The optimally diluted antibody was applied to the tissues and allowed to stand overnight in the refrigerator at 4°C. After incubation, the next day the excess antibody was washed with TBS buffer (Tris buffered saline) pH 7,4.

EnVision + Single Reagents /HRP Rabbit (Dako Corporation, Carpinteria, CA, USA) was used to visualize fibronectin. The incubation of the polymer system lasted 30 minutes. It was performed at room temperature in a humid chamber, after which the tissues were washed with TBS buffer. Specific immunostaining became visible after five minutes of incubation with 3,3 ' – diaminobenzidine tetrahydrochloride chromogen (DAB chromogen). After washing the excess chromogen to show the complete tissue structure, the tissue sections were contrasted with Mayer's Hematoxylin (30 seconds), washed with water, dehydrated in increasing ethanol concentration (70%, 90%, and 100%), clarified in xylene and permanently incorporated into Canada balsam. After that, the finished preparations were analyzed on a Leica DM2000 light microscope and photographed with a camera connected to a microscope.

The rats were divided into two experimental groups (Exp_14 and Exp_30) and one control group (control). The first (Exp_14) group consisted of 16 rats (256 molars and premolars of the upper and lower jaw) with experimentally-induced DM that received lead in water in the course of 14 days at a concentration of 1500 ppm. The second (Exp_30) group consisted of 16 rats (256 molars and premolars of the upper and lower jaw) with experimentally-induced DM that received lead in water in the course of 30 days at a concentration of 1500 ppm. The control consisted of 10 healthy rats.

In the rats of the first and the second group (Exp_14 and Exp_30) body weight and the level of blood sugar had been recorded before the experiment started. Using Alloxan the first and second group of rats (Exp_14 and Exp_30) were brought into experimentally induced DM. Alloxan solution was administered intraperitoneally at a dose of 100 mg per kilogram of body weight of the rats. The protocol was repeated every other day until the measured glycemic values exceeded 200 mg/dcl. Glycemia was measured using a device for glycemia measurement (ACCU ACEH, Roche, Basel, Switzerland) from the blood of the tail vein.

### Protocol for lead intoxication

Intoxication of adult rats with lead-acetate at a concentration of 1500 ppm was performed via drinking water *ad libitum*. Lead intoxication lasted 14 days in Exp_14, and 30 days in Exp_30. All the procedures performed on animals were done in accordance with the Guide for the Care and Use of Laboratory Animals [14]. After the period determined for intoxication by lead, animals were sacrificed and the upper

and lower jawbones were separated from the soft tissues and stored in the fixative (10% neutral buffered formalin).

In the laboratory of the Department of Pathology of the University Clinical Center Banja Luka preparation for pathohistological and immunohistochemical analysis was performed. The tooth samples were painted by using standard hematoxylin–eosin (HE) method and analyzed by using a light microscope (Leica DM2500, Leica Biosistems, Wetzlar, Germany).

On prepared dental pulp tissues, the immunohistochemical detection of targeted antigens was performed using the reaction of horseradish peroxidase applying Rabbit polyclonal Anti-Fibronectin antibody (Rabbit polyclonal Anti-Fibronectin antibody, PA1-23693, Thermo Fischer Scientific Waltham, MA, USA). For visualization of fibronectin, EnVision + Single Reagents / HRPRabbit (Dako Corporation, Carpinteria, CA, USA) was used. Final preparations were analyzed using light microscopy Leica DM2500

Expression was determined in the pulp (loose connective tissue of the pulp, pulp fibroblasts), odontoblasts, predentin and dentin (odontoblast extensions – Tomes' fibers located in predentin and dentin). The expression estimation of the examined antibodies was performed in accordance with personal semi-quantitative scale (Table 1).

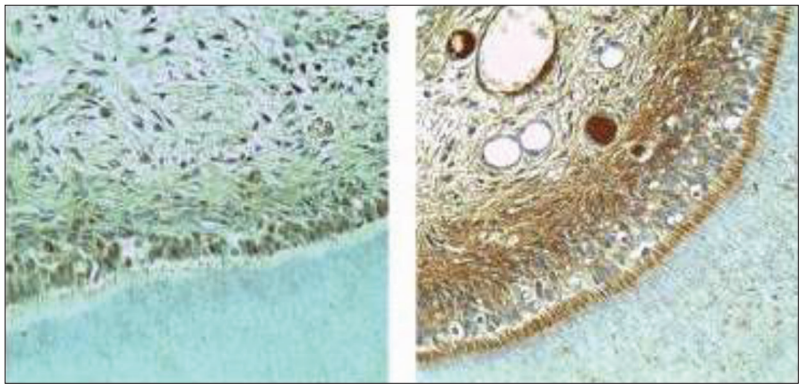

**Figure 1.** Cross section of the teeth of rats which received lead in the course of 14 days: a) representation of moderate expression (grade 2) of fibronectin in cytoplasm of odontoblast (400×); b) representation of high expression (grade 3) of fibronectin in cytoplasm of odontoblast (400×)

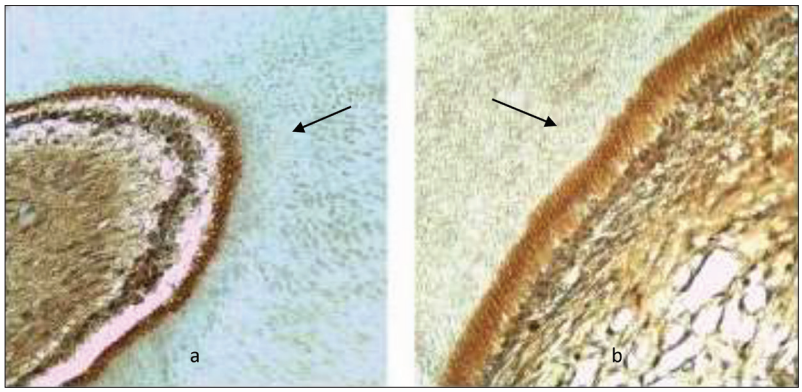


**Figure 2.** Cross section of the teeth of rats which received lead in the course of 30 days: a) representation of high expression (grade 3) of fibronectin in cytoplasm of odontoblast (200×); b) representation of high expression (grade 3) of fibronectin in cytoplasm of odontoblast 400×)

**Table 1.** Criteria used for semi-quantification of expression

| Grade | Parameter |
|---|---|
| 0 | Absence of staining, all analyzed structures without staining |
| 1 | Weak or moderate, focal positivity / diffuse weak positivity |
| 2 | High focal positivity / diffuse moderate positivity |
| 3 | Diffuse high positivity |

## Statistical analysis

The statistical analysis was done by using the IBM SPSS20. The following descriptive statistical parameters were determined: frequency, percentage, arithmetic mean, standard deviation, median, minimum and maximum. A $\chi^2$ test was used as the analytical statistical method ($\alpha = 0.05$).

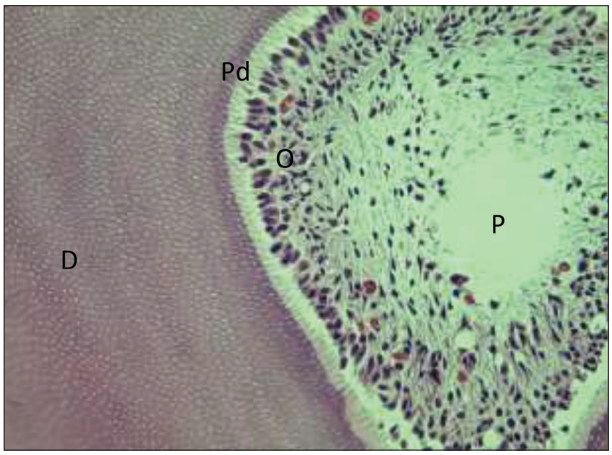


**Figure 3.** Tooth cross section – control group; pulp, odontoblast layer, predentin, and dentin without morphological changes can be seen (H&E, 400×);
P – pulp; O – odontoblast layer; Pd – predentin D – dentin

## RESULTS

The results of immunohistochemical analysis are shown in Table 2 and Figures 1, 2, and 3.

In the first experimental group (Exp_14), high diffuse positivity of fibronectin (grade 3) was noted in 63.6% of the cases (Figure 1), high focal positivity (grade 2) in 27.3% of the cases, weak or moderate (grade 1) in 9.1% of the cases while absence of staining (grade 0) was not registered in either case (Table 2).

In the second experimental group (Exp_30), high diffuse positivity of fibronectin (grade 3) was noted in 24% of the cases (Figure 2), high focal positivity (grade 2) in 48% of the cases, weak or moderate (grade 1) in 24% of

**Table 2.** Expression of fibronectin in the study groups

| Study groups | | | Fibronectin expression | | | | Total |
|---|---|---|---|---|---|---|---|
| | | | Absence of staining | Weak or moderate focal positivity | High focal positivity | High diffuse positivity | |
| Group | Pb 14 days | n | 0 | 1 | 3 | 7 | 11 |
| | | % | 0% | 9.1% | 27.3% | 63.6% | 100% |
| | Pb 30 days | n | 1 | 6 | 12 | 6 | 25 |
| | | % | 4% | 24% | 48% | 24% | 100% |
| | Control | n | 0 | 1 | 5 | 6 | 12 |
| | | % | 0% | 8.3% | 41.7% | 50% | 100% |
| Total | | n | 1 | 8 | 20 | 19 | 48 |
| | | % | 2.1% | 16.7% | 41.7% | 39.6% | 100% |

There was no statistically significant difference in the expression of fibronectin between tested groups ($\chi^2 = 6.864$; $p = 0.345$)

the cases, while the absence of staining was registered in one case (Table 2).

In the control group in healthy rats (without hyperglycemia and without lead intoxication) high diffuse positivity of fibronectin (grade 3) was noted in 50% of the cases (Figure 3), high focal positivity (grade 2) in 41.7% of the cases, weak or moderate (grade 1) in 8.3% of the cases, while the absence of staining was not registered in either case. There was no statistically significant difference in the expression of fibronectin among the examined groups ($\chi^2 = 6.864$, $p = 0.345$) (Table 2).

## DISCUSSION

DM is a condition of chronic hyperglycemia and predisposing factor for caries, enamel hypoplasia in infants from mothers with diabetes, gingivitis, periodontitis, oral candidiasis, xerostomia and many other oral cavity diseases [15, 16, 17].

Fibronectin represents one of the most significant glycoproteins which has a major influence on the odontogenesis process. It maintains the connection between cells and components of the extracellular matrix in healthy and infected tissues; affects the migration and polarization of odontoblasts and is considered to be a trigger for differentiation of odontoblasts [18–21].

The results of this study have shown that high diffuse expression of fibronectin was the largest in the group of teeth which belonged to the rats with experimentally induced DM that were intoxicated with lead through water in the course of 14 days. High focal expression of fibronectin was the highest in the group of teeth of rats that were intoxicated with lead in the course of 30 days.

These results can be explained by the fact that the group of rats (Exp_14) received lead through drinking water in a shorter period than the other group (Exp_30) which at a different level stimulated the reparative dentinogenesis i.e., fibronectin expression. Reduced expression of fibronectin of high diffuse positivity in the group of rats that were intoxicated with lead in the course of 30 days could be related to the fact that in the case of prolonged hyperglycemia and lead intoxication, the odontogenesis process was modified. Previous studies confirmed that there were changes in the small and large blood vessels in the dental pulp of rats with DM, especially the basement membrane thickening, which affects the leukotaxis of polymorphonuclear leukocytes and cellular immune system elements. These changes are most pronounced in the central zone of pulp which could be the reason for the confirmed low expression of fibronectin of high diffuse positivity in the second group of rats in this study [12, 22, 23].

High expression of fibronectin was noted in pulp cells after non-specific inductive effects of certain factors, such as bacterial and mechanical factors, on the pulp resulting in the formation of a fibronectin-rich matrix. This matrix serves as a source of growth factors, which are considered to be signaling molecules for odontoblast differentiation which may explain fibronectin expression in the control group of rats [19, 20, 21].

The results of this study were confirmed by Yoshiba et al. [11] examining the distribution of fibronectin in pulp of developing tooth and finalized growth tooth by using indirect immunofluorescence, confocal microscopy. In the apical region of developing tooth, fibronectin was found within the basal membrane and in the first formed predentin layer. A similar representation of fibronectin in the teeth with the finished growth was noted in the odontoblastic layer. Positive fibrous structures between odontoblasts correspond to von Korff's fibers and are associated with differentiation of odontoblasts and dentinogenesis. The authors have indicated that fibronectin was present in the odontoblast layer throughout all phases of dentinogenesis, which could explain its reduced expression in dental pulp of rats with experimentally induced diabetes that were intoxicated with lead in the course of 30 days [11].

An experimental study by Đeri [23], conducted on Wistar rats in which the hyperglycemia was experimentally caused by intraperitoneal administration of alloxan, affirmed a slower metabolism of pulp in diabetic rats than in healthy rats and weaker effects of direct covering of the pulp regardless of the material used, mineral trioxide aggregate (MTA) or calcium hydroxide. In her study she also concluded that microangiopathy in the dental pulp is probably one of the reasons for these findings, giving that the blood vessels with the increased lumen were noticed on the large number of sections of the dental pulp of rats [23].

It has been confirmed that matrix molecules, including fibronectin, growth factors and progenitor pulp cells as well as their interaction mainly stimulate dentinogenesis [20, 24].

Functional researches support the hypothesis that TGF-β1 plays an important role in stimulation of odontoblast metabolism. In *in vitro* conditions it passes through dental papilla and increases the secretion of collagen type I and fibronectin. It is considered not to affect the polarization of peripheral cells of the dental papilla. The synthesized and released growth factors are embedded in the dentin matrix and remain inactive until it dissolves or demineralizes. The

inductive influence of TGF-β1 has been noted in those studies where heparin was replaced by fibronectin, which also has affinity for TGF-β1 [9, 25].

Tziafas et al. [25] explained in more details the structure of pathological dentin, which was also noted in the teeth of rats with experimentally induced DM in this study. The aforementioned authors observed cells different from odontoblasts and pointed out that there were specific stimuli – growth factor TGF-β1 and fibronectin in the pulp tissue surrounding, which initiate and lead this process.

Ignotz and Massague [26] pointed out that the effect of TGF-β1 on fibronectin expression was rapid, selective, specific and stable. In the research conducted on the fibroblasts of 12 days old chicken embryos, a significant increase in fibronectin has been noted in the extracellular matrix under the influence of TGF-β1 growth factor.

In experimental research on dogs where biomatrix within the tooth's pulp chamber served as a sample, Tziafas et al. [25] explained the molecular basis of induction of the creation of reparative dentin. Namely, the pulp tissue of the experimental animals was implanted with Millipore filter soaked with fibronectin, which affected the formation of dentin matrix around the implant and the appearance of cells similar to odontoblasts. Wieser et al. [10] in their study came to the same conclusion that the pulp cells covered with fibronectin can be differentiated into cells similar to odontoblasts. In this case, fibronectin binds growth factors, in particular TGF-β1 and affects the initiation of dentinogenesis. These findings are in accordance with the results of our immunohistochemical examination.

Some studies showed that pulp microcirculation is an important factor in metabolic activity and dentinogenesis [12, 13, 26, 27, 28]. Using pathohistological analysis Madani et al. [15] studied changes in the pulp after direct covering in the Wistar rats which were induced into diabetes. Intense inflammatory response was found in the pulp that was covered with calcium-enriched mixture (CEM) cement compared to the pulp of the teeth treated with MTA.

In various phases of dentinogenesis, Linde et al. [29] examined the expression of fibronectin by using indirect fluorescence. Fibronectin and its expression were established in the basement membrane between the inner enamel epithelium and the primary dental mesenchyme and in the predentin layer, but were not represented in the predentin during further, circumpulpal formation of dentin. That indicates that the fibronectin molecules were not directly involved in mineralization. Fibronectin was localized in the odontoblast layer.

*In vivo* animal models used in this study, provide an acceptable alternative to human models, although differences between species should be taken into account while interpreting tissue response [30].

## CONCLUSION

Expression of fibronectin was higher in the group of rats with experimentally-induced DM that received lead through drinking water in the course of 14 days compared to the group of rats that received lead in the course of 30 days. Reparative dentinogenesis and fibronectin expression were reduced under the influence of DM, but also insufficiently stimulated in rats that had been intoxicated by lead through drinking water for a shorter period.

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**Conflict of interest:** None declared.

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# Експресија фибронектина у пулпи зуба пацова интоксикованих оловом са екперименталнo изазваним дијабетесом мелитусом

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**САЖЕТАК**

**Увод/Циљ** Изложеност организма олову представља један од значајнијих фактора који утичу на опште здравље укључујући орално здравље, те има утицај на настанак глеђних и дентинских дефеката.

Циљ овог рада је био да се имунохистохемијском анализом одреди експресија медијатора одонтогенезе фибронектина у пулпи зуба пацова са екперименталнo изазваним дијабетесом мелитусом, после излагања животиња олову.

**Методе** Истраживање је спроведено на 42 пацова соја вистар. Интоксикација адултних пацова оловним ацетатом је урађена путем воде за пиће *ad libitum*. Прву експерименталну групу (*Exp*_14 ) чинило је 16 пацова који су добијали олово у води 14 дана, другу групу (*Exp*_30) 16 пацова који су добијали олово у води 30 дана у истој концентрацији (1500 *ppm*), док је контролну групу чинило 10 здравих пацова. Групе пацова *Exp*_14 и *Exp*_30 су уведене у дијабетес мелитус помоћу раствора алоксана, који је апликован интраперитонеално. Патохистолошком и имунохистохемијском анализом одређивана је експресија фибронектина у пулпи, одонтобластима, предентину и дентину зуба експерименталних животиња.

**Резултати** Висока дифузна позитивност фибронектина у групи *Exp*_14 је уочена у 63,6% случајева, у групи *Exp*_30 у 24% случајева, док је у контролној групи уочена у 50% случајева. Није уочена статистички значајна разлика у експресији фибронектина између испитиваних група.

**Закључак** Интоксикација оловом путем воде за пиће, у периоду од 14 и 30 дана, имала је утицај на експресију фибронектина у пулпи, одонтобластима, предентину и дентину зуба животиња са екперименталнo изазваним дијабетесом мелитусом.

**Кључне речи**: фибронектин; одонтогенеза; дијабетес; олово

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

# Analysis of biochemical markers in the saliva and correlation with clinical parameters in patients with aggressive periodontitis, before and after the therapy

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**SUMMARY**

**Introduction/Objective** Aggressive periodontitis (AP) is a progressive disease that damages the periodontal tissues.

The aim of the study was the analysis of intracellular enzymes: aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), acid phosphatase (ACP) and electrolytes in the saliva of patients with AP and their correlation with clinical parameters before and after the therapy.

**Methods** The study included 30 patients with AP (experimental group) and 35 patients with healthy parodontium (control group). Intracellular enzymes and electrolytes were analyzed in an unstimulated saliva of subjects with AP, before and after the therapy and in saliva of the control group. The analysis of biochemical markers was carried out using kinetic methods with commercial reagents.

**Results** Concentrations of the biochemical markers AST (28.18 ± 25.16), ALT (5.48 ± 5.14), ALP (31.13 ± 37.79), ACP (17.53 ± 14.77), calcium (2.80 ± 1.97), phosphate (4.43 ± 1.92) in the saliva of subjects of the experimental group were statistically significantly higher in relation to the control group ($p = 0.000$; $p = 0.001$). Significant correlation was found between AST values, debris index ($\rho = -0.444$; $p = 0.026$) and calculus index ($\rho = -0.513$; $p = 0.009$), and between the plaque index and ALP level in the saliva after therapy ($p = 0.020$).

**Conclusion** The investigation will contribute to a better understanding and standardization of biomarkers in the saliva that mayhelp in diagnosing the AP and evaluation of the applied therapy.

**Keywords:** aggressive periodontitis; intracellular enzymes; saliva

## INTRODUCTION

Aggressive periodontitis (AP) is a disease followed by rapid and severe destruction of the periodontal tissue. Destruction of the periodontal tissue begins in the area of the first molars and incisors, and with aging it can involve the periodontium of adjacent teeth. It affects the peridontal tissue of at least three permanent teeth, except the first molars and incisors [1].

In the etiology of AP, virulence factors of microorganisms in dental biofilm play a key role including the presence of risk factors and genetic predisposition. In the periodontal lesions, *Aggregatibacter actinomycetemcomitans* is a major causative agent, and its role is in regulating the inflammatory response in the aggressive periodontal inflammatory process [2].

The diagnosis of AP is based on patient's medical history, clinical examination and radiographic analysis [3]. However, all these diagnostic methods tell us about the disease only when there are clear clinical symptoms. Thus, in most cases, the disease has already significantly advanced at the time of the diagnosis.

Over the past two decades, with the progress of technological development, saliva has increasingly been analyzed as a sample of biological material, which might be used to establish diagnosis and to clarify the pathogenesis of oral diseases. It provides evidence and gives useful information on various enzymes and biomolecules, the indicators of pathological processes in periodontal tissue [4, 5]. Literature data show that the concentration of intracellular enzymes responsible for the metabolic processes in the cells has been significantly increased in the saliva of patients suffering from periodontitis compared to healthy subjects [6].

The objective of this study was to analyze the activity of intracellular enzymes of aspartate aminotransferase (AST), alanine aminotransferase (ALT), acid phosphatase (ACP) and alkaline phosphatase (ALP), and calcium and phosphate concentrations in the saliva of patients with AP, before and after the therapy, as well as and correlation of the mentioned biochemical markers with clinical indicators of the condition of periodontal tissues.

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## METHODS

The study included 65 subjects.The experimental group consisted of 30 patients suffering from AP who were coming to the Clinic for Periodontology and Oral Medicine at the Military Medical Academy in Belgrade.The control group consisted of 35 subjects with the clinically healthy periodontium. The study excluded all persons who had a systemic illness, used medicines that could impair the condition of periodontium, consumed alcohol, as well as females during the pregnancy, lactation or menstrual cycle.

Clinical evaluation of the periodontal tissue condition was performed of the following parameters: gingival index (GI), plaque index (PI), probing depth (PD), debris index (DI), calculus index (CI), bleeding index (BI). Measurements were performed usingthe dental mirror and graduated periodontal probe (Periodont Sex Cp 11).

Patients suffering from AP underwent, a basic / causal therapy, such as: removal of soft deposits with brushes and the Vantal (Galenika) toothpaste, removal of supragingival deposits (supragingival calculus) with the ultrasonic apparatus (Kavo, Sonicflex 2000 N, Biberach an der Riss, Germany), removal of subgingival deposits and treatment of the tooth root with special curettes (Gracey, Kohler, Austria). The free content of the periodontal pocket was removed by washing with chlorhexidine digluconate (12%). After clinically visible signs of inflammation reduced, a surgical intervention was performed (Modified Widman flap surgery). Eight weeks (two months) after basic and surgical therapies were performed, clinical parameters were again measured to determine the condition of periodontal tissues.

### Determination of levels of intracellular enzymes and electrolytes in saliva samples

From the patients (control and experimental groups), during the first visit, samples of mixed unstimulated saliva were taken using special tubes (Salivette; Sarstedt AG & Co. KG, Nümbrecht, Germany). Eight weeks after the basic and surgical therapy, saliva samples were taken only from the experimental group.According to the protocol, saliva was taken in the morning hours at the same time.Centrifugation of samples lasted 10–20 minutes at 3000–5000 rpm and there after they were deposited at -80°C until the beginning of analysis.

The activity of enzymes in the saliva was determined by kinetic methods on the spectrophotometer (Secomam Basic, Ales, France) with commercial reagents (Human, Wiesbaden, Germany). Concentrations of calcium and phosphate in unstimulated saliva were determined by a colorimetric method, commercial reagents (Human, Germany) were used. All analyses were done in the Laboratory for Biochemistry and Hematology at the School of Dental Medicine Belgrade, according to the recommendations of the International Federation for Clinical Chemistry, IFCC.

### Statistical analysis of data

In order to perform necessary statistical testing, IBM SPSS Statistics for Windows, Version 27.0 (IBM Corp, Armonk, NY, USA) was used. All variables were described by measures of central tendency and measures of variability: mean, median, standard deviation, minimum and maximum values.

To test the difference between subjects in the observed groups, the Mann–Whitney U test was used for the nonparametric data and the t-test for independent samples for the parametric data. The Wilcoxon test was used to compare the values of the observed clinical and biochemical parameters before and after the therapy. The correlation between clinical and biochemical parameters was examined by the Spirman's correlation coefficient.

The factors of difference between the subjects with healthy parodontium and the patients with AP disease were established by logistic regression analysis. Statistical significance was defined as $p < 0.05$. The study was approved by the Ethics Committee of the Faculty of Medical Sciences, University of Kragujevac (No. 01-4798).

## RESULTS

### Descriptive characteristics of the subjects

The basic descriptive data of the subjects in the control and experimental groups are shown in Table 1.

**Table 1.** Descriptive characteristics of the subjects in the control and experimental groups

| Patients' characteristics | | Control group | Experimental group | Significance |
|---|---|---|---|---|
| Number of patients (n) | | 35 | 30 | |
| Age (X ± SD (Med., min.–max.)) | | 27.06 ± 3.72 (26; 22–38) | 42.30 ± 7.69 (40; 30–57) | p = 0.000* |
| Sex n (%) | Male | 12 (34.3%) | 18 (60%) | p = 0.038* |
| | Female | 23 (65.7%) | 12 (40%) | |
| Smoking n (%) | Yes | 5 (14.3%) | 15 (50%) | p = 0.002* |
| | No | 30 (85.7%) | 15 (50%) | |

*statistically significant difference (p-value < 0.05)

The control group included 35 subjects, with an average age of 27.06 ± 3.72 years. In the experimental group 30 subjects had AP, average age was 42.30 ± 7.69 years, 18 were males (60%) and 12 females (40%).

Intergroup analysis found that male subjects were statistically significantly more represented in the group of patients with AP than in the control group ($p = 0.038$; Table 1). The average number of years statistically significantly differed between the groups, and the subjects in the experimental group were older than those in the control group ($p = 0.000$; Table 1).

### Analysis of clinical parameters of subjects diagnosed with AP before and after the therapy

By analyzing the obtained results, a statistically significant correlation between the mean values of the clinical parameters of patients with AP, before and after the therapy ($p = 0.000$; Table 2) was established.

**Table 2.** Values of clinical parameters in the group of patients with aggressive periodontitis before and after the therapy

| Clinical parameters X ± SD (Med., min.–max.) | Aggressive periodontitis | | Significance |
|---|---|---|---|
| | Before therapy | After therapy | |
| GI | 1.66 ± 0.65 (2; 0.25–2.5) | 0.82 ± 0.56 (0.94; 0–1.75) | ap = 0.000* |
| PI | 1.69 ± 0.71 (2; 0.25–2.71) | 0.97 ± 0.51 (1; 0.25–2) | ap = 0.000* |
| BI | 1.47 ± 0.66 (1.5; 0.25–2.14) | 0.66 ± 0.39 (0.79; 0–1.2) | ap = 0.000* |
| PD | 5.05 ± 1.08 (5; 3.8–6.95) | 4.08 ± 0.98 (4; 2.8–5.9) | ap = 0.000* |
| DI | 1.4 ± 0.61 (1.5; 0.25–2.2) | 0.59 ± 0.36 (0.68; 0–1) | ap = 0.000* |
| CI | 0.02 ± 0.71 (0; 0–0.25) | 0.1 ± 0.14 (0; 0–0.5) | ap = 0.000* |

GI – gingival index; PI – plaque index; BI – bleeding index; PD – probing depth; DI – debris index; CI – calculus index;
*statistically significant difference;
[a]Whilcoxon test

Two months after the therapy a statistically significant decrease in the value of all observed clinical parameters was noticed, i.e., there was an improvement of periodontal health.

## Correlation of biochemical markers values in the saliva between the subjects of the control group and patients with aggressive periodontitis before and after the therapy

The activity of intracellular enzymes in the unstimulated saliva of the patients with AP was different from the one in the control group (Table 3).

**Table 3.** Values of biochemical markers in the saliva of healthy subjects and patients with aggressive periodontal disease before and after the therapy

| Biochemical markers | Aggressive periodontitis | | Significance |
|---|---|---|---|
| | Before therapy | After therapy | |
| AST (U/L) | 28.18 ± 25.16 (17.59; 1–98) | 26.57 ± 23.1 (16.99; 1.74–96) | ap = 0.845 |
| ALT (U/L) | 5.48 ± 5.14 (3.8; 1–23) | 5.45 ± 6.75 (4; 1–29) | ap = 0.442 |
| ALP (U/L) | 31.13 ± 37.79 (18.36; 7.59–178) | 17.61 ± 11.38 (16.98; 3.53–61.0) | ap = 0.100 |
| Ca (mmol/L) | 2.80 ± 1.97 (2.075; 0.66–10.31) | 2.98 ± 2.67 (2.13; 0.74–15.48) | ap = 0.643 |
| P (mmol/L) | 4.43 ± 1.92 (4.31; 1.75–9.46) | 3.87 ± 1.42 (3.9; 0.17–7.04) | bp = 0.158 |
| ACP (U/L) | 17.53 ± 14.77 (14.58; 2.54–80.97) | 15.44 ± 16.08 (11.45; 1.15–87) | ap = 0.309 |

AST – aspartate aminotransferase; ALT – alanine aminotransferase; ALP – alkaline phosphatase; ACP – acid phosphatase;
*statistically significant difference;
[a]Whilcoxon test;
[b]t-test for bound samples

AST was decreased in subjects with AP (28.18 ± 25.16 U/L) compared to the subjects in the control group (29.2 ± 32.67 U/L), but without statistical significance (Figure 1). In contrast to AST, the ALT level in the saliva of subjects in the experimental group (5.48 ± 5.14 U/L) was statistically significantly higher than in the saliva of subjects

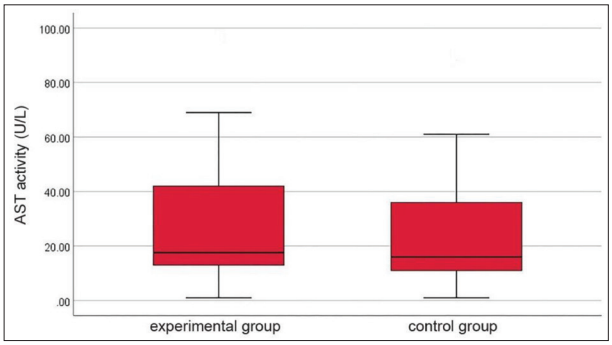


**Figure 1.** Value of aspartate aminotransferase (AST) in saliva of control and experimental group before the therapy

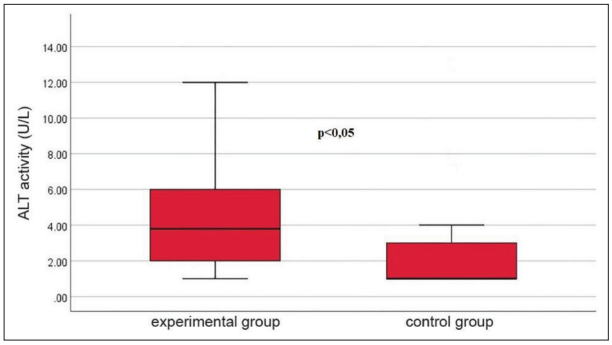


**Figure 2.** Alanine aminotransferase (ALT) level in the saliva of subjects in the experimental group is statistically significantly higher than in the saliva of subjects with healthy parodontium

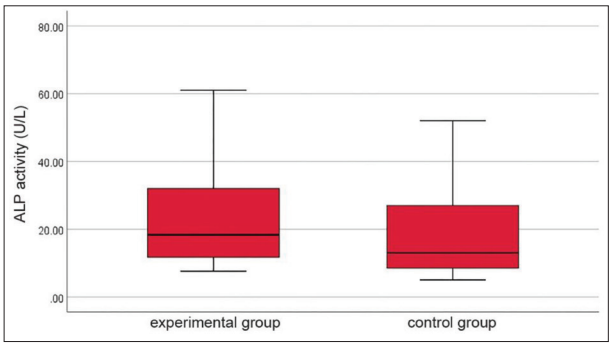


**Figure 3.** Value of alkaline phosphatase (ALP) in saliva of control and experimental group before the therapy

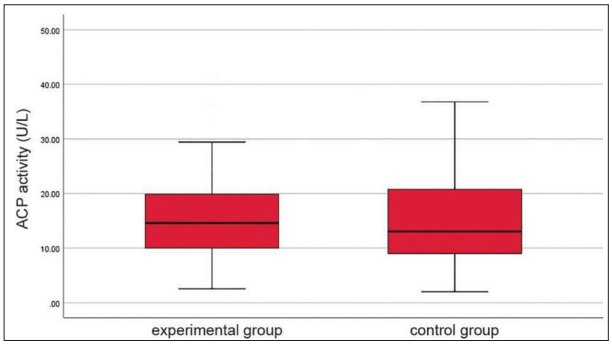


**Figure 4.** Value of acid phosphatase (ACP) in saliva of control and experimental group before the therapy

with healthy parodontium (2.40 ± 2.51 U/L) (p = 0.000; Table 3; Figure 2). Mean values of the activity of the enzymes ALP (31.13 ± 37.79 U/L), ACP (17.53 ± 14.77 U/L) in the saliva of subjects in the experimental group were

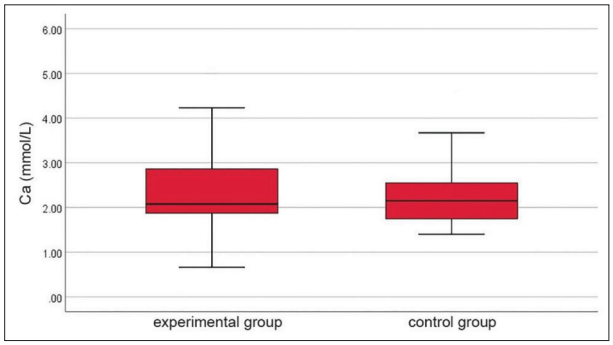


**Figure 5.** Concentration of calcium in saliva of control and experimental group before the therapy

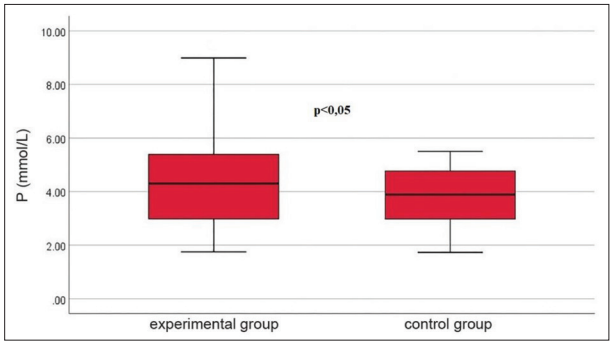


**Figure 6.** The concentration of phosphate in the experimental group was statistically significantly higher than in the control group

higher compared to the subjects in the control group (18.31 ± 12.39 U/L, 15.62 ± 8.52 U/L), but without statistically significant difference (Figures 3 and 4). The mean calcium concentration in the saliva of the subjects in the experimental group (2.80 ± 1.97) was higher compared to the subjects in the control group (2.25 ± 0.69) (Figure 5). Unlike calcium, the mean concentration of phosphate in the experimental group (4.43 ± 1.92) was statistically significantly higher than in the control group (3.87 ± 1.31) (p = 0.001; Table 3; Figure 6).

Eight weeks after the basic and surgical therapy, the activity of the enzymes (AST, ALT, ALP, ACP) in the saliva of the subjects with AP was reduced but without statistical significance (Table 3).

## Correlation of clinical parameter values and biochemical markers in patients with aggressive periodontitis before the therapy

Analyzing the interconnection, no statistical correlation was found between the values of GI, PD, PI, BI and the values of the analyzed markers (AST, ALT, ALP, ACP, Ca, P) in the saliva of subjects with AP, before the therapy (Table 4).

However, the Spirman correlation test showed a statistically significant correlation between the AST values in the saliva and DI values (q = - 0.444; p = 0.026) and CI values (ρ = -0.513; p = 0.009). The coefficient of correlation shows that with the increase in the values of CI and DI, there is a decrease in AST values in the saliva of patients with AP before the therapy (Table 4).

## Correlation of the values of clinical parameters and biochemical markers in patients with aggressive periodontitis

No statistically significant relationship was found between the values of clinical and biochemical parameters, in the saliva of the experimental group after the therapy. Unlike these data, a statistically significant correlation was observed between the PI value and the ALP level in the saliva (p = 0.020; Table 5). By the univariate regression analysis, the ALT has been found as statistically significant, thereforethe elevated values of this enzyme are always present in patients with AP.

**Table 4.** Correlation of the values of clinical parameters and biochemical markers in the saliva of subjects with aggressive periodontitis before the therapy

| Biochemical markers | Gingival index | Plaque index | Bleeding index | Probing depth | Debris index | Calculus index |
|---|---|---|---|---|---|---|
| AST | ρ = -0.326 | ρ = -0.236 | ρ = -0.280 | ρ = 0.076 | ρ = -0.444* | ρ = -0.513* |
| ALT | ρ = 0.023 | ρ = -0.063 | ρ = -0.071 | ρ = 0.147 | ρ = -0.024 | ρ = -0.063 |
| ALP | ρ = -0.167 | ρ = -0.035 | ρ = -0.043 | ρ = 0.140 | ρ = -0.112 | ρ = -0.069 |
| Ca | ρ = -0.143 | ρ = 0.010 | ρ = -0.069 | ρ = 0.167 | ρ = -0.109 | ρ = -0.080 |
| P | ρ = -0.285 | ρ = -0.029 | ρ = -0.147 | ρ = -0.002 | ρ = -0.248 | ρ = -0.238 |
| ACP | ρ = -0.205 | ρ = 0.086 | ρ = -0.094 | ρ = 0.110 | ρ = -0.178 | ρ = -0.156 |

ALT – alanine aminotransferase; ALP – alkaline phosphatase; ACP – acid phosphatase;
ρ – Spirman's coefficient of correlation; AST – aspartate aminotransferase;
*statistically significant linkage

**Table 5.** Correlation of the values of clinical parameters and biochemical markers in the saliva of patients with aggressive periodontitis after therapy

| Biochemical markers | Gingival index | Plaque index | Bleeding index | Probing depth | Debris index | Calculus index |
|---|---|---|---|---|---|---|
| AST | ρ = -0.010 | ρ = 0.078 | ρ = 0.057 | ρ = 0.309 | p = 0.238 | p = 0.165 |
| ALT | ρ = -0.118 | ρ = 0.089 | ρ = 0.080 | ρ = -0.125 | p = 0.097 | p = 0.185 |
| ALP | ρ = 0.394 | ρ = 0.463* | ρ = 0.327 | ρ = 0.018 | p = -0.180 | p = 0.058 |
| Ca | ρ = 0.204 | ρ = 0.293 | ρ = 0.043 | ρ = 0.094 | p = 0.238 | p = 0.123 |
| P | ρ = 0.086 | ρ = -0.062 | ρ = -0.053 | ρ = 0.158 | p = -0.259 | ρ = 0.024 |
| ACP | ρ = -0.062 | ρ = 0.166 | ρ = -0.027 | ρ = 0.247 | p = -0.157 | p = 0.196 |

ρ – Spirman's coefficient of correlation; AST – aspartate aminotransferase;
ALT – alanine aminotransferase; ALP – alkaline phosphatase; ACP – acid phosphatase;
*statistically significant linkage

## DISCUSSION

In patients with AP, the condition of periodontal tissues before and after the therapy was analyzed, based on the values of clinical parameters and the presence of biochemical markers in the saliva.

During the periodontal infection, various enzymes from stromal, epithelial and inflammatory cells are released into the saliva, gingival fluid and blood [7, 8]. Chambers et al. [9] published the first study that indicated an increase in the AST levels in gingival fluid in dogs during the experimental periodontitis. Since that time, many researchers have found that the AST activity in the

saliva is proportionate to the degree of tissue damage during the chronic periodontitis [10, 11] and gingivitis [12]. Due to the pathological process in periodontal tissues, the integrity of the cells is impaired, the permeability of their membranes is disturbed, and thus the AST is increasingly released from the cytoplasm into the saliva. Also, the AST values in the saliva [13] and gingival fluid [14] were in correlation with the values of the Community Periodontal Index of Treatment Needs. Our study has not proved that there is a correlation between the values of clinical parameters and the AST activity in the saliva.

In patients with chronic periodontitis, three months [11] or one month [12], after the basic therapy, there was a decrease in the AST levels in the saliva. Similar results were obtained in the gingival fluid [14]. Results of this study indicate that the mean value of the AST activity in the saliva of patients with AP was reduced two months after the basic and surgical therapy. Some authors believe that the decrease in AST activity in the saliva after the periodontal therapy, is a consequence of periodontal tissue reparation [8, 15]. In our study, in patients with AP, the mean PD was 5.05 ± 1.08, but there was a PD decrease (4.08 ± 0.98), after the basic and surgical therapy. This decrease in the PD was followed by a decrease in the AST levels in the saliva, but without statistical significance. After the mechanical therapy, in subjects with the chronic periodontal disease the decrease in AST activity was in correlation with the GI values and depth of the periodontal pocket [11]. The reduced GI value and AST levels indicate that the gingival health has improved [16]. In contrast to these studies, the AST level in the gingival fluid [17] and saliva [18] was not in correlation with the GI and PI values. Similar results have been obtained in our research. However, we have observed a statistical correlation between the increase in the DI and CI values and the decrease in the AST activity. The reason is in that the dental deposits are a predisposing factor which can lead to the development of gingivitis and periodontal disease.

Unlike the AST, the mean value of ALT (5.48 ± 5.14 U/L) in the saliva of experimental group was statistically significantly higher than in the control group (2.40 ± 2.51 U/L) ($p = 0.000$). By univariate analysis, it was found that the elevated ALT levels in the saliva are always present in subjects suffering from AP. In addition, this enzyme can be an indicator of the damage degree to the gingival tissuebecause the GI values were increasing linearly with increasing ALT levels in the saliva [10]. Also, in patients with chronic periodontitis and gingivitis, a correlation was found between the ALT level and the values of the depth of periodontal pocket, the level of clinical attachment and the number of periodontal-pathogenic bacteria in the saliva [15]. Data of this study indicate that there is no statistical correlation between the ALT levels and clinical parameter values. After applying the conventional therapy, in patients with chronic periodontitis, the level of ALT in the saliva [10] was significantly reduced. This is in line with our results. For the stabilization of periodontal tissue in patients with AP, it is necessary to apply the mechanical/surgical and antimicrobial treatment over a longer period [19]. Thus, the appropriate periodontal therapy for AP significantly changes the condition of the supporting structure of teeth, resulting in the level of ALT in the saliva. The authors consider that intracellular enzymes, especially the ALT, can serve as biochemical markers of acute damage of soft periodontal tissue during AP.

The ALP is an enzyme that catalyses the hydrolysis of the monophosphate ester bond in the alkaline environment. Its increased presence in the saliva reflects changes during the inflammation and destruction of periodontal tissue [20]. In the localized and generalized form of AP, a positive correlation between the pathological changes in periodontal tissues and the ALP concentration in the gingival fluid was demonstrated [21]. In our study, statistically significantly higher ALP values were measured in the group of subjects with AP compared to those with a healthy periodontium. Also, compared to healthy subjects, the level of ALP in the saliva and gingival fluid was increased in patients with chronic periodontitis [22, 23, 24]. The authors believe that, due to the tissue inflammation, there was an accumulation of polymorphonuclear leukocytes (PMN) that release the ALP into oral fluids [24]. Another study demonstrated a greater ALP activity in the gingival fluid in cases of chronic periodontitis, compared to patients with AP [24]. This is probably due to an impaired PMN function in patients with AP. In line with the intensity of the pathological process in periodontal tissue, the release of ALP also correlated. In patients with chronic periodontitis, the GI values and the depth of the periodontal pocket were statistically correlated with the level of ALP in the saliva and gingival fluid [10, 25]. The authors believe that the ALP enzyme can be a predictor of progression of periodontal diseases. In our investigation, no statistical correlation was found between the values of clinical parameters and the ALP concentration in the saliva.

After the basic and surgical therapy, the mean values of the ALP concentrations in the saliva of patients with AP (17.61 ± 11.38) were lower than the mean value of concentration (31.13 ± 37.79) before the therapy. Similar results were obtained in the gingival fluid in subjects with AP and patients with chronic periodontitis, after nonsurgical treatment [24, 26]. The authors believe that the reduction in ALP levels in the saliva can be a useful biomarker to monitor the effectiveness of the applied therapy.

In this study, the ACP as an important marker of remodelling the bone tissue was analyzed in saliva. The mean value of the ACP enzyme activity (17.53 ± 14.77 U/L) in the saliva samples of AP subjects was higher than the mean value in the control group (15.62 ± 8.52 U/L). Similar results were obtained in subjects with chronic periodontitis [10]. As periodontal disease is progressing, destructive processes develop in the alveolar bone, and ACP is released as a result of an increased osteoclastic activity [22]. Our results show that the ACP activity in the saliva of patients with AP was reduced, two months after the basic and surgical therapy, but without a statistical correlation. Reduction of the ACP levels in the saliva after the conventional periodontal therapy with a statistically significant correlation was observed in subjects with chronic periodontitis [10, 22].

Changes in the macro and trace element composition of saliva might be indicative for pathological changes in periodontal tissues [27]. Based on literature data, increased concentrations of calcium and phosphate in the saliva are the risk factors for the development of periodontitis [28]. Various studies have shown an increased concentration of calcium in the saliva of patients with chronic periodontitis compared to the group of subjects with healthy periodontium [27]. Also, our results demonstrate an increased calcium concentration in the saliva of subjects with AP, which is in agreement with other studies [27, 29]. Unlike the calcium concentration, a statistically higher phosphate concentration in the saliva of subjects with AP (4.43 ± 1.92) was observed compared to the subjects in the control group (3.87 ± 1.31). It has been proven that with the progression of periodontal disease, the levels of calcium and phosphate in the saliva are correlated with the values of clinical parameters (PI and GI) [29]. This is contrary to our results because the calcium and phosphate levels in the saliva were not in the statistical correlation with clinical parameters of the subjects with AP. Increasing the concentration of electrolytes in the saliva leads to mineralization of the dental plaque (tooth stones), which makes it difficult to clean, especially in the area of periodontal sulcus.

## CONCLUSION

Based on the obtained results, it can be concluded that the increased level of intracellular enzymes (AST, ALP, ALT, ACP) in the saliva of patients with AP is aconsequence of release from damaged cells and / or metabolic changes in periodontal tissues. After the basic and surgical therapy, the values of these enzymes were reduced, and thus they can be used to evaluate the effectiveness of the applied therapy.

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**Conflict of interest:** None declared.

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# Анализа биохемијских маркера у пљувачки и корелација са клиничким параметрима код оболелих од агресивне пародонтопатије, пре и после терапије

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**САЖЕТАК**

**Увод/Циљ** Агресивна пародонтопатија (АП) је прогресивно обољење које доводи до оштећења пародонталних ткива. Циљ истраживања био је анализа интраћелијских ензима аспартат-аминотрансферазе, аланин-аминотрансферазе, алкалне фосфатазе, киселе фосфатазе и електролита (калцијума, фосфата) у пљувачки испитаника са АП и њихова корелација са клиничким параметрима, пре и после терапије.

**Методе рада** У студију је укључено 30 пацијената са АП (експериментална група) и 35 испитаника са здравим пародонцијумом (контролна група). Интраћелијски ензими (аспартат-аминотрансферазе, аланин-аминотрансферазе, алкалне фосфатазе, киселе фосфатазе) и електролити (калцијум, фосфати) анализирани су у нестимулисаној пљувачки испитаника са АП, пре и после терапије (базичне и хируршке) и у узорцима пљувачке испитаника контролне групе. Анализа биохемијских маркера је вршена кинетичким методама, помоћу комерцијалних реагенаса.

**Резултати** Концентрације биохемијских маркера аспартат-аминотрансферазе (28,18 ± 25,16), аланин-аминотрансферазе (5,48 ± 5,14), алкалне фосфатазе (31,13 ± 37,79), киселе фосфатазе (17,53 ± 14,77), калцијума (2,80 ± 1,97), фосфата (4,43 ± 1,92) у пљувачки испитаника експерименталне групе биле су статистички значајно веће у односу на контролну групу ($p = 0,000$; $p = 0,001$). Вредности клиничких параметара су биле статистички значајно ниже након спроведене пародонталне терапије ($p = 0,000$). Спирмановим тестом уочена је статистичка корелација између вредности аспартат-аминотрансферазе и вредности индекса меких наслага ($\rho = -0,444$; $p = 0,026$) и индекса чврстих наслага ($\rho = -0,513$; $p = 0,009$). Након терапије је постојала корелација између вредности плак индекса и нивоа алкалне фосфатазе у пљувачки ($p = 0,020$).

**Закључак** Спроведено истраживање допринеће бољем разумевању и стандардизацији биомаркера у пљувачки који би помогли у дијагностиковању и праћењу спроведене терапије код оболелих од АП.

**Кључне речи:** агресивна пародонтопатија; интраћелијски ензими; пљувачка

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

# Effects of the light tip position on the degree of conversion and dentin bond strength of a universal adhesive

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**SUMMARY**
**Introduction/Objectives** To measure the degree of conversion (DC), immediate and long-term microshear bond strength (μSBS) to dentin of a universal adhesive relative to the light tip position and adhesive application protocol.
**Methods** Mid-coronal flat dentin of 48 human third molars was exposed and split in halves. Single Bond Universal (SBU; 3M) adhesive was applied to each half following 'total-etch' (TE) or 'self-etch' (SE) approach. Depending on the light tip (Bluephase G2, Ivoclar Vivadent, Schaan, Liechtenstein) angle and distance from adhesive surface, three groups were compared: "1 mm_90°" (control); "8 mm_90°" and "8 mm_60°". Cylindrical composite build-ups (ø1.7mm, Filtek Z250, 3M) were prepared in each half. DC was measured using Raman spectroscopy. μSBS was measured after 24 hours and six months storage in distilled water at 37°C. Fracture types were analyzed.
**Results** No significant difference in DC was detected between groups "1 mm_90°" (89.1 ± 6.2%) and "8 mm_90°" (94.6 ± 1.2%) ($p > 0.05$), both showing significantly higher DC ($p < 0.05$) than "8 mm_60°" group (74.9 ± 9.5%) ($p < 0.05$). Initially, there were no significant differences in μSBS between groups ($p > 0.05$). Group "1 mm_90°" TE (12.8 ± 4.3 MPa) and group "8 mm_60°" TE (14.7 ± 5.7 MPa) showed significantly lower μSBS after aging (8.4 ± 4.3 MPa and 9.2 ± 2.6 MPa, respectively) ($p < 0.05$). Adhesive fractures were predomintantly detected.
**Conclusion** Initially, both application protocols resulted in similar bond strength to dentin of a universal adhesive in suboptimal curing conditions. In the long-term, SE showed greater adhesive resistance to degradation resulting in smaller decrease in bond strength compared to TE. Light tip angulation affected DC and μSBS more than tip-to-surface distance.
**Keywords:** adhesive; scotchbond; degree of conversion; bond strength; light

## INTRODUCTION

Over the past few years, "universal adhesives" also known as "multi – mode adhesives" have been implemented in the dental practice. According to manufacturers, one of the most important benefits of universal adhesives is their versatility, as indications for use include bonding to tooth tissues but also to materials for indirect restorations, zirconia, glass-ceramics, and alloys. Furthermore, universal adhesives are recommended with any of the three currently accepted protocols for application to dental tissues: total-etch (TE), self- etch (SE) and selective – etch.

Two recent reviews concluded that mild universal adhesives yield the best results following a selective-etch protocol i.e., when adhesive is applied to previously acid etched enamel and un-etched dentin, but with limited success on indirect subtrates [1, 2]. Dentin etching with phosphoric acid as used in the TE protocol may improve bonding of ultra-mild universal adhesives, i.e., those with pH of > 3 compared to the SE protocol [1].

Enamel etching improves immediate and long-term bond strength of universal adhesives [1]. The same beneficial effect of enamel etching with phosphoric acid is evident for universal adhesives, as was previously seen with other adhesive groups [3]. As would be expected, long-term bonding to dentin is not so consistent and depends on adhesive pH [1]. Mild adhesives with pH between 2 and 3 showed greater degradation resistance irrespective of the application protocol than ultra-mild and intermediately strong which were associated with significantly reduced bond strength after aging [1]. Dentin etching has shown a detrimental effect on adhesive bond strength of several universal adhesives [4, 5]. Collagen degradation and resin hydrolysis were associated with biodegradation of universal adhesives and the resulting decrease in bond strength to dentin [4].

It is widely accepted that curing regimes, characterized by such factors as light irradiance, curing time and distance, affect curing efficiency in all light-cured materials. Light energy is directly related to the degree of conversion (DC) of light-cured resin-based materials, as shown on a model BisGMA/TEGDMA composite [6]. In clinical conditions, curing characteristics, especially the distance and tilt angle of the light tip from the material, may vary considerably, thus influencing light energy delivered to the material. Apart from various clinical conditions hampering an ideal position of the light source,

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**Table 1.** Materials used in the study

| Material (Code) | Manufacturer | Type | Composition |
|---|---|---|---|
| Single Bond Universal (SBU) | 3M ESPE, St. Paul, MN, USA | Universal adhesive | BisGMA, HEMA, DMDMA, ethanol, water, reaction products with 1,10-decanediol and P2O5, silane treated silica, copolymer of acrylic and itaconic acid, camphorquinone, dimethylaminoethyl methacrylate, ethyl-dimethyalaminobenzoat |
| Filtek Z250 (Z250) | | Microhybrid composite | BisGMA, UDMA, TEGDMA, BisEMA6, silane treated ceramic, benzotriazol, ethyl-dimethyalaminobenzoat |

BisGMA – Bisphenol A diglycidyl ether dimethacrylate; HEMA – 2 hydroxyethyl methacrylate; DMDMA – decamethylene dimethacrylate; UDMA – diurethane dimethacrylate; TEGDMA – triethylene glycoldimethacrylate; BisEMA6 – Bisphenol A polyethylene glycol diether dimethacrylate

Price [7] showed differences between irradiance delivered to restorations in two groups of clinicians, and also the importance of light curing instructions to achieve optimal polymerization. A study of light energy transfer using a MARC patient simulator revealed that as much as 31% of light energy was attenuated at a tilt angle of 20°. A wide difference in the amount of energy transferred to the material was seen with increasing distances of the light tip, this difference being highly dependent on the light-curing unit [8]. Other factors influencing the amount of delivered light energy in a clinical setting include inter-incisal opening, cavity location and operator experience with up to 17% difference between operator groups and 32% difference between anterior and posterior cavities [9].

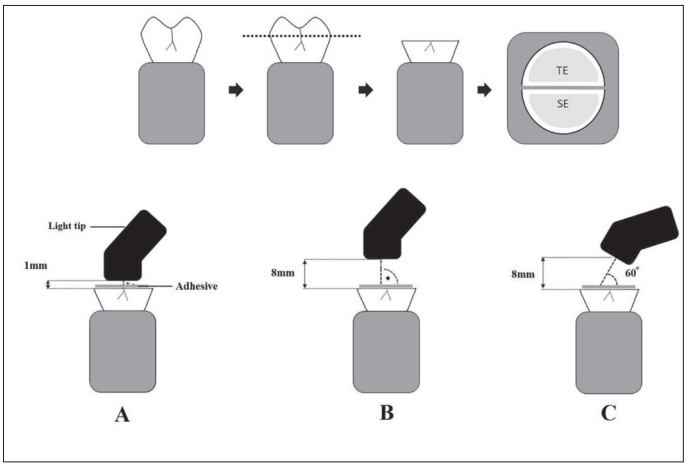


**Figure 1.** Specimen preparation A – Group "1mm_90°" (control); B – Group "8mm_90°; C – Group "8mm_60°";
TE – total-etch; SE – self-etch; a thick adhesive layer is presented for easy identification in this schematic and does not accurately reflect the actual thickness of the adhesive layer

Previous studies reported conflicting results on bond strength to dentin of adhesives following different application protocols [10, 11]. The differences could be related to multiple factors, such as the type of dentin, adhesive composition, application protocol, light-curing unit, and/or bond strength testing methods. Current literature lacks data on the effect of variable curing conditions, namely tip-to-surface distance and angle, on the DC, immediate and long-term bond strength to dentin of universal adhesives.

Therefore, the aim of this study was to measure the DC, immediate and long-term microshear bond strength (μSBS) of a universal adhesive to dentin depending on curing distance and angle of the light tip. The null hypotheses were:

1. there are no significant differences in DC of a universal adhesives cured at different tip-to-surface distances and angles of the light-curing unit;
2. there are no significant differences in μSBS to dentin of a universal adhesive cured at different tip-to-surface distances and angles of the light-curing unit;
3. there are no significant differences in the μSBS to dentin of a universal adhesive following artificial aging.

## METHODS

### Specimen preparation and bond strength testing

Forty-eight intact human third molars, extracted for orthodontic reasons were used in this study. Ethical approval was granted by the School Ethics Committee to use such teeth for research purposes. Following extraction, the teeth were cleaned from debris and refrigerated at +4°C in 0.2% thymol until the beginning of the experiment.

Teeth were embedded in gypsum up to the enamel-cementum junction. Enamel and superficial dentin were removed to expose mid-coronal flat dentin of each tooth. A 2 mm deep notch was made parallel to the long axis using a slow-speed diamond saw (Isomet 4000, Buehler, Lake Bluff, IL, USA), thus splitting the exposed dentin surface into two halves. All surfaces were inspected using a magnifying glass to ensure the absence of residual enamel.

The materials used in this study are shown in Table 1. SBU adhesive was applied to one dentin half following the TE and the other dentin half following the SE approach (Figure 1). In the TE protocol, dentin was first etched with 37% phosphoric acid for 15 seconds, rinsed and blot-dried. In the SE protocol, no acid etching was performed on dentin. SBU was applied to dentin for 20 seconds, according to manufacturer's instructions. Depending on the position and distance between the light tip and adhesive surface, prepared specimens were allocated to three groups (N = 8/ group) (Figure 1).

After adhesive polymerization, a silicone mold (ø1.7 mm × 2 mm) was placed on dentin and filled with the micro-hybrid composite (Z250) to produce cylinder composite build-ups with 2.27 $mm^2$ of adhesive surface area. In each group composite was light cured for 20 seconds at 1 mm distance using a high-intensity LED light-curing unit (Bluephase G2, Ivoclar Vivadent, Schaan, Liechtenstein).

Before μSBS testing, half of the teeth in each group were stored in distilled water for 24 h and the other half was stored in distilled water at 37°C for six months.

Irradiance of the LED light-curing unit was measured using a radiometer (Bluemeter II, Ivoclar Vivadent, Schaan, Liechtenstein). A metal foil with an 8×8mm window was placed on the radiometer sensor. The light tip was placed and oriented against sensor according to corresponding group.

A universal testing machine (PCE-200, PCE Group, Southampton, UK) was used for μSBS testing at 1 mm/min cross-head speed until specimen fracture. μSBS (MPa) to dentin was calculated by dividing maximum force at fracture (*N*) with bonded surface area ($mm^2$). Fracture types were analyzed under a stereomicroscope at 30× magnification and classified as:

1. adhesive – fracture occurring within the adhesive layer with no composite or dentin involved;
2. cohesive – fracture occurring within either composite of dentin;
3. mixed – fracture involving areas of adhesive layer extending into composite and/or dentin.

### Raman spectroscopy

The Raman spectra were recorded at room temperature with a DXR Raman Microscope (Thermo Scientific, Waltham, MA, USA). The samples were excited by the 532 nm emission line of a diode laser with 10 mW of power focused on a 2.1-μm spot on the surface of the sample using an objective magnification of 10×. The scattered light was analyzed by the spectrograph with a 900 lines $mm^{-1}$ grating. The spectrum was obtained as an average of three measurements on different places on the sample surface (10 exposures, 30 seconds each, per spot). All the Raman spectra were corrected for fluorescence by the OMNIC software (Thermo Scientific).

The DC was calculated using the following formula: DC = (1−R*cured*/R*uncured*) × 100 where *R* is the ratio of peak heights at 1639 $cm^{-1}$ and 1609 $cm^{-1}$ in cured and uncured material which was used as reference. The 1639 $cm^{-1}$ peak in the Raman spectrum is associated with the aliphatic C = C double bonds whilst the 1609 $cm^{-1}$ peak is associated with the aromatic C = C double bonds in cured/uncured material.

### Statistical analysis

Data were statistically analyzed in the software package Minitab 16 (Minitab Inc., State College, PA, USA). Two-way analysis of variance (ANOVA) was used to test the effects and interaction of the factors 'application protocol' and 'curing regime'. Intragroup comparison was done using paired t-tests. The level of significance was set at $\alpha = 0.05$.

## RESULTS

Light irradiance depending on the light-curing tip position is presented in Table 2. About 86% and 74% of the maximum irradiance observed in the control group was recorded in the 8mm_90° and 8mm_60° groups, respectively.

**Table 2.** Mean and standard deviation values of light irradiance in each group

| Group | 1mm_90° | 8mm_90° | 8mm_60° |
|---|---|---|---|
| Light irradiance ($mW/cm^2$) | 1195 ± 7 | 1017 ± 19 | 884 ± 11 |

Regarding DC, no significant difference ($p > 0.05$) was detected between control (89.1% ± 6.2%) and "8mm_90°" group (94.6% ± 1.2%). Those two groups showed significantly higher DC than "8mm_60°" group (74.9% ± 9.5%) ($p < 0.05$) (Figure 2).

Regarding μSBS, no significant differences in μSBS of the tested groups were detected initially ($p > 0.05$) (Figure 3). After aging, control TE group (8.4 ± 4.3 MPa) and "8mm_60°" TE group (9.2 ± 2.6 MPa) showed significantly lower μSBS than "8mm_60°" SE group (12.6 ± 4.2 MPa) ($p < 0.05$). Generally, all initial μSBS values were higher than those after aging irrespective of the application protocol. Nevertheless, greater differences between initial and long-term bond strength in all tested groups were associated with the TE protocol. These differences reached statistical significance in the control group and "8mm_60°" group ($p < 0.05$).

In all groups, predominantly adhesive fractures were detected (Figure 4). It is worth noting that the percentage of adhesive fractures increased and mixed fractures decreased

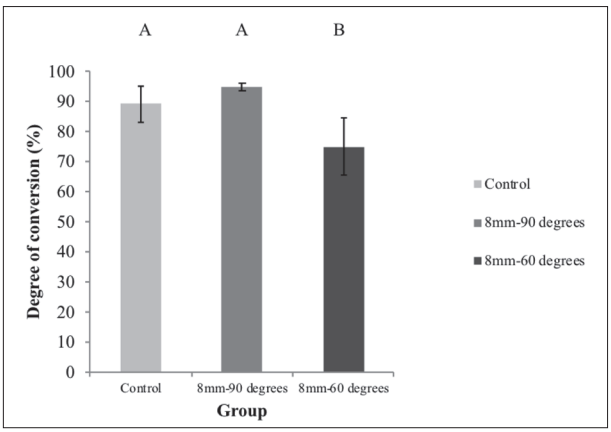


**Figure 2.** Mean and standard deviation values of the degree of conversion of Single Bond Univeral adhesive

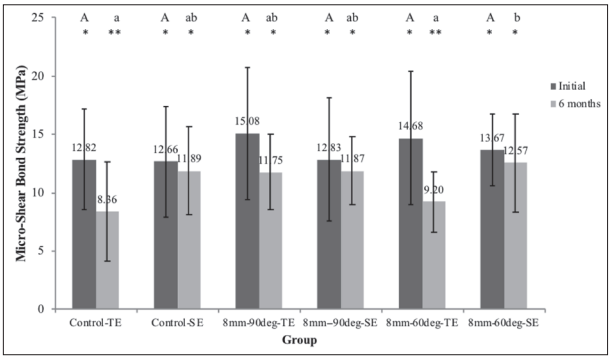


**Figure 3.** Mean and standard deviation values of the μSBS to dentin of Single Bond Universal adhesive; upper case letters-initial-intergroup comparison; lower case letters-6months-intergroup comparison; Asterisk-initial vs. six months-intragroup comparison ($p < 0.05$)

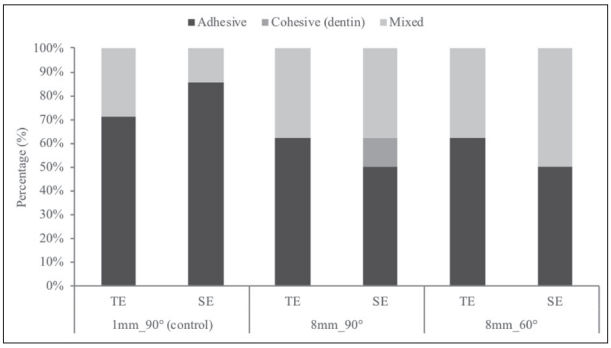


**Figure 4a.** Distribution of fracture types after 24 hours (baseline) measurements;
TE – total-etch; SE – self-etch

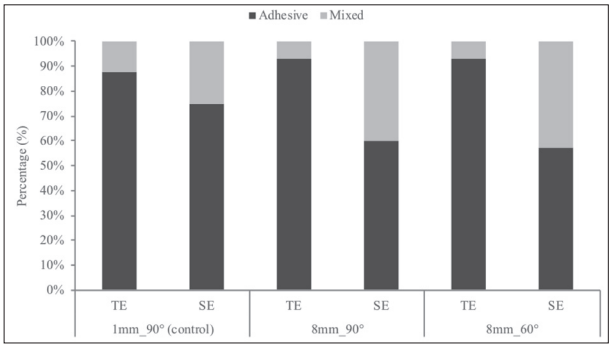


**Figure 4b.** Distribution of fracture types after six months measurements; TE – total-etch; SE – self-etch

in the aged specimens compared to those tested initially. This increase in adhesive fractures was more extensive in groups following the TE than SE protocol.

## DISCUSSION

All three tested hypotheses were rejected. At least one tested group showed significantly different DC ($H_1$). Although no differences were found between groups initially, long-term bond strength values of at least one group differed significantly compared to other curing conditions ($H_2$). Aging resulted in significantly lower bond strength values in two of three tested groups associated with the TE protocol compared to initial values ($H_3$).

SBU, a universal adhesive containing 10-MDP in its monomer mixture, was used in the present study due to its wide use in clinical practice. 10-MDP bonds chemically to the residual hydroxyapatite, thus creating secondary chemical bonding (nanolayering) in addition to micromechanical interlocking [12]. The influence of nanolayering between 10-MDP monomer and hydroxyapatite on adhesive-dentin bond strength and its longevity is questionable, especially in commercial adhesives, due to the scarcity of this phenomenon at the adhesive-dentin interface [13].

In everyday clinical practice, dentists may not achieve a minimal distance or perpendicular light tip position due to cavity depth, light-curing unit design or curing malpractice resulting in large variations of the energy of the light source delivered to the material and its photo-initiators [9]. With this in mind, two different distances (1 mm and 8 mm) and two different positions between the light tip and tooth surface (90° and 60°) were selected in order to examine if similar results can be achieved with less-than-ideal curing conditions. A high-intensity polywave light-curing unit was chosen as these units are recommended for photocurable materials containing both camphorquinone and alternative photoinitiators due to emission spectra compatible to absorption spectra of various photoinitiators [14].

The lowest light irradiance was detected in the 8mm_60° group, about 25% reduction compared to the control maximum irradiance. Irradiance was also reduced, by about 15% in the 8mm_90° group, but still remained above 1000 mW/cm$^2$. Irradiance indicates radiant power (flux) incident on a known surface area and is expressed as an average value over the surface area. The difference in irradiance between 8mm_60° and 8mm_90° groups clearly shows the adverse effect of light tip tilting which disrupts the beam profile against the irradiated surface. Light tip tilt causes inhomogeneous incident light beam reaching the irradiated surface resulting in spots with higher and lower radiant power.

Shear load was employed instead of tensile because of the complexity of experimental design i.e., dentin halves were used for different application protocols. The current test may be considered μSBS as it was stated by Van Meerbeek et al. [15] that "macro" bond tests are those with bonding areas exceeding 3 mm$^2$. Bonding area in the present study was 2.27 mm$^2$, larger than usual 1 mm$^2$, to mitigate a potential adverse effect of a thicker adhesive layer on very small composite build-up diameter resulting "in considerable bending and variable and non-uniform loading conditions" [15]. Although microtensile bond strength (μTBS) seems to be the preferred testing method [16], shear bond strength (SBS) or μSBS were also used in previous studies involving universal adhesives, mostly on enamel [17, 18] and less frequently on dentin [19]. Furthermore, Bracher and Özcan [20] reported no significant differences in adhesion to dentin between testing methods.

In line with irradiance recordings, the results of DC measurements indicated that 60° tilt of the light tip had a more detrimental effect on conversion than 8 mm tip-to-surface distance. The latter actually showed slightly higher mean DC values compared to the control group, albeit with no statistical significance. These findings may be related to lower light energy delivered to the material with the tilted light tip, whilst the effect of distance was dependent on the light-curing unit, as suggested in a previous study [8]. Somewhat lower DC in the control ("1mm_90°") than "8mm_90°" group could be associated with greater solvent evaporation due to higher temperatures generated in the adhesive when the light tip was held at 1 mm than 8 mm distance. It has been recently shown that a high-intensity LED light-curing unit exhibits about 10°C lower temperature at a distance of 4–5 mm compared to the temperature at the light tip [21]. Greater heat generated with the light tip held close to the adhesive surface may have facilitated solvent evaporation to the point where it adversely affected the final DC. A study on model adhesives showed that the absence of solvent actually reduced the final DC compared to a system with 10–20% solvent [22].

Furthermore, greater SD values in the control group compared to "8mm_90°" group indicated greater heterogeneity in monomer conversion, which could be associated with uneven solvent evaporation.

The present results showed that the protocol of application of a universal adhesive, with or without phosphoric acid etching, had no significant influence on the initial bond strength to dentin, no matter which polymerization regime was used. That is similar to other *in vitro* studies, whether a composite resin [23, 24] or ceramic materials [25] were bonded to dentin. Universal adhesives showed similar shear [25], microshear [26] or microtensile bond strength to dentin for both TE and SE protocols [27]. The present results indicate sufficient capacity of acidic monomers in SBU for partial dentin demineralization in the SE protocol as is achieved with phosphoric acid in the TE protocol.

Aging in water for six months significantly reduced bond strength to dentin of SBU following the TE protocol in the control group and "8mm_60°" group, whilst the values in the "8mm_90°" group were also lower but did not reach statistical significance. Similar reduction in bond strength to dentin of universal adhesives after six or 12 months of storage was reported in recent studies [25, 26, 28]. This may be explained by adhesive bond degradation within the hybrid layer. Acid etching of dentin and subsequent adhesive application create a zone in which collagen fibers are not incapsulated with resin because of shallower penetration of adhesive than previous acid etching [29]. This aggressive procedure exposes collagen fibers to degradation by matrix metalloproteinases activated in acidic environment such as the one caused by phosphoric acid etching and self-etching primers [30]. Furthermore, the present study indicated lower DC in the adhesive following curing at a distance (8 mm) and tilted angle of the light tip (60°) which could have also contributed to a hybrid layer more prone to degradation due to resin hydrolysis. The present results suggest that clinicians should accept with caution manufacturer's recommendation that SBU adhesive may be applied equally efficiently with or without phosphoric acid on dentin. Although initial bond strength may be comparable for both protocols, aging is associated with more pronounced bond deterioration when dentin is etched with phosphoric acid prior to adhesive application (TE protocol) compared to adhesive application to non-etched dentin (SE protocol). In line with other studies on the subject in which ideal curing conditions are applied, the present results indicate that the SE application protocol may be clinician's protocol of choice especially having in mind likely clinical deviation from the ideal curing conditions.

In line with changes in bond strength associated with TE protocol after storage is the notion that a greater increase in adhesive fractures and decrease in mixed fractures occurred in all TE groups compared to SE groups. It is generally known that adhesive fractures are associated with lower bond strengths than mixed fractures which occur at higher loads. Adhesive fracture was the predominant type for other universal adhesives as well as for SBS and μSBS tests to dentin [1, 20].

Relatively large standard deviations could be viewed as a limitation of the study. However, this commonly occurs in bond strength studies, especially testing adhesive-dentin bond strength [20, 25, 26]. Likely reasons for rather inhomogeneous results of bond strength testing could be variations in the sensitive dentin substrate as well as operator variability during specimen preparation.

## CONCLUSION

In general, angle tilt of 60° showed a greater adverse effect on the DC of a universal adhesive and its bond strength to dentin than tip-to-surface distance of 8 mm when cured with a high-intensity light-curing unit. TE and SE adhesive application protocols gave comparable results regarding initial bond strength to dentin of a universal adhesive. After six months of water storage, the SE application protocol was associated with greater adhesive resistance to degradation resulting in smaller decrease in bond strength compared to the TE protocol. A high-intensity LED unit allows some departure from an ideal curing position without jeopardizing adhesive bond strength to dentin as long as the incident light is perpendicular to the surface and the adhesive is applied without acid etching.

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**Conflict of interest:** None declared.

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## Утицај растојања и положаја светлосног извора на степен конверзије и јачину везе универзалног адхезива

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**САЖЕТАК**

**Увод/Циљ** Циљ рада је измерити степен конверзије (СК) универзалног адхезива и јачину везе за дентин тестом смицања (ЈВС) после 24 сата и шест месеци зависно од положаја светлосног извора и протокола апликације.

**Методе** Дентин у пределу екватора крунице 48 интактних хуманих умњака експониран је пресецањем дијамантском тестером, после чега је сваки зуб пресечен на пола. *Single Bond Universal* (*SBU*; *3M*) адхезив је апликован на једну половину по протоколу '*total-etch*' (ТЕ), док је на другој коришћен '*self-etch*' (СЕ) протокол. Према ангулацији и растојању светлосног извора (*bluephase G2*, *Ivoclar Vivadent* Шан, Лихтенштајн) од адхезива формиране су три групе: "1 *mm*_90°" (контрола); "8 *mm*_90°" и "8 *mm*_60°". Композитна надоградња (ø1,7 *mm*; *Filtek Z*250, *3M*) израђена је на сваком узорку. СК је мерен Рамановим спектрофотометром, ЈВС је мерена после 24 сата и шест месеци старења у дестилованој води на 37°*C*. Анализиране су врсте фрактура.

**Резултати** Групе "1 *mm*_90°" (89,1 ± 6,2%) и "8 *mm*_90°" (94,6 ± 1,2%) показале су статистички значајно виши степен конверзије ($p < 0,05$) у односу на групу "8 *mm*_60°" (74,9 ± 9,5%), док између њих није уочена статистички значајна разлика ($p > 0,05$). Након 24 сата није било статистички значајне разлике у ЈВС између група ($p > 0,05$). Групе "1 *mm*_90°" ТЕ (12,8 ± 4,3 *MPa*) и "8 *mm*_60°" ТЕ (14,7 ± 5,7 *MPa*) показале су значајно нижу ЈВС након старења него иницијално (8,4 ± 4,3*MPa* односно 9,2 ± 2,6 *MPa*) ($p < 0,05$). Адхезивне фрактуре су биле најзаступљеније.

**Закључак** Иницијално, оба протокола нагризања су показала сличну ЈВС. После старења СЕ протокол се показао ефикаснијим у одупирању деградацији хибридног слоја, што је резултовало већом ЈВС него ТЕ протокол. Ангулација врха лампе је имала више утицаја на степен конверзије и ЈВС него раздаљина од површине адхезива.

**Кључне речи:** адхезив; степен конверзије; јачина везе; полимеризација; удаљеност

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

# Prediction and prognosis of acute myocardial infarction in patients with previous coronary artery bypass grafting using neural network model

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**SUMMARY**

**Introduction/Objective** The aim of this study was to analyze the usefulness and accuracy of artificial neural networks in the prognosis of infarcted patients with previous myocardial surgical revascularization.
**Methods** The 13 predictor variables per patient were defined as a data set. All the patients were divided into two groups randomly: the training group and the test group, of 1090 patients each. The evaluation of the neural network performance was organized by using the original data, as well as the complementary test data, containing patient data not used for training the network. In generating the file of comparative results, the program compared the actual outcome for each patient with the predicted one.
**Results** All the results were compared with 2 × 2 contingency table constructed from sensitivity, specificity, accuracy, and positive–negative prediction. The network was able to predict the outcome with the accuracy of 96.2%, sensitivity of 78.4%, specificity of 100%, positive predictivity of 100%, and negative predictivity of 96%. There was not efficient prognosis of infarcted patients previously operated on using linear discriminant analysis (accuracy 68.3%, sensitivity 66.4%, and positive predictivity 30.2%).
**Conclusion** This study suggest that a neural network was better for almost all parameters in outcome prognosis of infarcted patients with previous myocardial surgical revascularization.
**Keywords:** artificial intelligence; prognosis; acute myocardial infarction; revascularization

## INTRODUCTION

Patients with previous coronary artery bypass grafting (CABG) represent a substantial percentage of the total population of patients with acute myocardial infarction (AMI) [1, 2, 3]. The timing of CABG for AMI remains a controversial topic [4–7]. The benefit persist in most patients during the first few years after surgery, but the progression of coronary artery disease in the ungrafted coronary arteries and the development of atherosclerosis in the vein grafts are important mechanisms by which angina and/or myocardial infarction can recur, detracting from the primary effect of revascularization [3, 7, 8, 9].

Prognosis of the future disease expression is an important part in the follow-up of patients with previous CABG. Prognosis can be expressed in symptom-free period, quality of life, but the most common type of prognosis is survival. Linear discriminate analysis, multilinear regression analysis, and logistic regression analysis have been used extensively for evaluation in medical prognosis. It is well known that outcome of patients with previous CABG is influenced by many abnormalities. In processing medical classification, linear methods cannot be appropriate because many medical patterns can be classified only by more complex nonlinear decision making. Each of these methods has inherent limitations when applied to a complex biological process, and a high degree of predictive accuracy has yet to be achieved.

Neural networks are a form of artificial intelligence and may obviate some of the problems associated with traditional statistical techniques, and they represent a major advance in predictive modeling [10–13]. Neural networks can find seamingly hidden features in input patterns, not visible by conventional statistical methods. There are some studies that have shown that connectionist models can be used for the prediction of outcome in patients with coronary heart disease, the onset of diabetes, and other medical problems [14–19].

The aim of this study is to analyze the usefulness and accuracy of an artificial neural network in AMI expression and its five-year prognosis in patients with previous CABG, using a complex mixture of predictor variables.

## METHODS

The baseline characteristics and clinical data were recorded in 2180 consecutive patients (13.8% women, mean age 63.4 ± 4 years) with previous

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CABG who were later determined to have a definite AMI. The patients with early perioperative AMI were excluded from the study. The diagnostic parameters and protocol were identical for all the patients. For inclusion, the patients were required to have at least two of the three following criteria: chest discomfort and/or symptoms suggestive of myocardial infarction lasting ≥ 20 minutes, electrocardiography changes suggestive of evolving myocardial infarction according to the Minnesota coding system, and typical elevation of at least one of three cardiac enzymes to at least twice the upper limit of the normal reference range. The follow-up period was 13.8 years (range 1.5–15). Information regarding survival (new coronary event – NCE) or circumstances of death during the follow-up period was obtained by control clinical examination or by letter or telephone interview.

### Predictor variables

The data set contained 13 predictor variables per patient (Table 1). All the patients were divided into two groups randomly, the training group and the test group, each containing 1090 patients. The original data included five continuous predictor variables [age, body mass index (BMI), C/T index, number of grafts, and ejection fraction] and eight binary predictors (sex, history of hypertension, smoking, hypercholesterolemia, diabetes mellitus, previous angina pectoris, previous AMI, and type of previous AMI) (Table 1). For use by the neural network, all the input variables were automatically standardized into the interval [0, 1] (Table 1).

**Table 1.** Clinical variables and input nodes

| Clinical variables (n = 13) | No. of input nodes (n = 14) |
|---|---|
| 1. Sex (male) (yes/no) | 1 |
| 2. Age (< 40 years, 40–65 years, 65 years) | 2 |
| 3. Hypertension (yes/no) | 1 |
| 4. Smoking (yes/no) | 1 |
| 5. Hypercholesterolemia (yes/no) | 1 |
| 6. High BMI (yes/no) | 1 |
| 7. Diabetes mellitus (yes/no) | 1 |
| 8. Previous angina (yes/no) | 1 |
| 9. Previous AMI (yes/no) | 1 |
| 10. Q-wave previous AMI (yes/no) | 1 |
| 11. No. of grafts > 1 (yes/no) | 1 |
| 12. Enlarged C/T index (yes/no) | 1 |
| 13. EF ≤ 40% (yes/no) | 1 |

BMI – body mass index; AMI – acute myocardial infarction; EF – ejection fraction

### Neural network architecture

The artificial neural network was created using commercial desktop PC software Neural Planner 4.5, running under Microsoft Windows (Microsoft Corporation, Redmond, WA, USA). Inputs into the neural network included 13 clinical variables, giving a total of 14 input nodes (variable age was standardized as binary variable) (Table 1). The neural network had 29 hidden nodes in one layer. The layer of 29 hidden nodes is a layer that connects only to the output nodes. There were six output nodes: AMI expression after CABG (yes/no), time interval from CABG to AMI (≤ 5 years after CABG, > 5 years after CABG), localization of AMI (anterior, inferior, lateral) after CABG, type of AMI (Q wave, non-Q wave AMI), and time of cardiac death expression after AMI (in the first, second, third, fourth, or fifth year of the follow-up period), if NCE was expressed in the follow-up period. All six outcomes after a five-year observation period were coded as follows: 0 = free of NCE; 1 = non-free of NCE. Patients were randomly enrolled into the test group or the training group. The learning method was error backpropagation and the transfer function was sigmoid. The method of presentation of examples during the training was randomized and the method of weight updating was continuous.

Artificial neural network performance was evaluated using the original data set for each network, as well as its complementary test data set, containing patient data not used for training the network. Generating a file of comparative results, program compared each patient's actual outcome with the predict one. At the end, the results from this file were analyzed and compared, on the basis of a 2 × 2 contingency table constructed from expected or obtained statistics (accuracy, sensitivity, specificity, and positive/negative predictivity), as well as on the basis of receiver operating characteristic (ROC) areas [20, 21].

Ethical approval for this study was obtained from the Cardiology Clinic Review Board, Clinical Center of Serbia (approval number 1973/2020)

## RESULTS

The study group included 2180 consecutive patients (301 female, 1879 male), age range being 26–82 years, mean age 63.4 ± 4 years, divided into training and testing sets. Clinical characteristics of patients in training and testing sets are shown in Table 2.

**Table 2.** Clinical characteristics of patients in training and testing sets

| Clinical variable | Training set (n = 1090) | Testing set (n = 1090) | p |
|---|---|---|---|
| Sex (male) | 952 (87.3%) | 861 (79%) | 0.0001 |
| Age < 40 years | 2 (0.2%) | 8 (0.7%) | 0.0572 |
| Age 40–65 years | 856 (78.5%) | 821 (75.4%) | 0.0966 |
| Age 65 years | 232 (21.3%) | 261 (23.9%) | 0.1098 |
| Hypertension | 370 (33.9%) | 382 (35%) | 0.5887 |
| Smoking | 162 (14.9%) | 240 (22%) | 0.0001 |
| Hypercholesterolemia | 510 (46.8%) | 571 (52.4%) | 0.0090 |
| High BMI | 229 (21%) | 207 (19%) | 0.2388 |
| Diabetes mellitus | 290 (26.6%) | 305 (28%) | 0.4708 |
| Previous angina | 468 (42.9%) | 414 (38%) | 0.0185 |
| Previous AMI | 500 (45.9%) | 371 (34%) | 0.0001 |
| Previous Q wave AMI | 457 (41.9%) | 391 (36%) | 0.0037 |
| No. of grafts > 1 | 796 (73%) | 730 (67%) | 0.0020 |
| Enlarged C/T index | 205 (18.8%) | 245 (22.5%) | 0.0343 |
| EF ≤ 40% | 135 (12.4%) | 168 (15.4%) | 0.0411 |

BMI – body mass index; AMI – acute myocardial infarction; EF – ejection fraction

**Table 3.** Prognosis of new coronary event expression: linear discriminant analysis

| Output variables | Data set | Accuracy (%) | Sensitivity (%) | Specificity (%) | Positive predictivity (%) | Negative predictivity (%) |
|---|---|---|---|---|---|---|
| 1. AMI expression after CABG | Full set | 68.3 | 66.4 | 68.8 | 30.2 | 90.4 |
| 2. Time-interval from CABG to AMI | Full set | 66.8 | 63.8 | 65.2 | 26.8 | 88.2 |
| 3. Localization of AMI | Full set | 62.8 | 60.2 | 60.4 | 22.4 | 84 |
| 4. Type of AMI | Full set | 65.6 | 64 | 65.2 | 24.6 | 86.6 |
| 5. Time of cardiac death expression after AMI | Full set | 60.8 | 60.4 | 62.2 | 20 | 80.2 |

AMI – acute myocardial infarction; CABG – coronary artery bypass grafting

**Table 4.** Prognosis of new coronary event expression: neural network analysis

| Output variables | Data set | Accuracy (%) | Sensitivity (%) | Specificity (%) | Positive predictivity (%) | Negative predictivity (%) |
|---|---|---|---|---|---|---|
| 1. AMI expression after CABG | Training set | 96.2 | 78.4 | 100 | 100 | 96 |
| | Test set | 86.2 | 56.2 | 96.3 | 68 | 92 |
| 2. Time-interval from CABG to AMI | Training set | 100 | 100 | 100 | 100 | 100 |
| | Test set | 88.6 | 72 | 90.2 | 60.5 | 94 |
| 3. Localization of AMI | Training set | 93.8 | 73.4 | 98.7 | 90.2 | 96 |
| | Test set | 84 | 34.2 | 90.6 | 48.6 | 88 |
| 4. Type of AMI | Training set | 96.8 | 86.0 | 100 | 100 | 96 |
| | Test set | 76.6 | 44.8 | 84.2 | 36.3 | 86 |
| 5. Time of cardiac death expression after AMI | Training set | 98 | 100 | 100 | 100 | 100 |
| | Test set | 82.8 | 46.4 | 84.2 | 34.2 | 48.2 |

AMI – acute myocardial infarction; CABG – coronary artery bypass grafting

## Statistical analysis

The results of univariate statistical analysis of study variables are shown in Table 2. Categorical variables significantly different between training and testing sets were sex, smoking, hypercholesterolemia, previous angina, previous AMI, previous Q wave AMI, number of grafts more than one, enlarged C/T index, and ejection fraction ≤ 40%.

The linear discriminate analysis (Table 3) was not efficient enough to distinguish NCE expression; the accuracy for AMI expression after CABG was only 68.3%, with the sensitivity of 66.4%; for the time-interval from CABG to AMI, the accuracy was 66.8%, with sensitivity of 63.8%; for the localization of AMI, the accuracy was 62.8%, with sensitivity of 60.2%; for the type of AMI, the accuracy was 65.5%, with sensitivity of 64%; and for the time of cardiac death expression after AMI, the accuracy was only 60.8%, with sensitivity of 60.4%. The weakest feature of the linear discriminate analysis solution was positive predictivity, which was only 30.2% for AMI expression after CABG; 26.8% for the time-interval from CABG to AMI; 22.4% for the localization of AMI; 24.6% for the type of AMI; and only 20% for the time of cardiac death expression after AMI. Negative predictivity was excellent for AMI expression after CABG (90.4%), for the time-interval from CABG to AMI (88.2%), for the localization of AMI (84%), for the type of AMI (86.6%), and for the time of cardiac death expression after AMI (80.2%). These results show that a statistical linear model is not able to perform class separation in multidimensional space and that a nonlinear approach is justified.

## Neural network analysis

The artificial neural network results are summarized in Table 4. The network was able to predict outcome with the accuracy of 96.2% for AMI expression after CABG for the training data set *vs.* 86.2% for the test data set, with a sensitivity of 78.4% *vs.* 56.2%, specificity 100% *vs.* 96.3%, positive predictivity 100% *vs.* 68%, and negative predictivity 96% *vs.* 92%. For the time-interval from CABG to AMI, the network was able to predict the outcome with the accuracy of 100% for the training data set *vs.* 88.6% for the test data set, with a sensitivity of 100% *vs.* 72%, specificity 100% *vs.* 90.2%, positive predictivity 100% *vs.* 60.5%, and negative predictivity 100% *vs.* 94%. For the localization of AMI, the network was able to predict the outcome with the accuracy of 93.8% for the training data set *vs.* 84% for the test data set, with a sensitivity of 73.4% *vs.* 34.2%, specificity 98.7% *vs.* 90.6%, positive predictivity 90.2% *vs.* 48.6%, and negative predictivity 96% *vs.* 88%. For the type of AMI, the network was able to predict the outcome with the accuracy of 96.8% for the training data set *vs.* 76.6% for the test data set, with a sensitivity of 86% *vs.* 44.8%, specificity 100% *vs.* 84.2%, positive predictivity 100% *vs.* 36.3%, and negative predictivity 96% *vs.* 86%. For the time of cardiac death expression after AMI, the network was able to predict the outcome with the accuracy of 98% for the training data set *vs.* 82.8% for the test data set, with a sensitivity of 100% *vs.* 46.4%, specificity 100% *vs.* 84.2%, positive predictivity 100% *vs.* 34.2%, and negative predictivity 100% *vs.* 48.2%. The least reliable variable for all outcome variables was always sensitivity, denoting a relative inability to correctly predict the number of patients with different NCE expressions.

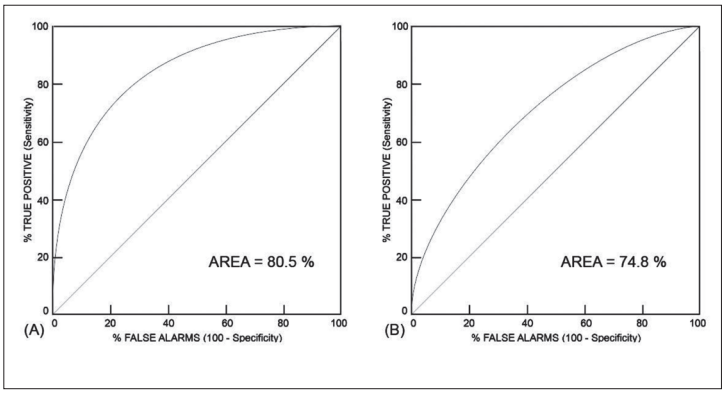


**Figure 1.** Receiver operating characteristic curve for committee classifier; (A) area (C-index) for neural network analysis 80.5%; (B) area (C-index) for linear discriminant analysis 74.8%

The ROC areas (C-index) for both prediction models after training (using training data) and final testing (using testing data) are provided in Figure 1. ROC areas (C-index) are all about 74.8% for logistic regression and vary 1.4 percentage points (range 73.4–76.2%). For artificial neural network model, ROC areas (C-index) are all about 80.5% and vary 1.5 percentage points (range 79–82%).

## DISCUSSION

The performance of artificial neural networks in the prognosis of acute myocardial infarction in patients with previous CABG can be rated as very good if we consider that a large number of input variables is associated with outcome and input variables present a large variability among patients of the training and the testing set. The number of training examples may be too low in relation to problem dimensionally, but large enough for very good accuracy and specificity of artificial neural network analysis. In particular, the accuracy will increase with the increase in the number of training sets and the number of hidden layers. A nearly optimal combination of high sensitivity and specificity was achieved with the network model for time interval from CABG to AMI variable [22, 23, 24]. The accuracy value of 88.6% achieved with the test data demonstrates that this neural network was able to give a decision surface with acceptable prognostic power for the prediction of the time interval for AMI expression after CABG. In analyzing the performance of the neural network and linear discriminate analysis in outcome prognosis of AMI in patients with previous CABG, it is clear that neural network was better for almost all parameters in outcome prognosis for all analyzed variables.

Some of the previous studies have shown that ACS patients with prior CABG have an increased risk of early mortality. In the Global Registry of Acute Coronary Events (GRACE), prior CABG was associated with increased in-hospital mortality [26] and was a univariable predictor of six-month mortality [25, 26]. The same results were presented with the neural network prediction model in our study.

Prior CABG was an independent predictor of cardiovascular death, AMI, and heart failure. The GRACE registry findings proved the findings of an earlier Canadian cohort study of 410 AMI patients with or without prior CABG. In these patients, a history of CABG was associated with a higher crude rate of ischemic cardiac events at five years [27, 28]. Our prognostic model has shown very similar results in the five-year interval prediction. Some other previous studies have demonstrated that patients with prior CABG have more extensive native vessel coronary artery disease [28]. The higher expression of previous AMI and left ventricle dysfunction in patients with prior CABG may explain the reduced capacity of these patients to withstand recurrent myocardial ischemia or infarction and their increased risk of cardiac morbidity and mortality [28].

As in our study, the VALIANT (Valsartan in Acute Myocardial Infarction) trial also showed that a history of prior CABG was a univariable but not a multivariable predictor of all-cause mortality [29]. This result is consistent with similar results from the GRACE registry [25].

It's well known that a major problem among artificial neural network is overtraining [30, 31]. Because of that, when an artificial neural network is overtrained, it models the test group so well that it becomes poor at predicting outcomes when new cases are presented. This problem in prediction and prognosis of AMI in patients with previous CABG was probably resolved with a relatively high number of analyzed patients and long follow-up period. Further prospective validation of this neural network approach, with a more prolonged follow-up period, may be useful.

## CONCLUSION

In this clinical situation, artificial intelligence appears to be superior to linear methods for prediction and prognosis of AMI in patients with previous CABG.

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# Примена неуронских мрежа у предвиђању појаве акутног инфаркта миокарда код болесника са претходном хируршком реваскуларизацијом миокарда

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**САЖЕТАК**

**Увод/ Циљ** Циљ ове студије је да испита сензитивност и специфичност неуронских мрежа у предвиђању појаве акутног инфаркта миокарда код болесника са претходном хируршком реваскуларизацијом миокарда.

**Методе** Скуп података се састоји од 13 предиктивних параметара по болеснику. Посматрани болесници подељени су у две групе: група болесника за обучавање неуронске мреже (1090 болесника) и група болесника за тестирање неуронске мреже (1090 болесника). Неуронска мрежа је обучавана употребом оригиналних података за сваки појединачни параметар, док је њена специфичност и сензитивност тестирана новим сетом оригиналних података болесника који нису коришћени за обучавање неуронске мреже.

**Резултати** На крају испитивања, резултати обучавања неуронске мреже контролисани су мерењем прецизности, сензитивности, специфичности и позитивне/негативне предиктивности. Неуронска мрежа приказала је статистички значајне вредности у прогнози ових болесника са тачношћу од 96,2%, сензитивношћу од 78,4%, специфичношћу 100%, позитивним предвиђањем 100% и негативним предвиђањем 96%. Линеарна дискриминантна анализа, као статистички модел предвиђања и прогнозе појаве акутног инфаркта миокарда код оперисаних болесника, показала се као лошији предиктивни модел у поређењу са неуронском мрежом (тачност 68,3%, сензитивност 66,4%, позитивно предвиђање 30,2%).

**Закључак** Анализирањем употребе неуронске мреже у предвиђању појаве и прогнозе акутног инфаркта миокарда код болесника са претходном хируршком реваскуларизацијом миокарда показало се јасно да је неуронска мрежа бољи предиктивни модел у односу на све статистичке параметре који се користе за анализирање предиктивних параметара.

**Кључне речи:** вештачка интелигенција; прогноза, акутни инфаркт миокарда; реваскуларизација

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

# A complete versus inducible ischemia-guided revascularization after a culprit-only primary percutaneous coronary intervention in multivessel coronary artery disease – a pilot study

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**SUMMARY**

**Introduction/Objective** Revascularization in multivessel coronary artery disease (MVD) in patients with ST elevation myocardial infarction (STEMI) is a matter of debate. We sought to compare outcomes between revascularization strategies based on angiographic lesion severity or inducible ischemia.

**Methods** In prospective study, first ever STEMI patients with MVD, defined as > 70% stenosis in non-culprit vessel, treated with culprit only primary PCI were randomized to: A. Complete revascularization of all non-culprit significant lesions during initial hospitalization; B. Complete revascularization after 30 days, or C. Revascularization based on non-invasive testing for inducible ischemia. The study explored occurrence of major adverse cardio-cerebral events (MACCE) (cardiac death, repeated MI, cerebrovascular event).

**Results** The study enrolled 120 patients with door to balloon time within appropriate limits (A 51 ± 26 *vs*. B 47 ± 33 *vs*. C 44 ± 29 min, p = 0.604) The patients in group A underwent complete revascularization at 6 [4–7] days after primary PCI, while in the group B it was 35 [32–39] days. In group C, 16/43 (37.2%) patients underwent PCI at 82 [66–147] days after infarction (p < 0.001). The patients were followed for 2.7 ± 0.8 years. The events occurred less frequently in patients that underwent planned complete revascularization compared to those who underwent ischemia testing (7.8 *vs*. 20.9%, p = 0.040). Kaplan–Meier analysis favored complete delayed revascularization (MACCE A 8.8 *vs*. B 6.9 *vs*. C 20.9%, log rank p = 0.041).

**Conclusions** Planned, angiography guided, complete revascularization after initial event may be favorable strategy compared to single stress test for MVD in STEMI.

**Keywords:** coronary artery disease; myocardial infarction; stress echocardiography

## INTRODUCTION

The multivessel coronary artery disease (MVD) is a common finding in patients presenting with ST elevation myocardial infarction (STEMI). It is estimated that around 40–50% of patients with STEMI present with MVD [1]. Previously, culprit only primary percutaneous coronary intervention (PCI) was indicated, but this was challenged by several studies suggesting benefits of immediate, complete revascularization at initial PCI or during initial hospitalization [2, 3, 4]. Although these studies enrolled relatively small number of participants with heterogeneous definition of MVD and composite end points, their results have caused important concern regarding the need for complete revascularization in patients with STEMI and MVD. Based on their results, guidelines for treatment of STEMI have been updated [5]. An observational, retrospective study has demonstrated benefit of staged PCI within 60 days of index intervention [6]. The strategy of dobutamine stress echocardiography testing after myocardial infarction seems feasible and safe and can predict serious adverse events during short term follow up [7].

We sought to investigate the appropriateness of staged, complete revascularization during hospitalization or after 30 days from initial PCI based on angiographic lesion severity compared to intervention based on outpatient non-invasive ischemia testing, aiming to reduce major adverse cardio-cerebral events (MACCE).

## METHODS

This study was prospective, randomized, single center, open label study in patients with STEMI with MVD, initially treated with culprit only primary PCI. After successful culprit only primary PCI, patients were randomly assigned to one of three treatment arms: staged, complete revascularization of all non-culprit significant lesions in a single session during initial hospitalization; staged, complete revascularization of all non-culprit significant lesions in a single session after 30 days from initial hospitalization for STEMI and revascularization or deferral of revascularization of non-culprit coronary artery lesions based on ischemia testing using dobutamine stress echocardiography. The study was

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approved by the institutional committee on ethics and was done in accordance with the Helsinki declaration (Clinical trials identifier: NCT 02756000).

### Patient population

All patients admitted with clinical and electrocardiographic signs of first ever STEMI (chest pain lasting less than 12 hours with persistent ST elevation of ≥ 1 mm in two contiguous leads on ECG recording) and MVD on initial coronary angiogram, defined as visually assessed stenosis of more than 70% of any of the non-culprit vessels, were treated with primary PCI of infarct related artery (IRA) only. Within 24 hours after completion of primary PCI, after obtaining a written informed consent, they were randomly assigned 1:1:1 to one of the treatment arms. The hemodynamically unstable patients, defined as presence of Killip class IV, need for mechanical circulatory support and/or ventilation prior, during and after primary PCI, presence of significant valvular disease or decision that patient needs to be treated with coronary artery bypass grafting (CABG) and/or valvular replacement or reconstruction surgery after initial culprit only PCI were not considered for the study. Patient were excluded from the study if myocardial infarction was caused by stent thrombosis or there was a chronic total occlusion of any of the coronary arteries on initial angiogram. Patients previously treated by CABG or having estimated life expectancy less than one year were also excluded from the study. Patients unsuitable for dobutamine stress echocardiography because of poor acoustic windows were also excluded from the study.

### Randomization

Patients were randomized in a 1:1:1 fashion to one of the treatment arms according to computer generated algorithm (GraphPad Software, Inc., San Diego, California, US) after completion of primary PCI and signed informed consent form. Crossover between treatment arms was allowed only in case of persistent chest pain or patient's hemodynamic instability that requires immediate coronary angiography and/or intervention that was further acknowledged as study endpoint. Vascular access, PCI technique, use of guiding catheters, coronary guidewires, thrombus aspiration, predilatation and stent implantation were used according to operators' preference, both at primary PCI and at repeated intervention.

### Medical treatment

After establishing diagnosis of STEMI, patients were pretreated with loading dose of aspirin (300 mg) and ticagrelor (180 mg) or clopidogrel (600 mg), while heparin (80–100 IU/ kg iv.), was given before insertion of coronary guidewire. After PCI aspirin, 100 mg per day, was given indefinitely with ticagrelor 90mg twice a day or clopidogrel, 75 mg per day. Recommended duration of dual antiplatelet therapy was 12 months. Patients were treated with beta blocking agents, ACE inhibitors and statins according to the current guidelines for STEMI [5].

Patients were seen in an office visit one month after final PCI or Dobutamine stress echocardiography – vital and clinical status along with prescribed medications were assessed, ECG and arterial blood pressure measurement done. Angina status assessment was done according to the Canadian Cardiovascular Society (CCS) Classification of angina or Braunwald angina classification.

Vital and clinical status, presence of angina, medications, hospitalization for any reason, myocardial infarction, repeated PCI or CABG were assessed at one year after initial procedure or at the end of follow up (telephone interview or office visit).

### Dobutamine stress echocardiography

Dobutamine stress echocardiography (DSE) was performed in all stable patients assigned to this study arm at least 30 days after the coronary event according to the guidelines. In patients with suboptimal parasternal echo windows, the test was considered valid for interpretation if all apical views are obtained and suitable for analysis. The test was considered positive for inducible ischemia in the presence of new or worsening wall motion abnormalities in two or more adjacent segments [8].

### Definitions

A culprit artery was defined as an artery with an identifiable thrombus and/or significant lesion on angiogram corresponding to ischemic ECG changes. Significant lesion was defined as coronary artery stenosis with narrowing of the lumen of more than 70% assessed by quantitative coronary angiography (QCA) software (Leonardo multimodality workstation, Siemens, Erlangen, Germany).

A repeated revascularization was considered clinically indicated if angiography during follow-up showed a diameter stenosis greater than or equal to 50 percent at any point in the coronary artery previously treated and if one of the following occurred: 1) a positive history of recurrent angina pectoris, presumably related to the target vessel; 2) objective signs of ischemia at rest (ECG changes) or during exercise test (or equivalent), presumably related to the target vessel; 3) abnormal results of any functional diagnostic test (e.g. stress echocardiography, fractional flow reserve); 4) a revascularization with a diameter stenosis greater than 70% even in the absence of the above-mentioned ischemic signs or symptoms.

Death was regarded as cardiac in origin unless obvious non-cardiac causes could be identified. Any death during the index hospitalization for STEMI was regarded as cardiac death. Sudden death was defined as unexplained death in previously stable patients. Myocardial infarction (MI) is defined according to the Fourth universal definition of myocardial infarction [9]. Procedure-related MI is regarded as present with creatinine kinase (CK) MB fraction ≥ 3 times upper limit of normal after PCI procedure or total CK ≥ 3 times upper limit of normal in the absence of CKMB measurement. Bleeding is defined according to the Bleeding Associated Research Consortium criteria [10].

Cerebrovascular accident was defined as sudden onset of vertigo, numbness, aphasia, or dysarthria due to vascular lesions of the brain such as hemorrhage, embolism, thrombosis, or rupturing aneurysm that persists > 24 hours.

### Statistical analysis

Categorical variables were presented as numbers and percentages and were compared using chi square test. Continuous variables were expressed as mean ± standard deviation (SD) or medians with interquartile ranges. Continuous variables were compared using the one-way ANOVA or Kruskal Wallis' test based on their distributions. Clinical outcomes were analyzed according to the intention-to-treat principle. Each endpoint was assessed by the Kaplan–Meier method and compared by log-rank test. Statistical analysis was done using IBM SPSS Statistics 20.0 software (IBM Corp. Armonk, New York, US). A p value of $< 0.05$ was regarded as statistically significant.

## RESULTS

The study included 120 patients in Clinical Hospital Centre Zemun, high volume university PCI center from June 2016 to January 2019. The study was prematurely stopped due to slow enrollment and after interim analysis revealed a potential futility in the ischemia testing arm of the study. The 182 patients were evaluated for the study after meeting the inclusion criteria. Due to poor acoustic window 21 patients (11.5%) could not be randomized to stress echocardiography, 14 patients (7.7%) have not signed an informed consent form to participate in the study and surgical revascularization was recommended in 27 patients (14.8%).

The patients included in the study had high incidence of hypertension, dyslipidemia and smoking. There was borderline difference in body mass index (BMI) between the groups (Table 1). Three vessel disease was seen in over 40% patients in the group of patients randomized to complete revascularization during initial admission, which was higher incidence than in other groups, but the difference was insignificant. Thrombus aspiration was used as a first intervention in only 1/4 to 1/3 of patients while the balloon angioplasty was used in more than half of patients in all groups. The interventions were deemed successful in almost all patients with restoration of TIMI III flow (Table 2).

**Table 1.** Clinical characteristics of the patients in the study groups

| Variable | In hospital (n = 34) | After 30 days (n = 43) | Ischemia testing (n = 43) | p |
|---|---|---|---|---|
| Age (years) | 61 ± 8 | 60 ± 11 | 60 ± 9 | 0.877 |
| Male gender (%) | 70.6 | 74.4 | 65.1 | 0.640 |
| Heredity (%) | 41.1 | 44.2 | 44.2 | 0.956 |
| Smoking (%) | 44.1 | 53.5 | 65.1 | 0.179 |
| Hypertension (%) | 79.4 | 72.1 | 83.7 | 0.418 |
| Dyslipidemia (%) | 52.9 | 55.8 | 69.7 | 0.255 |
| Diabetes mellitus (%) | 23.5 | 32.5 | 21 | 0.439 |
| PAD (%) | 0.0 | 4.6 | 2.3 | 0.429 |
| CKD (%) | 2.9 | 2.3 | 4.6 | 0.826 |
| COPD (%) | 8.8 | 7 | 7 | 0.942 |
| BMI (kg/m$^2$) | 29 ± 4 | 27 ± 4 | 27 ± 4 | 0.047 |
| LVEF (%) | 43 ± 7 | 43 ± 8 | 45 ± 7 | 0.963 |

BMI – body mass index; CKD – chronic kidney disease; COPD – chronic obstructive pulmonary disease; LVEF – left ventricular ejection fraction; PAD – peripheral arterial disease

**Table 2.** Primary PCI characteristics in the study groups

| Variable | In hospital (n = 34) | After 30 days (n = 43) | Ischemia testing (n = 43) | p |
|---|---|---|---|---|
| Prehospital time (min) | 295 ± 199 | 225 ± 196 | 241 ± 220 | 0.531 |
| D2B time (min) | 51 ± 26 | 47 ± 33 | 44 ± 29 | 0.604 |
| Radial access (%) | 55.9 | 51.2 | 46.5 | 0.715 |
| Triple vessel disease (%) | 41.2 | 23.2 | 23.2 | 0.146 |
| LAD culprit (%) | 41.2 | 37.2 | 30.3 | 0.593 |
| Cx culprit (%) | 17.6 | 18.6 | 13.9 | 0.832 |
| RCA culprit (%) | 41.2 | 44.2 | 55.8 | 0.382 |
| Thrombus aspiration (%) | 35.3 | 25.6 | 25.6 | 0.568 |
| Predilatation (%) | 50 | 65.1 | 60.4 | 0.398 |
| GP IIbIIIa inhibitor (%) | 20.6 | 18.6 | 20.9 | 0.959 |
| Total contrast load (ml) | 157 ± 71 | 156 ± 63 | 153 ± 42 | 0.963 |
| TIMI III flow (%) | 100 | 97.7 | 97.7 | 0.669 |

Cx – circumflex artery; D2B – door to balloon time; GP – glycoprotein; LAD – left anterior descending; RCA – right coronary artery; TIMI – thrombolysis in myocardial infarction

**Table 3.** Incidence of MACCE events in the study groups

| Variable | In hospital (n = 34) | After 30 days (n = 43) | Ischemia testing (n = 43) |
|---|---|---|---|
| Death, n (%) | 0 (0) | 0 (0) | 2 (4.6) |
| Repeated MI, n (%) | 1 (2.9) | 1 (2.3) | 2 (4.6) |
| Repeated PCI, n (%) | 2 (5.9) | 2 (4.6) | 4 (9.2) |
| CVI, n (%) | 0 (0) | 0 (0) | 1 (2.3) |
| MACCE, n (%) | 3 (8.8) | 3 (6.9) | 9 (20.9) |

CVI – cerebrovascular insult; MI – myocardial infarction; PCI – percutaneous coronary intervention; MACCE – major adverse cardio-cerebral event

The patients randomized to complete revascularization during initial hospitalization underwent the procedure at a median of 6 [4–7] days after primary PCI, while the median time to complete PCI was 35 [32–39] days in a group randomized to staged intervention. In ischemia testing group patients underwent dobutamine stress echocardiography at 36 [31–46] days after initial admission for STEMI. Of these patients 16/43 (37.2%) were treated with PCI based on positive test results at the median of 82 [66–147] days after infarction ($p < 0.001$). All patients with positive stress test result were treated with PCI of the non-culprit vessels, according to the study protocol.

The patients were followed for median of 1046 [734–1220] days and the adverse events occurred infrequently in all groups. The incidence of stable angina class CCS II and higher was similar in all study groups (group I 1/34, group II 3/43, group III 3/43, $p = 0.137$). Kaplan–Meier freedom from angina curves demonstrated relatively late onset of angina, similar in all study groups (log rank $p = 0.309$) (Figure 1). The MACCE events occurred more frequently in patients assigned to ischemia testing after initial culprit only primary PCI (table 3). Adverse cardiovascular events

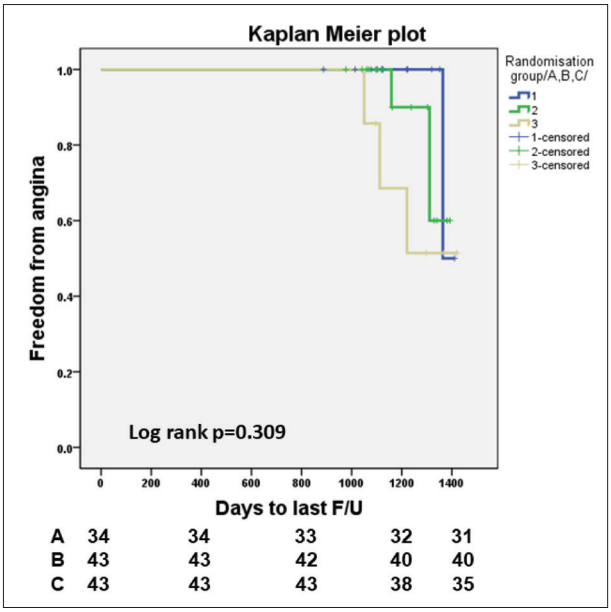


**Figure 1.** Kaplan–Meier plot representing freedom from angina CCS II during follow up in days after initial event (study groups A – immediate complete revascularization; B – delayed complete revascularization; C – revascularization based on stress-echocardiography)

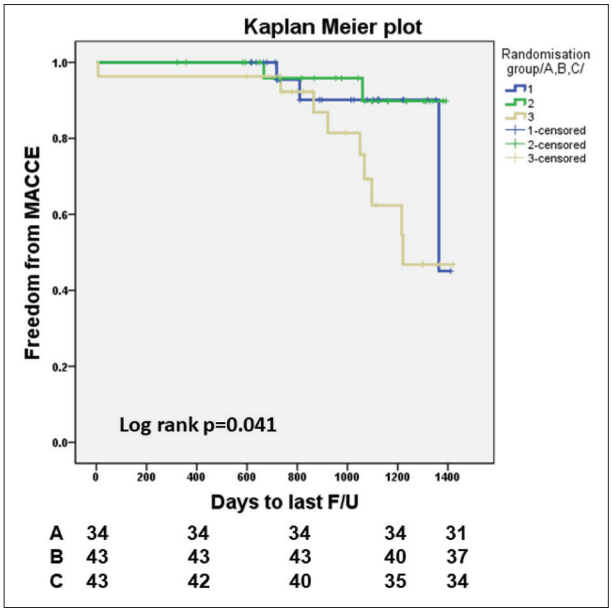


**Figure 2.** Kaplan–Meier plot representing freedom from major adverse cardio-cerebral events (MACCE) during follow up in days (study groups A – immediate complete revascularization; B – delayed complete revascularization; C – revascularization based on stress-echocardiography result)

occurred less frequently in patients that underwent planned revascularization either at the initial hospitalization or after 30 days from initial primary PCI, compared to those who underwent ischemia testing (7.8 *vs.* 20.9%, p = 0.040). Kaplan–Meier survival analysis favored complete delayed revascularization (log rank p = 0.041) (Figure 2).

## DISCUSSION

This pilot randomized study, done in a single, high volume PCI university center, has demonstrated that strategy based on single dobutamine stress echocardiography test to detect ischemia in non-culprit vessels territory after culprit only primary PCI was associated with increased incidence of adverse cardiovascular events compared to complete staged, angiography guided revascularization after primary PCI for first ever STEMI in patients with MVD during long term follow up.

The decision when to do staged PCI after primary PCI can be affected by many factors. A prothrombotic and inflammatory milieu related to possible stent thrombosis, large myocardial territory at risk with multivessel PCI in STEMI along with the procedural risks (increased radiation and contrast load) can all lead to decreased benefit of immediate complete revascularization [11, 12]. In addition, an estimate of severity of the non-culprit lesions could be jeopardized by spasm of the entire coronary tree leading to unnecessary PCI procedures [13]. Older age, overt heart failure, decreased renal function and additional medical conditions requiring attention can be the reasons to avoid complete revascularization during initial hospitalization that could translate to increased incidence of adverse events [14, 15]. The staged PCI for STEMI can be beneficial compared to culprit only PCI as Cui and al. has showed in retrospective analysis of more than 1000 patients. The staged procedure was done within 30 days of primary PCI and after propensity matching of patients, had lower incidence of MACCE. However, the same study failed to demonstrate the benefit of this strategy for diabetic patients [16]. The registry by Hannan et al. [6] has demonstrated a benefit in terms of reduced mortality at 12 months for staged PCI within 60 days after initial primary PCI compared to culprit only PCI within the initial hospitalization. The study also demonstrated increased in-hospital mortality for multivessel PCI at the initial procedure [6]. Recently published large trial that included more than 4000 patients has demonstrated consistent benefit of complete revascularization in MVD patients with STEMI compared to culprit only primary PCI during long term follow up. The complete revascularization was done either during initial hospitalization or within 45 days from initial event [17]. A large Korean registry data also supported the strategy of delayed complete revascularization during initial hospitalization in MVD [18]. The findings in our study support complete revascularization at initial hospitalization or after 30 days based on lesion severity assessed at initial coronary angiogram, showing reduced incidence of MACCE.

The strategy based on non-invasive ischemia testing, in our study, was associated with increased incidence of MACCE. Kaplan–Meier curves separate late in the follow up, when there was higher incidence of events in the ischemia testing group. The reason for this could be the progression of atherosclerotic disease that was not detected as significant ischemia burden on the early dobutamine stress echocardiographic study. Atherosclerosis is a progressive disease and it has been demonstrated that patients that suffered an event would have high incidence of repeated events despite the revascularization and medical treatment [19]. Also, stress testing after revascularization usually yields very few

repeated revascularization procedures irrespective of the test results [20]. However, positive test results in terms of inducible ischemia usually are related to increased incidence of adverse events. In the meta-analysis by Harb et al. [21], that analyzed the studies where stress echocardiography was used to detect ischemia after revascularization, it has been demonstrated that inducible ischemia was associated with increased incidence of adverse cardiovascular events. This study pointed out that older age and time interval between initial revascularization and the positive stress test were predictors of worse outcomes, meaning that longer the time interval between the revascularization and the test, more adverse events occurred. The authors stressed that the impact of time interval was caused by progression of coronary artery disease and more events occurring during longer follow up period [21]. The study by Sicari et al. [7], has pointed out that early stress test after MI could be helpful in predicting adverse events during short term follow up, up to one year, but thereafter progression of atherosclerotic disease in a non-culprit vessels may provoke new ischemic events, as shown in large Swedish registry of patients with MI undergoing culprit only primary PCI [8, 22]. Patients in our study underwent stress echocardiography early after initial event and additional tests were not planned, so the higher incidence of events was probably due to further progression of atherosclerosis and/or restenosis of previously treated lesions. Late onset of angina as a sign of progressing atherosclerosis, long after the test was done, further supports the idea that the higher rate of MACCE events in the ischemia-testing group was due to progression of atherosclerotic lesions that, albeit present, did not provoke myocardial ischemia at the time of the test. Also, stress tests in asymptomatic patients yield few repeated revascularization procedures, a fact that had to be accounted for [23]. On the other hand, in other two groups we might have treated with PCI some intermediate lesions that were not physiologically significant or causing ischemia, despite perceived as significant on coronary angiogram. Due to small cohort of patients the benefits of such treatment probably exceeded the potential "costs" in terms of complications and restenosis [24, 25].

How to treat MVD patient after primary PCI in STEMI remains an open question. Complete revascularization based on angiographic estimate of stenosis severity has proven benefits but comes with a risk of unnecessary interventions producing more restenosis and stent thrombosis. A strategy of repeated noninvasive ischemia testing after initial revascularization may discern between the patients deemed for revascularization and the ones who should be treated medically avoiding the risks of inappropriate invasive procedures. The timing and the interval between repeated test need to be investigated further.

### Study limitation

The study included small number of selected patients (first ever myocardial infarction in native coronary artery, no CTO, preserved LVEF, good echocardiographic windows) with MVD and STEMI, therefore its conclusions may not be applicable to general population. In the study we used dobutamine stress echocardiography to assess ischemia. This test with its inherent limitations in detecting ischemia may influence decisions to perform further revascularization.

## CONCLUSION

The ischemia-guided strategy based on early single dobutamine stress echocardiography test may be inferior to complete revascularization based on angiographic estimate of stenosis severity in multivessel disease patient within thirty days after primary PCI for first ever STEMI. The repeated testing may improve detection of atherosclerosis progression and allow institution of appropriate treatment.

**Conflict of interests:** None declared.

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# Комплетна у односу на исхемијом вођену реваскуларизацију после примарне перкутане коронарне интервенције артерије одговорне за инфаркт у вишесудовној коронарној болести – пилот студија

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**САЖЕТАК**

**Увод/Циљ** Реваскуларизација у вишесудовној коронарној болести код болесника са инфарктом миокарда sa *ST* елевацијом (*STEMI*) представља изазов. Упоредили смо клиничке исходе између различитих стратегија реваскуларизације руковођене ангиографски процењеним степеном сужења или на основу теста оптерећења срца.

**Методе** У проспективном истраживању, болесници са првим *STEMI*-јем и вишесудовном коронарном болешћу (више од 70% сужења артерије која није одговорна за инфаркт) рандомизовани су после примарне перкутане коронарне интервенције на болеснике са: А. комплетном перкутаном реваскуларизацијом свих лезија током иницијалне хоспитализације; Б. комплетном реваскуларизацијом после 30 дана; В. реваскуларизацијом на основу провокабилне исхемије. Студија је бележила настанак нежељених кардиоваскуларних догађаја – срчане смрти, поновљеног инфаркта миокарда, цереброваскуларног догађаја.

**Резултати** Укључено је 120 болесника код којих је време до реперфузије било у границама препоручених вредности (А 51 ± 26 *vs*. Б 47 ± 33 *vs*. В 44 ± 29 минута, $p = 0,604$). Болесници у групи А су подвргнути комплетној реваскуларизацији 6 (4–7) дана после примарне перкутане коронарне интервенције, док су у групи Б реваскуларизовани после 35 (32–39) дана. У групи В, 16/43 (37,2%) болесника је подвргнуто перкутаној коронарној интервенцији после 82 [66–147] дана од инфаркта ($p < 0,001$). Болесници су праћени током 2,7 ± 0,8 година. Нежељених догађаја је било мање код болесника који су подвргнути комплетној реваскуларизацији у односу на тестиране на исхемију (7,8 *vs*. 20,9%, $p = 0,040$). Каплан–Мејеровом анализом показана је предност комплетне реваскуларизације у току иницијалне хоспитализације (нежељени кардиоваскуларни догађаји А 8,8 *vs*. Б 6,9 *vs*. В 20,9%, *log rank* $p = 0,041$).

**Закључак** Планирана, ангиографијом вођена комплетна реваскуларизација, после примарне перкутане коронарне интервенције, може бити боља стратегија у односу на појединачно тестирање на исхемију код вишесудовне коронарне болести.

**Кључне речи:** коронарна болест; инфаркт миокарда; тест оптерећења срца

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

# Electrocardiographic predictors of five-year mortality in chronic obstructive pulmonary disease patients

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**SUMMARY**

**Introduction/Objective** Cardiovascular disease is one of the most common comorbidities among subjects with chronic obstructive pulmonary disease (COPD). The aim of this study is to evaluate electrocardiogram (ECG) parameters and mortality predictors in COPD patients.

**Methods** A total of 835 consecutive patients were included. The patients were classified to suffer from COPD if the forced expiratory volume in the first second (FEV1) and forced vital capacity (FVC) was < 70% in three consecutive postbronchodilator measurements. The following ECG changes were observed: axis, P wave, low ORS complex, transitional zone, left bundle branch block (LBBB), right bundle branch block (RBBB), incomplete RBBB, S1S2S3 configuration, negative T in V1–V3. The patients were followed up for mortality over a five-year period.

**Results** Both survivors and non-survivors were of similar age, sex, and COPD status. FVC and FEV1, as well as Global Initiative for Chronic Obstructive Lung Disease stadiums were significantly higher in the survivor group ($p < 0.016$, $p < 0.001$, $p < 0.001$, respectively). Normal axis was in significantly higher percentage in non-survived patients ($p = 0.020$). RBBB and incomplete RBBB are more frequent findings in patients who died ($p < 0.001$, $p < 0.05$, respectively). LBBB, S1S2S3 configuration is in significantly higher percentage present in non-survivors ($p < 0.016$, $p < 0.001$, respectively). In the multivariable logistic model, patients with LBBB have two times higher chance of mortality compared to patients without LBBB. In contrast, patients with RBBB have 1.6 times lower chance of having death outcome.

**Conclusion** The main ECG predictors of COPD patients' five-year mortality are LBBB and RBBB, but according to statistical model, ECG should be further explored and possibly obligatory involved in a routine clinical practice as an easy and low-cost screening method.

**Keywords:** chronic obstructive pulmonary disease; electrocardiography; mortality

## INTRODUCTION

Chronic obstructive pulmonary disease (COPD) is a very common, preventable and treatable disease, characterized by respiratory symptoms and airflow limitation due to airway and/or alveolar abnormalities, caused by significant exposure to noxious particles or gases [1]. It is the fourth leading cause of death worldwide, exceeded only by myocardial infarction, malignancy, and stroke [2]. Among chronic high morbidity and mortality diseases throughout the world, many aged people suffer from COPD and die prematurely [2, 3]. COPD complexity and mortality are increased by its exacerbations and co-morbidities [4]. Along with pulmonary involvement, there are significant extra pulmonary effects in COPD [3, 4, 5]. COPD can influence electrocardiographic (ECG) changes variously.

COPD is often associated with cardiovascular diseases, thus representing one of the most frequent and clinically important coexisting conditions. An accumulating body of evidence indicates COPD association with coronary artery disease (CAD), chronic heart failure, hypertension, and cardiac arrhythmias, independent of shared risk factors [6, 7]. Apart from the common risk factors presence (age, smoking habit, environmental pollutants, sex, and diet), it appears that multiple pathophysiologic abnormalities contribute to both the COPD and CAD development and progression. COPD and CAD association is characterized by specific ECG abnormalities [5, 6, 7].

Changes of Sokolow–Lyon index and clockwise rotation of the horizonal QRS axis are some of the changes. ECG changes can be found in different stages of COPD and can be associated with the increased death risk. An increased burden of cardiac arrhythmias has also been recognized recently [5]. In COPD, various mechanisms can influence ECG diversely, independent of a possible CAD [6]. The most consistent patterns reported have been vertical axes for P and QRS and increased P wave amplitude (P-pulmonale). The QRS amplitudes are often reduced. Previous studies have related ECG findings with obstruction and emphysema and

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increased pulmonary vascular pressure or right ventricular hypertrophy (RVH). However, none of these studies have linked their findings to the combined effects of obstruction, emphysema, and increased afterload [5, 6]. COPD comorbidities have been infrequently studied, mostly in evaluating relationships between COPD and some specific diseases.

The current study was aimed at finding a correlation between ECG changes and COPD, as well as at mortality in relation to ECG [6, 7]. Regular pulmonologist examination does not include ECG. However, ECG could be the first marker of COPD – approachable, easy to perform, and inexpensive, but extremely helpful.

## METHODS

A prospective study was conducted from January 2009 to February 2014 at the Zemun Clinical Hospital Center and included 835 COPD patients. COPD cases were diagnosed and selected from patients who were attending the Outpatient Department of Respiratory Diseases for treatment of various respiratory problem. Exclusion criteria were any kind of cardiovascular diseases (previous myocardial infarction, arterial hypertension, angina pectoris, congenital cardiovascular disease, heart failure, etc.) Also, patients who were not able to adequately perform spirometry were excluded.

Spirometry was performed using Turninac pneumotach Pony FX (Cosmed Srl, Rome, Italy), following the American Thoracic Society / European Respiratory Society (ATS/ERS) recommendations using postbronchodilator values in order to overestimate the prevalence of COPD [8]. The ATS/ERS recommendations for chronic obstructive lung disease have defined airflow obstruction if forced expiratory volume in the first second (FEV1) and forced vital capacity (FVC) ratio is < 0.7 of predicted. Airflow obstruction severity was graded according to Global Initiative for Chronic Obstructive Lung Disease (GOLD) 2011 as Stage 1 – mild, Stage 2 – moderate, Stage 3 – severe, and Stage 4 – very severe; characterized by FEV1 the values are > 80% of predicted, 50–80% of predicted, 30–50% of predicted, and < 30% of predicted, respectively [8]. All the patients were smokers.

All of the patients underwent ECG. A 12-lead ECG (CARDIOVIT AT-102; SCHILLER Americas Inc., Doral, FL, USA), including three bipolar limb leads, three unipolar limb leads, and six unipolar precordial leads, was performed. All necessary precautions advised for ECG were observed. ECG was done in the supine position. ECG parameters were measured using the Minnesota Code, by two independent persons, and their final judgement was achieved by consensus in case of disagreement [9].

The P value axis and QRS complex were calculated according to the Cabrera system [9]. The following parameters were monitored: high, peaked wave ≥ 2.5 mm tall in leads II, III, aVF; RVH – right axis deviation of + 110° or more; dominant R wave in V1 (> 7 mm tall or R/S ratio > 1); dominant S wave in V5 or V6 (> 7 mm deep or R/S ratio < 1); QRS duration < 120 ms); left bundle branch block (LBBB) (the QRS duration ≥ 120 ms, QS or rS complex in lead V1, notched ('M'-shaped) R wave in lead V6), incomplete RBBB (RSR' pattern in V1–V3 with QRS duration < 120 ms), abnormal transitional zone (poor R wave progression or "poor anterior R wave progression"), S1S2S3 pattern (presented with S waves in leads I, II, and III and negative T wave changes in V1–V3). The patients were followed up for five years for the mortality prediction calculations.

The study protocol was approved by the Ethics Committee of the Faculty of Medicine, University of Belgrade. All the procedures were performed in accordance with the Helsinki declaration. All the patients have given their written informed consent to participate in the study.

### Statistical analysis

The results are presented as count (%), mean ± standard deviation or median (25–75th percentile). Group differences were analyzed using parametric (independent samples t-test) and non-parametric tests (Mann–Whitney U-test, Pearson's $\chi^2$ test, and Fisher's exact test). Logistic regression analysis was performed to assess independent predictors of mortality. All data were analyzed using IBM SPSS Statistics, Version 20.0 (IBM Corp., Armonk, NY, USA). All p-values < 0.05 were considered statistically significant.

## RESULTS

The patients were divided in relation to exitus into two groups – those who survived the five-year period and those who died, as well as according to their sex. The minimum observed age was 42 years, the maximum age was 84 years, and the mean age (SD) was 63.4 (8.8) years. FVC was statistically higher in non-survivors (70.9 ± 14.8) with regard to surviving patients (68 ± 15.3). Also, FEV1 was statistically higher in persons who died than in the surviving ones (44.3 ± 10.7, 41.4 ± 11.7, respectively). FEV1/FVC ratio was statistically higher in non-survivors than in survived patients (63.8 ± 60.4–65.8; 63.1 ± 56.9). There was no statistical difference in MEF 25–75 between the two groups. GOLD stadiums 2, 3, and 4 were statistically higher in the surviving group. General characteristics and lung function of COPD patients separately for five-year survivors and non-survivors are presented in Table 1.

As shown in Table 1, both survivors and non-survivors are of similar age, sex, and COPD status. In contrast, FVC and FEV1 are significantly higher in the survivor group. FEV1/FVC is significantly higher in the survivor group, but the difference in medians is small. Most of the patients were in GOLD stadium 3, in whom higher mortality than in GOLD stadiums 2 and 4 was observed.

Normal axis was statistically higher in non-survived patients. Left axis deviation was found in 24.4.9% of non-survivors and in 75.6% of patients who survived the five-year period. Tall, peaked P wave > 2.5 mm in height was described in inferior leads for 30.7% of non-survivors and in 69.3% of survivors. The patients who had no RVH had statistically lower percentage of five-year mortality. In contrast, RBBB, as well as incomplete RBBB, was a statistically higher finding in patients who died. Low QRS was found in

**Table 1.** General characteristics of chronic obstructive pulmonary disease patients in relation to exitus

| Characteristics | Death in five years | | p |
|---|---|---|---|
| | No | Yes | |
| Age (years) | 63.3 ± 8.3 | 63.8 ± 9.1 | 0.409[a] |
| Sex | | | |
| male | 78 (29.5%) | 186 (70.5%) | 0.918[b] |
| female | 203 (29.2%) | 492 (70.8%) | |
| FVC (%) | 70.9 ± 14.8 | 68.0 ± 15.3 | 0.007[a] |
| FEV1 (%) | 44.3 ± 10.7 | 41.4 ± 11.7 | < 0.001[a] |
| FEV1/FVC | 63.8 (60.4-65.8) | 63.1 (56.9–65.4) | < 0.001[c] |
| MEF (%) | 13.0 (10.5–19) | 14.6 (11.4–19) | 0.149[c] |
| GOLD | | | |
| 2 | 98 (35.4%) | 179 (64.6%) | < 0.001[c] |
| 3 | 156 (30.1%) | 363 (69.9%) | |
| 4 | 27 (16.6%) | 136 (83.4%) | |

MEF – maximum expiratory flow-volume; FEV1 – forced expiratory volume in the first second; FVC – forced vital capacity
[a]t test;
[b]Pearson $\chi^2$ test;
[c]Mann–Whitney U-test;
results are presented as count (%), mean ± SD or median (25–75th percentile)

**Table 2.** Electrocardiographic characteristics of chronic obstructive pulmonary disease patients in relation to the five-year mortality

| Characteristics | Death in five years | | p |
|---|---|---|---|
| | No | Yes | |
| Axis | | | |
| normogram | 210 (31.4%) | 458 (68.6%) | 0.028[a] |
| left | 71 (24.4%) | 220 (75.6%) | |
| p > 2.5 | | | |
| no | 147 (28.2%) | 375 (71.8%) | 0.396[a] |
| yes | 134 (30.7%) | 303 (69.3%) | |
| RVH | | | |
| no | 269 (28.8%) | 664 (71.2%) | 0.056[a] |
| yes | 12 (46.2%) | 14 (53.8%) | |
| RBBB | | | |
| no | 184 (26.2%) | 519 (73.8%) | < 0.001[a] |
| yes | 97 (37.9%) | 159 (62.1%) | |
| Incomplete RBBB | | | |
| no | 225 (30.9%) | 502 (69.1%) | 0.047[a] |
| yes | 56 (24.1%) | 176 (75.9%) | |
| Low QRS | | | |
| no | 120 (31.3%) | 263 (68.7%) | 0.260[a] |
| yes | 161 (28%) | 415 (72%) | |
| Transitional | | | |
| no | 58 (27.6%) | 152 (72.4%) | 0.544[a] |
| yes | 223 (29.8%) | 526 (70.2%) | |
| LBBB | | | |
| no | 270 (30.3%) | 622 (69.7%) | 0.016[a] |
| yes | 11 (16.4%) | 56 (83.6%) | |
| QRS < 0.12 s | | | |
| no | 252 (28.9%) | 621 (71.1%) | 0.345[a] |
| yes | 29 (33.7%) | 57 (66.3%) | |
| S1S2S3 | | | |
| no | 276 (29%) | 677 (71%) | 0.010[b] |
| yes | 5 (83.3%) | 1 (16.7%) | |
| Negative T in V1/V3 | | | |
| no | 269 (29.1%) | 655 (70.9%) | 0.509[a] |
| yes | 12 (34.3%) | 23 (65.7%) | |
| QT < 400 ms | | | |
| no | 2 (16.7%) | 10 (83.3%) | 0.525[b] |
| yes | 279 (29.5%) | 668 (70.5%) | |

[a]Pearson $\chi^2$ test;
[b]Fisher's exact test
LBBB – left bundle branch block; RBBB – right bundle branch block

28% of non-survivors and in 72% of survivors, transitional ECG zone was found in 29.8 % of non survivors and in 70.2% of surviving patients. LBBB was statistically higher in non-survivors. S1S2S3 configuration was statistically higher in non-survivors even in a small sample; V1–V3 leads negative T wave was found in 34.3% of the patients who died and in 65.7% survived patients; QRS duration ≤ 0.12 seconds was found in 33.7% of the non-survivors and in 66.3% of the surviving patients. QT < 0.12 was found in 29.5% of the patients who died and in 70.5% of the survived ones. ECG characteristics of COPD patients in relation to a five-year death outcome are presented in Table 2. Significant correlation with the five-year mortality is observed with normal axis, RVH, incomplete RBBB, LBBB, and S1S2S3. Patients with normal axis, incomplete RBBB, and LBBB had significantly higher percentage of mortality. In contrast, the patients with RVH and RBBB had significantly lower percentage of the five-year mortality. Only six patients had positive S1S2S3 and they will not be included in the multivariate model due to the small sample size for this analysis.

Logistic regression analysis is performed to assess significant predictors of mortality adjusted for potential confounders. Univariable and multivariable logistic regression analysis results are presented in Table 3. Only variables with p < 0.2 are presented in the univariate analysis.

Univariate analysis revealed that lung functions (FVC, FEV1, FEV1/FVC, GOLD) are significant predictors of mortality. Since all of them are highly multi-correlated, FEV1/FVC will was used for multivariate analysis as an adjusting variable for ECG parameters. RVH, RBBB, incomplete RBBB, LBBB, and S1S2S3 are also significantly correlated with the outcome. Since S1S2S3 is positive in only six patients, this variable was not used in multivariable modeling due to small sample size. The final model was performed using logistic regression backward method. Four steps were performed to obtain the final model, which was compared to the first model (all variables at the beginning) and the results of the Hosmer–Lemeshow goodness of fit test and Nagelkerke $R^2$ reveal similar characteristics of both models. We decided to use simple (final, backward model) as the model of five-year mortality predictors. The final model revealed two significant predictors of five-year mortality – RBBB and LBBB. Patients with LBBB had two times higher chance of mortality compared to patients without LBBB. In contrast, RBBB is a protective factor and patients with RBBB have 1.6 times lower chance of having death outcome.

## DISCUSSION

COPD and cardiovascular diseases have common risk factors, including smoking and ageing. Also, both diseases are

**Table 3.** Regression model with exitus as outcome

| Characteristics | Univariate | | Multivariate (backward method) | |
|---|---|---|---|---|
| | OR (95% CI) | p | OR (95% CI) | p |
| FVC | 0.988 (0.978–0.997) | 0.007 | | |
| FEV1 | 0.978 (0.966–0.990) | < 0.001 | | |
| FEV1/FVC | 0.949 (0.926–0.973) | < 0.001 | 0.951 (0.928–0.974) | < 0.001 |
| MEF 25–75 | 1.005 (0.992–1.018) | 0.436 | | |
| Gold | 1.545 (1.247–1.916) | < 0.001 | | |
| Axis | 1.421 (1.038–1.944) | 0.028 | | |
| RVH | 0.473 (0.216–1.035) | 0.061 | | |
| RBBB | 0.581 (0.429–0.787) | < 0.001 | 0.610 (0.448–0.830) | 0.002 |
| Incomplete RBBB | 1.409 (1.003–1.978) | 0.048 | | |
| LBBB | 2.210 (1.140–4.284) | 0.019 | 2.027 (1.039–3.956) | 0.038 |
| S1S2S3 | 0.082 (0.009–0.701) | 0.022 | | |

MEF – maximum expiratory flow-volume

presented with pro-inflammatory mechanisms and oxidative stress. Sedentary lifestyle in COPD may contribute to cardiovascular disease developing, as well [3, 4, 10]. A number of studies have reported ECG abnormalities and cardiac arrhythmias in COPD patients [5, 10]. The majority of ECG abnormalities are associated with COPD, which is mostly presented with a combination of two factors: pulmonary hypertension and anatomic changes. Pulmonary hypertension, however, is always the underlying pathologic mechanism for right ventricular hypertrophy in cor pulmonale and altered electrical conduction. Also, hyperinflation causes a thorax heart displacement position. Abnormalities in conduction usually occur late in COPD patients, after the right ventricle hypertrophy has developed to such an extent that its electrical forces overcome those of the left [11]. In our study, we have found no arrhythmias, despite the fact that almost half of the investigated groups had developed the COPD terminal stadium.

We included 835 cases with a stabile phase of COPD, evaluated by spirometry and electrocardiography. Some authors have reported higher age in men as a significant risk factor associated with FEV1 decline with age, so that the advanced disease stage tends to reduce the FEV1/FVC ratio [11, 12]. In our study, both sexes were of similar age, with more female participants, which is contrary to the fact that men suffer from COPD more frequently [11, 12, 13]. Some authors have investigated vertical QRS axis as a single criterion for a COPD disease screening in an adult hospital population, concluding that vertical QRS axis can detect COPD with 89% sensitivity and 96% specificity [10, 11]. In fact, vertical QRS axis is a synonym for COPD and its severity known as a "hanging heart" [10, 13]. It is explained by reduced Sokolow–Lyon index for left ventricular mass by obstruction and afterload, presumably reflections which both increased the right-sided and decreased the left-sided QRS amplitudes by the combined anatomical and electrical remodeling of the heart [12]. In COPD patients, hyperinflation of the lungs leads to the depression of the diaphragm, and this is associated with clockwise rotation of the heart along its longitudinal axis. This clockwise rotation means that the transitional zone (defined as the progression of rS to qR in the chest leads) shifts towards the left with the persistence of an rS pattern as far as V5 or even V6. This may give rise to a "pseudoinfarction" pattern, with deep S waves in the right precordial leads simulating the appearance of the QS waves and poor R wave progression seen in anterior myocardial infarction.

Other studies have shown the QRS right axis deviation dominance with clockwise rotation [10–13]. Unlike the study by Alter et al. [10] with the vertical QRS axis predominance, the normal axis is a more common finding in our study. In fact, it is a highly statistically important factor in patients who did not survive the five-year follow-up. Also, we had no patient with right axis on ECG. Our study has shown that vertical axis in COPD patients is not "the holy grail", always connected with COPD. The evaluation of ECG abnormalities' significance as COPD prognostic factors has started in 1975; it was reported by Kok-Jensen that an ECG p-II amplitude of at least 0.2 mV is related to poor prognosis [9]. Our study has shown that P wave ≥ 2.5 mm in height has no predicted value in mortality of COPD patients. In the present study, peaked P wave (amplitude > 2.5 mm) was recorded in 52.33% of the cases with COPD. In a Spodick's series, 13.9% of COPD patients had P-wave equal or greater than 2.5 mm [14]. Carid and Wilcken found incidence of P-pulmonale in 15.5% of their COPD patients, while another group of authors recorded the incidence of 32.7% in their respective studies [13, 14]. However, there was no statistical significance between survivors and non-survivors considering P wave height, which is not in concordance with previous studies [12, 13, 14].

Patients who had RVH recorded on ECG had lower five-year percentage of mortality. In our study, most participants belonged to GOLD stadium 3, which is characterized as severe. They had higher mortality percentage over the five-year period compared to GOLD 2 and 4 stadiums, despite the fact that patients in GOLD 4 stadium had very severe airway obstruction.

Other researchers have found variable COPD pulmonary hypertension and right ventricle remodeling prevalence, increased by disease progression, which is in concordance with our results [15, 16]. Although the exact prevalence is unknown, RVH appears to be a common complication of chronic lung disease, and more frequently complicates advanced lung disease [14, 16]. Generally, our study has shown earlier ECG COPD presentation as compared to previous studies, presented as early as GOLD 2 stadium, but mostly in GOLD 3 stadium. In COPD, chronic pulmonary hypertension accompanied by right ventricular work increase results in uniform RVH. Several mechanisms including pathophysiology of pulmonary hypertension could lead to COPD, chronic cor pulmonale, and consecutively to the right heart failure. In patients with COPD, P-pulmonale and the RVH ECG evidence are not shown unless FEV1 < 45% of predicted is presented (GOLD 2) [12, 13]. This led to the conclusion that RVH

develops faster than expected, which could be applicable to our study [12, 13, 16]. The appearance of complete and incomplete RBBB in otherwise healthy individuals is believed to be benign, but several cardiac and pulmonary diseases are known to be associated with RBBB and incomplete RBBB. In our study, they were more frequent in non-survivors and presented a mortality risk. Investigation of the pathophysiology of this pattern presents a challenge for future prospective studies**.** Investigators from Denmark have explored this issue in the Copenhagen City Heart Study prospective database with almost 19,000 subjects in the 1976–2003 period [17]. Patients with prior myocardial infarction, heart failure, or LBBB were excluded while 18,441 were included to be followed up until 2009. Primary end points were all-cause mortality, major cardiovascular events, and admission for COPD [17]. The original purpose of the study was to focus on prevention of coronary heart disease and stroke and to maximize the likelihood of identifying disease causes. Still, there are no explanations for a lot of questions [4, 16, 17, 18]. Thus, finding of incomplete or complete RBBB in COPD patients should not be neglected, and in these cases pulmonologists should order more cardiology examinations.

LBBB has been proposed as a risk factor for cardiovascular morbidity and mortality [16, 17]. LBBB in the absence of a clinically detectable heart disease is associated with new-onset heart failure and death from cardiovascular diseases. Further study is warranted to determine if additional diagnostic testing or earlier treatment in patients with asymptomatic LBBB can decrease cardiovascular morbidity or mortality [19]. Our study had shown that LBBB is a predictor of mortality in COPD patients. There are no facts to suggest a connection between the two. Possible explanations are age, smoking, hypoxemia atherosclerosis, diabetes.

It is common that patients with COPD show low ORS complex (LQRSV), particularly in the limb leads, because of an increased heart/chest wall distance from the lung hyperinflation, which, if not offset, would be expected to augment QRS potentials by the increased electrical impedance [6, 15, 16]. The amplitude of the QRS complexes may be small in patients with COPD as lung hyperinflation leads to poor electrical conductors. Our study registered low ORS voltage in 69% of the patients, but this had no influence of the outcome in COPD patients.

T wave may be inverted in leads V1, V2, or V3 due to RVH, commonly found due to varying effect of lung hyperinflation, axis deviation, and enlargement of the right ventricle. Generalized ST depression with T wave inversion may also be seen [17, 18]. In our study, negative T wave had no predictive value in COPD mortality.

The point of our study is to find out which ECG pattern could be a predictive model for five-year mortality in COPD patients. We used logistic regression analysis to assess significant predictors of mortality adjusted for potential confounders. Final model discovered two significant predictors – RBBB and LBBB. In fact, patients with LBBB registered on ECG have two times higher chance of mortality compared to patients without LBBB. This finding is not surprising considering that LBBB in not a finding that should not be easily dismissed in any patient or disease. In contrast, RBBB is a protective factor and patients with RBBB have 1.6 times lower chance of fatal outcome. To date, RBBB is not considered a dangerous condition and many people have it unknowingly, without any symptoms, and its discovery is usually incidental [17, 18]. 'Silent' RBBB in COPD patient is a very frequent finding. The exact explanation for this does not exist, but further studies could elucidate electrical activity of the heart in COPD patients [19, 20].

The ECG findings were found to be 35.7% sensitive and 95.6% specific in the diagnosis of COPD among patients with respiratory problems. Hence, there is a chance of false negative but not of false positive findings in detecting COPD cases by ECG. Based on the findings of the study, positive predictive value was found to be 71.4%, meaning thereby that the chances of COPD are high among patients having ECG changes. Similarly, negative predictive value was 83%, meaning thereby that the chances of not having COPD among patients not having ECG changes are also quite high [6, 16, 17, 20, 21].

Improving survival of COPD patients has been the central theme for years. Most of the conducted studies put the focus on therapy and rehabilitation, giving no attention to ECG screening. According to our study, ECG screening in COPD patients should be obligatory, followed-up with regular ECG monitoring, especially if the normal axis, incomplete RBBB, and LBBB were found. Those patients should be monitored more frequently by both the pulmonologist and the cardiologist.

## CONCLUSION

COPD patients with normal axis, incomplete RBBB, and LBBB have significantly higher percentage of five-year mortality. Apart from that, patients with LBBB have two times higher chance of mortality than those without it. On the other hand, patients with RBBB have lower risk of mortality and according to our study this pattern is a possible protective characteristic in COPD patient. Main ECG predictors of COPD patients' five-year mortality are LBBB and RBBB, but according to the statistical model, ECG should be further explored and possibly obligatory in the routine clinical practice as an easy and low-cost screening method. It is extremely important to emphasize that patients who have both cardiovascular and pulmonary disease are at a higher risk of mortality and that both diseases should be treated in parallel and independently as recommended by the GOLD guidelines.

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# Електрокардиографски предиктори петогодишњег морталитета оболелих од хроничне опструктивне болести плућа

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**САЖЕТАК**

**Увод/Циљ** Најчешћи коморбидитети међу оболелим од хроничне опструктивне болести плућа (ХОБП) су обољења кардиоваскуларног система.

Циљ ове студије је процена параметара електрокардиографије (ЕКГ) и предиктора смртности код болесника са ХОБП-ом.

**Методе** У студију је укључено укупно 835 болесника. Болесници су класификовани да болују од ХОПБ-а ако је у три узастопна мерења постбронходилататорна вредност *FEV*1/*FVC* била < 70%. Праћене су следеће промене у ЕКГ-у: осовина, П-талас, комплекс с ниским *ORS*-ом, прелазна зона, блок леве гране Хисовог снопа (*LBBB*), блок десне гране (*RBBB*), непотпуни блок десне гране снопа, конфигурација *S1S2S3*, негативан *T* у *V*1–*V*3. Праћен је морталитет оболелих у периоду од пет година.

**Резултати** И преживели и преминули били су сличног узраста, пола и статуса ХОПБ-а. *FVC* и *FEV*1, као и *GOLD* стадијум, значајно су већи у групи која је преживела ($p < 0,016$, $p < 0,001$, $p < 0,001$, респективно). Нормална осовина била је у знатно већем проценту код умрлих болесника ($p = 0,020$). Десни *RBBB* и непотпуни *RBBB* су чешћи налаз код болесника који су умрли ($p < 0,001$, $p < 0,05$, респективно). Конфигурација *LBBB*, *S1S2S3* је у знатно вишем проценту код умрлих болесника ($p < 0,016$, $p < 0,001$, респективно). У мултиваријабилном логистичком моделу болесници са *LBBB* имају двоструко већу шансу за смртност у поређењу са болесницима без *LBBB*. Супротно, болесници са *RBBB* имају 1,6 пута мању шансу да изгубе живот.

**Закључак** Главни ЕКГ предиктори петогодишњег морталитета код ХОПБ-а су *LBBB* и *RBBB*, али према статистичком моделу, електрокардиограм треба додатно истражити и евентуално обавезно укључити у рутинску клиничку праксу као једноставан и приступачан метод скрининга.

**Кључне речи:** хронична опструктивна болест плућа; електрокардиограм; морталитет

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

# Association between epidermal growth factor receptor mutation status, clinicopathological characteristics and TTF-1 expression in lung adenocarcinoma – a single center study

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**SUMMARY**

**Introduction/Objective** The presence of epidermal growth factor receptor (EGFR) mutations is the best predictor of response for therapy with tyrosine kinase inhibitors. In this study, we investigate association between EGFR mutations and clinicopathological characteristics and thyroid transcription factor (TTF-1) expression in lung adenocarcinomas (AD).

**Methods** We analyzed 142 surgical samples from patients with histologically confirmed lung AD from January 2010 to December 2015. All tumor tissues were reclassified according to the World Health Organization criteria and EGFR mutations detected by real-time polymerase chain reaction. TTF-1 expression was detected by immunohistochemistry in 83 out of 142 cases. The association between EGFR and TTF-1 expression was analyzed using the $\chi^2$ test or Fisher's exact test with SPSS software version 20.0.

**Results** This study included 78 male and 64 women with a median age of 61.6 (range, 42–82) years. Acinar (ACN) and solid (SOL) were the most common histological types (47.9% and 38.7%, respectively). TTF-1 expression was present in 69 of 83 (83%) ADs. The EGFR mutation was found in 7%, more frequently in women, and patients with smoking history, and acinar type of AD, whereas it had no association with age and pathological stage and TTF-1 expression.

**Conclusion** In conclusion, the results of this study demonstrate that the presence of EGFR mutations is associated with some clinical characteristics and histologic type of ADs, but not with TTF-1 expression.

**Keywords:** adenocarcinoma; EGFR mutation; clinicopathological characteristics; TTF-1 expression

## INTRODUCTION

Epidermal growth factor receptor (EGFR) consists of 486 amino acids and 170 kDa in size. It is part of the ErbB family of structurally related receptor tyrosine kinases: EGFR (HER1, ErbB1), HER2 (Neu, ErbB2), HER3 (ErbB3) and HER4 (ErbB4), which are involved in signal transduction pathways and play a key role in the regulation of cellular proliferation and apoptosis. Molecular analysis of the mutation status for EGFR is critical for treatment of tyrosine kinase inhibitors (TKIs), which show improved progression-free survival (PFS) and overall survival in inpatients with adenocarcinomas (AD), which is second most frequent histological type of lung cancer found in surgically treated lung cancer patients in Serbia [1, 2, 3]. According to international guidelines, conventional identification of EGFR genotype requires tissue/cytologic samples (Ti/Cy), but in the last five years, EGFR testing can be performed by analyzing circulating-free tumor DNA (cfDNA) in peripheral blood samples. EGFR mutations are more frequent in tumors with ADs histology, in never-smokers or light smokers, in female, and in patients with East Asian ethnicities. The most frequent EGFR mutations are in-frame deletions of exon 19 and the exon 21 L858R mutation [4, 5]. Previous investigations have demonstrated that EGFR gene mutation is mainly detected in patients with lepidic (LP), papillary (PAP), micropapillary (MPP), and acinar (ACN) types, whereas the mutation rate is extremely low in patients with the solid (SOL) histological type [6–9].

Thyroid transcription factor 1 (TTF-1), is a homeodomain nuclear protein that belongs to the NK2 family of transcription factor. TTF1 is recommended as one of a panel of lineage-specific immunohistochemical markers for AD differentiation and may modulate lung cancer biology. Clinicopathologic features such as age, sex, smoking status, histological type, and pathological stage were similar between TTF-1-positive and TTF-1-negative tumors. TTF-1-positive tumors have more commonly EGFR mutations, as well as better response to EGFR TKIs comparing to TTF-1-negative tumors, but TTF-1 negativity should not be the exclusion criteria for EGFR testing [10, 11, 12].

In the present study, we investigated the association between EGFR mutation status, clinicopathological characteristics, and TTF-1 expression in lung AD.

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## METHODS

Surgical samples of 142 patients with lung AD admitted to the Institute for Lung Diseases of Vojvodina (Sremska Kamenica, Serbia) between January 2010 and December 2015 were retrospectively analyzed.

Clinicopathological parameters including age, sex, smoking history, pathological stage, and histological type were recorded. The histological classification was done based on 2015 WHO classification system and all samples were divided into five groups (ACN, PAP, MPP, LP, or SOL).

One hundred forty-two surgical samples were fixed in 10% formalin, embedded in paraffin, cut on four-micron-thick sections and stained with routine H&E staining. Eighty-three of them were deparaffinized and incubated in a citrate buffer (10 mM sodium-citrate monohydrate, pH 6.5) at 120°C for 20 minutes in an autoclave. The sections were reacted for one hour with antibody of TTF-1 (monoclonal antibody, Denmark DAKO products) and then incubated with a commercially available detection kit (DAKO EnVision Plus-HRP, Dako, Glostrup, Denmark) following the manufacturer's instructions. Positive and negative controls were used as appropriate. TTF-1 expression was rendered semiquantitatively on a score 0–2. Tumors were scored according to TTF-1 expression into following scores: 0 (lack of expression), score 1 (< 50%) and score 2 (≥ 50%) based on the percentage of positively stained tumor cells of any intensity.

The EGFR mutations (in exons 18, 19, 20, and 21) was done with the Cobas EGFR Mutation Test (Roche, Basel, Switzerland) "Real time PCR." The Cobas Sample Preparation Kit (Roche) was used for the sample preparation and DNA extraction. Automatic amplification and detection were done on the Cobas z 480 Analyzer (Roche).

### Statistical analysis

Pearson's $\chi^2$ test and Fisher's exact test were used to compare frequencies of clinicopathological variables; p-values < 0.05 were considered statistically significant. All analyses were performed using IBM SPSS Statistics, Version 21.0 (IBM Corp., Armonk, NY, USA).

The study was done in accord with standards of the institutional committee on ethics.

## RESULTS

One hundred forty-two patients diagnosed with infiltrative AD (Figure 1) which had been surgically resected were included in this study. The median age of all patients was 61.6 years (ranging 42–82). The majority of patients were males (78; 54.9%), and 13 of 142 (9.2%) had no-smoking history (Table 1).

Most common histological types were ACN (68; 47.9%) and SOL (55; 38.7%), with most frequent pathological stage IIA according to the 7th TNM classification system (Table 2).

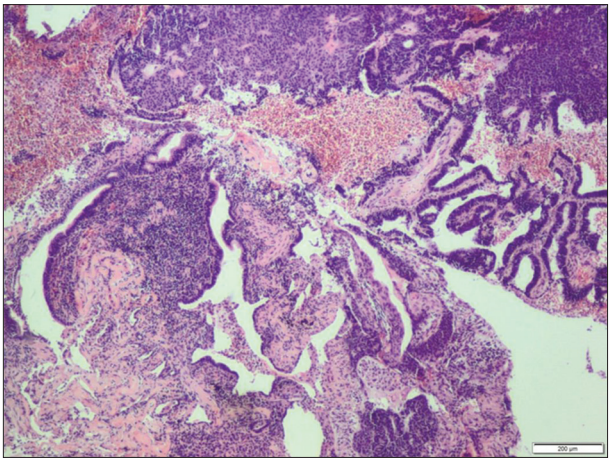

**Figure 1.** Acinic type adenocarcinoma (H&E, 10×)

**Table 1.** Patient characteristics and epidermal growth factor receptor (EFGR) mutation status

| Clinical characteristics | Frequency | EGFR status, n (%) | | p |
|---|---|---|---|---|
| | | positive | negative | |
| Total | 142 | 10 (7) | 132 (93) | |
| Age, years<br>Median<br>Range | <br>61.6<br>42–82 | <br>65.5<br>46–82 | <br>61.3<br>42–82 | |
| Sex<br>Male<br>Female | <br>78 (54.9)<br>64 (45.1) | <br>3 (2.1)<br>7 (4.9) | <br>75 (52.8)<br>57 (40.1) | 0.114 |
| Smoking history<br>Never<br>Former/current | <br>13 (9.2)<br>129 (90.8) | <br>3 (2.1)<br>7 (4.9) | <br>10 (7)<br>122 (85.9) | 0.05 |

**Table 2.** Histopathological characteristics of adenocarcinomas and epidermal growth factor receptor (EFGR) mutation status

| Histopathological characteristics | Frequency | EGFR status, n (%) | | p |
|---|---|---|---|---|
| | | positive | negative | |
| Histological type | | | | |
| LP | 6 (4.2) | 0 (0) | 6 (4.2) | 0.32 |
| ACN | 68 (47.9) | 8 (5.6) | 60 (42.3) | |
| PAP | 9 (6.3) | 0 (0) | 9 (6.3) | |
| SOL | 55 (38.7) | 2 (1.4) | 53 (37.3) | |
| MPP | 4 (2.8) | 0.(0) | 4 (2.8) | |
| Stage | | | | |
| IA | 32 (22.5) | 3 (2.1) | 29 (20.4) | 0.46 |
| IB | 24 (16.9) | 1 (0.7) | 23 (16.2) | |
| IIA | 35 (24.6) | 2 (1.4) | 33 (23.2) | |
| IIB | 28 (19.7) | 4 (2.8) | 24 (16.9) | |
| IIIA | 21 (14.8) | 0 (0) | 21 (14.8) | |
| IV | 2 (1.4) | 0 (0) | 2 (1.4) | |
| Total | 142 (100) | 10 (7) | 132 (93) | |

LP – lepidic; PAP – papillary; MPP – micropapillary; SOL – solid; ACN – acinar

**Table 3.** Association between TTF-1 expression and epidermal growth factor receptor (EFGR) mutations

| TTF-1 expression | Frequency | EGFR status, n (%) | |
|---|---|---|---|
| | | positive | negative |
| Score 0 | 14 (16.9) | 2 (2.4) | 12 (14.5) |
| Score 1 | 3 (7.2) | 0 (0) | 6 (7.2) |
| Score 2 | 63 (75.9) | 3 (3.6) | 60 (72.3) |
| Total | 83 (100) | 5 (6) | 78 (94) |

Sixty-three (75.9%) of the 83 cases of lung AD showed TTF-1 expression levels corresponding to score 2 (Figure

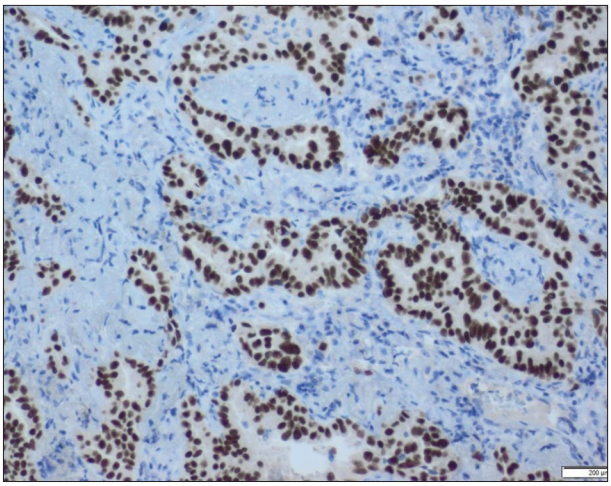

**Figure 2.** TTF-1-positive expression in adenocarcinoma cells (IHC, 10×)

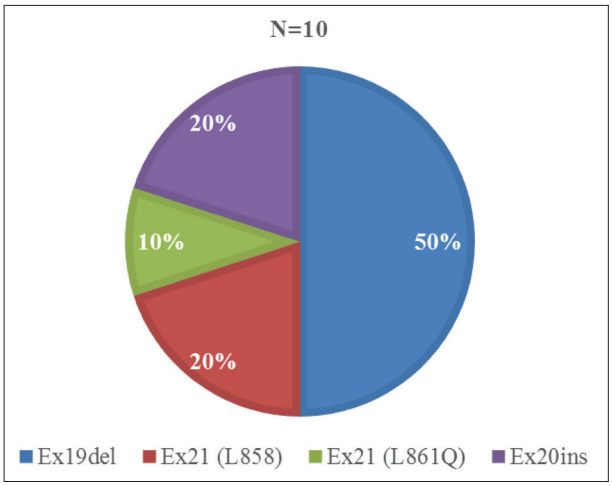


**Figure 3.** The proportion of epidermal growth factor receptor mutations

2), 3 (7.2%) to score 1, and 14 (16.9%) cases lack of TTF-1 expression (Table 3).

EGFR mutations in exons 18, 19, 20, or 21 were examined in 142 cases of lung AD. The overall frequency of EGFR mutation was 7% (10 of 142). Five cases had an in-frame deletion in exon 19, three cases had exon 21 substitutions (L858R, L861Q), and two cases exon 20 insertions. Multiple mutations (≥ 2) and other EGFR mutations were not observed (Figure 3).

Three male and seven female patients harbored EGFR mutation ($p = 0.114$). Three patients with EGFR gene mutation were non-smokers. ($p = 0.05$) (Table 1). The EGFR mutations were found in eight ADs with ACN histological type and in two with SOL histological type ($p = 0.32$). Other histological types were without EGFR mutations. Pathological stage with EGFR mutations were: IIB, IIA, and IB (4, 3, and 1; $p = 0.46$) (Table 2). Association between TTF-1 protein expression and EGFR mutation status is shown in Table 3. Three cases with TTF-1 expression (score 2) had EGFR mutations. Also, EGFR mutations were present in two cases lack of TTF-1 expression (score 0) (Table 3).

## DISCUSSION

In recent years, significant progression has been obtained in the molecular biological research of lung ADs with EGFR mutation. EGFR mutation is a protein on cell surface with intracellular tyrosine kinase (TK) activity due to targetable activating mutations. These tumors are susceptible to TKIs such as gefitinib, erlotinib, or afatinib [9].

Ti/Cy samples are mostly obtained by sampling of the primary tumor or a metastatic lymph node. The median EGFR test turnaround time for Ti/Cy samples was 11 days for Europe and eight days for Japan. When tumor samples are unavailable, cfDNA is a feasible sample for EGFR mutation analysis because the overall concordance of EGFR mutation status between matched Ti/Cy and plasma samples was 89%. It is important to conduct mutation testing in specialized laboratories, using sensitive mutation testing methods to ensure accuracy of the results. In Europe, 43 laboratories perform the Ti/Cy testing, while in five laboratories both analysis of Ti/Cy and cfDNA in peripheral blood samples are performed [11, 13, 14]. At our institution, both methods of EGFR mutation testing have been implemented in clinical practice three years ago.

Since computed tomography (CT) is routinely used in lung cancer diagnosis, as CT imaging is more readily available than biopsies, many researchers proposed analysis CT imaging for predicting EGFR mutations [15]. The presence of emphysema or airway abnormality predicts a wild type status of EGFR, while the presence of any ground glass component indicates EGFR mutations [16]. Recently, the deep learning model provides a non-invasive and easy to use method for predicting the EGFR mutation status. Wang et al. [17] retrospectively collected data from 844 lung AD patients with pre-operative CT images, EGFR mutation, and clinical information from two hospitals. The deep learning score demonstrated significant differences in EGFR-mutant and EGFR-wild type tumors ($p < 0.001$).

ADs are the most common histological subtypes of lung carcinoma, and it has been shown that this is associated with activating mutations in the EGFR gene in 15% of European and 47% of Japanese patients [18]. The most common EGFR mutations include exon 19 deletion. Other recurrent mutations, in exon 18-point mutations in position G719, in exon 21 L861Q mutation, and in-frame exon 19 insertions are rare (3%; 2%, and < 1%) [19–22]. In our study, EGFR mutations were detected in 10 of 142 (7%) cases of AD, five of 10 were deletion in exon 19, similar to results in other studies.

The association between EGFR mutations with patients' age, sex, and smoking status has been demonstrated in numerous studies with a variety of cases [23–26]. In this investigation, EGFR gene mutation was mainly observed in patients aged > 60 years, which is consistent with previous findings.

Mutations were found more frequently in women (69.7%), in patients who had never smoked (66.6%), and

in those with adenocarcinomas (80.9%) ($p < 0.001$) [22, 26]. Contrary to these results, in our study, EGFR mutations were more frequently detected in smokers (seven of 10 cases). These differences are probably due to different lifestyles.

Many authors have studied the association between EGFR mutations and histologic type of AD and TTF-1 expression, as well as pathologic stage of the disease [27–30]. Villa et al. [31] reported that the most common histologic type seen in the EGFR-mutant-positive ADs was LP (44%). Contrary to these results, Zhang et al. [22] reported that ACN type most frequently correlated with EGFR mutation, which is consistent with our results. In our study, ACN (7/10) and SOL (3/10) type were independent predictors of EGFR mutation. EGFR-mutated ADs may develop through a distinct carcinogenetic pathway, in which the MPP element may play an important role in promoting progression and has prognostic value [32]. In our results, MPP histologic type was detected in four of 142 (2.8%) cases with wild type EGFR ADs. Pi et al. [27] showed that EGFR mutation was significantly higher in stage IA than in stage IIB ($p = 0.002$). In our study, there was no difference in EGFR mutations between stage IA and stage IIB, probably because it was a single center study with a small number of cases.

TTF-1 is expressed in the distal bronchial epithelium, including type II alveolar epithelial cells and terminal respiratory epithelial cells, as well as in lung carcinoma: frequently in small cell carcinoma and in ADs [10]. Sixty-six of 83 cases (79.5%) of lung ADs included in this study showed score 2 (75.9%) of TTF-1 expression, while three (7.2%) showed score 1. These results suggest that lung ADs expressed TTF-1. In recent years, many studies have mentioned the association between TTF-1 expression and EGFR mutations [10, 12, 32, 33]. The TTF-1 positivity staining was strongly correlated with the presence of EGFR mutations ($p < 0.001$) and TTF-1 negativity was said to be a good predictor of EGFR wild type mutations [32]. The results of the Svaton et al. [29] study suggested that patients with EGRF wild type lung ADs and a lack of TTF-1 expression may have significantly lower PFS and overall survival, and TTF-1 expression may be a useful predictor of TKIs efficacy in patients with EGFR wild type lung ADs. The patients, TTF-1-positive or -negative ADs, could benefit from the first-line chemotherapy [30]. Therefore, a lack of TTF-1 expression should not exclude patients from EGFR testing.

To our knowledge, this is the first study that investigates the association between EGFR mutation and TTF-1 expression in Serbia. Among tumors in which immunohistochemical analysis to TTF-1 antibody was performed ($n = 83$), EGFR mutations were detected in five cases: in three tumors with score 2 of TTF-1 expression and in two tumors a lack of TTF-1 expression (Table 3).

## CONCLUSION

The results of this study demonstrate that the presence of EGFR mutations is associated with some clinical characteristics and histologic type of AD, but not with TTF-1 expression.

**Conflict of interest**: None declared.

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# Удруженост мутационог статуса рецептора епидермалног фактора раста са клиничкопатолошким карактеристикама и експресијом ТТФ-1 у аденокарциному плућа – студија једног центра

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**САЖЕТАК**

**Увод/Циљ** Присуство мутација рецептора епидермалног фактора раста (РЕФР) најбољи је предиктор одговора на терапију инхибиторима тирозинске киназе. У овој студији смо истраживали удруженост мутација РЕФР-а са клиничкопатолошким карактеристикама и експресијом тироидног транскрипционог фактора 1 (ТТФ-1) у аденокарциномима плућа.

**Методе** Анализирана су 142 хируршка узорка болесника са хистолошки потврђеним аденокарциномом плућа у периоду од јануара 2010. до децембра 2015. године. Сви туморски узорци су рекласификовани према критеријумима СЗО и код свих су мутације РЕФР-а детектоване методом ланчане реакције полимеразе у реалном времену. Експресија ТТФ-1 је одређена имунохистохемијски у 83 од 142 случаја. Асоцијација мутација РЕФР-а и експресије ТТФ-1 је анализирана употребом $\chi^2$ теста или Фишеровог теста.

**Резултати** У студију је било укључено 78 мушкараца и 64 жене просечне старости 61,6 (од 42 до 82) година. Ацинарни и солидни типови су били најчешћи хистолошки типови (47,9% и 38,7%). Експресија ТТФ-1 била је присутна у 69 од 83 (83%) аденокарцинома. Мутације РЕФР-а су детектоване у 7% случајева, чешће код жена, бивших и активних пушача, са ацинарним хистолошким типом и нису биле повезане са годинама, патолошким стадијумом болести и експресијом ТТФ-1.

**Закључак** Резултати ове студије показују да је присуство мутација РЕФР-а удружено са неким клиничким карактеристикама и хистолошким типом аденокарцинома, али не и са експресијом ТТФ-1.

**Кључне речи:** аденокарцином; мутације рецептора епидермалног фактора раста; клиничкопатолошке карактеристике; експресија ТТФ-1

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

# Clinical and radiological evaluation of fracture union in pathologic fractures after closed intramedullary nailing and adjuvant radiotherapy – a retrospective study

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**SUMMARY**

**Introduction/Objective** Pathologic fractures are devastating complications in metastatic bone disease. Treatment of these condition varies, and includes systemic therapies and surgical interventions. Lack of evidence still exists for standardized care.
The aim of this study is to analyze radiological healing response and clinical outcomes after intramedullary nailing (IMN) and adjuvant radiotherapy in complete pathologic fractures of femur or humerus

**Methods** A total of 19 patients who presented with pathological fracture were retrospectively reviewed. Data regarding demographic characteristics, clinical outcomes and radiologic images were obtained from hospital records. All patients in this cohort were treated with closed, unreamed IMN and adjuvant radiation treatment.

**Results** Pain relief and full range of motion was obtained in all patients. The mean postoperative Musculoskeletal Tumor Society scores at last follow-up were 69% (range 50–85). All patients demonstrated complete radiographic healing between 2 and 6 months. Only one patient required reoperation for refracture at the tip of the nail which was revised with a longer nail.

**Conclusion** Our study demonstrated that pathologic fractures managed with closed unreamed IMN and adjuvant multifractional 20 Gy dose radiotherapy yielded good clinical outcomes with complete radiologic response regardless of patient's life expectancy, adjuvant treatments and overall condition. Closed unreamed IMN was also associated with decreased surgical time in these high-risk patients.

**Keywords:** pathologic fracture; intramedullary nailing; adjuvant radiotherapy; bone healing

## INTRODUCTION

Prolonged survival in patients with carcinoma has increased the overall frequency of metastatic disease. Bone is the third most common site for metastatic disease, after lung and liver. Metastatic bone disease commonly involves the spine, followed by femur and humerus [1].

Pathologic fractures are one of the disabling complications in metastatic bone disease and comprise 10% of all metastatic bone lesions. These fractures cause severe pain, morbidity and even mortality [2]. Conservative treatment usually is not enough to reduce the pain and provide functional improvement [3]. With the improvement in cancer treatment modalities, implant technologies and surgical fixation, there is an overall decrease in complications and the ability to satisfy the treatment goals for these subsets of patients with complex needs. Pathologic fractures should be managed appropriately, so that the patient can receive relevant oncological treatment as soon as possible after surgery. The treating orthopedic surgeon must be aware of the compromised healing characteristics of the pathologic bone, increased infection rate and other associated perioperative complications such as thromboembolism and thereby direct treatment accordingly. The primary goal is to obtain immediate functional recovery without causing a delay in application of appropriate adjuvant treatments. This in turn requires an optimal surgical procedure that minimizes postoperative surgical and systemic complications such as pulmonary embolism, implant failure and disease progression. After primary diagnosis of pathologic fracture has been clearly established, timing of surgery and receiving chemotherapy or radiotherapy (RT) often need to be addressed in a multidisciplinary approach. Preoperative planning should include patient's expected survival by considering possible complications of available surgical options ranging from stabilization with an intramedullary nailing (IMN) and plate osteosynthesis to resection and endoprosthetic reconstruction (EPR).

Among these, intramedullary fixation has emerged as a preferred surgical technique in

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the treatment of metastatic bone disease, although it has been reported that healing may not be accomplished. [4, 5] Previous studies demonstrated improved clinical outcomes for surgical fixation and adjuvant radiotherapy; however convincing data regarding radiological fracture healing is limited to small case series [3, 6].

The aim of this study is to analyze radiological and clinical improvements after unreamed IMN and adjuvant radiotherapy in terms of bone healing and clinical outcomes.

## METHODS

Between 2016 and 2019, 19 patients with pathological fractures due to solid organ metastases or multiple myeloma were treated with locked IMN at our tertiary teaching hospital. This study was approved by the institutional review board. A retrospective chart review was carried out to collect demographic data (age, sex), type of primary lesion, previous history of pathologic fracture and radiotherapy, metastatic status, location of the lesion within the bone, nail dimensions, length of hospital stay, postoperative complications, postoperative survival and functional and radiological outcomes. Inclusion criteria included patients with multiple metastases with pathologic humerus and femur fractures that underwent IMN. Exclusion criteria were endoprosthetic reconstruction, inadequate follow-up, incomplete data due to death within two months after operation.

Before developing an impending or a complete pathologic fracture, all cases except two patients had a routine follow up by a medical oncology division and appropriate systemic therapy was administered according to treatment protocol of primary disease. Positron emission tomography-computed tomography was used to identify any other skeletal and visceral metastases in our patients. Pathologic fracture with pain was primary indication for surgery, in accordance with Mirels' criteria. Patients who were deemed stable with reasonable life expectancy (> 3 months) based on PATHFx estimation, eligible for surgery were operated. Our cases included multimetastatic patients and they were all evaluated by our multidisciplinary tumor board before surgery. Biopsy was preferred for investigating the impending or completely fractured bone lesions as the last step in our diagnostic algorithm. We had first obtained routine laboratory tests and performed radiological investigations. If the patient had an unknown origin of primary lesion (two patients in our study cohort), percutaneous needle biopsy under general anesthesia was performed. One week after, if the pathology was confirmed as metastatic bone lesion, we proceed with IMN. In patients with a known primary malignancy, tissue specimen was obtained for frozen pathological evaluation. If the result was confirmed as metastatic carcinoma, then we performed IMN as previously planned. No preoperative embolization was performed for relatively vascular lesions like renal cell carcinoma, angiosarcoma and myeloma. Nailing was performed for all metadiaphyseal fractures of the humerus and femur. Fractures involving femoral head and distal end of humerus were excluded.

Follow-up duration was defined from completion of RT to last clinical/radiologic evaluation. Every patient was followed up for a minimum of two months (range: 2–16 months). The median age at the time of the surgery was 65.5 years (range 53–86 years). None of the patients had any history of prior RT before surgery. All patients received postoperative bisphosphonate treatment after radiotherapy.

Clinical assessment was made using Musculoskeletal Tumor Society rating scale score. Radiologic assessment was made based on plain radiographs according to radiological response criteria as described by Harada et al. [7], complete response, partial response, no change, and progressive disease.

### Surgical technique:

**IMN of the femur:** The patient was placed on a traction table in a supine position. Fracture reduction was achieved under fluoroscopic guidance. A 2–3-centimeter incision was made proximal to the greater trochanter and the fascia was split so as to palpate the tip of the greater trochanter. Entry point is determined on the medial face of the greater trochanter. After guidewire was inserted, intramedullary nail (Trigen InterTan; Smith and Nephew, Memphis, TN, USA) was inserted with appropriate length and size by using template X-rays. No intramedullary reaming was performed, and no cement was used. Proximal and distal locking was performed. Patients were allowed to bear weight as tolerated immediately after the surgery. Postoperative external beam radiation (20 Gy in five fractions) to the affected long bone was administered 14 days after the stitches were removed.

**IMN of the humerus:** Patient was placed in beach-chair position. Fracture was reduced under scopy control. Anterolateral approach was made to expose the site of the nail entry. Entry point of the nail was at the center of humeral head just posterior to bicipital groove. Unreamed technique was performed according to manufacturer's instructions. Nail was inserted with appropriate length and diameter by using template X-rays. Proximal locking was made using two or three screws. Distal locking was performed using endopin technique (InSafeLock, TST Medical Devices, Istanbul, Turkey). Patients were immobilized in a sling. Gentle pendulum exercises were begun as tolerated. External beam radiation (20 Gy in five fractions) to the affected long bone was administered 14 days after the stitches were removed.

## RESULTS

Details regarding pathologic fractures in humerus and femur are shown in Tables 1 and 2. Lung (n = 5) and breast carcinoma (n = 6) were the most common primary lesions, followed by renal cell (n = 2), prostate (n = 2), multiple myeloma, malignant epithelioma, angiosarcoma and nasopharyngeal carcinoma (one patient for each type). All patients had multiple bone metastasis or lesions. There was no concomitant pathological fracture in another extremity,

**Table 1.** Details about the pathologic humeral fractures

| Case | Age (years) | Sex | Follow-up (months) | Primary lesion | Localization | Dimension of intramedullary nail (cm × mm) | Duration of operation (min.) | Length of hospital stay (days) | Complication | MSTS score (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 56 | male | 3 | myeloma | proximal | 240 × 8 | 35 | 3 | none | 70 |
| 2 | 79 | male | 4 | lung | proximal/ diaphyseal | 280 × 7 | 30 | 4 | none | 70 |
| 3 | 53 | female | 5 | malignant epithelial carcinoma | diaphysis | 200 × 7 | 20 | 5 | none | 85 |
| 4 | 57 | male | 16 | nasopharyngeal carcinoma | Proximal | 220 × 7 | 25 | 2 | none | 85 |
| 5 | 56 | female | 6 | angiosarcoma | piaphysis | 220 × 8 | 25 | 3 | none | 80 |
| 6 | 86 | male | 4 | lung | diaphysis | 280 × 9 | 35 | 6 | none | 70 |
| 7 | 86 | female | 5 | breast | diaphysis | 220 × 9 | 45 | 7 | none | 70 |
| 8 | 72 | male | 3 | renal cell | diaphysis | 240 × 8 | 30 | 3 | none | 75 |
| 9 | 71 | female | 3 | breast | diaphysis | 240 × 7 | 20 | 5 | none | 75 |

MSTS – Musculoskeletal Tumor Society rating scale score

**Table 2.** Details about the pathologic femoral fractures

| Case | Age (years) | Sex | Follow-up (months) | Primary lesion | Localization | Dimension of intramedullary nail (cm × mm) | Duration of operation (min.) | Length of hospital stay (days) | Complication | MSTS score (%) |
|---|---|---|---|---|---|---|---|---|---|---|
| 10 | 62 | male | 8 | lung | subtrochanteric | 400 × 10 | 65 | 3 | none | 65 |
| 11 | 69 | male | 13 | prostate | subtrochanteric | 250 × 11 | 75 | 4 | none | 70 |
| 12 | 77 | female | 14 | breast | diaphysis | 360 × 11 | 80 | 5 | none | 60 |
| 13 | 58 | female | 2 | breast | diaphysis | 340 × 10 | 55 | 2 | none | 50 |
| 14 | 59 | female | 3 | breast | diaphysis | 60 × 9 | 45 | 3 | none | 60 |
| 15 | 80 | male | 2 | lung | intertrochanteric | 250 × 12 | 75 | 6 | none | 70 |
| 16 | 84 | male | 3 | prostate | intertrochanteric | 220 × 10 | 40 | 7 | none | 55 |
| 17 | 62 | female | 3 | breast | subtrochanteric | 360 × 10 (right: impending left: pathologic) | 85 | 6 | none | 60 |
| 18 | 71 | male | 15 | lung | intertrochanteric | 220 × 10 | 45 | 7 | exchange nail | 70 |
| 19 | 62 | male | 16 | renal cell | subtrochanteric / diaphysis | 360 × 10 | 75 | 5 | none | 75 |

MSTS – Musculoskeletal Tumor Society rating scale score

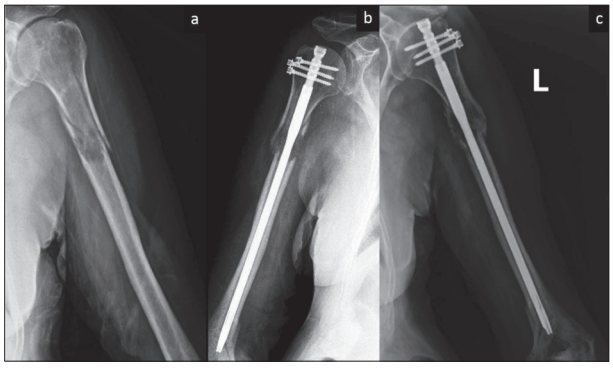


**Figure 1.** Case 2: a) Anteroposterior view of the pathologic humerus fracture due to lung carcinoma (79-year-old male); b) Postoperative view; c) four-month follow-up; Note callus formation at the fracture site, indicating complete response to adjuvant radiotherapy

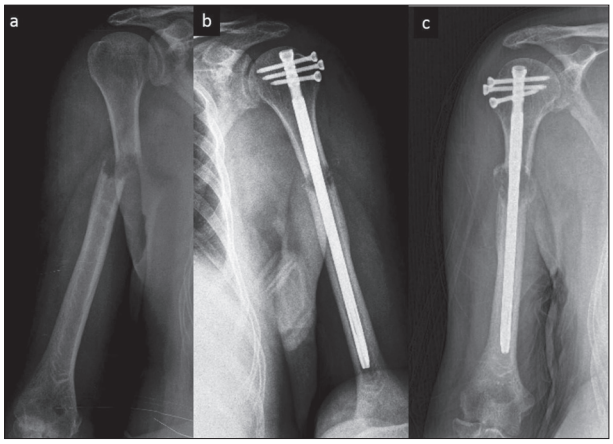


**Figure 2.** Case 8: a) Anteroposterior view of the pathologic humerus fracture due to renal cell carcinoma (72-year-old male); b) Postoperative view; c) three-month follow-up; Note callus formation at the fracture site, indicating complete response to adjuvant radiotherapy

except one patient with bilateral pathologic humeral fractures (case 9). Patients also had an expected survival of at least three months according to PATHFx model [8].

The median hospital stay was three days (range 1–7 days). No complication was observed related to RT (i.e., wound dehiscence, pathologic fracture, infection). No re-irradiation was performed.

Pain relief was obtained in all lesions. All patients regained preoperative mobility at their last follow-up. All lesions achieved complete radiological response with a median of four months (range: 2–16 months) after radiotherapy (Figures 1 and 2). One patient with left pathologic

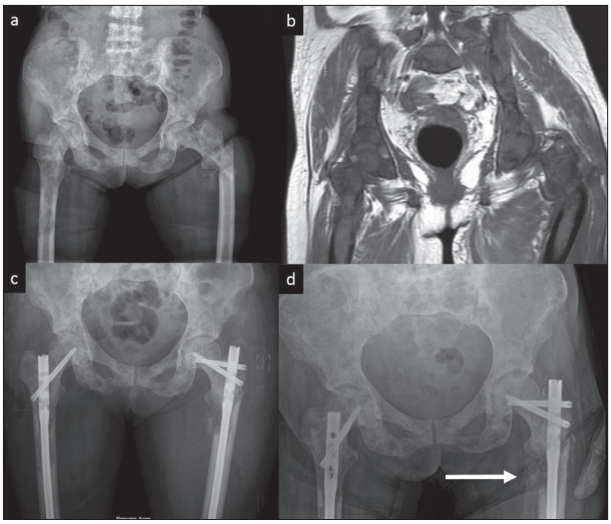


**Figure 3.** Case 17: a) Anteroposterior view of the bilateral femoral metastatic lesions with left impending and right complete fracture (62-year-old female, breast carcinoma); b) Magnetic resonance imaging view of the bilateral femoral metastatic lesions; c) Postoperative view of the bilateral femur; d) three-month follow-up; Callus formation at fracture site, indicating complete response to adjuvant radiotherapy (white arrow)

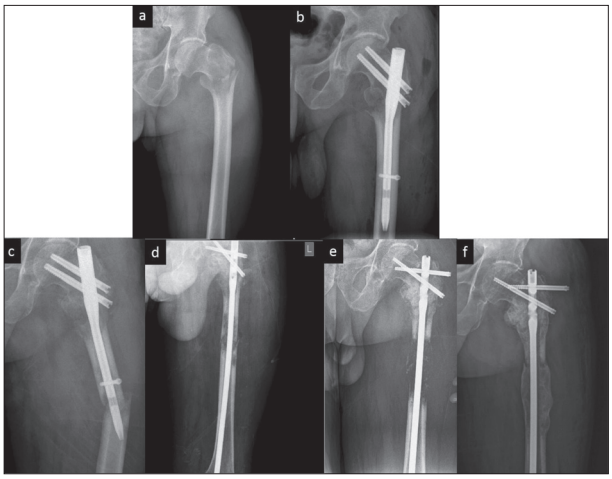


**Figure 4.** Case 18: a) Anteroposterior view of the femoral metastatic lesion with a pathologic fracture of the proximal third of femur which is fixed with intramedullary nail (71-year-old male, lung carcinoma); b) Postoperative radiograph; c) At three-month follow-up, patient presented with fixation failure due to short intramedullary implant; anteroposterior radiograph demonstrated stress riser effect of the short nail; d) Postoperative view of long revision intramedullary nailing; e) Radiotherapy was given due to progression; f) 15-month follow-up; Pain relief and satisfactory clinical improvement was obtained; Screw-out was observed but this complication did not interfere with patient's clinical outcome

femur fracture underwent prophylactic fixation for right impending femur fracture (Figure 3). The only complication requiring reoperation was a refracture distal to short proximal femoral nail due to tumor recurrence. This was also revised with a long intramedullary implant with bony union thereafter (Figure 4). All patients were alive at the time of last follow-up.

## DISCUSSION

There are only few studies evaluating outcomes and bone healing after fixation of pathological fractures and adjuvant radiotherapy for treatment of metastatic bone lesions [3, 6, 9]. Previous studies generally put emphasis on surgical decision making based on survival, clinical outcomes and perioperative complications [10].

The data on bone healing potential after surgical fixation of complete pathologic fractures dates back to early 1980s. Apart from this, clinical effects of radiotherapy in pathologic fractures are also inconclusive [1]. It is generally assumed that postoperative radiotherapy will increase the likelihood of delayed union and nonunion; however, adjuvant multifraction RT has been recommended to accelerate bone healing, control disease progression and avoid implant failure in the literature [10, 11].

Harada et al. [7] suggested that healing of the metastatic lesions can be accomplished with only radiotherapy in impending fracture cases and non-progressive metastatic bone disease.

In complete pathologic fractures, bone healing can be improved with internal fixation and adjuvant radiotherapy. Gainor and Buchert [6] demonstrated that internal fixation of pathologic fractures resulted in improved union in cases who survived six months or longer. They also added that union rate in patients receiving adjuvant radiotherapy was found to be higher in internal fixation group compared to cast immobilization. Additionally, internal fixation was recommended as necessary for patients whom received greater than 30 Gy dose due to its inhibitory effect on callus formation [6].

Townsend et al. [12] compared clinical results of 29 patients who underwent surgery alone with 35 patients who received postoperative adjuvant radiotherapy. The median dose of RT was 30 Gy. On multivariate analysis, postoperative RT has been found to be an independent positive factor for functional improvement with decreased secondary surgery rates; however, they did not evaluate union [12].

Redmond et al. [3] administered adjuvant radiotherapy on 11 cases with 14 humerus pathologic fractures whom underwent static IMN. They obtained good to excellent results with osseous healing in seven of eleven fractures whom survived at least three months. No major complication except one case who underwent screw removal due to irritation was noted. [3] Atesok et al. [9] reported on 22 pathologic humeral fractures managed with IMN 20 of whom received adjuvant RT. Union was observed in 88% (15/17) of all the patients who survived at least three months after the procedure. Ofluoglu et al. [13] treated 23 patients with pathological humerus fractures who underwent IMN and low dose adjuvant RT. Four weeks following the surgery, 20 patients were alive and 12 cases had complete union. Van Geffen et al. [14] reported that they experienced similar pain scores with remarkable less complication in radiotherapy group relative to non-irradiated cases after IMN although there are few RT cases (21% irradiated *vs.* 14% not irradiated). Moura et al. [15] reported on 82 patients with pathologic humerus fractures treated with IMN and adjuvant radiotherapy. They stated that closed

unreamed static locked nailing was a fast, safe, and effective surgery with low morbidity. He also emphasized that closed IMN decreased the risk of impaired healing after adjuvant radiotherapy.

Moon et al. [16] performed IMN in 40 patients with sarcoma metastasis. In total, 11 patients received either preoperative or postoperative radiotherapy. Fracture union was not achieved in the majority of cases; however, they concluded that multimetastatic patients with primary bone and soft tissue sarcomas and poor survival had palliative benefit [16].

Our findings are in accordance with these studies. Clinical improvement and radiological healing were achieved in the short term, regardless of over-all disease specific survival from primary disease. All these studies indicate that benefits of multifraction RT outweigh the risks reported in literature. According to radiological outcomes of the current study, it is possible that this regimen will boost bone healing after surgical fixation of pathologic fractures. Compared to preoperative RT, postoperative RT is more advantageous in terms of lower risk of wound complications and availability of pathologic evaluation for individualized adjuvant treatment. To minimize these potential risks, our study group received low dose postoperative RT (20 Gy) after intramedullary stabilization with a complete radiologic response. Compared to endoprosthetic reconstruction and plate fixation, these patients may benefit from closed unreamed IMN with less postoperative wound problems in a manner that will allow patients for immediate commencement of radiotherapy and medical oncology treatment.

Another important issue is that proximal and distal locking should be performed to ensure enhanced stability. The only revision was due to a short proximal femoral nail in our study cohort. This representative case demonstrated that stabilizing the entire length of the long bone obviates the need of re-surgery due to disease progression. Protecting the entire bone has also been associated with increased survival in a recent study [17]. Like femoral lesions, all humeral lesions have been satisfactorily managed with IMN. The same technical rules were applied for these lesions. Proximal and distal static locking was performed. Although cementation of the fractured fragments provides initial stability, in long term implant failure risks increase as the fracture does not heal due to cement [18]. Intramedullary reaming in pathologic fracture is another important point. This issue is controversial and we did not prefer reaming due to possible tumoral contamination and vascular tumoral spread. In line with our opinion, a recent study by Younis et al. [19] supported the use of unreamed IMN in pathologic humerus fractures with the advantages of less blood loss, systemic complications and decreased hospital stay.

For femoral neck and head lesions, endoprosthetic reconstruction should be preferred. Nevertheless, given their high implant costs, fixation with long IMN may be a more cost-effective option for pathologic fractures in metaphyseal and diaphyseal long bone lesions by avoiding additional surgeries due to complications specific to arthroplasty (i.e., dislocation, intraoperative bleeding, infection) [20].

Osteosynthesis with plate fixation is less preferred for pathological fracture fixation as quality of bone stock proximal and distal to fracture is abnormal and reliable fixation may be harder to achieve. Hoellwarth et al. [21] analyzed 105 interventions due to pathologic humerus fractures which were managed by photodynamic therapy, IMN, and plate fixation. Although reoperation rates were similar at each time point, IMN had lowest rate of broken implants compared to plate fixation. This study supports our preference of IMN against plate fixation.

Furthermore, the intramedullary nail stabilizes the full metaphyseo-diaphyseal length of the bone and is a load sharing device compared to a plate which is a load bearing device. Lastly, one important point is that solitary or oligo bone lesions due to solid organ metastases deserve a different approach. Wide resection as is the norm for the primary malignant bone tumors, that may prolong survival and be curative in selected cases. Prior to pathological fracture stabilization, the surgeon should be sure about the histologic subtype of the malignant cells. Diagnostic work-up for these lesions should follow the established orthopedic oncology principles.

Limitations of this study include a small sample size and retrospective study design. Although femur and humerus are most commonly affected long bones, tibia is another common site for pathologic fractures where IMN is advocated. There is no control group for comparison and further studies comparing IMN to plate fixation with adjuvant radiotherapy or RT alone in patients who are not eligible for surgery will be very helpful.

## CONCLUSION

In multimetastatic cases, closed unreamed IMN of humeral and femoral diaphyseal pathologic fractures with adjuvant low dose RT offered good osseous healing with minimal complications and improved quality of life as reflected in their Musculoskeletal Tumor Society rating scale scores.

**Conflict of interest:** None declared.

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# Клиничка и радиолошка евалуација спојених патолошких прелома после затвореног интрамедуларног закивања и помоћне радиотерапије – ретроспективна студија

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**САЖЕТАК**

**Увод/Циљ** Патолошки преломи су тешке компликације код метастатске болести костију. Лечење ових стања варира и укључује системске терапије и хируршке интервенције. Мањак доказа и даље постоји за стандардизовану негу. Циљ ове студије је да се анализира одговор на радиолошко лечење и клиничке исходе после интрамедуларног закивања, и помоћне радиотерапије у комплетним патолошким преломима бутне или надлактичне кости.

**Методе** Ретроспективно је прегледано 19 болесника који су имали патолошки прелом. Подаци о демографским карактеристикама, клиничким исходима и радиолошким сликама добијени су из болничких картона. Сви болесници у овој студији лечени су затвореним, неримованим интрамедуларним закивањем (НИЗ) и помоћним третманом зрачења.

**Резултати** Ублажавање бола и пуни опсег покрета постигнути су код свих болесника. Средњи постоперативни резултати Друштва за мишићно-скелетне туморе на последњем праћењу били су 69% (распон 50–85%). Сви болесници су показали потпуно радиографско зарастање после два–шест месеци. Само једном болеснику је била потребна реоперација ради прелома на врху клина, који је замењен дужим клином.

**Закључак** Наша студија је показала да су патолошки преломи управљани затвореним неинфицираним НИЗ и адјувантном мултифракционом радиотерапијом од 20 *Gy* дали добре клиничке резултате са потпуним радиолошким одговором без обзира на очекивани животни век болесника, адјувантне третмане и укупно стање. Затворени неримовани НИЗ такође је повезан са смањеним временом операције код ових високоризичних болесника.

**Кључне речи:** патолошки прелом; интрамедуларно закивање; помоћна радиотерапија; зарастање костију

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

# Alcohol abuse as a risk factor for developing thyroid cancer

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**SUMMARY**

**Introduction/Objective** Alcohol abuse influence on developing thyroid cancer is controversial. While some studies consider it a protective factor, others deny any impact on thyroid cancer.
The objective of the paper was to establish a possible link between alcohol abuse and certain types of thyroid cancers.

**Methods** The retrospective study included 502 patients with thyroid cancer and a control group of 600 patients with benign forms of thyroid diseases (e.g. nodular, multinodular, and toxic nodular goiter). Thyroid cancer patients were divided into four groups: I – papillary, II – medullary, III – anaplastic, and IV – follicular carcinoma, and grouped by sex, age (< 30 years; > 30 years) and alcohol abuse, as defined by the World Health Organization.

**Results** Thyroid cancer patients were predominantly male of younger age. This distribution difference was statistically significant in groups I and II ($p < 0.001$). Of total 10 (0.9%) patients with chronic alcohol abuse, eight (1.6%) had thyroid cancer, while two (0.3%) belonged to the control group ($p < 0.001$). In thyroid cancer patients, chronic alcohol abuse was absent from groups III and IV. Distribution in groups I and II was six (1.6%) and two (2%), respectively ($p < 0.001$).

**Conclusion** Alcohol abuse deserves to be considered as a risk factor for papillary and medullary forms of thyroid cancer, while it does not stay the same for anaplastic and follicular thyroid cancers.

**Keywords:** thyroid cancer; papillary cancer; medullary cancer; anaplastic cancer; follicular cancer; alcohol abuse

## INTRODUCTION

Apart from the social, mental, and behavioral disturbances, chronic alcohol abuse (CAA) causes and/or affects many serious somatic diseases, including cancer [1, 2]. Among the surgical patients, different drinking patterns may also affect specific features in the management of anesthesia, patient behavior, and different complications in the perioperative period [2, 3, 4].

Alcohol abuse was addressed as a possible cause or contributing factor for thyroid cancers and other non-cancerous thyroid diseases by many observational studies. The results of these studies are different, sometimes inconclusive, or even conflicting [5, 6]. Yet, the abundant evidence of the increasing incidence of thyroid cancers, attributed mainly to increased detection of papillary thyroid cancer, deserves a careful analysis of all possible risk factors, including CAA [7, 8].

We designed a retrospective, cross-sectional study to determine a possible influence of CAA on thyroid cancer incidence.

The objective of this study was to determine if CAA was a risk factor for thyroid cancer in general, as well as for different types of thyroid cancer (i.e. I – papillary, II – medullary, III – anaplastic, and IV – follicular carcinoma).

## METHODS

A total of 1102 consecutive patients who underwent thyroid surgery at the Center for Endocrine Surgery, Clinical Center of Serbia, during three consecutive years were analyzed. The study group included 502 patients with different forms of thyroid cancer and the control group included 600 patients with benign or degenerative diseases of the thyroid gland. Thyroid cancer patients were divided according to histopathological findings into four groups: I – papillary carcinoma (380 patients, 75.7%), II – medullary carcinoma (102 patients, 20.3%), III – anaplastic carcinoma (10 patients, 2%), and IV – follicular carcinoma (10 patients, 2%). The control group consisted of patients with thyroid nodule (233 patients, 38.8%), multinodular goiter (337 patients, 56.2%), and toxic adenoma (30 patients, 5%) (Table 1). All patients with autoimmune thyroid diseases were excluded from the study.

Patients' records were used to collect demographic (age, sex) and clinical data (present and past diseases and surgeries) as well as socio-epidemiological questionary (exposures, habits, abuses) with a particular accent on CAA (type, dose, pattern), as defined by the World Health Organization [9]. For this study, CAA was

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defined at least as moderate alcohol intake and/or alcohol dependence. Accordingly, moderate alcohol intake implies daily consumption of 1–2 (women) or 3–4 (men) standard drinks. Standard drink implies 0.03 L of distilled beverage or 0.2 L of vine or 0.3 L of beer. Alcohol dependence is present if at least 3/7 criteria were present in the past year: craving, the irresistible need for alcohol; increased tolerance; loss of control; abstinence syndrome; use of the same or of related substances to relieve the withdrawal syndrome; progressive neglect of alternative pleasures (socializing, hobbies, sports, etc.) and specific drinking pattern.

All data were collected into an electronic database (IBM SPSS Statistics for Windows, Version 26.0; IBM Corp., Armonk, NY, USA) and presented in tables. Pearson's $\chi^2$ test was used to compare the difference between categorical variables, and the p-value was set at < 0.05.

This study was approved by the Ethical Committee of the Faculty of Medicine, University of Belgrade (decision No. 1575/7).

## RESULTS

Papillary carcinoma was the most common form in thyroid cancer group (75.7%) while multinodular (56.2%) and nodular goiter (38.8%) made 95% of the control group pathologies (Table 1).

The mean age of patients was similar in the group with thyroid cancer (50.34 years) and the control group (50.88 years). Patients under the age of 30 were significantly more represented in the cancer than in the control group (13.3% *vs.* 6.8%, p < 0.001). The same is true for the male sex distribution (19.7% *vs.* 11.7%, p < 0.001) (Table 2).

**Table 1.** Distribution of patients by diseases

| Disease | n (%) |
|---|---|
| Thyroid cancer group | |
| I – papillary cancer | 380 (75.7) |
| II – medullary cancer | 102 (20.3) |
| III – anaplastic cancer | 10 (2) |
| IV – follicular cancer | 10 (2) |
| Total | 502 (100) |
| Control group | |
| Nodular goiter | 233 (38.8) |
| Multinodular goiter | 337 (56.2) |
| Toxic nodular goiter | 30 (5) |
| Total | 600 (100) |

**Table 2.** Distribution of patients by age and sex

| Characteristics | Thyroid cancer (n = 502) n (%) | Control group (n = 600) n (%) | p |
|---|---|---|---|
| Age | | | |
| ≤ 30 years | 67 (13.3) | 41 (6.8) | < 0.001 |
| > 30 years | 435 (86.7) | 559 (93.2) | n.s. |
| Sex | | | |
| Male | 99 (19.7) | 70 (11.7) | < 0.001 |
| Female | 403 (80.3) | 530 (88.3) | n.s. |

n.s. – non-significant

Group I (i.e. papillary carcinoma), compared with the control group, had significantly more patients under the age of 30 (14.5% *vs.* 6.8%, p = 0.000) and patients of male sex (18.7% *vs.* 11.7%, p = 0.002). Group II (i.e. medullary carcinoma) had no age difference but did show a significant male predominance, compared to control (26.5% *vs.* 11.7%, p = 0.000). All patients from group III (i.e. anaplastic carcinoma) were over the age of 30, but this fact provided no statistically significant difference to the control group (100% *vs.* 93.2%, p = 0.392). Group IV (i.e. follicular carcinoma) had no significant difference in age (p = 0.695) and sex (p = 0.251) distribution, compared with the control group, despite old age and female predominance (Table 3).

There was an overall significant difference in CAA distribution between the cancer and the control group (1.6%, 8/502 patients *vs.* 0.3%, 2/600 patients, p < 0.001). The presence of CAA was recorded only in groups I and II of thyroid cancer patients, with incidences significantly higher than the control group (1.6% and 2% *vs.* 0.3%, p = 0.034 and p = 0.044). There were no records of CAA in groups III and IV. The incidence in these groups was significantly lower than that of the control group (p = 0.001) (Table 4).

## DISCUSSION

Almost 10% of men in Serbia had alcohol use disorders, compared to 2.1% of women [10]. Alcohol consumption is an attributable risk for 5.1% of all-cause deaths in our country [11]. There is epidemiological evidence that alcohol causes cancer at seven sites in the body (oropharynx, larynx, esophagus, liver, colon, rectum, and breast), although without exact and complete knowledge of underlying biological mechanisms [1]. Pandemic increase in thyroid cancer incidence over the past two decades resulted in significant efforts towards early detection and therapy, but also deeper analyses of possible toxic, environmental, and socio-economic causes [12].

Many studies, so far, have addressed alcohol abuse as a possible risk factor for thyroid cancer [6, 13, 14]. A recent and, so far, the most comprehensive meta-analysis of 33 observational studies which involved a total of 7725 thyroid cancer patients and 3,113,679 participants without cancer suggested that alcohol intake may decrease the risk of thyroid cancer. In a subgroup meta-analyses by geographic region, alcohol intake was associated with a decreased risk of thyroid cancer in the American, but not in the European or Asian regions [6]. Previous studies of risk factors for thyroid cancer published in Serbia also have not found any correlation with CAA [15, 16].

However, our study has shown that younger (under the age of 30 years) male patients with history of CAA were at a higher risk for overall and particularly for papillary (group I) and medullary (group II) forms of thyroid carcinoma, compared to the control group of non-cancerous thyroid patients.

Results of a study from South Korea, which has the highest incidence of thyroid cancers in the world, based on data collected from 12,276 individuals, among others, reveals CAA (OR: 1.89; 95% CI: 1.08–3.32) as a significant risk factor for thyroid cancer [14]. Data from the Thyroid

**Table 3.** Distribution of patients by age and sex according to the type of thyroid cancer

| Parameter (p-value) | Control n = 600 n (%) | I – Papillary n = 380 n (%) | II – Medullary n = 102 n (%) | III – Anaplastic n = 10 n (%) | IV – Follicular n = 10 n (%) |
|---|---|---|---|---|---|
| Age | | | | | |
| ≤30 | 41 (6.8) | 55 (14.5) | 11(10.8) | 0 (0) | 1 (10) |
| > 30 | 559 (93.2) | 325 (85.5) | 91 (89.2) | 10 (100) | 9 (90) |
| p | | (0.000) | (0.159) | (0.392) | (0.695) |
| Sex | | | | | |
| Male | 70 (11.7) | 71 (18.7) | 27 (26.5) | 1 (10) | 0 (0) |
| Female | 530 (88.3) | 309 (81.3) | 75 (73.5) | 9 (90) | 10 (100) |
| p | | (0.002) | (0.000) | (0.871) | (0.251) |

**Table 4.** Alcohol abuse among thyroid cancer patients

| Alcohol abuse | All cancers n = 502 n (%) | I – Papillary n = 380 n (%) | II – Medullary n = 102 n (%) | III – Anaplastic n = 10 n (%) | IV – Follicular n = 10 n (%) |
|---|---|---|---|---|---|
| Yes | 8 (1.6) | 6 (1.6) | 2 (2) | 0 (0) | 0 (0) |
| No | 494 (98.4) | 374 (98.4) | 100 (98) | 10 (100) | 10 (100) |
| p* | < 0.001 | 0.034 | 0.044 | 0.001 | 0.001 |

*Statistical significance was measured against the control group incidence of alcohol abuse;
**bold** – significantly higher incidence;
***bold-italic*** – significantly lower incidence

Cancer Longitudinal Study on 2258 thyroid cancer patients and 22,580 healthy individuals showed that acute high-dose and chronic lifetime exposure (> 31 years) to alcohol are linked to an increased risk of developing thyroid cancer [13]. In addition to these findings, another study from the same country, comparing health behaviors of 942 thyroid cancer survivors with 9420 matched non-cancer controls, found that clustering of smoking, drinking, and physical inactivity is more often present in male thyroid cancer survivors [12].

Inconsistent reports from different studies of CAA and thyroid cancer are commonly based on a small number of patients with cancer involved (i.e. less than 500), restriction to certain patient sub-population (e.g. postmenopausal females), and failure to evaluate the effect modifiers (e.g. cigarette smoking, obesity, physical inactivity, etc.) [6, 12, 13, 14].

Rare studies have precisely defined thresholds of alcohol intake (i.e. amount, duration) in terms of thyroid cancer risk. Honnamurthy et al. [17] revealed a significant influence of alcohol consumption duration, but not alcohol dependence on thyroid function tests. Hwang et al. [13] report a reduction in thyroid cancer risk with decreased alcohol consumption (25 g or less) per event (i.e. mild to moderate consumption) and a drinking duration of less than 10 years, compared to never-drinkers. In contrast, acute heavy alcohol consumption (151 g or more per event), consumption of alcohol for 31 or more years, was associated with an increased risk for thyroid cancer in both men and women [13]. Our study has set the threshold of alcohol intake to moderate and higher levels, which may explain similar results in terms of a positive correlation between CAA and thyroid cancer.

The precise mechanism by which alcohol possibly induces thyroid oncogenicity remains unclear. It has not yet been firmly established whether the alcohol at certain blood levels and duration of exposure has a direct toxic effect on thyroid cells, but abnormal functioning of the hypothalamic–pituitary–thyroid axis has been observed in chronic alcoholics. Experimental data have shown that chronic ethanol exposure in rats elevated thyroid-releasing hormone messenger RNA in hypothalamic neurons. Whether this effect, in the long term, may produce hyperproliferation and/or cancerogenesis, remains unclear [18, 19, 20].

This study has several limitations. Being a retrospective cross-sectional study, its results necessitate further validation in a wider scope, prospective study, including a larger number of patients and variables to allow statistics that are more powerful.

## CONCLUSION

The results of our study suggest that the CAA is positively correlated with the appearance of papillary and medullar forms of thyroid carcinoma, whereas in anaplastic and follicular forms this correlation was absent. Further prospective investigations are needed to confirm these findings.

**Conflict of interest:** None declared.

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# Злоупотреба алкохола као фактор ризика за развој рака штитне жлезде

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**САЖЕТАК**

**Увод/Циљ** Утицај злоупотребе алкохола на развој рака штитне жлезде је контроверзан. Док неке студије то сматрају заштитним фактором, друге негирају икакав утицај на рак штитне жлезде.

Циљ рада је анализа могуће везе између хроничне злоупотребе алкохола и одређених врста рака штитне жлезде.

**Методе** Ретроспективна студија обухватила је 502 болесника оболела од рака штитне жлезде и контролну групу од 600 болесника са доброћудним облицима болести ове жлезде (нпр. нодуларна, мултинодуларна и токсична нодуларна струма). Оболели од рака штитне жлезде подељени су у четири групе: I – папиларни, II – медуларни, III – анапластични и IV – фоликуларни карцином и груписани по полу, узрасту (< 30 год.; > 30 год.) и злоупотреби алкохола, у складу са дефиницијом СЗО.

**Резултати** Оболели од рака штитне жлезде били су претежно мушкарци млађег узраста. Ова разлика у расподели је статистички значајна у групама I и II ($p < 0,001$). Од укупно 10 (0,9%) болесника са хроничном злоупотребом алкохола, 8 (1,6%) њих је имало рак штитне жлезде, док су 2 (0,3%) припадала контролној групи ($p < 0,001$). Код оболелих од рака штитне жлезде хронична злоупотреба алкохола није забележена у групама III и IV. Дистрибуција у групама I и II је била 6 (1,6%), односно 2 (2%) ($p < 0,001$).

**Закључак** Злоупотреба алкохола заслужује да буде размотрена као фактор ризика за папиларне и медуларне облике рака штитне жлезде, док то исто не важи за анапластични и фоликуларни рак штитне жлезде.

**Кључне речи:** рак штитњаче; папиларни рак; медуларни рак; анапластични рак; фоликуларни рак; злоупотреба алкохола

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# Analysis of the venomous snakebite patients treated in the Užice General Hospital (Western Serbia) between 2006 and 2018

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**SUMMARY**

**Introduction/Objective** A countrywide survey of venomous snakebites was never conducted in Serbia: the making of a central register was initiated only in 2018. We collected and analyzed the available data regarding venomous snakebites in the Užice region (Western Serbia). The previous analysis from this hospital was published in 1968.

**Methods** We retrospectively analyzed the data regarding the venomous snakebite patients treated at the Užice General Hospital between 2006 and 2018 and compared these with the data from the 1960s, from one more hospital in Serbia, and from two ex-Yugoslav countries.

**Results** In 13 years, 249 persons were treated. Of all cases, 10.4% were with inconspicuous symptoms (mild pain at the place of bite), 68.7% were with mild to moderate symptoms, and 20.9% were more or less severe. No fatalities were recorded.

**Conclusion** Although usually not a life-threatening issue, venomous snakebites are quite common and can cause serious complications. With proper education, many can be avoided. Also, bearing in mind not only the biodiversity *per se* but also the importance of snakes' venoms for the making of various medically important products, we emphasize the need for proper protection of all three venomous snake species in Serbia, namely *Vipera ammodytes* (nose-horned viper), *V. berus* (European adder), and *V. ursinii* (meadow viper).

**Keywords:** envenomation by *Vipera ammodytes* and *Vipera berus*; interdisciplinary cooperation and education; paucity of information; protected species conservation

## INTRODUCTION

As in other countries of former Yugoslavia (except Slovenia), only three autochthonous venomous snake species exist in Serbia, *Vipera ammodytes* (nose-horned viper), *V. berus* (adder), and *V. ursinii* (meadow viper), and all are protected by law [1]. To the best of our knowledge, in the surroundings of the city of Užice (Western Serbia, 43.859° N, 19.849° E, 10×10 km Universal Transverse Mercator, UTM square DP05), only the nose-horned viper can be found (1 provides occurrence maps and lists of localities). The nearest localities (UTM squares in brackets) in South-western and Western Serbia where the presence of adder was confirmed [1] or where suitable habitats exist (boreal forest or/and alpine pastures), are the mountains Zlatibor (CP74, along with *V. ammodytes*), Zlatar, Golija (DP20), Javor (DP21, with *V. ammodytes*), Kamena Gora (CN89, with *V. ammodytes*), and Jadovnik (DN09, with *V. ammodytes*) [1]. The third viper species was found only in remote places in the southwestern margin of the country, at altitudes over 1600 m [1].

Venoms of *Vipera* species are combinations of proteins, polypeptides and enzymes with specific chemical and biological activities, primarily used to subdue prey. Oxidases, proteases, esterases, hemolysins, neurotoxins, cardiotoxins, myotoxins and factors that modify the coagulation system act on muscles, cardiovascular and neural systems, blood cells, leading to various damages of organs and organ systems [2–8]. Clinical manifestations of envenomation range from negligible to fatal; however, the latter are very rare and often occur in particular cases of bites, for instance, to the neck or directly to blood vessels, in very young patients or as a complication of some chronic disease in the elderly [6–12]. In high contrast to the popular belief, European vipers "are not considered fatal" because the lethal doses for average humans are higher than the amounts of venom the vipers can produce [12]. According to the national Statistical Office, between 2008 and 2017 only four people in Serbia died after being bitten by venomous snakes.

Although Serbia is nowhere near any of the seriously affected areas [13], bites by venomous snakes do occur in our country – and their occurrences are not being analyzed. We found only two papers from Serbia with multi-year data regarding venomous snakebites: 155 cases

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from Užice (1960–1968) and 264 from Priština (1981–1997) [2, 8]. Three other papers we obtained present nine cases in total [14–16]. Also, a report exists of an anaphylactic reaction following the bite of a non-venomous snake (*Zamenis longissimus*): although "examines excluded toxic effects of snakebite," in the description the author mentions "two punctiform wounds from bites," which raises suspicion [17]. Contrary to some of the most severely affected regions of the world [e.g. 13], in Serbia, importantly [18], the antivenom is produced in a national institute [19, 20], it is readily available and adequately used.

The work on the epidemiology of Viperidae snakebites in Central and Southeastern Europe started recently. However, at the time of this manuscript preparation, the data regarding Serbia was not available [7, 21]. Therefore, our intention was not only to present the information from a single hospital but also to inspire medical workers in other parts of Serbia to collect, analyze and publicize the data they have an insight into. With a better overview of the outcomes of the snake–human encounters, we can better design the necessary education of laypeople regarding snakes. Also, medical workers could expand their knowledge of the distribution of vipers in Serbia and novel approaches in the treatment of venomous snakebites so they could standardize and improve the procedures not only of therapy but also of data gathering, severity score grading, etc. [6, 7].

## METHODS

We inspected medical records of the patients treated in the Department for Infectious and Tropical Diseases of the General Hospital in Užice (UGH) between 2006 and 2018. No personal information was used except for the sex and ages of the patients, therefore, the approval of the ethics committee was not necessary.

The UGH is responsible for a population of approximately 300,000 persons. During the entire period covered in this overview, the hospital and its local ambulances were well supplied with antivenom and medications needed for symptomatic therapy. All physicians are well informed and prepared to properly react in the case of a venomous snakebite.

Snakebites were diagnosed according to anamnesis data, through clinical monitoring, and according to the information provided by the patients regarding the snakes that inflicted the bites [22]. We considered three main types of data:

**Epidemiological data:** sex, age, month in a year, and activity of patients at the time of the bite, the area/locality where the bite occurred, and the reported snake species. Activities of bitten persons were categorized as follows: people who were performing their usual everyday activities related to agriculture (in crop fields or gardens); local persons who were bitten during walks or picnicking in suburban/rural areas (including the picking of berries, fishing, etc.); and tourists from other parts of Serbia, i.e., foreigners.

**Clinical data:** localization of the bites (part of the body), the severity of the clinical picture, and the administered therapy. The grading of the clinical courses of the disease was made according to the Severity score of snakebites [23], with grades from 0 (no envenomation; fang marks and minimal pain) to 4 (very severe envenomation).

**Laboratory analyses:** We considered blood parameters, coagulation status, urea and creatinine, sodium, potassium, alanine aminotransferase, and aspartate aminotransferase; urine was also analyzed. We performed the standard laboratory tests used in Serbia.

Where appropriate, statistical analyses were performed, in Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA) or using an online calculator (www.quirks.com/tools/calculator). All graphs were made in Excel.

## RESULTS

### Epidemiology, treatment, and outcomes

The data about 249 snake envenomation cases were collected during the 13 year period, 19.15 on average per year. Of those, 30 patients (12%) were treated in primary health care, and 219 (88%) were hospitalized. Antivenom was given to 234 (94%) bitten persons. In all cases, we used the Viekvin® equine antiserum produced by the Torlak Institute of Virology, Vaccines and Sera (5 mL vials). According to the producer, 1 mL of the preparation can neutralize at least 100 $LD_{50}$ of *V. ammodytes* venom and 50 $LD_{50}$ of *V. berus* venom [20]. The serum was administered subcutaneously or intramuscularly to all the patients. In 115 (46.2%) cases, symptomatic therapy was administered (antiedematose and analgesic medications). Antibiotics were given to 132 (53%), and corticosteroids to 54 (21.2%) patients. Anti-tetanus protection was given to all patients who had not previously been vaccinated (204: 81.9%). In five cases (2%), local necroses developed, which were surgically treated. The 21 (8.4%) patients with the most severe symptoms were treated at the Intensive Care Unit for 24–48 hours, and were later transferred to the ward for further observation.

In Table 1, we provided the numbers and percentages of the main manifestations of snake venom poisonings. Almost a quarter of patients experienced nausea, vomiting, and diarrhoea. Thrombocytopenia ($< 150 \times 10^9$/L) was the second most frequent manifestation of envenomation. Acute renal failure was accompanied by elevated urea and creatinine, hypokalemia, and proteinuria. Liver damage manifested as

**Table 1.** Symptoms and signs in organ systems, and abnormalities in laboratory analyses, in the patients treated for snakebites in the Užice General Hospital

| Symptoms | Numbers (percentages) |
|---|---|
| Gastrointestinal disorders | 58 (23.3%) |
| Thrombocytopenia | 43 (17.3%) |
| Acute renal failure | 12 (4.8%) |
| Coagulation disorder | 10 (4%) |
| Shock | 9 (3.6%) |
| Liver damage | 7 (2.8%) |
| Visible bleeding | 7 (2.8%) |
| Neurotoxic symptoms | 4 (1.6%) |

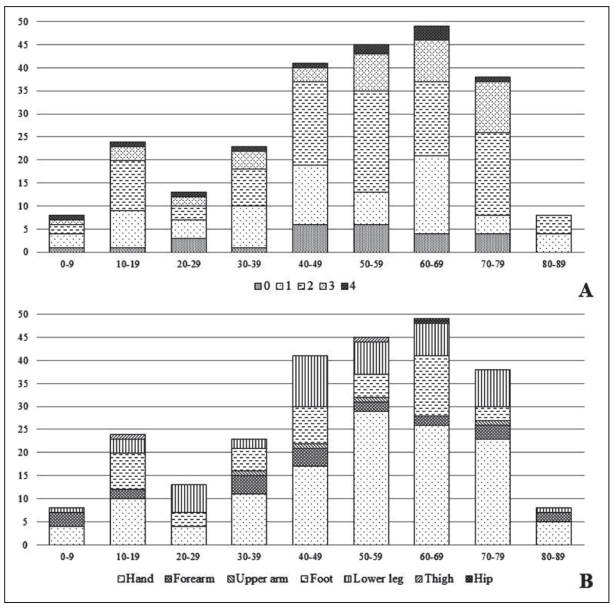


**Figure 1.** (A) Numbers of bites and 0–4 severity grades in 10-year age groups; (B) Bite sites (bitten body parts) in different age categories

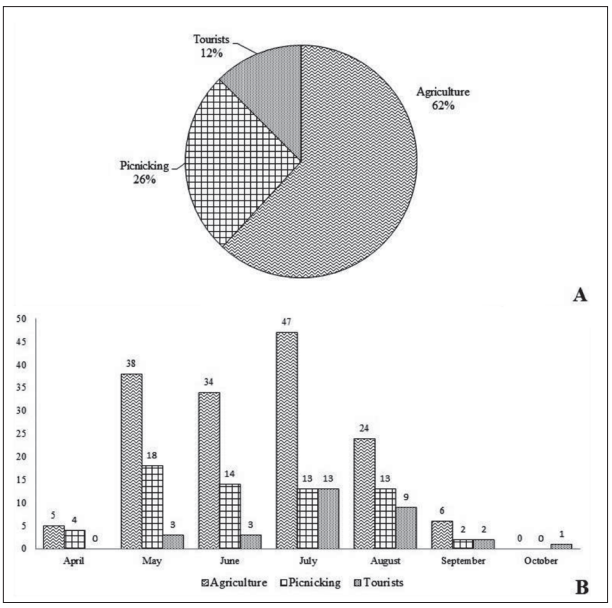


**Figure 2.** (A) Percentages of patients according to their activity/occupation; (B) Seasonal variations in numbers of bites according to the patients' occupation/activity

elevated levels of liver enzymes alanine aminotransferase and aspartate aminotransferase. Visible bleeding expressed as petechiae and hematomas, rarely as hematuria; in only one patient bleeding from the digestive tract was recorded (hematemesis and melena). Neurotoxic symptoms expressed as transient limb pareses at the bites sites.

More male (59.04%) than female (40.96%) patients were bitten (one-sample t-test between percents): $t_{248} = 2.901$, $p = 0.004$.

The average time elapsed between the bite and antivenom administration was 53.15 minutes (range 15–300 minutes). Milder clinical pictures, e.g. grade 1, developed after shorter times to antivenom administration (37.61 minutes on average) compared to grade 4, which developed in patients treated on average 99.54 minutes after the bite. The Pearson's coefficient R was 0.5365, with a high statistical significance of correlation ($p < 0.000$) between severity grades and minutes to antivenom.

On average, the patients were hospitalized for 2.5 days (range 0–9). All patients with the symptoms grade 4 were held for six or more days.

Calculated against the 300,000 population, the annual incidence of venomous snakebites per 100,000 persons ranged from 4.3 to 8.7, with an average of 6.4.

## Severity grades and ages of the patients

The percentages of 0–4 severity grades were 10.4, 27.7, 41.0, 16.5, and 4.4. Not a single death was recorded as a consequence of a venomous snakebite. The youngest patient was a baby boy less than a year old (grade 4), and the oldest a man of 88 (grade 2). The correlation between ages and severity grades was not significant (Pearson's $R = 0.0628$, $p = 0.324$). Both the bites inflicted certainly by the nose-horned vipers and those possibly made by adders caused the symptoms of all grades.

In Figure 1A, we graphed the numbers of bitten individuals in 10-year age groups, with their symptoms severity grades. The highest number of patients were the people in their 60s. Among those younger than 40, the most bites were recorded in the 10–19 years age group. In all age categories, more bites were inflicted on the upper extremities (Figure 1B). Significantly more people had bites on hands and arms (62.2% of the whole sample) compared to feet and legs (37.80%): $t_{248} = 3.97$, $p < 0.001$ (one-sample t-test between percents).

## Percentages of bites related to activity/occupation

Almost 62% of our patients were people engaged in some agricultural activity; a quarter were local people picnicking, and the lowest number of snakebites was recorded in tourists (Figure 2A).

In the "agriculture" group, significantly more people were bitten in the upper compared to lower extremities ($t_{153} = 7.041$, $p < 0.001$). Of those who spent time outdoors picnicking in a suburban/rural environment (25.7%) similar numbers of patients were bitten in upper and lower extremities ($t_{63} = 0.256$, $p = 0.803$), and among tourists, presumably hikers (12.4%), the majority were bitten in lower extremities ($t_{30} = 2.572$, $p = 0.015$). One-sample t-tests between percents were used.

The numbers of bites among seasons varied differently in the three occupations/activity categories (Figure 2B). Agricultural activities and hiking were the riskiest in July while picnicking appeared hazardous in May.

## Annual and seasonal distribution of venomous snakebites

In the analyzed 13-year-long period, up to two-fold oscillations in the annual numbers of bites occurred, in intervals

of approximately four-five years (Figure 3A). The first bites were recorded in April, and their numbers were the highest in July; October was the last month to have records of snakebites (Figure 3B).

### Snake identification

In 38.96% of cases, the snakes were not identified by the bitten persons. Of those who were sure that some venomous snake bit them, 44.58% claimed it was an adder. An exceptionally low number of patients reported nose-horned vipers (16.47%). Of the four patients with neurological symptoms, only one claimed that an adder bit him; the rest reported *V. ammodytes*.

## DISCUSSION

In the past several years, venomous snakebites and their treatment, as well as the respective education/training of both the general population and medical workers, are gaining global attention, and actions have been announced/undertaken to reduce their impact [6, 7, 18, 24]. Although mortality due to European *Vipera* spp. bites is generally low, the fear and disgust regarding snakes is widespread and deeply rooted [7–12, 25].

We compared our data with those previously published from the UGH and with four other publications depicting high numbers of venomous snakebites: 264 from Serbia (hospital in Priština), 542 and 93 from Croatia, and 341 from and Bosnia and Herzegovina, during 17, 21, 11, and 24 years, respectively [2, 8, 9, 10, 26].

All aspects of envenomations treated in the UGH correspond to those from the comparable studies, from the duration of hospitalization through severity grades and average incidences per 100,000 inhabitants, to the most severely affected groups of people and the antivenom application frequencies [2, 8, 9, 10, 26]. A recent overview for Europe showed similar trends [7]. Annually, we treated more patients on average (19.15) than reported in the 1960s (17.22) and in Priština (15.53) [2, 8]. In contrast to the 1960s (and Priština), in our study more people were bitten to the hands: $t_{402} = 4.359$, $p < 0.001$, while previously significantly more bites to the feet were recorded: $t_{402} = 5.733$, $p < 0.001$ (t-tests between percents). Previously "children and pupils" dominated [2], while nowadays the most bites were inflicted on people over 40 (Figure 1A). In Priština, 0–20-year-old patients were the dominant age group [8]. Such differences were already observed, and they depend on the prevalent activities in the target groups [8, 9, 10]. Contrary to two neighboring countries [9, 10], where the gender ratios among the bitten persons were equal, in Serbia, more males suffered the bites – in our study almost every year (Figure 3A) – similarly to the data obtained for Europe [7, 8]. This can be related either to their occupation or to the lack of fear.

Low incidence of local necroses (2%) can be attributed to the fact that all emergency stations in the entire Užice region, including local ambulances in villages, possess and

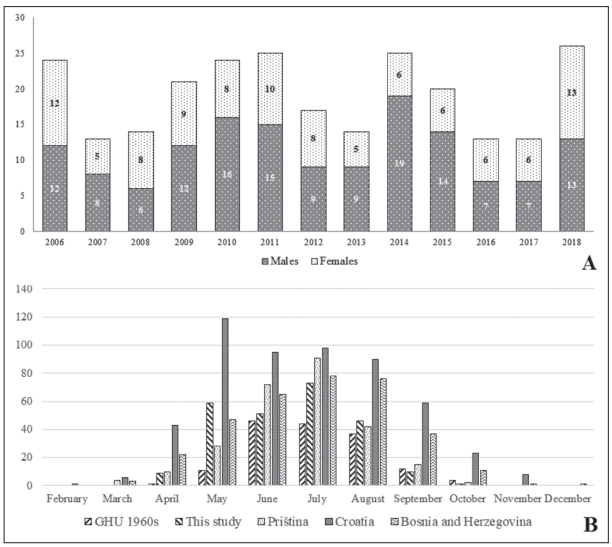


**Figure 3.** (A) Annual numbers of bites in the present study: male and female patients; (B) Seasonal distribution of bites reported in this study compared to previously published data from the same hospital, and the data from Priština, Croatia, and Bosnia and Herzegovina


**Figure 4.** (A) and (B): Typically colored female and male *Vipera ammodytes*; (C) and (D): typically colored female and male *V. berus*; (E): atypically colored female *V. ammodytes*, and (F) unusually colored male *V. berus* (Photos: Aleksandar Simović)

correctly utilize the anti-viperine serum. For certain snake species, or in certain cases of *Vipera sp*. bites, intravenous antivenom application was recommended [6]. To all our patients the serum was administered subcutaneously or intramuscularly: that was probably the reason no adverse reactions developed. Antibiotics therapy was given to one-half of our patients (the cases of complicated infections). This is in accordance with the existing recommendations and usual practice in the adjacent countries [6, 8, 9, 10].

According to the physicians' notes, all analyzed bites were legitimate (hazardous), often occupational, contrary to some previous reports [15, 16] and our suspicions. Illegitimate bites (by the snakes which are observed and provoked or irritated intentionally), although highly probable in natural surroundings, often are inflicted by captive,

sometimes exotic species and can result in more severe envenomation compared to bites on laymen by autochthonous snakes [27, 28]. Judging solely by the frequencies of bitten persons and locations of the bites in various environments/activities, we suppose that those engaged in some agricultural work either did not see the snakes or tried to remove them by hand hence significantly more bites to the upper extremities. For the local people on a picnic, no conclusion could be made regarding their behaviour in contact with snakes. We presume that most of the tourists did not see the snakes and provoked them unintentionally.

Figure 3B presents seasonal variations in the numbers of bites in our study and four other analyses [2, 8, 9, 10]. In the 1960s in Užice, the most bites were recorded in June, but in our study as well as the one made in Priština, this occurred in July. All studies showed variations in months and years with the highest numbers of bites: this can probably be attributed to differences in climate, but also in human activities. This is also consistent with the findings for Europe [7].

As noted already in 1968, the prevailing weather conditions influence the activity of snakes i.e., the numbers of bites to people. People reported that in rainy years the snakes were more abundant [2]. Comparatively humid conditions favour the growth of vegetation, which can lead to increased numbers of small mammals, the snakes' main prey.

We sought some regularity regarding the severity of symptoms due to snakebite and the time of the year when the bite was inflicted. In May, the most bites of grades 3 and 4 happened. However, for sound conclusions, we need more information (besides the snake identity), including the data regarding the snakes' ecology and behavior [29]. There were findings that during winter, *V. ammodytes* venom contains less of the lethal components [30] and that in spring and summer viper venom is more potent [31]. It was speculated [8] that during the snakes' winter inactivity, the venom accumulates in their venomous glands hence its amount at the first bite is large, which results in severe clinical pictures in persons bitten in spring. To the best of our knowledge, there is no proof for such an assumption.

Many of our patients had no or only mild symptoms. This can, *inter alia*, be due to bites of non-venomous snakes (in almost 40% of cases the snakes were not identified) or to 'dry' bites by venomous species.

In other analyses, only a few fatal cases were reported and very small numbers of serum sickness and anaphylactic reactions developed [8, 9, 10]; we had none. In all three countries, *Vipera ammodytes* inflicted the most bites. Like in our case, in the cited studies often the snakes could not be identified, and most probably many cases remained unreported. The latter is a global problem though [7, 32].

In our study, many people reported being bitten by adders (111 of 249, 44.58%), which could result from the common misidentification stemming from great variability within and similarities between adders and nose-horned vipers (Figure 4). Another source of confusion may be the differences in local names of snake species. In as many as 97 cases (38.96%), the snake was not identified, and in the remaining 41 (16.47%) people reported nose-horned vipers. According to expert opinion (given before the publication of exact localities where the adders were recorded), bites by adders were possible in only 40 (16.1%) cases. However, when we compared the reported localities of bites by "adders" with the published [1] UTM squares where adders were recorded (eight in total, in four together with *V. ammodytes*), no overlap was found. Nevertheless, we cannot exclude the possibility that adders are present in certain places that are still formally unknown.

In all localities noted in medical records available for our study, nose-horned vipers were previously recorded, and in some places (Zlatibor, Zlatar, Uvac – Nova Varoš) they are abundant in the places frequented by tourists, mountaineers, recreationists, or farmers. Encounters with adders are possible in some parts of the Zlatar mountain, and in the surroundings of Sjenica, where the terrain and climate suit them. Even in places where they are present, adders are comparatively scarce and spend most of the daytime hiding in low vegetation. The periods when they can be seen are early morning and/or late afternoon. They can be more easily encountered at the end of summer (second half of August) when people collect forest fruits. Also, people working in places 1000 meters above the sea level can come across adders. These snakes often hide in piles of cut trees hence the workers can get bitten while manipulating trunks. Also, adders can be transported with logs/timber to the places they naturally do not occur in. For these reasons, it is important to precisely record the localities where the bites occur.

Importantly, in four places in South-western and Western Serbia, *Vipera ammodytes* and *V. berus* live in sympatry. Nevertheless, as the effects of their venoms differ [3], it is possible to deduce which species inflict bites. However, it would be best if the perpetrator animals could be identified and left alive [24].

## CONCLUSION

In certain parts of the world, venomous snakebites present a severe threat to people. Nevertheless, in Serbia (like elsewhere in the Balkans and Europe), venomous snakes are neither as numerous nor as dangerous as for instance, in Asia or Africa. We are deeply convinced that people in Serbia can be properly educated regarding the three venomous snake species (out of the total ten) and that many snakebites can be prevented or avoided.

Variations in seasonal/annual numbers of bites – and the changes thereof – highlight the fact that more investigation at the ecology of venomous snakes has to be undertaken and that information should be exchanged between medical professionals and professional biologists to create adequate education, advice, and preventive measures. Also, the vipers' distribution data could be filled in more detail. A series of lectures should be organized to inform the physicians on the necessity for a more thorough approach to this issue so both the collection of data and medical treatment could be standardized and improved. In this way, both people and snakes would be protected.

## ACKNOWLEDGEMENT

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**Conflicts of interests:** None declared.

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# Анализа болесника лечених од уједа змија отровница у Општој болници Ужице (Западна Србија), у периоду од 2006. до 2018. године

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**САЖЕТАК**

**Увод/Циљ** До сада није сачињен преглед уједа отровних змија у целој Србији: са прављењем централног регистра започето је тек 2018. године. Прикупили смо и обрадили доступне податке везане за уједе отровних змија на подручју Ужица (Западна Србија). Претходна анализа из ове болнице објављена је 1968. године.

**Методе** Ретроспективно смо анализирали податке о пацијентима леченим у Општој болници Ужице након уједа отровних змија, у периоду 2006–2018. године, и упоредили их са подацима из 1960-их, са студијом из још једне болнице у Србији и са подацима из две земље бивше Југославије.

**Резултати** Током 13 година збринуто је 249 особа. Од свих случајева, 10,4% је било са неупадљивим симптомима (благ бол на месту уједа), 68,7% је прошло са благим до умереним симптомима, а мање или више озбиљних је било 20,9%. Смртних случајева није било.

**Закључак** Иако углавном нису животно угрожавајући, уједи отровних змија су релативно чести и могу довести до озбиљних компликација. Уз одговарајућу едукацију многи се могу избећи. Такође, имајући у виду не само биолошку разноврсност као такву него и значај отровâ змија за производњу различитих медицински значајних препарата, наглашавамо потребу за одговарајућом заштитом све три врсте отровница у Србији – поскока (*Vipera ammodytes*), шарке (*V. berus*) и шаргана (*V. ursinii*).

**Кључне речи**: међудисциплинарна сарадња и образовање; недостатак информација; очување заштићених врста; уједи *Vipera ammodytes* и *Vipera berus*.

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

# Diagnostic value of three simple and rapid dry eye tests – lid parallel conjunctival folds, tear meniscus height, and tear ferning

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**SUMMARY**

**Introduction/Objective** The objective of this paper was to assess the diagnostic value of three simple dry eye (DE) tests: lid parallel conjunctival folds (LIPCOF), tear meniscus height (TMH), and tear ferning (TF).

**Methods** LIPCOF, TMH, and TF diagnostic DE tests were performed in 100 patients. Eighty of them were referred to us by rheumatologists and general practitioners either during evaluation for Sjögren's syndrome, or because of DE symptoms. The control group was composed of 20 patients, with no DE-related symptoms. Ocular Surface Disease Index questionnaire was used for DE symptoms' evaluation. Results of LIPCOF, TMH, and TF tests were compared with results of the Copenhagen criteria DE tests i.e., tear fluorescein breakup time, Schirmer I and Rose Bengal tests. Ability of the tests to recognize DE in various grades according to Dry Eye Work Shop (DEWS) report score system was assessed.

**Results** Compared to the Copenhagen criteria, sensitivity of LIPCOF and TMH was high (92.8% and 83.5%, respectively), while specificity was low (34.4% and 49.2%, respectively). TF had low sensitivity (59.1%) but high specificity (82.7%). Mean values of both LIPCOF and TMH differed significantly ($F = 7.222$, $p < 0.001$ and $F = 11.802$, $p < 0.001$, respectively) between the control group and all DEWS grades, but not among different grades of DE.

**Conclusion** TMH and LIPCOF diagnostic tests showed high sensitivity, which makes them excellent screening DE tests. Low sensitivity of TF suggests that it is not truly a good screening test on its own, but its high specificity is of definite value.

**Keywords:** dry eye disease; lid parallel conjunctival folds; tear meniscus height; tear ferning

## INTRODUCTION

In the pool of diagnostic tests for dry eye (DE), no test is found to be both sensitive and specific enough on its own [1]. For reaching DE diagnosis in practice, there is a tendency to use a group of clinical tests, chosen at the examiners discretion, to complement overall clinical judgment. To state it otherwise, although there is a consensus of a group of experts on DE definition (Dry Eye Work Shop – DEWS), there is no consensus on a definite set of tests (nor their outcomes) for DE [2]. Also, symptoms often do not correlate with signs of DE nor do they correlate well with the stage of DE [3, 4]. A new report of the DEWS group from 2017 suggests evaluating symptoms with Ocular Surface Disease Index (OSDI) or the Five-Item Dry Eye (DEQ-5) questionnaire. Clinical tests for reaching the DE diagnosis in their opinion are non-invasive breakup time or fluorescein tear breakup time (FTBUT), tear osmolarity, or ocular surface staining. But for grading of the disease and assessing the type of DE they recommend other tests, like non-invasive tear volume measurement, assessing meibomian gland disfunction (MGD), and lipid thickness/dynamics [1].

While searching for any well-defined set of clinical DE tests, commonly used as a whole, rather than as an *ex tempore* formed group of tests, the Copenhagen criteria (CC) tests stand out as a very well defined and time-honored set. These tests combine acceptable levels of both sensitivity and specificity for non-Sjögren's syndrome (SS) DE though they were initially devised for SS-related DE [5]. They were, accordingly, used in our study as the criteria for DE diagnosis and a reference clinical standard for the comparison with single tests that we were interested in: lid parallel conjunctival folds (LIPCOF), tear meniscus height (TMH), and tear ferning (TF).

There is a rising number of people suffering from DE symptoms, seeking help from their eye doctors, who do not always have time or resources to apply sophisticated diagnostic tests. Epidemiological studies have demonstrated that DE has a prevalence of 5–45%, depending on the criteria and location [6–10]. In a study with over 20,000 glaucoma patients, Erb et al. [11] report TMH and LIPCOF as simple and noninvasive tests for DE. TF was suggested by the DEWS group as a potentially good screening test [1].

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Our aim was to compare LIPCOF, TMH, and TF tests with CC DE tests and to analyze their ability to recognize dry eye disease (DED) in it its various stages.

## METHODS

Out of 100 subjects we examined for DE (200 eyes) at the Clinic for Eye Diseases, Clinical Centre of Serbia, during 2013 and 2014, 88 were woman. The mean age ± SD was 50.17 ± 16.74 years. Thirty of them were referred to us by rheumatologists during evaluation for SS, and 50 were referred by general practitioners because of DE symptoms. The control group was made up of 20 patients, with no DE-related symptoms, examined during the evaluation for cataract surgery. The two groups were matched for age (no statistically significant difference between groups, $p = 0.21$) and sex ($p = 0.45$). Exclusion criteria in our study were any ocular surgery performed within one year, contact lens wear, topical eye therapy (if the only therapy was tear substitutes, they had to be suspended at least eight hours prior to the examination), entropion, ectropion, or other lid closure problems, ocular allergies, or the presence of anterior blepharitis. The study was approved by the Ethical Committee of the University of Belgrade, Faculty of Medicine. All the patients signed an informed consent form.

We performed the following clinical tests: Schirmer without anesthesia (Schirmer I), FTBUT, Rose Bengal (RB), LIPCOF, TMH, and TF. Eyelids were inspected for MGD. The symptoms were evaluated based on OSDI. Only the patients with OSDI score under 13 were enrolled into the control group.

To confirm DED in our study, we considered results from a group of three clinical tests. These three tests – Schirmer I, FTBUT, and RB – represent the ophthalmological part of testing for SS according to CC but also proved useful in diagnosing DE out of the SS context [5]. In order to be diagnosed with DE, the patient should be positive for two out of three CC tests in one or both eyes. According to CC, a positive result for Schirmer I test is value less than 10 mm, for the FTBUT test the value less than 10 seconds, and for the RB test score equal or greater than 4 according to Van Bijsterveld grading system [12]. Eighty of them had DED, since one or both eyes were positive in two out of three clinical tests. Twenty patients among this symptomatic group had some form of MGD. In the control group, no eye met these criteria. One patient from the control group had MGD, without signs or symptoms of DED. Bearing in mind that we separately analyzed both eyes, we found that 139 eyes were positive for DED. We also graded DE severity from 1 to 4, according to the DEWS report score system, where grade 1 is mild DE and 4 is the most severe form of the disease [13].

The tests were performed during one examination, by two examiners, in the morning. Patients' TMH and the presence of folds for LIPCOF test were examined by slit-lamp. We performed these tests at the beginning of examination to avoid blinking induced by prolonged gaze and also to avoid induced reflex tearing. For TMH, we registered values of 0.3 mm, 0.2 mm, 0.1 mm, and less than 0.1 mm. TMH was compared with variable slit-lamp beam height, which was regulated with a mechanical cylinder attached to the slit lamp. Once we adjusted the beam height, we read the value from the measuring scale connected to the cylinder. The lowest value on the measuring scale at our disposal was 0.2, followed by 0.3. When TMH was half of 0.2 mm beam height, we registered the value as 0.1, and if TMH was lower than half of 0.2 mm beam height, it was registered as lower than 0.1 mm. Measuring of TMH was done at the 6 o'clock position, where lower limbus was in the closest contact with the lid, in order to avoid influence of conjunctival folds on the measurement. For the LIPCOF test, we registered values only in the temporal zone as no folds, half of the fold (if the horizontal fold was not present completely throughout the temporal zone), one fold less than 0.2 mm in height, two folds 0.2 mm heigh, three folds or more of over 0.2 mm. These stages, although similar, are not completely analogous to those most commonly used, described by Höh et al. [14]. Instead of using the term normal meniscus tear height, we used the value of 0.2 mm as a cut-off value between stages. This value was considered as normal height for tear meniscus by other authors as well [15, 16]. In order to form four grades as is the case with the DEWS severity score system, we divided stage 1 by Höh into two stages. Then we performed Schirmer I, FTBUT, and RB tests. The Schirmer I test was performed by hooking the folded end of Schirmer paper over the temporal one-third of the lower lid margin. After a period of five minutes, we measured the length of wetting from the notch. For FTBUT, the dye was applied on the ocular surface with impregnated strips. Looking through cobalt blue filter, we measured the time needed for the dyed tear film to break up. After applying tetracaine eye drops, we instilled RB dye and scored result with the Van Bijsterveld grading system. Collecting tear sample from the inferior tear meniscus, for performing the TF test, was done by Eppendorf (Merck KGaA, Darmstadt, Germany) automatic micropipette with single-use 1–10 µl Eppendorf Tips. Tear sample was pipetted onto a clean microscope slide and allowed to air-dry for 10 minutes. Then it was observed by phase contrast light microscope at magnification levels of 20 × and 40 ×, and quantified according to the Rolando grading scale, based on the level of arborization, where grade 1 is characterized by uniformed large arborization, while in grade 4 there is no ferning [17].

We analyzed sensitivity (ability to recognize the disease), specificity (ability to rule out disease), positive and negative predictive value (PPV and NPV) of all clinical tests used in the study. By using one-way ANOVA and *post-hoc* test, we tested their ability to grade severity of DE according to the severity score system from the DEWS report. The data were statistically evaluated by using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY, USA).

## RESULTS

Most of the eyes (37.5%) diagnosed as dry in our study belong to grade 2 according to severity score system from

**Table 1.** Results of clinical tests from dry eye group and group of normal eyes

| Clinical test | Mean value dry eyes | 0.95 CI | Mean value normal eyes | 0.95 CI | t | p |
|---|---|---|---|---|---|---|
| Schirmer I | 15.61 | ± 1.469 | 25.125 | ± 1.989 | -7.74 | < 0.0001 |
| FTBUT | 5.08 | ± 0.457 | 10.6 | ± 0.573 | -11.47 | < 0.0001 |
| RB | 3.38 | ± 0.385 | 0.35 | ± 0.212 | 13.82 | < 0.0001 |
| TMH | 0.11 | ± 0.008 | 0.165 | ± 0.019 | -5.34 | < 0.0001 |
| LIPCOF | 1.41 | ± 0.117 | 0.625 | ± 0.222 | 6.26 | < 0.0001 |
| TF | 2.52 | ± 0.137 | 1.5789 | ± 0.212 | 7.52 | < 0.0001 |

CI – confidence interval; t – value of Student's t test; Schirmer I – Schirmer test without anesthesia; FTBUT – fluorescein tear breakup time; RB – Rose Bengal; TMH – tear meniscus height; LIPCOF – lid parallel conjunctival folds; TF – tear ferning;
p is statistically significant at the level < 0.01

the DEWS report. Fifty-four (27%) eyes belong to grade 1, 23 (11.5%) to grade 3, and only 11 eyes (5.5%) to grade 4.

All of the clinical tests that we used in this study were able to distinguish normal from DE. Mean value of parameters measured by these tests and significance of difference between test values for non-DE and DE groups are presented in Table 1.

When tested against the group of DE tests form CC, FTBUT had the highest sensitivity (95%), followed by LIPCOF and TMH (92.8% and 83.5%, respectively). RB and Schirmer I had 100% specificity, but TF also displayed high specificity (82.7%). Sensitivity and specificity of all the tests as well as PPV and NPV are presented in Table 2.

**Table 2.** Sensitivity, specificity, positive predictive value, and negative predictive value of clinical tests, each against dry eye tests from the Copenhagen criteria

| Parameters | FTBUT | RB | Sch I | LIPCOF | TMH | TF |
|---|---|---|---|---|---|---|
| Se (%) | 95 | 48.9 | 33.1 | 92.8 | 83.5 | 59.1 |
| Sp (%) | 80.3 | 100 | 100 | 34.4 | 49.2 | 82.7 |
| PPV | 0.92 | 1 | 1 | 0.76 | 0.79 | 0.89 |
| NPV | 0.85 | 0.46 | 0.44 | 0.68 | 0.57 | 0.47 |

Se – sensitivity; Sp – specificity; PPV – positive predictive value; NPV – negative predictive value; DE – dry eye; FTBUT – fluorescein tear breakup time; RB – Rose Bengal; Sch I – Schirmer I; LIPCOF – lid parallel conjunctival folds; TMH – tear meniscus height; TF – tear ferning

**Table 3.** Mean fluorescein tear breakup time values in different dry eye severity groups

| Groups | n | Mean | SD | SE | 95% confidence interval for mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower bound | Upper bound | | |
| 0 | 37 | 10.59 | 1.94 | 0.32 | 9.95 | 11.24 | 4 | 15 |
| 1 | 54 | 6.96 | 2.72 | 0.37 | 6.22 | 7.71 | 3 | 11 |
| 2 | 75 | 5.08 | 2.3 | 0.27 | 4.55 | 5.61 | 2 | 10 |
| 3 | 23 | 3.48 | 2.48 | 0.52 | 2.40 | 4.55 | 0 | 10 |
| 4 | 11 | 0.55 | 0.93 | 0.28 | -0.08 | 1.17 | 0 | 2 |
| Total | 200 | 6.18 | 3.49 | 0.25 | 5.69 | 6.66 | 0 | 15 |

FTBUT – fluorescein tear breakup time; n – number of eyes; Mean – average parameter value of tested eyes of different grades; SD – standard deviation; SE – standard error

We analyzed mean FTBUT values between different grades of severity according to DEWS (Table 3). By using ANOVA, we found that the average FTBUT value differs between the groups (F = 62.474, p < 0.001). *Post-hoc* test allowed us to establish that this difference was statistically significant for every group compared to all the other groups (Figure 1).

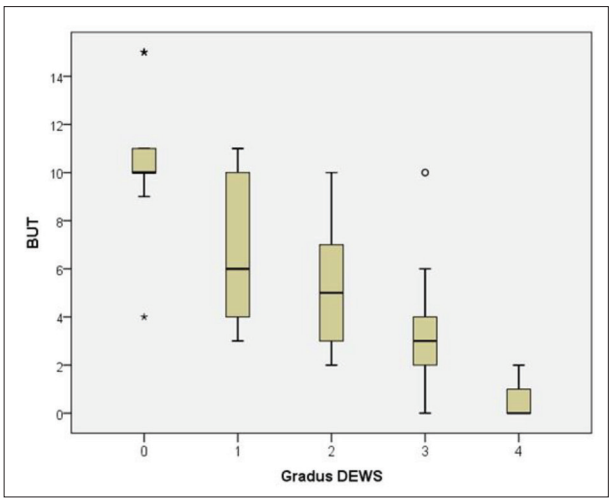


**Figure 1.** Mean fluorescein tear breakup time values in different dry eye severity groups;
average fluorescein tear breakup time value differs between the groups tested with ANOVA (F = 62,474, p < 0,001); difference is statistically significant for every group compared to all the other groups analyzed with the post-hoc test; the mean difference is significant at the 0.05 level;
FTBUT – fluorescein tear breakup time; Gradus DEWS – grades by the Dry Eye Work Shop report score system [2]

When we analyzed mean values between different DEWS grades with ANOVA (Table 4), we found that there is a statistically significant difference for the TF test (F = 18.192, p < 0.001). Analyzed with the *post-hoc* test, we found a significant difference between all the groups, except between the second and the third, and the third and the fourth grade.

**Table 4.** Mean tear ferning values in different dry eye severity groups

| Groups | n | Mean | SD | SE | 95% confidence interval for mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower bound | Upper bound | | |
| 0 | 34 | 1.59 | 0.701 | 0.120 | 1.34 | 1.83 | 1 | 3 |
| 1 | 53 | 2.11 | 0.847 | 0.116 | 1.88 | 2.35 | 1 | 4 |
| 2 | 72 | 2.54 | 0.786 | 0.093 | 2.36 | 2.73 | 1 | 4 |
| 3 | 21 | 2.81 | 0.680 | 0.148 | 2.5 | 3.12 | 1 | 4 |
| 4 | 10 | 3.50 | 0.527 | 0.167 | 3.12 | 3.88 | 3 | 4 |
| Total | 190 | 2.33 | 0.897 | 0.065 | 2.2 | 2.46 | 1 | 4 |

TF – tear ferning; n – number of eyes; Mean – average value of tested eyes of different grades; SD – standard deviation; SE – standard error

LIPCOF and TMH tests' mean values also differed significantly (Table 5 and Table 6) between the groups (respectively F = 7.222, p < 0.001; F = 11.802, p < 0.001). With the *post-hoc* test we established that this was due to the significant difference between the control group and all other severity grade groups, including mild DE grade, for both tests (cut-off value was 0.19 for TMH and 0.97 for LIPCOF). The difference was not significant among different grades of DE.

**Table 5.** Mean values for tear meniscus height in different dry eye severity groups

| Groups | N | Mean | SD | SE | 95% confidence interval for mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower bound | Upper bound | | |
| 0 | 37 | 0.17 | 0.06 | 0.01 | 0.15 | 0.19 | 0.1 | 0.3 |
| 1 | 54 | 0.12 | 0.06 | 0.01 | 0.10 | 0.14 | 0.05 | 0.3 |
| 2 | 75 | 0.11 | 0.05 | 0.01 | 0.10 | 0.13 | 0.05 | 0.3 |
| 3 | 23 | 0.09 | 0.03 | 0.01 | 0.07 | 0.1 | 0.05 | 0.2 |
| 4 | 11 | 0.09 | 0.07 | 0.02 | 0.05 | 0.14 | 0 | 0.2 |
| Total | 200 | 0.12 | 0.06 | 0.00 | 0.11 | 0.13 | 0 | 0.3 |

TMH – tear meniscus height; n – number of eyes; Mean – average parameter value of tested eyes of different grades; SD – standard deviation; SE – standard error

**Table 6.** Mean values for lid parallel conjunctival folds in different dry eye severity groups

| Groups | n | Mean | SD | SE | 95% confidence interval for mean | | Min. | Max. |
|---|---|---|---|---|---|---|---|---|
| | | | | | Lower bound | Upper bound | | |
| 0 | 37 | 0.73 | 0.72 | 0.12 | 0.49 | 0.97 | 0 | 2 |
| 1 | 54 | 1.19 | 0.82 | 0.11 | 0.96 | 1.41 | 0 | 3 |
| 2 | 75 | 1.39 | 0.75 | 0.09 | 1.21 | 1.56 | 0 | 3 |
| 3 | 23 | 1.65 | 0.65 | 0.13 | 1.37 | 1.93 | 1 | 3 |
| 4 | 11 | 1.55 | 0.69 | 0.21 | 1.08 | 2.01 | 1 | 3 |
| Total | 200 | 1.25 | 0.8 | 0.06 | 1.14 | 1.36 | 0 | 3 |

LIPCOF – lid parallel conjunctival folds; n – number of eyes; Mean – average parameter value of tested eyes of different grades; SD – standard deviation; SE – standard error

We analyzed separately patients with DE who in the course of this study were diagnosed with SS according to revised international criteria [18]. Comparing average values of Schirmer I test between DE of the patients with SS (11.79 mm), and patients without SS (18.23 mm), we found that the first group, expectedly, had significantly lower values ($t = -4.25$, $p < 0.001$). Average FTBUT value of 4.15 seconds in SS patients was also significantly lower than 5.64 in non Sjögren DE ($t = -3.13$, $p = 0.002$), and the RB in average was significantly higher (4.06 in SS group versus 2.98 in non-SS group, $t = 2.64$, $p = 0.009$). Eyes of the patients with SS had in average more folds in LIPCOF test (1.52 in SS group versus 1.33 in non-SS group, $t = 1.57$, $p = 0.06$), but there was no difference between the groups when it comes to TF test and TMH (respectively, $t = 0.27$, $p = 0.39$; $t = -0.39$, $p = 0.35$). Eyes of the patients with SS were statistically more in higher grades of severity ($t = 4.02$, $p < 0.0001$).

## DISCUSSION

According to DEWS Diagnostic Methodology Subcommittee we should be aware of the difference between DE tests used for screening, where high sensitivity is preferable and group of diagnostic tests for DED with high overall accuracy along with good sensitivity [1]. Screening tests that the DEWS group suggested are TMH and TF, especially the first one, being rapid and simple, and also with good sensitivity, as confirmed by other studies [11]. In our study, both LIPCOF and TMH had good sensitivity, compared to the CC DED clinical tests group (92.8%, 83.5%). Their ability to distinguish normal from mild DE makes them especially convenient for screening. García-Resúa et al. [19] found that there is a good correlation between osmolarity and subjective grading of TMH as well as measuring of TMH using open-source software (NIH ImageJ) [19]. Both tear osmolarity and tear meniscus optical coherence tomography (OCT) measurements comply with the DEWS grading system as previously reported by Tukenmez-Dikmen et al. [20] Mean values of TMH and LIPCOF between different grades of DE did not show statistically significant difference, so according to our result they are not convenient for grading DE. In our study, the mean value of TMH in the group without DE was 0.17 mm. It is somewhat lower than the one published by Imamura et al. [15] measured on slit-lamp with graticule for three different age groups of patients without DE (from younger to older; group 1: 206 μm; group 2: 209 μm; group 3, 226 μm). One would expect that the average value in the older group would be lower as in their study, but they assumed that the obstruction of lacrimal drainage that occurs with age may have influenced the results in their study. When comparing the average value of TMH measured with slit-lamp and with OCT in normal subjects, Imamura et al. [15] found no statistical differences. Since variability in measurement was less shown with the slit-lamp method, they suggest slit-lamp measuring of TMH may still be one of the most useful clinical methods to evaluate tear meniscus. With the cut off value of 0.19 mm, the sensitivity of TMH in our study was 83.5%. With the cut-off value of 205 μm, Singh et al. [21] found that sensitivity of TMH measured with OCT was 98.3%. As reported in the study by Erb et al. [11], we also found that LIPCOF has high sensitivity with the cut-off value of 0.97, but its ability to rule out diagnosis where DE was not recognized by other clinical tests was low (33.9%). Specificity of TMH compared to CC DE tests was also low (49.2%). TF has been reported previously by Rolando as a valuable test and the grading scale he devised, as the most popular one, has been used by other authors [17, 22]. The TF test shows strong correlation with osmolarity as reported by Versura et al. [23], statistically significant for each DE subgroup. In our study, TF did not have high sensitivity and could not distinguish between all DE subgroups, but had good specificity.

Values of Schirmer I and FTBUT tests of patients with SS were significantly lower than those in the group of patients with no SS. The average value of RB was higher for eyes of the patients with SS, as reported in other studies as well [24]. One would expect that the average value of TMH would be lower in the SS group, but that was not the case in our study. On the other hand, there were more conjunctival folds in the LIPCOF test in eyes of patients with SS. TF showed no difference between the two groups.

New methods of meniscometry have been developed to facilitate simple and dynamic visualization of the tear meniscus. OCT assessment of the tear meniscus and conjunctival folds has been extensively studied in the last decade [25, 26]. Spectral-domain OCT meniscometry has shown

high reproducibility, but can be biased by conjunctivochalasis and LIPCOF in the same way as with slit-lamp measurements of the tear meniscus [1]. Measuring TMH at the 6 o'clock position is, in our opinion, optimal when using slit-lamp, but the same position is suggested by other authors as the preferred one when using swept-source OCT [15]. Whether we observe tear meniscus or the presence of conjunctival folds, analysis of the image acquired with OCT may be complex, time-consuming, and operator-dependent. Therefore, we think that slit-lamp measurements of TMH and LIPCOF are preferred as screening test available in everyday ophthalmology practice.

## CONCLUSION

TMH and LIPCOF diagnostic tests are rapid and simple DE tests, whose high sensitivity and ability to recognize even mild cases, in spite of lacking the strength to rule out disease where other tests are negative, makes them excellent screening DE tests. Due to low sensitivity in our study, TF seems not to be a very good screening test. In our study, FTBUT showed remarkably high sensitivity and ability to correctly distinguish between all DED severity groups, which makes it a good screening test, but also a good test for grading and monitoring the effects of therapy for DED.

## NOTE

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**Conflict of interest:** None declared.

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# Дијагностичка вредност три једноставна и брза теста за суво око – набори конјунктиве паралелни ивици капка, висина менискуса суза и тест гранања суза

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**САЖЕТАК**

**Увод/Циљ** Циљ овог рада је да се процени дијагностичка вредност три једноставна теста за суво око: набори конјунктиве паралелни ивици капка (НКПИК), висина менискуса суза (ВМС) и тест гранања сузе (ГС).

**Методе** Дијагностички тестови НКПИК, ВМС и ГС су изведени код 100 пацијената, од којих нам је 80 упућено на преглед од стране реуматолога и надлежних офталмолога, током испитивања на Сјогренов синдром или због симптома сувог ока. Контролну групу је чинило 20 пацијената без симптома сувог ока. Симптоми су евалуирани применом упитника о индексу болести површине ока. Резултати тестова НКПИК, ВМС и ГС су упоређени са вредностима резултата тестова за суво око по Копенхашким критеријумима, а то су: време прекида сузног филма обојеног флуоресцеином, мерење секреције суза без анестезије током пет минута Шимеровом траком (*Schirmer I*) и бојење површине ока виталном бојом *Rose Bengal*. Такође је процењена способност тестова да препознају различите стадијуме по систему градирања болести *Dry Eye Work Shop* (*DEWS*).

**Резултати** Поређењем са групом тестова по Копенхашким критеријумима, НКПИК и ВМС су показали високу сензитивност (92,8% и 83,5%), док им је специфичност била ниска (34,4% и 49,2%). ГС је имао ниску сензитивност (59,1%), али високу специфичност (82,7%). Просечне вредности тестова НКПИК и ВМС се статистички значајно разликују између контролне групе и свих стадијума болести по градацији *DEWS*, али не и између различитих стадијума болести сувог ока.

**Закључак** Тестови ВМС и НКПИК су показали високу сензитивност, што их чини одличним тестовима за скрининг болести сувог ока. Ниска сензитивност теста ГС га не сврстава у добре скрининг тестове, али његова висока специфичност му даје дијагностичку вредност.

**Кључне речи:** болест сувог ока; набори конјунктиве паралелни ивици капка; висина менискуса суза; тест гранања сузе

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ORIGINAL ARTICLE / ОРИГИНАЛНИ РАД

# The effect of a mobile application for learning about traumatic dental injuries during the COVID-19 pandemic

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**SUMMARY**

**Introduction/Objective** University teachers have a challenging task in finding creative ways to present educational content. One of them is to create applications dedicated to educational purposes, which students can use on their mobile phones any time.
The aim of this study was to evaluate the impact of mobile learning of dentistry students during COVID-19 pandemic.

**Methods** The prospective study involved 56 students from two medical faculties in the Balkans, who continued to study online after the declaration of the COVID-19 pandemic. Online teaching was based on material in the form of PowerPoint presentations. In order to provide an additional educational tool, a step-by-step mobile application for managing traumatic dental injuries was developed. After one week of using that mobile application, all students completed a questionnaire in electronic form concerning teaching satisfaction.

**Results** Over 90% of the respondents stated that the application facilitated a learning process, improved their understanding of the teaching unit, and provided a great convenience in terms of access to information. Median value of the total score concerning clinical protocol by the use of application was 20 (16–20), which was significantly higher than the neutral value ($p < 0.001$). Median value of the total score concerning the use of conventional PowerPoint presentations did not differ significantly from the neutral value ($p = 0.284$).

**Conclusion** Mobile learning resulted in improved knowledge of dental traumatology diagnostics and treatment among undergraduate dentistry students during COVID-19 pandemic.

**Keywords:** COVID-19; mobile learning; dental traumatology

## INTRODUCTION

Preventive measures instituted to limit a spread of the COVID-19 among population, such as social distancing and self-isolation, have initiated the closure of primary, secondary and higher educational institutions around the world [1]. In their efforts to mitigate the immediate impact of school closures, many universities and faculties have replaced the traditional methods of teaching with distance teaching (and learning) [2]. As it was impossible to predict the duration of the self-isolation period, distance learning has been based primarily on electronic communication between students and teachers, i.e., via e-learning. E-learning can be defined as any use of computers and the Internet in education, where teaching content is sent in electronic form. With this technology, teachers can visually present educational content in a digital environment, trying to make the content inspiring and filled with interesting material, motivating students as much as possible. Students, on the other hand, can learn at their convenience [3].

With growing utilization of smartphone technology, both for personal and professional purposes [4], mobile learning (m-learning) has developed as a new research branch of e-learning, in which mobile devices are used during the learning process [5]. With numerous entertainment-oriented applications available online, teachers have a challenging task in finding creative ways to display educational content. One of them is creating educational tools that students can use on their mobile phone at any time. Numerous mobile applications are constantly being developed that allow mobile learners to have access to a wide variety of learning resources [6, 7].

These are particularly valuable for topics that do not ordinary happen in everyday dental practice, such as managing dental trauma (traumatic dental injuries [TDI]) [8]. As the prognosis of traumatized teeth depends on immediate and appropriate treatment, the dentist must be conscious of the best clinical protocol at all times. However, several reports have been published showing students having insufficient knowledge on how to manage a TDI,

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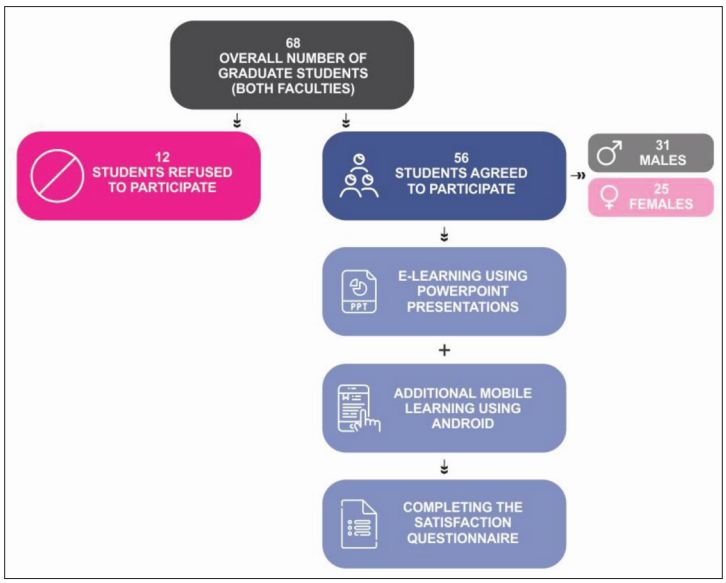


**Figure 1.** Research protocol

and indicating the need for more effective educational programs [9–12].

The aim of this study was to evaluate the impact of mobile learning during COVID-19 pandemic, using an application for managing TDIs.

## METHODS

### Participants

This prospective study involved 56 fifth-year students in a five-year-long schooling program (average age is 23 years) at the Department of Dentistry, the Faculty of Medicine, University of Priština (Kosovska Mitrovica) and the Faculty of Medicine, University of East Sarajevo (Foča), who continued to operate online after the declaration of the COVID-19 pandemic (12 of 68 students refused to participate) (Figure 1). Online teaching was based on materials in the form of PowerPoint presentations (Office, Microsoft Corporation, Redmond, WA, USA) (the presentations were of the same content for both faculties), distributed to students *via* e-mail or the *Moodle* e-learning platform. All participants in the study went through all stages of the educational process and signed the consent form electronically.

### Additional mobile learning

In the light of the COVID-19 pandemic and the transfer to online teaching methods, a curriculum-aligned mobile application dedicated to dental injuries teaching (Dent.IN JURY) was developed by the corresponding author (based on recommendations of the International Association of Dental Traumatology and faculties curriculum) [13]. All students downloaded the Dent.IN JURY application from the Google Play Store, available in Serbian and English (Figure 2).

### Satisfaction questionnaire

After completing the online working week according to the curriculum, all students filled out an electronic satisfaction questionnaire (Google Forms). Twelve items were quantified with a five-point Likert scale (1 = strongly disagree, 2 = disagree, 3 = neutral, 4 = agree, and 5 = strongly agree) [14]. The questionnaire contained three sections that referred to the following: a) student experience and

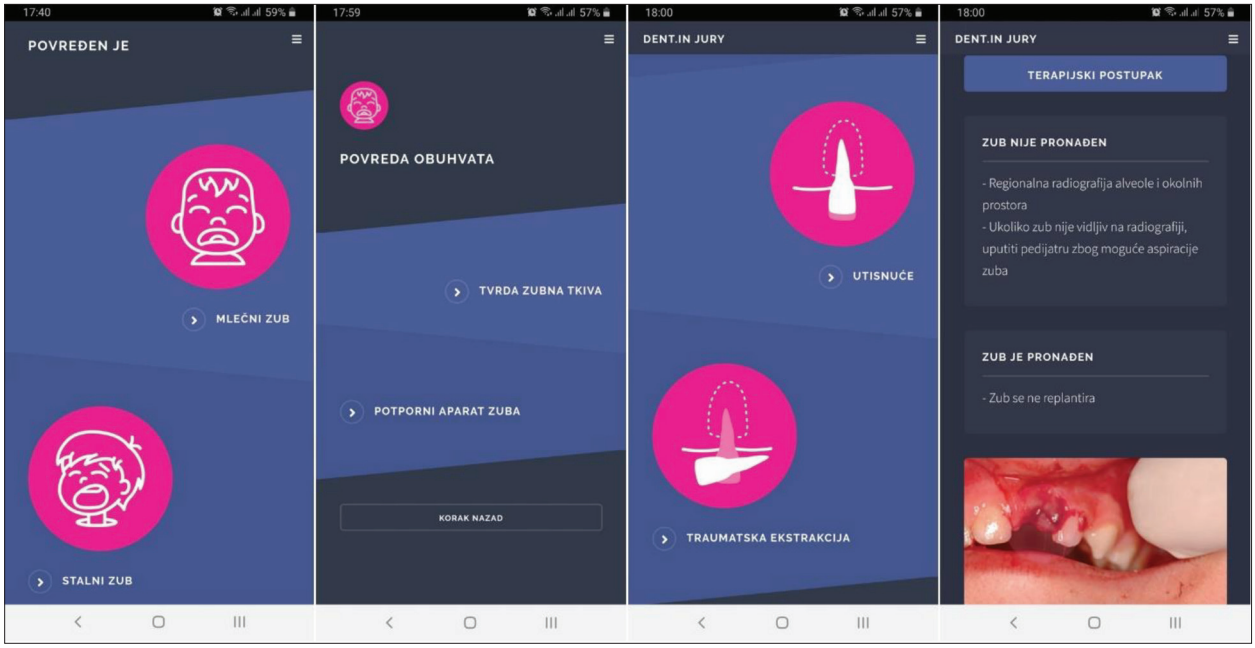


**Figure 2.** Mobile phone application (Dent.IN JURY) screenshots

**Table 1.** Satisfaction questionnaire

| No. | Question | Section |
|---|---|---|
| 1. | Mobile phone application helped me master the dental injury teaching unit | a |
| 2. | Mobile phone facilitated my understanding of clinical protocol following dental injuries | a |
| 3. | The advantage of the Mobile phone application is its accessibility at any time using a smartphone and ease of use | a |
| 4. | This type of additional learning can be very helpful in learning in other disciplines as well | a |
| 5. | I have mastered dental injuries through Mobile phone application | b |
| 6. | PowerPoint presentations were more beneficial in mastering dental injuries compared to Mobile phone application | c |
| 7. | I have mastered the treatment following injuries to primary teeth by learning through Mobile phone application | b |
| 8. | PowerPoint presentations were more beneficial in mastering the treatment following injuries to primary teeth compared to Mobile phone application | c |
| 9. | I have mastered the treatment following injuries to permanent teeth with incomplete root growth by learning through Mobile phone application | b |
| 10. | PowerPoint presentations were more beneficial in mastering the treatment following injuries to permanent teeth with incomplete root growth compared to Mobile phone application | c |
| 11. | I have mastered the treatment following injuries to permanent teeth by learning through Mobile phone application | b |
| 12. | PowerPoint presentations were more beneficial in mastering the treatment following injuries to permanent teeth compared to Mobile phone application | c |

a – student experience and satisfaction with mobile learning environment; b – student satisfaction with learning process using the mobile application Dent.IN JURY; c – student satisfaction with learning process using conventional online teaching tools

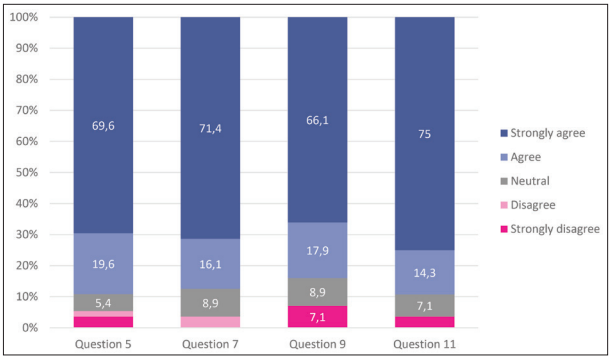


**Figure 3.** Student experience and satisfaction with mobile learning

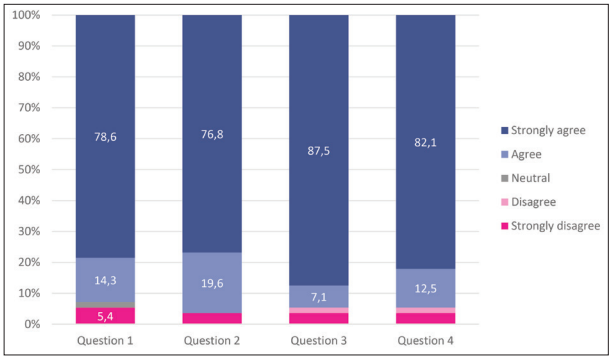


**Figure 4.** Student satisfaction with learning process using Dent.IN JURY mobile application

satisfaction with mobile learning environment (Questions 1–4); b) student satisfaction with learning process using the mobile application Dent.IN JURY (Questions 5, 7, 9, 11); and c) student satisfaction with learning process using conventional online teaching tools (PowerPoint) (Questions 6, 8, 10, 12). To ensure confidentiality, participants signed a separate consent form.

## Statistical analysis

Data are presented as n (%) or median (Q1-Q3). To test whether the median of the sample was equal to a known standard value, we used the Wilcoxon one-sample test. Statistical hypotheses were tested at the level of statistical significance of 0.05. All data were processed in the R programming environment (R Core Team, 2019).

## RESULTS

Over 90% of the respondents believed that the mobile phone application facilitated the learning process, improved their understanding of the teaching unit, and provided great convenience in terms of information access (Figure 3).

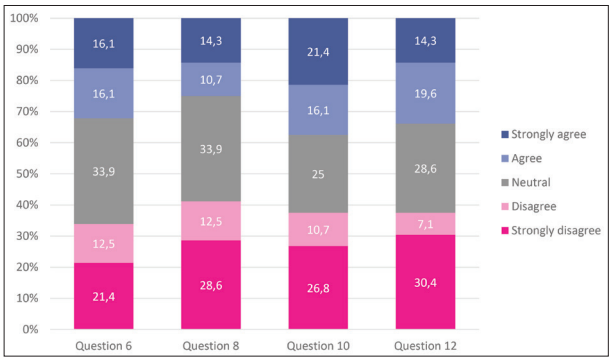


**Figure 5.** Student satisfaction with learning process using PowerPoint presentations

Over 85% of respondents believed that they have mastered the treatment following dental injury, mostly concerning injuries of permanent teeth with completed growth (Figure 4).

The data show that the respondents chose neutral option for items 6 and 8 of the questionnaires (33.9%), whereas they completely disagree with statements 10 and 12 (26.8% and 30.4%). Only 1/3 of the respondents indicated agreement with these statements (Figure 5).

The median score for statements related to satisfaction with the learning application was 20 (18.5–20) and

significantly higher than the neutral value ($p < 0.001$). The median score for statements related to mastering of clinical protocol using the application was 20 (16–20) and significantly higher than the neutral value ($p < 0.001$). The median score for statements related to mastering of clinical protocol using conventional PowerPoint presentations was 12 (7–15) and did not differ significantly from the neutral value ($p = 0.284$).

## DISCUSSION

In the last decade, there has been a rapid expansion of educational resources available for medical students. As well as traditional resources, students are increasingly accessing mobile technology and online tools for learning [15]. As it provides learning flexibility and autonomy, the concept of mobile learning attracts the interest of both students and researchers [16]. Smartphones are becoming a more suitable tool for advancing education in developing countries [17, 18, 19].

Given that in normal conditions the average person spends up to 5.5 hours a day on their phone [20], we can assume that in quarantine conditions, during a state of emergency due to the COVID-19 pandemic, this number increased greatly. According to the available data, no mobile application for managing TDIs is available in Serbian. In order to animate students during the pandemic and provide them with a better insight into dental traumatology, we developed a mobile application (Dent.IN JURY) for the purposes of this study. Creating educational applications is a unique opportunity for developers, but it also comes with numerous obstacles and challenges. The biggest problem for the developers is to understand the educators' requirements for the application to be relevant to the end user. In our case, this was not a problem because the corresponding author of this article developed the application [13]. Over 90% of respondents believed that the application facilitated the learning process and improved their understanding of the teaching topic. It seems to have precedence over the classic PowerPoint presentation as it enables better accessibility to information and ease of use. Respondents agreed that it can possibly be of great help in learning process in other areas as well. The results of our study confirm the fact that additional mobile learning methods are important for students, and can reduce educational difficulties in remote areas or during emergencies [21].

The opportunity for dentistry students to encounter a traumatic injury during undergraduate education depends on a variety of factors, whereas incidents are managed by specialists or post-graduate students, leaving the dentistry students with very little opportunity to be directly involved in the treatment process, and to acquire sufficient clinical competency prior to graduation [22]. The step-by-step concept used in the mobile application in this research helped students to improve their understanding on management of injuries of both deciduous and permanent teeth. Students found the interactive approach to learning more efficient compared to the less inspiring PowerPoint presentations. The positive effects of mobile learning were also reported by Machado et al. [9], who noticed that dentistry students showed greater affinity to a mobile application for managing TDIs compared to printed material.

Having knowledge of proper diagnosing and treating a dental injury is also important for medical practitioners, and especially for those who work in rural areas, where dental practice is not well supported. Medical students receive little or no formal dental trauma assessment and management during medical study, and in order to educate this target group, numerous educational models for learning were recommended [23]. Positive results of our study support the fact that the teaching application can be easily adapted to the needs of medical practitioners and applied in everyday practice, making it easier for them to provide first aid. Conventional teaching, despite serious and thorough research, seems uninspiring for students. In order to improve the transfer of knowledge, it is necessary to incorporate new technologies into learning process. It is very important that educators keep up with the times and provide students with the latest methods of learning and working as much as they can, thus bringing them closer to their interests [24].

One of the limitations of this application is that it is Android exclusive, so it is necessary to adapt it to other platforms in order to be available to all users (iOS, Windows Phone).

## CONCLUSION

Supplementary mobile learning has improved the knowledge in diagnostics and therapy of dental trauma in undergraduate students in quarantine conditions caused by the COVID-19 pandemic. The development of dedicated applications is extremely important for providing better access to information and facilitating learning process for dentistry students.

## ACKNOWLEDGEMENTS

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### Consent for publication

Written informed consent was obtained from all study participants via Google Forms.

**Conflict of interest:** None declared.

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# Ефекат мобилне апликације за учење о повредама зуба током пандемије *COVID*-19

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**САЖЕТАК**

**Увод/Циљ** Наставници имају озбиљан задатак у проналаску креативних начина приказивања едукативног садржаја. Један од њих је и израда наменских апликација у образовне сврхе које студенти могу користити на свом мобилном телефону у сваком тренутку.

Циљ ове студије је био да се процени утицај додатног мобилног учења мобилном апликацијом за денталну трауматологију за време онлајн наставе узроковане пандемијом *COVID*-19.

**Методе** У проспективној студији учествовало је 56 студената са два медицинска факултета (одсека за стоматологију) на Балкану, која су после проглашења пандемије *COVID*-19 наставила рад онлајн наставом. Онлајн настава се заснивала на материјалу у виду *PowerPoint* презентација. У циљу додатне едукације за потребе ове студије развијена је мобилна апликација за учење денталне трауматологије корак по корак. После завршене онлајн радне недеље, сви студенти су попунили електронски упитник који се односи на задовољство наставом.

**Резултати** Преко 90% испитаника сматра да им је апликација помогла у учењу, олакшала разумевање, да има предност услед доступности у сваком моменту и да може бити од велике помоћи у учењу и у другим дисциплинама. Медијана укупног нумеричког скора питања везаних за савладавање терапијских поступака коришћењем апликације статистички значајно је виша од медијане укупног скора за неутралан став ($p < 0,001$). Медијана укупног нумеричког скора питања везаних за боље савладавање терапијских поступака коришћењем конвенционалних *PowerPoint* презентација статистички значајно се не разликује од медијане укупног скора за неутралан став ($p = 0,284$).

**Закључак** Мобилно учење обезбедило је боље познавање дијагностике и терапије денталне трауматологије студената основних студија за време онлајн наставе узроковане пандемијом *COVID-19*.

**Кључне речи:** *COVID*-19; мобилно учење; дентална трауматологија

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

# Radical antegrade modular pancreatosplenectomy – report of two cases and review of the literature

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**SUMMARY**

**Introduction** The radical antegrade modular pancreatosplenectomy (RAMPS) procedure was introduced as a modification of standard retrograde pancreatosplenectomy (SRPS). It was designed to establish a new surgical approach, with intension to increase possibility of achieving negative posterior (retroperitoneal) resection margin, as well as to provide complete N1 lymph node clearance.

**Outline of cases** We present two cases with diagnosed left-sided pancreatic tumors, who were surgically treated in our hepato-pancreato-biliary department. Both patients underwent posterior RAMPS procedure. Postoperative course was uneventful in both patients.

**Conclusion** RAMPS is a safe procedure because it provides complete vascular and bleeding control. It is a superior procedure in oncologic terms compared to SRPS, as it increases the rate of R0 resection, and provides larger number of lymph nodes harvested. Furthermore, RAMPS is associated with better overall survival.

**Keywords:** pancreatic carcinoma; left-sided pancreatic tumors; distal pancreatectomy; RAMPS

## INTRODUCTION

Pancreatic cancer is one of the most lethal and aggressive tumors in human pathology, with median survival of 3–6 months in untreated cases, and a five-year survival rate that ranges 6–9% [1, 2]. Left-sided pancreatic cancer is often asymptomatic and more commonly diagnosed at an advanced stage. Surgical resection, often combined with chemo- and/or radiation therapy, is the only method which gives a chance of curing this disease. The first distal pancreatic resection was performed by Trendelenburg in 1882, and was standardized by Mayo in 1913 [3]. It is now well understood and widely accepted that R0 resection is the key factor in the improvement of the long-term survival [4]. Therefore, it has always been a goal and a challenge for pancreatic surgeons to increase the rates of R0 resections and reduce the recurrence rates. RAMPS procedure was designed as an answer to those tendencies in modern pancreatic surgery. It has been performed since 1999 and established by Strasberg et al. [5] as a novel technique in 2003. The three main principles of the operation are N1 lymph node dissection, modular setting of the posterior plan of dissection to improve the probability to achieve negative posterior resection margins, and right-to-left dissection for early and optimal vascular/bleeding control. The posterior plane of dissection can be directly on the left adrenal gland and Gerota's fascia (anterior RAMPS) or can be posterior to the adrenal and Gerota's fascia (posterior RAMPS), depending on the extent of penetration of the tumor on computed tomography (CT) scan (Figure 1). This new procedure and technique has shifted focus from pancreatic head tumors to less frequent but equally aggressive and even more sinister left-sided pancreatic tumors.

## REPORT OF CASES

### Patient 1

A 66-year-old female patient was admitted to our hospital for upper abdominal pain and discomfort, followed by a weight loss of around 10 kg for the last two months. Laboratory findings and tumor-marker serum levels (CEA, CA 19-9) were within the reference range. Abdominal multi-detector computed tomography (MDCT) and magnetic resonance imaging (MRI) detected a large tumor mass (65 × 35 × 45 mm) located in the tail of the pancreas with involvement of the greater curve of the stomach, the spleen, and the left adrenal gland, after which a final decision for surgical resection was made (Figure 2).

The patient underwent posterior RAMPS with wedge resection of the greater curve of the stomach (Figure 3).

It was the very first RAMPS performed at the Clinical Center of Serbia in Belgrade. The

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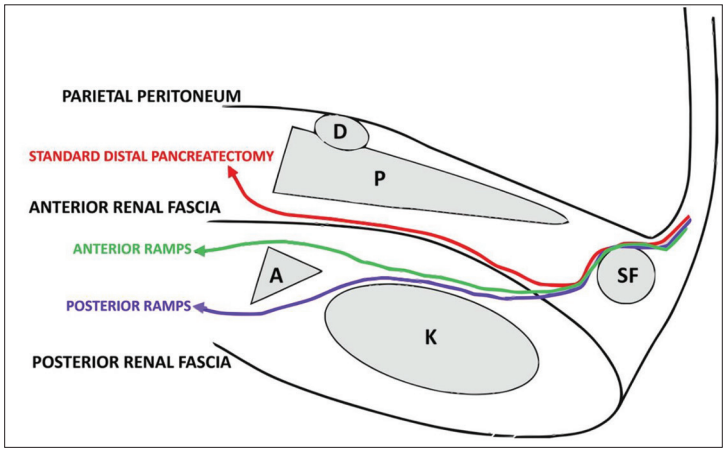


**Figure 1.** Left retroperitoneum plane of posterior margin of the radical antegrade modular pancreatosplenectomy procedure; A – left adrenal gland; SF – splenic flexure of colon; D – fourth part of duodenum; K –left kidney; P – pancreas

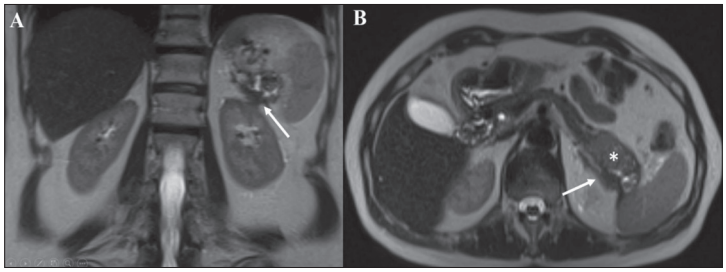


**Figure 2.** Coronal T2-weighted magnetic resonance image (A) shows propagation of the pancreatic tail tumor in perirenal fat plane with infiltration of the renal capsule (arrow); axial T2-weighted MR image in the same patient (B) shows infiltration of the left adrenal gland (arrow); the pancreatic tail tumor is shown on B (asterisk)

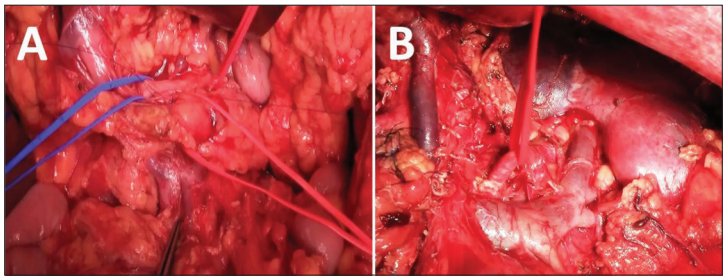


**Figure 3.** A) Early vascular control; B) retroperitoneal plane after removal of the specimen

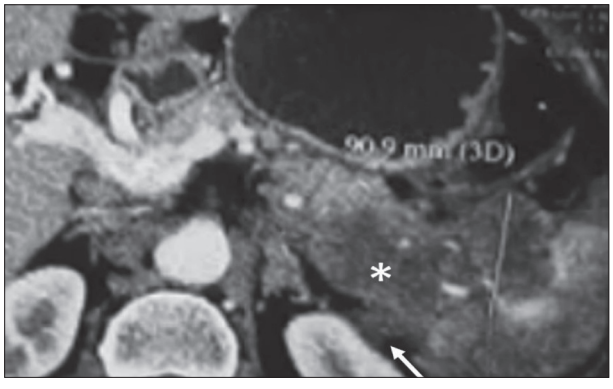

**Figure 4.** Coronal reformatted computed tomography image in the late arterial phase shows a large tumor (asterisk) with infiltration of the perirenal fat on the left adrenal capsule (arrow); also note infiltration of the hilus of the spleen with consequent infarction of the splenic parenchyma

procedure lasted around 250 minutes and no blood transfusions were given. The histopathologic analysis revealed a ductal invasive adenocarcinoma. The postoperative course was uneventful and the patient was discharged from the hospital after 11 days. Regular check-ups were scheduled for every three months during the first postoperative year. The patient received adjuvant chemotherapy (gemcitabine). One year after surgery, three liver metastases were detected on MDCT of the abdomen, two of which in the right liver lobe and one in the left. Palliative chemotherapy treatment was started. Three months later, multiple pulmonary metastases were detected with chest CT, and three months later the patient died from hepatic failure in the terminal stage of the malignant disease.

### Patient 2

The other patient was a 64-year-old female who was admitted after a large tumor (45 × 35 × 32 mm) was detected in the tail of the pancreas with abdominal MDCT and MRI. Imaging techniques showed extrapancreatic tumor propagation with infiltration of the splenic artery, splenic hilum, left adrenal gland, and superior pole of the left kidney (Figure 4). Tumor-marker CA 19-9 serum level was elevated with a value of 383 nmol/L. After a preoperative physical status assessment, a decision for a radical surgical procedure was made, and a patient underwent posterior RAMPS with left nephrectomy. Operative time was around 300 minutes and no blood transfusions were given. After histologic examination of the specimen by a pathologist, a diagnosis of pancreatic ductal invasive adenocarcinoma was established. Tumor stage was T3N1(3/27), and resection status was R1. The postoperative course was uneventful and the patient was discharged from the hospital on the 12th postoperative day. The patient is currently receiving the first course of adjuvant chemotherapy (gemcitabine).

All procedures performed involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

## DISCUSSION

Distal pancreatectomy is the standard surgical approach for left-sided pancreatic cancer. However, long-term survival of these patients remains unsatisfactory, with a median survival time of 10–28 months, and a five-year overall survival of 6–30% [6, 7]. In recent years, new surgical

approaches for resectable or borderline-resectable pancreatic head cancer, including the artery-first approach, superior mesenteric vein/portal vein resection and reconstruction, intraoperative radiotherapy and preoperative chemo-radiotherapy, have been increasingly combined with pancreaticoduodenectomy. Despite the highly aggressive nature of the disease, and early regional lymph node metastasis, adenocarcinomas of the body and tail of the pancreas have attracted significantly less clinical attention. However, in 2003, Strasberg et al. [5] described a new distal pancreatectomy technique, termed RAMPS, to achieve negative posterior resection margins and to remove the N1 lymph nodes completely. In the RAMPS procedure, the lymph nodes along the superior and inferior borders of the left-sided pancreas (10, 11, and 18 according to Japan Pancreas Society classification), the celiac lymph nodes (9) and nodes along the front and left side of superior mesenteric artery (14p, 14d) are considered N1 lymph nodes, and are completely removed. In the standard left-pancreatectomy, only lymph nodes 10, 11, and 18 are removed. Further, RAMPS is based on the anatomical architecture of the posterior pancreatic peritoneal fusion fascia (Gerota's fascia, Treitz' fascia, and Toldt's fascia). Using Kocher approaches, the inferior vena cava and the left renal vein along the Treitz' fascia level, behind the Gerota's fascia, the left renal vein, the renal capsule, and the left adrenal gland, are separated to achieve a complete resection of the nerve fiber connective tissue of the tail, the spleen, and lymph nodes, enhancing the rate of R0 resection of the posterior peritoneum.

In the past decade, the RAMPS procedure has been increasingly performed, particularly in Japan and Korea. Multiple studies from different centers have compared RAMPS to SRPS, evaluating postoperative complications, R0 resection rates, and long-term survival after each type of procedure [7, 8]. A large meta-analysis from 2019 compared RAMPS to SRPS [9]. Seven studies containing 474 patients have been enrolled in this meta-analysis, including 168 patients who underwent RAMPS and 306 patients who underwent SRPS. Three were prospective and four were retrospective studies. The studies were conducted in five countries, China, Italy, Japan, Korea, and the USA. The pooled analysis showed that RAMPS patients had better overall survival compared to the SRPS group of patients. This, however, did not apply to disease-free survival (DFS), which did not improve in the RAMPS patient group. Further, blood loss in the RAMPS group was significantly less than in the SRPS group, emphasizing the importance of early vascular control of major blood vessels in the RAMPS technique. Regarding the number of harvested lymph nodes, significantly more lymph nodes were harvested in the RAMPS than in the SRPS group. It is calculated that at least 21 lymph nodes should be removed and analyzed, to ensure a reliable assessment of the nodal status. Although it has been showed that extended lymphadenectomy does not improve survival, more harvested lymph nodes may result in more accurate node and tumor staging, thus more precisely identify the group of patients who could benefit from postoperative chemotherapy. Recurrence rate in the RAMPS group is significantly lower than that in the SRPS group. Since RAMPS uses a so-called "no-touch isolation technique," it is fair to assume that this might result in the reduction of distant tumor cells' spread. Surprisingly, this meta-analysis, in contrast to that of Cao et al. [10] from 2017, did not show any significant difference in R0 rate between the RAMPS and the SRPS patient groups. Meta-analysis and systemic review by Cao et al. [10] included six retrospective cohort studies with a total of 378 patients. RAMPS was done in 152 patients and 226 patients underwent standard procedure. In this study, R0 resection rates were significantly higher in the RAMPS group. However, no statistically significant difference between the groups was detected with respect to the recurrence rate. Furthermore, there was no significant difference regarding the OS rate between the two groups of patients, which also applies to the comparison of DFS between the groups. As expected, the number of lymph nodes harvested in RAMPS patients was significantly higher than in those in the standard group. Despite of higher multivisceral resection rate in RAMPS patients, incidence of postoperative complications did not increase. Also, there was no significant difference in the length of hospital stay, when comparing the two groups of patients [10]. RAMPS procedures required greater technical skills, as well as longer operative times, but not in the terms of statistical significance; RAMPS group exhibited a tendency towards improvement of a median survival but no improvement in recurrence rates. Also, DFS rates were similar in the two groups. It should be stated here that laparoscopic or robotic RAMPS have also been performed with satisfactory oncological results and survival outcomes [11, 12]. However, this approach should be limited to highly selective cases. Lee et al. [11] proposed Yonsei criteria by which only the following groups of patients should be treated with minimally invasive RAMPS: a) tumor confined to the pancreas, b) intact fascia layer between the distal pancreas and the left adrenal gland and kidney, and c) tumor is localized at least 1–2 cm from the celiac axis.

RAMPS is a safe surgical procedure providing superior vascular and bleeding control compared to SRPS. RAMPS is also a superior procedure in oncologic terms compared to SRPS since it increases the rate of R0 resections, and provides a larger number of lymph nodes harvested. Further, RAMPS does not increase the rates of postoperative complications. Also, there seemed to be an improvement in the overall survival with the RAMPS technique. However, further randomized controlled clinical trials of high quality are needed to draw more solid conclusions regarding the long-term survival benefit.

**Conflict of interest:** None declared.

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# Радикална антероградна модуларна панкреатоспленектомија – приказ два болесника и преглед литературе


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**САЖЕТАК**

**Увод** Радикална антероградна модуларна панкреатоспленектомија (РАМПС) уведена је као модификација стандардне ретроградне панкреатоспленектомије (СРПС). Осмишљена је као нови хируршки приступ са намером да се повећа могућност за постизање негативне постериорне (ретроперитонеалне) ресекционе маргине, као и са циљем комплетног уклањања свих лимфних нодуса *N*1.

**Прикази болесника** Приказујемо два болесника са туморима тела и репа панкреаса дијагностикованим мултидетекторском компјутеризованом томографијом и магнетном резонанцом која су оперисана на нашем одељењу за хепато-билио-панкреатичну хирургију. Код оба болесника урађен је задњи РАМПС. Постоперативни ток код оба болесника протекао је без компликација.

**Закључак** РАМПС је безбедна хируршка процедура зато што омогућава потпуну контролу васкуларних елемената и могућег крварења. То је онколошки супериорна техника у односу на СРПС зато што резултира знатно већим стопама R0 ресекција и знатно већим бројем уклоњених лимфних нодуса. Такође, РАМПС је удружен са бољим дугорочним преживљавањем у односу на СРПС.



**Кључне речи:** карцином панкреаса; тумори тела и репа панкреаса; дистална панкреатектомија; РАМПС

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

# Laparoscopic enucleation of a neuroendocrine tumor on the posterior aspect of the pancreas – case report and literature review

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**SUMMARY**
**Introduction** Neuroendocrine tumors of the pancreas are rare neoplasms. They are divided into two groups: functional and non-functional. Non-functional tumors represent a diagnostic challenge, given that they often remain asymptomatic and are diagnosed as an incidental finding.
**Case outline** We present a patient in whom the tumor was discovered at the junction of the body and the tail of the pancreas, on the dorsal side. The patient had no specific symptomatology, there was no loss in body weight. Considering the diagnostic procedures conducted and the condition of the patient, we decided to perform laparoscopic enucleation. The procedure was carried out in a safe and efficient manner, so that operative and postoperative recovery was uneventful. The definitive histopathological examination confirmed the finding of a non-functional pancreatic neuroendocrine tumor.
**Conclusion** Laparoscopic enucleation is an effective and safe treatment modality for neuroendocrine tumors of the pancreas with well-known advantages, as compared to open surgery, but there is always a tendency to improve the already existing results and thus to contribute, not only to treatment, but to the greater comfort of the patient.
**Keywords:** pancreas; neuroendocrine tumor; laparoscopic enucleation

## INTRODUCTION

Neuroendocrine tumors of the pancreas (pNETs) represent rare neoplasms. They are divided into two basic groups: functional (F-pNETs) and non-functional (NF-pNETs). NF-pNETs can often secrete chromogranin A, neuron-specific enolase, calcitonin, or other peptides, but they are mainly with no characteristic symptomatology [1, 2].

Pre-operative imaging diagnostics is needed for the evaluation and detection of the location of the tumor, and, in this sense, computed tomography (CT) of the abdomen, endoscopic ultrasonography (EUS), and magnetic resonance imaging (MRI) are applied. The octreotide scanner is particularly useful to determine the relevancy or the affinity of the tumor for somatostatin, as well as for the detection of possible tumor foci that were not observed by means of the above-mentioned radiological diagnostics [3].

Modalities of the NF-pNET surgical treatment, depending on the size and the localization of tumors, range from enucleation and atypical pancreatectomy to typical pancreatectomy with lymphadenectomy, including splenectomy; in the case of tumors localized in the distal part of the pancreas [4, 5].

## CASE REPORT

A 56-year-old male patient was admitted to our clinic for diagnostics and treatment. At admission, he was asymptomatic and reported no previous loss in body weight. Three months before hospitalization at our clinic, a focal mass on the pancreas was detected, first with CT scanning, and then by positron emission tomography (PET/CT), which completed the diagnostics (Figure 1). The PET scan showed a radioactive

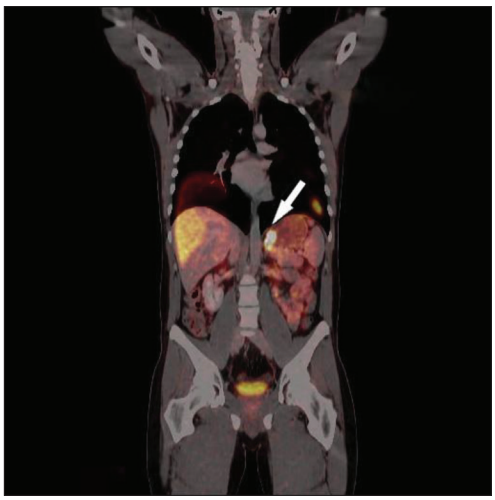

**Figure 1.** Gallium-68 PET/CT DOTATATE scan – with the presented radioactive focus in the part of the body towards the tail of the pancreas

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focus on the posterior aspect of the junction between the body and the tail of the pancreas. The tumor lesion was 18 mm in size and did not invade the intrahepatic duct nor disrupt the contour of the pancreas.

Taking into account the patient's general status and previously conducted diagnostic and clinical examinations, it was decided that the patient should undergo laparoscopic enucleation of the pancreatic tumor.

The patient was put under general endotracheal anesthesia, pneumoperitoneum was created, after which working ports were placed on the sites typical for this type of procedure. Exploration of the abdomen showed a normal finding. The omental bursa was first opened via the intercoloepiploic access, the posterior wall of the stomach was freed, and the upper edge of the pancreas was then accessible. The body and the tail of the pancreas were completely mobilized from the retroperitoneum, wherein the splenic artery and vein were identified. The finding of an enclosed tumor with a capsule, 2 cm in diameter, was verified at the transition from the body to the tail of the pancreas, on the posterior aspect of the pancreas, in the vicinity of the splenic artery (Figure 2). The tumor did not give the impression of infiltrating the pancreatic tissue toward the front, nor having contact with the Wirsung canal. The enucleation of the tumor was performed in full, with the use of the LigaSure device (SurgRx, Redwood City, CA, USA), without damaging the capsule (Figure 3). Re-exploration did not verify lesions of the Wirsung canal. The *pars libera* of the greater omentum was placed into the cavity of the removed tumor, wherein the cavity was completely obliterated. Instead of standard drains, two contact Foley catheters were placed in the vicinity of the cavity. A tissue sample of the tumor was sent for definitive histopathological analysis.

The patient's postoperative recovery was uneventful. Drains were removed on the third postoperative day. The patient was discharged from the hospital four days after surgery. One month after the surgery, abdominal ultrasound follow-up was performed, and the findings were normal. Six months after the surgery, MRI examination was carried out and abdominal findings were normal. The patient is still in the process of regular monitoring and medical follow-up.

Definitive PH: The histological organization of pNETs is predominantly pseudoglandular, insular and trabecular in the foci, but definitely well differentiated. The finding of this tumor was immunohistochemically confirmed, it did not demonstrate any production of hormones, and was assessed as NET-G2 as to its proliferative potential (Figure 4).

The aim of our study was to review laparoscopic enucleation, as an efficient, safe, and secure surgical approach in the treatment of non-functioning neuroendocrine tumors localized on the dorsal side of the pancreas.

All procedures performed involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Written consent to publish all shown material was obtained from the patient.

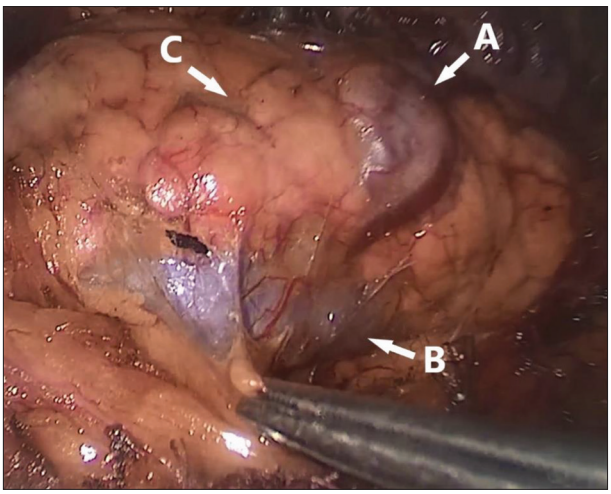


**Figure 2.** Intraoperative finding – the relationship of the tumor to the lienal artery; (A) tumor; (B) lienal artery; (C) posterior wall of the pancreas

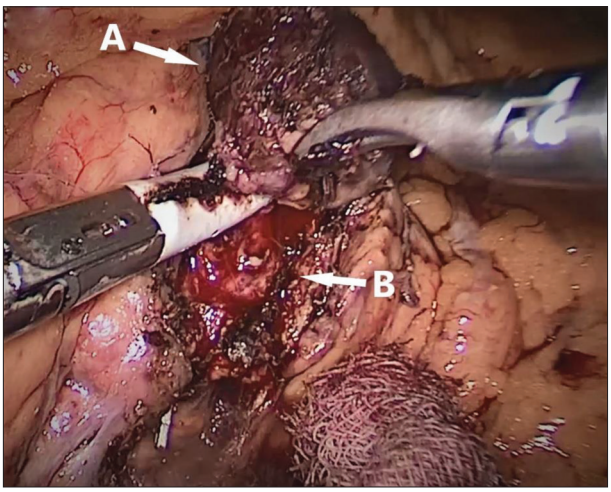


**Figure 3.** Intraoperative photo: tumor enucleated using LigaSure device; (A) tumor of the pancreas; (B) the cavum of the removed tumor

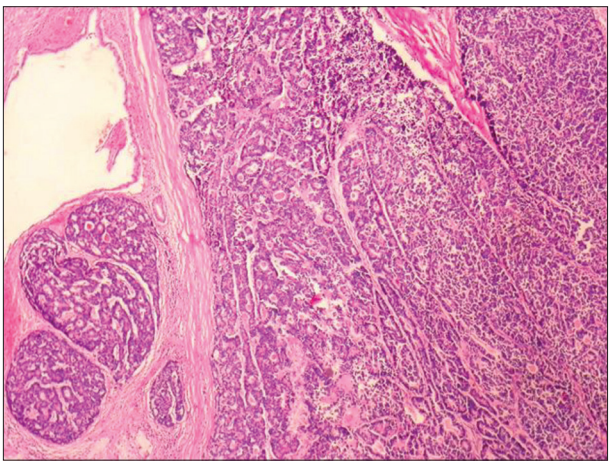

**Figure 4.** Total histomorphological and immunohistochemical findings correspond to well-differentiated neuroendocrine tumor of the pancreas, category (NET – G2)

## DISCUSSION

pNETs originate from cells of the pancreatic islets and represent a heterogeneous group of pancreatic neoplasms. Depending on their ability to secrete biologically active hormones, and whether they show typical clinical symptoms, pNETs are divided into F-pNETs and NF-pNETs [6].

NF-pNETs are generally asymptomatic. Their symptomatology is mostly related to the effect of the mass of the tumor itself on the pancreas or on the surrounding structures, as well as to their correlation with metastases [7]. When the symptomatology is present, it presents with abdominal pain, weight loss, jaundice, and less frequently with anorexia, nausea, fatigue, palpable masses in the abdomen, and other signs and symptoms [8]. Mainly, NF-pNETs present as asymptomatic and are accidentally discovered, most commonly as incidental findings within the diagnostics of other diseases [7, 8].

CT is an initial diagnostic examination in detecting pNETs. These tumors are usually clearly defined lesions. The presence of a hypoechogenic mass in the arterial phase of tomography and the presence of calcifications inside the mass detected is generally associated with a more aggressive form of the tumor, and therefore with a poorer prognosis [9]. The MRI has a higher sensitivity and specificity as compared to the CT scan. The PET/CT and octreotide scanner are the most sophisticated methods used to confirm the diagnosis, as well as to detect the possible presence of metastases or other tumor foci in the body, which were not detected with the previous imaging diagnostic. EUS fine-needle aspiration may be of great importance in determining the nature and localization of the tumor, preoperatively [4, 9].

In the patient that we have presented, preoperative diagnostics equivalent to the guidelines of the current literature was performed, and, after the completion of the preoperative evaluation, we were able to conclude, with great certainty, the diagnosis of NF-pNET, and we accordingly decided on the modality of treatment.

Surgical treatment is the only curative treatment modality for these tumors and, depending on the size of the tumor and its location, it ranges from organ preservation procedures to atypical and typical resection of the pancreas and accompanying lymphadenectomy [5, 10]. According to the literature, with pNETs whose diameter is < 3 cm, enucleation of the tumor can be safely applied. In the 1990s, Gagner performed the first successful laparoscopic procedure on the pancreas and presented his initial experiences and results [10]. Since then, there have been numerous studies and papers which, through their results, indicate the advantages of minimally invasive surgical approaches to the treatment of these tumors, with special emphasis on the enucleation of these lesions, which remains limited by the size of the tumor [11, 12].

Due to uncharacteristic clinical presentation, especially with NF-pNETs, which generally remain asymptomatic and are usually discovered as an incidental finding in the framework of other diagnostic targets, it should be kept in mind that early detection and surgical treatment have good immediate and long-term results in the treatment of this disease. Therefore, it is very important to apply a careful and multidisciplinary approach in each patient. Laparoscopic enucleation, although limited by the size of the tumor, provides an effective, safe, and secure access, regardless of the location of the tumor. The improvement of operational techniques and the introduction of new instruments and equipment provide an opportunity for improving current results.

**Conflict of interest:** None declared.

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# Лапароскопска енуклеација неуроендокриног тумора на задњој страни панкреаса – приказ болесника и преглед литературе

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**САЖЕТАК**

**Увод** Неуроендокрини тумори панкреаса су ретке неоплазме. Деле се у две групе: на функционалне и нефункционалне. Нефункционални тумори представљају дијагностички изазов, с обзиром на то да често остају асимптоматски и дијагностикују се случајно или као узгредни налаз.

**Приказ болесника** Представљамо болесника код кога је тумор откривен на месту спајања тела и репа панкреаса на дорзалној страни. Болесник није имао специфичну симптоматологију, био је без губитка телесне масе. С обзиром на спроведену дијагностику и опште стање болесника, одлучили смо се за лапароскопску енуклеацију. Овај поступак је примењен на сигуран и ефикасан начин, тако да је оперативни и постоперативни ток прошао без компликација. Дефинитивним хистопатолошким прегледом потврђено је да је реч о нефункционалном неуроендокрином тумору панкреаса.

**Закључак** Лапароскопска енуклеација је ефикасан и сигуран начин лечења ових тумора са добро познатим предностима у односу на отворену хирургију, али увек постоји тежња ка побољшању већ постојећих резултата и на тај начин се може допринети не само лечењу већ и бољем комфору болесника.

**Кључне речи:** панкреас; неуроендокрини тумор; лапароскопска енуклеација

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

# Simultaneous ipsilateral rhabdoid renal cell carcinoma and multifocal urothelial carcinoma of the ureter in a patient from the region of Balkan endemic nephropathy – case report and literature review

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**SUMMARY**

**Introduction** Simultaneous ipsilateral coexistence of renal cell carcinoma (RCC) and upper urinary tract urothelial carcinoma (UTUC) rarely occurs. Balkan endemic nephropathy (BEN) is a chronic degenerative tubulointerstitial renal disease, strongly associated with UTUC.

**Case outline** A 60-year-old man from the region of BEN was referred to our clinic due to right flank pain, fever, and purulent discharge from the cutaneous fistulous opening in the right lumbar area. Multislice computed tomography urography scan showed right-side pyonephrosis and nephrocutaneous fistulous tract between the kidney and the skin in the right lumbar region. Cystoscopy detected a papillary tumor protruding from the right ureteric orifice. Right-side nephroureterectomy with bladder cuff excision was performed. Histopathological examination revealed rhabdoid RCC of the kidney and multifocal urothelial carcinoma of the ureter.

**Conclusion** Our case report and literature review indicate that due to rising incidence of multiple primary malignant neoplasms (MPMNs), when treating patients with RCC or UTUC, and especially those from the region of BEN, one should keep in mind the likelihood of synchronous or metachronous occurrence of these tumors.

**Keywords:** rhabdoid renal cell carcinoma; urothelial carcinoma; Balkan endemic nephropathy; pyonephrosis; nephrocutaneous fistula

## INTRODUCTION

Renal cell carcinoma (RCC) represents the most common kidney tumor, accounting for approximately 85% of all kidney malignancies [1]. Although upper urinary tract urothelial carcinoma (UTUC), especially ureteric, occurs more frequently in some regions of the Balkans than in other areas of the world, its ipsilateral simultaneous coexistence with RCC is extremely rare [2, 3]. We report a rare case with simultaneous occurrence of ipsilateral rhabdoid RCC (RRCC) and UTUC of the ureter, in a 60-year-old man from the region of Balkan endemic nephropathy (BEN).

## CASE REPORT

A 60-year-old male patient from the region of BEN was admitted to our clinic due to right flank pain and fever up to 39°C. Physical examination revealed a palpable right flank mass, with purulent discharge from the cutaneous fistulous opening localized at the level of the middle axillary line, below the 12th rib (Figure 1).

Laboratory testing revealed elevated inflammatory markers, including white blood cell count of $19 \times 10^9$ cells/L, with 85.1% neutrophils, C-reactive protein of 58.4 mg/dL, procalcitonin of 16.25 ng/mL, and serum creatinine of 1.65 mg/dL. Multislice computed tomography urography revealed normal left kidney and enlarged, nonfunctioning right kidney, with high-density fluid within dilated renal collecting system and a fistulous communication to the skin of the right lumbar region. Cystoscopy detected a papillary tumor protruding from the right ureteric orifice.

Following adequate preparation of the patient, right side nephroureterectomy with bladder cuff excision was performed. Intraoperative findings showed enlarged right kidney, adherent to surrounding structures due to pyonephrosis, with dilated proximal ureter and a tumor in its distal part. The postoperative course was regular.

On gross inspection, the rhabdoid component of RCC appears as solid white areas at the peripheral part of the kidney parenchyma and

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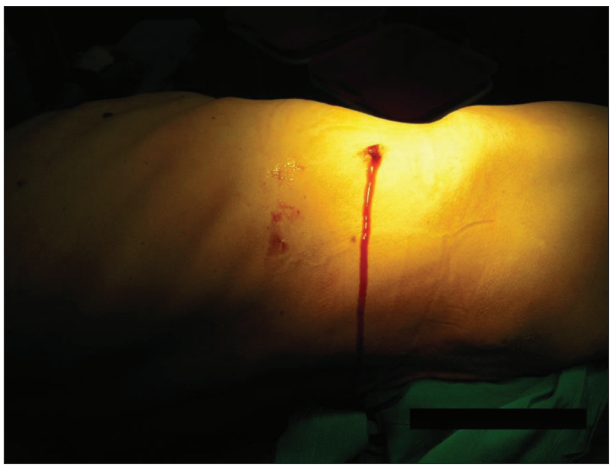

**Figure 1.** Nephrocutaneous fistula in the right lumbar area

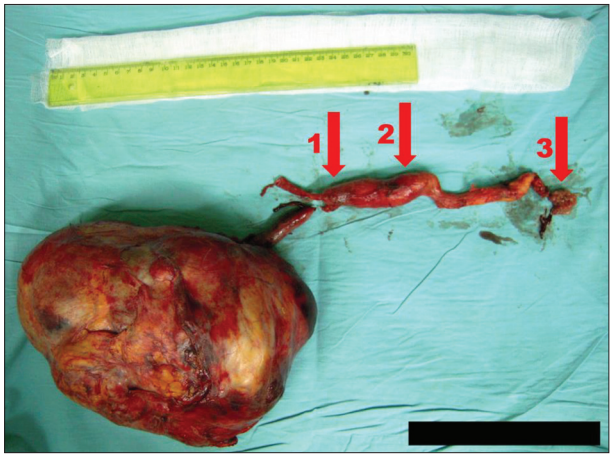


**Figure 2.** Operative nephroureterectomy specimen showing sites of two mid-ureteric tumors (1, 2) and the distal ureteric tumor (3)

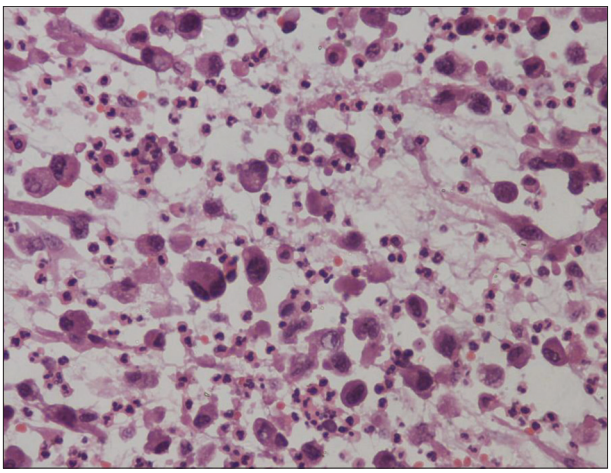

**Figure 3.** Rhabdoid component of renal cell carcinoma with myxoid stromal change (H&E, ×400)

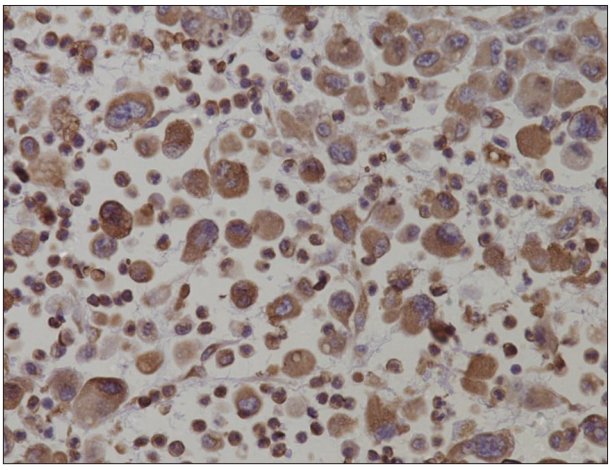

**Figure 4.** The representative immunohistochemical staining of vimentin in rhabdoid component of renal cell carcinoma (EnVision, ×400)

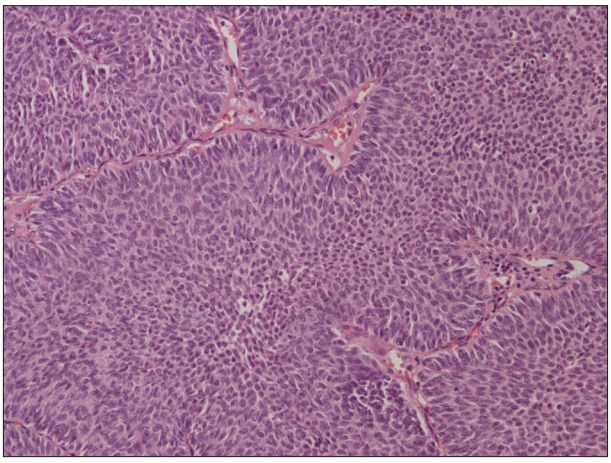

**Figure 5.** Ureteral-associated transitional cell carcinoma (H&E, ×200)

surrounding fatty tissue. The dominant finding was massive necrosis and hemorrhage. There was a total of three ureteric tumors, all with papillary configuration. One of them was located at the distal part, protruding into the bladder cuff, while the other two had occluded lumen of the upper ureter (Figure 2).

On microscopic examination, with standard hematoxylin and eosin (H&E) stain, the rhabdoid component of RCC was characterized by large round cells with globular eosinophilic paranuclear inclusion bodies, large eccentric vesicular nuclei with prominent nucleoli. Some cells had clear cytoplasm. The architectural growth pattern was alveolar with delicate fibrovascular septae encircling solid nests of cell. Stroma was scanty, with myxoid change in some parts of the tumor (Figure 3). The RRCC has been associated with hemorrhage and necrosis with neutrophil infiltration, with vascular and perirenal fat invasion (Fuhrman grade IV, pT3aNxMx). The rhabdoid component was dominant and had been intermixed with the sarcomatoid component. RRCC was analyzed using the immunohistochemical method with standard En vision system. Rhabdoid cells showed focal immunoreactivity with pancytokeratin and RCC, and vimentin in 100% of tumor cells (Figure 4). Both pancytokeratin and vimentin gave diffuse cytoplasmic staining, with strong accentuation in the globular cytoplasmic inclusion bodies. Despite their morphology, the rhabdoid cells showed no evidence of myoblast differentiation as immunostains for the muscle markers desmin and muscle-specific actin / smooth muscle actin had been negative. Nuclear staining for the Ki-67 nuclear proliferation antigen was strongly present in rhabdoid and

sarcomatoid components, in more than 90% of tumor cells. Other investigated immunohistochemical markers, including cytokeratin 7 and 20, were negative in all cells. Ureteric tumors showed classical features of urothelial carcinoma. One of them was high-risk (high-grade; pT2NxMx), while the other two showed a classical feature of low-grade, low stage urothelial carcinoma (pTaNxMx) (Figure 5).

Written consent to publish all shown material was obtained from the patient.

## DISCUSSION

Simultaneous ipsilateral RCC and UTUC rarely occur. Since the first case presented in the literature as far back as 1921 by Graves and Templeton, approximately 50 cases of simultaneous occurrence of RCC and UTUC in the ipsilateral kidney or ureter have been reported in the literature until today [3, 4]. The incidence of multiple primary malignant neoplasms (MPMNs) ranges 0.7–11.7%, with an increasing tendency [5, 6].

In respect to all histologic types of RCC in adults, clear cell is the most common, accounts for 70% of the total, while other types are less frequent, including papillary RCC in 10–15%, chromophobe in 4–6%, unclassified in 4–5%, and collecting duct carcinoma in less than 1% [6]. UTUCs occur infrequently, accounting for 5–10% of all urothelial carcinomas [7]. However, the incidence is increased in the region of BEN [2, 8].

Epidemiological data on simultaneous ipsilateral RCC and UTUC show extremely rare incidence of this phenomenon. In a review of more than 700 cases operated on due to RCC, Voneschenbach et al. [9] cited only one case (0.14%) of ipsilateral coexistence of RCC and UTUC of the renal pelvis. Although the exact mechanism and relationship of ipsilateral RCC and UUT development remains unclear, urinary stone disease, hydronephrosis, chronic irritation and smoking are referred to as the most common etiological factors [10]. The symptoms and demographics in these patients correspond to those that occur in patients with solitary RCC or UTUC, with hematuria as the most common symptom (90%), following by flank pain (19%) and a palpable flank mass (14%) [3, 11]. Compared to the right side, these tumors affect the left kidney and ureter three times more often [11]. As proposed by Rouprêt et al. [12], regardless of tumor location, the standard management in high-risk UTUC includes open radical nephroureterectomy with bladder cuff excision.

Spontaneous kidney to skin fistulous communication is a rare condition, occurring as a complication of renal surgery, stone disease, tumors, injuries, and perirenal abscess formation [13]. Usually, fistulous tract is directed to the weak anatomical sites of the lumbar region, opening into external space in the area of the lumbar (Petit's) triangle and the superior lumbar (Grynfeltt's) quadrilateral space [14].

BEN is a chronic degenerative tubulointerstitial renal disease, strongly associated with UTUC, typically occurring in villages of certain areas of the Danube tributaries within the region of the Balkans [8]. The pathology of BEN has been elaborated in several reports. However, one of the hypotheses, namely the chronic poisoning with aristolochic acid, a toxin produced by plants of the *Aristolochia* genus, has provided significant evidence as to the primary causative agent in BEN, and particularly in the role of developing BEN-associated cancer [15]. A possible cause of BEN development and also the urothelial cancer onset are the DNA adducts, since metabolic activation of the aristolochic acids leads to a reduction of the nitro group to create N-hydroxylaristolactams (N-hydroxyl-ALs) [16]. A recent study by Rosenquist and Grollman [17] has shown that aristolochic acid had been also associated with the occurrence of some tumors other than UTUC, including RCC, bladder tumors, and some liver tumors [17]. Patients with BEN also show a tendency towards certain metabolic alterations, which have not been observed in patients with other kidney diseases. Petković et al. [18] showed that the main risk factors for vascular calcification, including systolic blood pressure, as well as serum cholesterol and phosphorus levels, are less pronounced in patients with BEN.

In this case report, we present a patient with a rare and complex pathological substrate, with an atypical presentation. Interestingly, the existence of RCC was not verified by the diagnostic procedure, since it was hidden by the finding of pyonephrosis, which was a consequence of complete obstruction of the ureter by tumors.

Rhabdoid differentiation in clear cell RCC has a typical pattern of dedifferentiation, suggesting poor prognosis, as reported by Yang et al. [19]. Investigating the impact of rhabdoid and sarcomatoid differentiation on the prognosis in 264 patients operated on due to RCC, Kara et al. [20] reported that, unlike sarcomatoid, rhabdoid differentiation was not associated with an increased risk of lethality. Sarcomatoid differentiation is reported as an independent negative prognostic factor, in both localized and advanced disease, associated with higher grade and stage, with lower cancer-specific survival rates comparing to grade 4 RCC [20].

It is noteworthy to emphasize the possibility of misdiagnosing RRCC for transitional cell carcinoma with sarcomatoid differentiation, which can significantly affect the therapy and the prognosis. As for the simultaneous occurrence of RCC and UTUC, it cannot be concluded whether it is a synchronous or metachronous occurrence, due to a lack of data when each of these tumors appears. According to Moertel et al. [21], a malignant tumor is considered synchronous if it occurs within six months of the onset of the previous tumor, while a metachronous tumor occurs after that period. Terada et al. [22] state that neither the mechanism nor the causal relationship of ipsilateral simultaneous tumorogenesis of RCC and UTUC have been elucidated. Given that the presented patient is from the BEN region, one possible explanation is the impact of aristolochic acid as a mutagenic environmental factor on the genome, and further association with tumorogenesis of various tumors, including UTUC and RCC.

In regard to the follow-up, there is no standardized protocol, but postoperative evaluation is performed according to the protocols for UTUC and RCC, separately. At the first postoperative control after three months, the patient felt

well, with normal findings on urethrocystoscopy, abdominal ultrasound and MSCT of the lungs. To our knowledge, this is the only case report with the simultaneous occurrence of ipsilateral RRCC and multifocal urothelial carcinoma of the ureter, with a clinical presentation in the form of pyonephrosis with spontaneous nephrocutaneous fistula. Having in mind the nature of these tumors, above all RCC, we consider that in this particular case, rigorous postoperative monitoring is necessary, in order to detect early disease progression and achieve maximum therapeutic effects.

Our case report and literature review indicate that due to the rising incidence of MPMNs, when treating patients with RCC or UTUC, and especially those from the region of BEN, one should keep in mind the likelihood of synchronous or metachronous occurrence of these tumors.

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# Симултани ипсилатерални рабдоидни карцином бубрежних ћелија и мултифокални уротелни карцином уретера код болесника из региона балканске ендемске нефропатије – приказ болесника и преглед литературе

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**САЖЕТАК**

**Увод** Симултана ипсилатерална коегзистенција карцинома бубрежних ћелија и карцинома уротела горњег уринарног тракта ретко се јавља. Балканска ендемска нефропатија представља хроничну дегенеративну тубулоинтерстицијалну болест бубрега, снажно повезану са карциномом уротела горњег уринарног тракта.

**Приказ болесника** Шездесетогодишњи мушкарац из региона балканске ендемске нефропатије упућен је у нашу клинику због болова у десној слабини, повишене телесне температуре и гнојног пражњења из фистулозног отвора на кожи у пределу десне лумбалне регије. Вишеслојна компјутеризована томографија са урографијом показала је десностране пионефрозу и нефрокутани фистулозни тракт између бубрега и коже десне лумбалне регије. Цистоскопијом је откривен папиларни тумор који вири из орифицијума десног уретера. Урађена је десностранa нефроуретеректомија са ексцизијом манжетне мокраћне бешике. Хистопатолошким прегледом откривен је рабдоидни карцином бубрежних ћелија и мултифокални уротелни карцином уретера.

**Закључак** Наш приказ болесника и преглед литературе указују да због све веће учесталости вишеструких примарних малигних неоплазми, приликом лечења болесника са карциномом бубрежних ћелија или карциномом уротела горњег уринарног тракта, а посебно оних из региона балканске ендемске нефропатије, треба имати на уму вероватноћу синхроне или метахроне појаве ових тумора.

**Кључне речи:** рабдоидни карцином бубрежних ћелија; карцином уротела; балканска ендемска нефропатија; пионефроза; нефрокутана фистула

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

# Rotavirus gastroenteritis as a precipitating factor of celiac crisis in infancy – case reports and review of literature

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**SUMMARY**

**Introduction** Celiac crisis is a rare and life-threatening complication of celiac disease. Although it occurs in all ages, the most common affects children within the first two years.

**Outline of cases** We report three infants (two female, one male, age range 9–12) with celiac crises as an initial presentation of celiac disease precipitated with rotavirus gastroenteritis. Celiac crisis was preceded by failure to thrive caused by anorexia, occasional vomiting and frequent abundant stools for 4–8 weeks, and 1–2 days before admission with fever, frequent vomiting and profuse watery diarrhea. They were admitted in a very severe general condition, severely dehydrated, markedly malnourished, with an enormously distended abdomen, edema of the lower legs and feet, and perianal erythema. After correction of dehydration and hypoalbuminemia, they were placed on a gluten- and disaccharide-free diet and within the first two weeks on additional parenteral nutrition. The applied therapeutic measures resulted in stabilization and further rapid improvement of the patient's condition. In all three patients the latex agglutination test for rotavirus was positive, IgA anti-TTG antibodies elevated (58.6–78 U/ml) and all three were homozygous carriers of the HLA DQ2 gene. Enterobiopsy was performed two weeks after the admission and total villous atrophy (Marsh IIIc) was registered in all three patients. In the further course, the complete recovery of the patient was accomplished by a strict gluten-free diet.

**Conclusion** Our experience indicates that rotavirus gastroenteritis in timely unrecognized classical celiac disease in infants can lead to celiac crisis.

**Keywords:** celiac crisis; infants; rotavirus gastroenteritis

## INTRODUCTION

Celiac crisis (CC) is a rare life-threatening complication of celiac disease (CD), which is mostly seen in the first two years of life [1–9]. It is characterized by a spontaneous or induced acutization of chronic diarrheal disorder followed by severe hydroelectrolytic, acid-base and nutritive disbalance [2, 3, 4, 9–13]. Frequent precipitating factors of the disease in children involve interecurrent gastrointestinal or extraintestinal infections [1, 2]. We present three infants with CC, as an initial presentation of CD precipitated by rotavirus (RV) gastroenteritis. We present three infants hospitalized over the past 15 years due to CC as an initial presentation of CD precipitated by RV gastroenteritis.

## CASE REPORTS

### Case 1

A nine-month-old female infant with the data of frequent and abundant stools, loss of appetite, occasional vomiting and weight loss since age 7.5 months. Two days before admission febrile (up to 38.6°C, rectally), does not accept meals, often vomits and received profuse watery diarrhea. On admission severely dehydrated, noticeably malnourished, somnolent, adynamic, with cold and cyanotic acral regions, edema of the foot dorsum and lower limbs, and perianal erythema. Rectal temperature (RT) 36.1°C, heart rate (HR) 140/min, respiration rate (RR) 42/minute and blood pressure (BP) 60/35 mmHg. Body length (BL) P 50%, body weight (BW) -28%. Stool pH 5 and Clini test +++. Antibodies to tissue transglutaminase (AtTG) IgA 66.3 U/ml.

### Case 2

A 10-month-old male infant admitted due to severe dehydration, meteorism, and marked malnutrition followed by generalized hypoproteinic edema. According to parents, over the previous two months he had frequent, abundant, fatty or watery and loose stools, progressive food aversion, intermittent postprandial vomiting, apathy, irritability and weight loss. Two to three days before admission he

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developed a sudden increase of fever, persistent vomiting and profuse watery diarrhea. On admission somnolent, adynamic, afebrile (RT 36.6°C), HR 146/min, RR 46/min, deep, BP 55/30 min/Hg, BH P25% and BW -32%. Perianal erythematous ulcer changes. Stool pH 5, Clini test +++. AtTG IgA 78 U/ml.

### Case 3

A 12-month-old female infant admitted a day after a sudden deterioration of one-month diarrheal disorder followed by high fever**,** vomiting, profuse watery diarrhea, severe dehydration and meteorism**.** On admission adynamic, apathetic, irritable, subfebrile (RT 37.9°C), HR 148/min, RR 44/min, BP 70/40 mmHg, BH P25–50% and BW -24%, edematous dorsum of feet, perianogenital erythema. Stool pH 5.5, Clini test +++. AtTG IgA 58.6 U/ml.

Nutrition, onset of CD symptoms and blood laboratory values on admission of the infants with CC are shown in Tables 1 and 2.

By the method of latex agglutination, in all three children we confirmed RV in the stool. Stool examination for pathogenic bacteria and *Giardia lamblia* were negative in all three patients. Cases 1 and 2 had a mild hypertransaminasemia, without hepatomegaly**,** hyperbilirubinemia and increased activity of serum creatinine-phosphatase.

After correction of dehydration and hypoalbuminemia, patients were placed on a gluten- and disaccharide-free diet and within the first two weeks on additional parenteral nutrition. The therapeutic measures resulted in the stabilization of the patients' condition followed by gradual recovery. Due to marked secretory diarrhea, during the first ten days the prevention of dehydration was done intravenously and during the next seven to ten days i.e., until the normalization of stool frequency, with oral rehydration solution (Orosal® 65, Galenika). After two weeks of treatment, enterobiopsy was performed and in all three cases the stereomicroscopic and histological examination of the small intestinal mucosa showed a total villous atrophy (Marsh IIIc) (Figure 1). Also, all three were homozygous carriers of the HLA DQ2 gene. On a gluten-free diet and a four-month-long supplementation of iron and multivitamin preparations, all three patients completely recovered.

This case report was approved by the institutional ethics committee, and written consent was obtained from the patients' parents/caregivers for the publication of this case report and any accompanying images.

## DISCUSSION

We reported about three infants with CC as the initial presentation of CD precipitated by RV gastroenteritis. In all three cases CC was preceded by disturbances which evidently indicated the classical form of CD that was not timely recognized, while 1–2 days before admission the infants developed the typical signs of RV gastroenteritis: increased fever, frequent vomiting and profuse watery diarrhea, i.e., osmotic-secretory diarrhea [1, 14]. All three children were shortly breast fed and two were exposed to gluten too early, which, in the presence of genetic predisposition, led to early expression of CD [15–18]. On the other hand, as Serbian children are not vaccinated against rotavirus**,** RV infection occurred at the age 9–12 months, i.e., in the period of the loss of prenatally acquired passive immunity, which, associated with the absence of maternal milk protective effect, added to the development of a severe clinical form of infective enteritis [14, 19–23]. The common characteristic of both diseases was that the inflammatory changes of the small bowel mucosa were most expressed in the proximal portion of the small bowel, i.e., in the segment that plays the central role in the processes

**Table 1.** Nutrition and onset of first celiac disease (CD) symptoms in infants with celiac crisis

| Case | Duration of breast feeding (months) | Age at gluten introduction (months) | Age at onset of CD symptoms (months) |
|---|---|---|---|
| 1 | 1 | 3.5 | 7.5 |
| 2 | 1.5 | 3 | 8 |
| 3 | 0.25 | 4 | 11 |

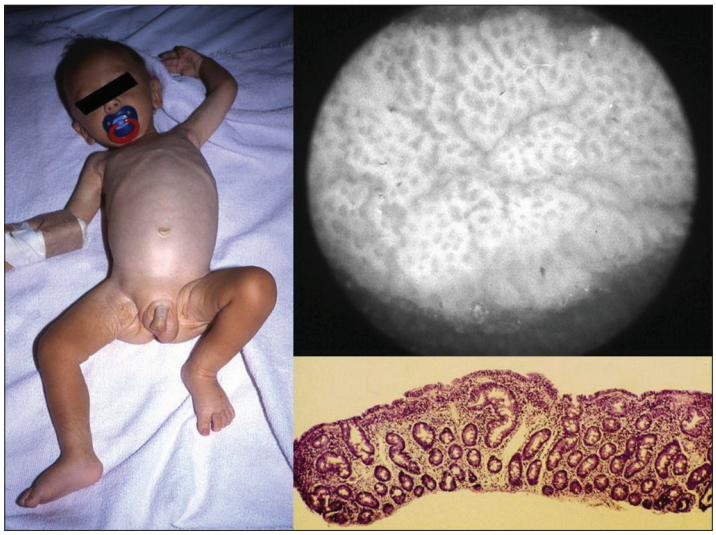

**Figure 1.** Male patient in the initial phase of recovery and stereomicroscopic and histological appearance of the patient's small intestinal mucosa

**Table 2.** Blood laboratory values on admission

| Case | Sodium (mmol/l) | Potassium (mmol/l) | pH* | Creatinin (μmol/l) | Calcium (mmol/l) | Magnesium (mmol/l) | Phosphate (mmol/l) | Albumin (g/l) | Hb (g/l) |
|---|---|---|---|---|---|---|---|---|---|
| 1 | 129 | 2.8 | 7.24 | 101 | 1.96 | 0.66 | 0.79 | 22 | 98 |
| 2 | 122 | 2.4 | 7.21 | 108 | 1.79 | 0.63 | 0.64 | 18 | 84 |
| 3 | 130 | 2.9 | 7.26 | 98 | 1.98 | 0.67 | 0.76 | 24 | 79 |

*Capillary blood

of food digestion and absorption [1, 14]. The joint action of two different types of inflammation, autoimmune and infective, resulted in the severe damage of the small bowel mucosa followed by the reduction of its functional surface and epithelial immaturity [24, 25]. Therefore, the initial diarrheal disorder in association with CD complicated by RV gastroenteritis suddenly resumed a severe clinical course to enter all three infants into CC. By the correction of hydroelectrolytic and acid-base disbalance, albumin deficit compensation, as well as gluten and disaccharide-free diet, the condition of the patients stabilized. However, due to the prolonged secretory diarrhea and poor nutritional state, all three patients required a 10-day intravenous hydroelectrolytic and a two-week parenteral nutritional support. Three weeks of treatment resulted in disaccharide tolerance, so that gluten-free diet was sufficient to achieve full recovery.

In conclusion, RV gastroenteritis and CD are characterized by identical localization of the small intestinal damage. Thus, this infection in infants with timely unrecognized classical CD can lead to CC.

**Conflict of interest:** None declared.

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# Ротавирусни гастроентеритис као преципитирајући фактор целијачне кризе код одојчади – приказ болесника и преглед литературе

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**САЖЕТАК**

**Увод** Целијачна криза је ретка и животно угрожавајућа компликација целијачне болести. Иако се јавља у свим добима, најчешће погађа децу унутар прве две године.

**Приказ болесника** Приказујемо три одојчета (два женског и једно мушког пола, узраста 9–12 месеци) са целијачном кризом као иницијалном презентацијом целијачне болести преципитирану ротавирусним гастроентеритисом. Целијачној кризи је претходио поремећај напредовања узрокован анорексијом, повременим повраћањем и честим обилним столицама 4–8 недеља и 1–2 дана пре пријема повишена температура, често повраћање и профузна водена дијареја. Одојчад су примљена у врло тешком општем стању, тешко дехидрирани, изразито потхрањени, са енормно дистендираним трбухом, едемом потколенице и стопала и перианалним еритемом. Након корекције дехидрације и хипоалбуминемије, стављени су на дијету без глутена и дисахарида и унутар прве две недеље на додатну парентералну исхрану. Примењене терапијске мере су резултирале стабилизацијом и у даљем току брзим побољшањем стања болесника. Код сва три болесника латекс аглутинациони тест на ротавирус био је позитиван, антитела *IgA* анти-*TTG* повишена (58,6–78 *U/ml*) и сва три су били хомозиготни носиоци гена *HLA DQ2*. Ентеробиопсија је урађена две недеље после пријема и код сва три болесника је регистрована тотална вилозна атрофија (*Marsh IIIc*). У даљем току, на строгој дијети без глутена, уследио је потпуни опоравак болесника.

**Закључак** Наша искуства указују да ротавирусни гастроентеритис код благовремено непрепознате класичне целијачне болести код одојчета може довести до целијачне кризе.

**Кључне речи:** целијачна криза; одојчад; ротавирусни гастроентеритис

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CASE REPORT / ПРИКАЗ БОЛЕСНИКА

# Management of fulminant mucormycosis of the maxillary sinus and orbit with an uncontrolled diabetic

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**SUMMARY**

**Introduction** Mucormycosis of paranasal sinuses is a rare life-threatening opportunistic fungal disease that requires urgent treatment. The commonly involved are the immunosuppressed and immunocompetent patients. Patients are presented with facial or orbital cellulitis, necrotic palate, paresthesia of facial or trigeminal nerves and loss of vision, signs of meningitis. Radiological examinations are not sensitive in the early stages of infection. Definitive diagnosis is established by biopsy and histological examination of the necrotic tissue.

**Case outline** In August 2017, a 52-year-old female diabetic was admitted to the Clinic for Maxillofacial surgery due to the swelling and pain in the right side of the face, headache, fever, restriction of ocular movements, purulent rhinorrhea lasting for one week. Computed tomography examination showed spreading cellulitis of the right side of the face, total right maxillary end ethmoid sinus heterogeneous occupation and osteitis of the maxillary walls. Radical surgical debridement was performed. Histopathology and microbial tests were consistent with the finding of invasive mucormycosis. Liposomal amphotericin B 5mg/kg per day for four weeks was administered and patient's glucose levels were controlled with injectable insulin and local status significantly improved. Patient was reoperated later due to the defect of the right maxilla.

**Conclusion** Early diagnosis and multidisciplinary approach including microbiology, pathology, radiology, surgery, hematology, infectious disease, intensive care and pharmacology is essential. Treatment of mucormycosis of paranasal sinuses requires prompt and aggressive treatment with antifungal agents, surgical debridement and control of predisposing factors.

**Keywords:** mucormycosis; paranasal sinuses; fungal infection; diabetes; opportunistic infection

## INTRODUCTION

Mucormycosis of paranasal sinuses is a rare, fulminant life threatening opportunistic fungal disease that requires urgent treatment because of its progressive and destructive nature [1, 2, 3]. The commonly involved are the immunosuppressed and immunocompetent patients. Poorly controlled diabetes is the major risk factor for this opportunistic fungal infection, however there is increasing number of reports of patients with mucormycosis undergoing chemotherapy, immunotherapy, and organ transplantation [1, 2, 3]. Due to anatomical factors and compromised immunity, infection from the paranasal sinuses can spread intracranially and is termed as rhinocerebral mucormycosis. Even in the modern medical age, death rates are very high, 40–80% even with antifungal therapy [1, 2, 3]. The infection is caused by the fungi of the order Mucorales which can be found as saprophytic microorganisms in the upper airway tract mucosa, with Rhizopus species most commonly found in the paranasal mucormycosis [1, 2, 3]. In immunocompromised patients due to impaired phagocytic function of granulocytes the fungi spores develop into a hyphae form, invade into the blood vessels, causing thrombosis and progressive soft necrosis and bone destruction [1, 2, 3]. Due to early invasion of blood vessels the infection can be disseminated, and rhinocerebral, pulmonary, gastrointestinal and cutaneous are the most often forms [4, 5].

The clinical manifestations at the early stages are not specific, but with the disease progression unilateral headache, facial pain, fever, numbness and nasal discharge are common. Patients are presented with facial or orbital cellulitis, necrotic palate, paresthesia of facial or trigeminal nerves and loss of vision, signs of meningitis with intracranial propagation [1, 2, 3]. Radiological examinations are not sensitive in the early stages of infection. Computed tomography (CT) shows osteitis of maxillary walls and occupation of maxillary sinus. Magnetic resonance imaging (MRI) is needed when intracranial propagation is suspected. Definitive diagnosis is established by biopsy and histological examination of the necrotic tissue.

Treatment of mucormycosis of paranasal sinuses must be aggressive and quick, consists of early diagnosis, control of the predisposing disease and aggressive surgical debridement and antifungal therapy.

The aim of this case report is to present a rare case of mucormycosis of maxillary sinus and orbit in patient with newly discovered

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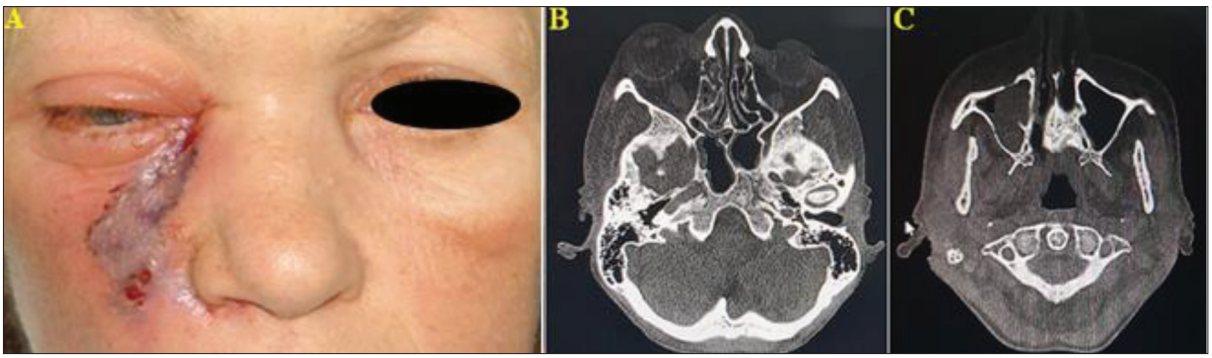


**Figure 1.** A) Patient presenting with cellulitis of the right side of the face and necrotic skin of paranasal area; B) computed tomography showing right ethmoid cells with heterogeneous density and osteitis; C) computed tomography showing right maxillary sinus with heterogeneous density and osteitis

diabetes, demonstrating the diagnostic dilemmas and treatment algorithm.

All procedures described in this paper involving were in accordance with the institutional ethical standards and with the 1964 Helsinki declaration. Informed consent was obtained from the patient for being included in the study and use of medical data and clinical pictures.

## CASE REPORT

In August 2017, 52-year-old female patient with newly discovered diabetes mellitus type II was admitted to the Clinic for Maxillofacial Surgery due to the swelling and pain in the right side of the face, headache, fever, restriction of ocular movements, purulent rhinorrhea lasting for one week (Figure 1). Because of the swelling propagation and suspicion on orbital cellulitis we performed CT examination which showed spreading cellulitis of the right side of the face, total right maxillary end ethmoid sinus heterogeneous occupation and osteitis of the maxillary walls. Because of the rapid progression of infection, ophthalmoplegia and necrosis of skin in infraorbital area, we suspected fungal infection and decided to perform radical surgical debridement consisting of removal of necrotic skin and subcutaneous tissue, partial maxillectomy and ethmoidectomy via intraoral approach and decompression of the orbit. The necrotic skin, mucosa of the maxillary and ethmoid sinuses and orbital preseptal tissue were sent for histopathological examination. Histopathology tests showed fragments of the necrotic tissue with spores and irregularly shaped hyphae with perivascular infiltration, infiltration by neutrophils consistent with the finding of invasive mucormycosis. Culture of biopsy specimens showed colonies were consistent with Mucorales. Antimicrobial susceptibility testing was performed and parenteral liposomal amphotericin B 5 mg/kg per day for four weeks was administered. During hospitalization, the patient's glucose levels were controlled with injectable insulin and local status significantly improved. On control CT exams, performed weekly, there were no signs of infection.

However, after two months, the patient returned to the clinic due to the cellulitis of the right side of the face and

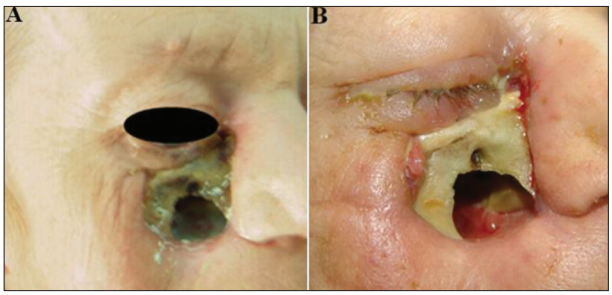


**Figure 2.** Relapse of the infection two months after first treatment; A) defect of the skin and right maxilla; B) necrotic bone of anterior maxilla, floor of the orbit and zygomatic bone

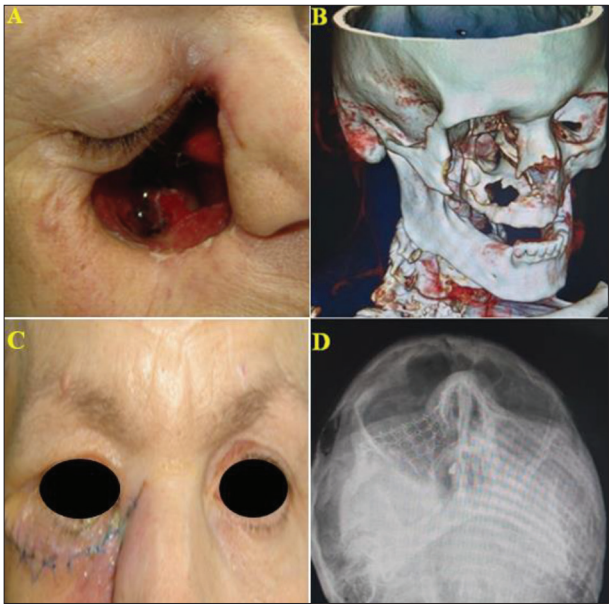


**Figure 3.** Reconstruction of the defect of the right maxilla six months after first treatment; A) clinical appearance of the defect; B) 3D reconstruction of the defect of the right maxilla; C) postoperative appearance of the patient after reconstruction of the floor of the orbit and skin; D) control radiographic picture

the antro-cutaneous fistula (Figure 2). Blood glucose levels were in normal range and vital signs were normal. The patient was reoperated and radical surgical debridement with removal of necrotic maxillary processes of zygomatic bone, floor of the orbit and medial orbital wall and necrotic skin of the right cheek was performed. The defect was partially reconstructed with local skin flap. Postoperatively

parenteral therapy included amphotericin B (5 mg/kg per day) and meropenem. The surgical specimens were sent for pathohistological analysis and bony and soft tissue specimens were positive for fungal spores and hyphae consistent for mucormycosis. Postoperative period was uneventful and patient was released from the hospital in good general health.

In February 2018, six months following the first infection, we decided to reconstruct the defect of the base of the orbit with titanium mesh and defect of the skin of the infraorbital region with local skin flap (Figure 3). After this surgery patient was reoperated in the April 2018, November 2018, and October 2019, because of the skin dehiscence and ectropion when the local skin flaps were used to reconstruct skin defect. At a recent follow-up two years after mucormycosis was diagnosed, there were no signs of recurrent infection.

## DISCUSSION

Although rare, mucormycosis is a life-threatening fungal infection that occurs in immunocompromised patients. Previous papers reported that there is tendency to an increase of incidence of mucormycosis mainly due to the increasing number of immunocompromised patients [2, 6, 7]. Treatment of mucormycosis of paranasal sinuses requires early diagnosis, prompt and aggressive treatment with antifungal agents, surgical debridement and control of predisposing factors. However, despite early diagnosis and the aggressive surgical and medical treatments, the mortality rate is very high and reports from literature show to be 40–80% [1, 2, 3]. Thus, early diagnosis and multidisciplinary approach including microbiology, pathology, radiology, surgery, hematology, infectious disease, intensive care and pharmacology is essential [2].

Rhinocerebral mucormycosis is typically presented with diabetic patients, while cutaneous and pulmonary forms are common in immunocompetent patients [7, 8]. Rhinocerebral mucormycosis commonly develops in paranasal sinuses, most often in maxillary sinus and subsequently involves orbit, brain and cranial bones. Localized sinus infection when early discovered and aggressively treated have good prognosis, while intracranial propagation, especially in immunocompetent patients, have very poor prognosis [1, 2, 3]. Immunological state, predominantly severe neutropenia, was found to be the most significant negative survival factor, regardless to the extent of the fungal infection [1, 2].

Surgical debridement with free margins is still the standard therapy in order to control infection and obtain tissue for histopathological and microbiological diagnosis. Postoperative sequels such as disfigurement, loss of vision, difficulties with oral function, and low quality of life are common with surviving patients [3].

**Table 1.** Treatment algorithm for rhinocerebral mucormycosis

| Suspected mucormycosis | |
|---|---|
| 1. Clinical picture | Facial pain, swelling, sinusitis, ophthalmoplegia, persistent fever, headache<br>Uncontrolled diabetic, neutropenic patient |
| 2. Cranial CT<br>Cranial MRI<br>Chest CT | Bone destruction, sinus involvement<br>Orbit, brain involvement<br>Respiratory symptoms |
| 3. Infected tissue biopsy/ endoscopy | Histopathology<br>Microbiology |
| 4. Radical surgical debridement | Obtain clean margins<br>Control the infection<br>Microbiological diagnosis |
| 5. Long term antifungal treatment | Amphotericin B 5–10 mg/kg – first line treatment<br>Posaconazole 200–1000 mg/day<br>Isavuconazole 200–1000 mg/day |
| 6. Control of predisposing factors | Diabetes<br>Neutropenia |
| 7. Control images | Treatment response assessment |
| 8. Delayed reconstruction | 6–12 months following local control of infection |

CT – computed tomography; MRI – magnetic resonance imaging

Thus, further research should be made to redefine the need for radical surgical debridement. First line of antifungal treatment is liposomal amphotericin B in doses 5–10mg/kg per day, with constant monitoring of serum creatinine concentrations. In cases of acute renal toxicity, azaconazole and posaconazole were found to be safe alternatives. Several reports demonstrated efficacy of posaconazole and amphotericin B combination, even without the need for surgical treatment [2, 3, 4]. Currently there is no relevant protocol regarding dosage and duration of antifungal treatment. Our patient received antifungal treatment with amphotericin B for four weeks during first hospitalization, but infection relapsed even with good control of diabetes and absence of infection on control CT scan. Thus, we feel that reconstructive surgery for these patients should be delayed for at least six months after treating the infection.

Treatment of rhinocerebral mucormycosis requires multidisciplinary approach. Facial swelling, orbital cellulitis, headache, ophthalmoplegia and sinusitis in diabetic patients should raise suspicion of rhinocerebral mucormycosis. Both CT and MRI examinations should be performed. Following radiological examinations, if sinusitis is diagnosed endoscopy or open biopsy should be performed. Surgical debridement in order to control infection and obtain clear margins should be radical in addition to systemic antifungal treatment. Antifungal treatment should be continued until complete local resolution of disease, complete response on radiological images and control of the predisposing factors (Table 1).

**Conflict of interest**: None declared.

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# Терапија фулминантне мукормикозе максиларног синуса и орбите код болесника са дијабетесом


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**САЖЕТАК**

**Увод** Мукормикоза параназалних синуса ретка је и по живот опасна опортунистичка гљивична инфекција која захтева хитно лечење. Оболели су најчешће имуносупресивни и имунокомпетентни болесници. У клиничкој слици среће се целулитис лица или орбите, некроза непца, парестезија фацијалног или тригеминалног живца, губитак вида и знаци менингитиса. Радиолошки прегледи нису осетљиви у раним фазама инфекције. Дефинитивна дијагноза се поставља биопсијом и хистолошким прегледом некротичног ткива.

**Приказ болесника** У августу 2017. године 52-годишња дијабетичарка примљена је на Клинику за максилофацијалну хирургију због отицања и бола на десној страни лица, главобоље, повишене температуре, офталмоплегије, гнојне ринореје у трајању од једне недеље. Компјутеризованом томографијом је уочен целулитис десне стране лица, засенченост максиларног и етмоидалних синуса и остеитис максиларних зидова. Учињени су радикални хируршки дебридман и некректомија. Хистопатолошка и микробиолошка микробна показала су инвазивну мукормикозу. Примењен је липосомални амфотерицин Б 5 *mg/kg* дневно током четири недеље, дијабетес је контролисан инсулином, а локални статус се значајно побољшао. Болесница је касније поново оперисана због дефекта десне максиле и пода орбите.

**Закључак** Рана дијагноза и мултидисциплинарни приступ који укључује микробиолога, патолога, радиолога, хирурга, хематолога, инфектолога болести, интензивну негу и фармаколога од суштинског су значаја. Лечење мукормикозе параназалних синуса захтева брзо и агресивно лечење антифунгалним агенсима, хируршки дебридман и контролу предиспонирајућих фактора.



**Кључне речи:** мукормикоза; параназални синуси; гљивична инфекција; дијабетес; опортунистичка инфекција

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REVIEW ARTICLE / ПРЕГЛЕД ЛИТЕРАТУРЕ

# Interdisciplinary crossover for rapid advancements – collaboration between medical and engineering scientists with the focus on Serbia

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**SUMMARY**
Over the past decades, development of engineering sciences has vastly contributed to advancements in medicine by production of numerous devices for diagnostics and treatment. In the middle of the 20th century, a new scientific field, biomedical engineering (BE), was established, which has developed into an extremely complex scientific discipline requiring a distinctive educational profile. Various study programs in BE have been established at universities around the world but also at several universities in Serbia. Also, intensive research in this field is performed at several scientific institutions in Serbia. In the present paper, short summaries of the research results of several groups of engineers and medical doctors are presented as an illustration of the wide field of BE research and possibilities of its application in diagnosis and therapy of various diseases.
**Keywords:** biomedical engineering; research; education; Serbia

## INTRODUCTION

The Academy of Medical Sciences of the Serbian Medical Society (AMS-SMS) and the Academy of Engineering Sciences of Serbia (AESS) signed the Protocol on Cooperation on February 26, 2020 with the aim to enable the exchange of experiences and knowledge in the fields of medicine and engineering sciences, encourage new multidisciplinary scientific research, and contribute to education of doctors and engineers.

Over the past decades, the development of engineering sciences has incredibly contributed to advancements in medicine by production of numerous devices for diagnostics and treatment of various diseases. However, it should be noted that certain technical devices were already in use in medicine back in ancient times. Nevertheless, the number of technical discoveries that contributed to the development of medicine increased significantly in the 19th century and almost unthinkably so in recent decades.

## BIOMEDICAL ENGINEERING

In the middle of the 20th century, a new scientific field, biomedical engineering (BE) (Figure 1), was established, which has developed into a largely expanded discipline divided into the following sub-disciplines [1, 2]:

*Biomechanics* – examines the kinetics and dynamics of healthy and diseased individuals in order to better understand and solve problems in various fields of medicine (orthopedics, traumatology, surgery, dentistry, etc.);

*Biomechatronics* – deals with intelligent electromechanical systems that help correct disturbed or lost body functions;

*Bioinformatics* – an interdisciplinary field, combining computer science, statistics, mathematics, and engineering for the analysis and interpretation of biological data;

*Biomaterials science* – directed towards surfaces or materials that come into permanent or temporary contact with human tissues, cells, or body fluids;

*Tissue engineering* – defined as "an interdisciplinary field that applies the principles of engineering and the life sciences towards the development of biological substitutes that restore, maintain, or improve tissue function" [3];

*Genetic engineering* – includes methods of direct manipulation of genetic material;

*Neuroengineering* – uses engineering techniques to investigate, explain, repair, replace, and improve the functions of nervous systems;

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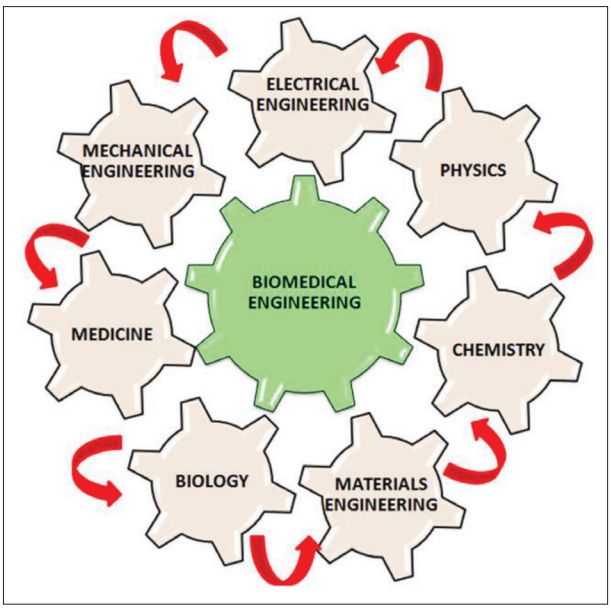


**Figure 1.** General scheme of integration of technical, medical and natural sciences in biomedical engineering

*Bio-instrumentation* – focuses on the development of instruments used in the diagnosis and treatment of diseases.

## EDUCATION IN BIOMEDICAL ENGINEERING

BE is an extremely complex and extensive scientific discipline, so it requires a special educational profile. Over the past decades, BE education has grown rapidly all over the world, as well as in Serbia. At technical faculties in Belgrade, Novi Sad, Niš, and Kragujevac there are several study programs in BE.

The University of Belgrade has united experts in medical, technical, and natural sciences working in the fields of reconstructive, preventive, and regenerative medicine and has formed an integrated doctoral study program titled *Biomedical Engineering and Technology*. Also, some faculties independently offer courses within master and doctoral study programs that belong to the BE field (e.g., tissue engineering, controlled release, biomaterials, biomedical informatics, etc.). At the University of Niš, within the framework of a Tempus project in 2014, the elective BE course was established within the doctoral studies.

Also, in the field of dentistry at the faculties in Serbia, new subjects have come to life: computerized dentistry, visualization techniques, etc. Most clinical subjects (oral implantology, maxillofacial surgery, prosthodontics, orthodontics) use 3D images, video clips, and simulation programs as teaching aids.

## BIOMEDICAL ENGINEERING RESEARCH IN SERBIA AND APPLICATION OF THE RESULTS IN PRACTICE

In Serbia, several scientific institutions and many scientists are engaged in research in various fields of BE. Also, much research in the field of engineering that is not primarily focused on medicine often contributes significantly to its progress [4, 5]. The main goal of the AMS-SMS and AESS cooperation is to encourage collaborations of all of these institutions and scientists and thus improve the research in BE and contribute to the promotion of the achieved results. This paper, prepared by several physicians and engineers, marks the beginning of our cooperation. The following summaries of some of their studies present only an illustration of the wide field of BE research and possibilities of its application in diagnosis and therapy of various diseases.

### Biomechanical study of fractured bone fixation

Great progress in orthopedic surgery has been achieved through multidisciplinary research in the field of biomechanical characterization of bone and skeletal system. In the early 1980s, a team was formed at the University of Niš, which compared the intensities of forces necessary for the fracture of human long bone in the laboratories at the Faculty of Mechanical Engineering. The results of this investigation led to the assumption that, if such a device for fixing a fractured bone was constructed, which would provide stability very similar to the natural stability of the human bone, fractures would heal much better, with fewer possible complications [6]. At that time, the conventional concept of external fixation implied fixing the fracture with a device with all components in one plane, which resulted in excessive stability in one and insufficient stability in the perpendicular plane. Application of the new concept resulted in the construction of new original implants and devices that proved to be significantly more effective in clinical practice compared to conventional implants. The first invention based on the new concept was an external skeletal fixator (Figure 2) [7, 8]. At the same time, it was the simplest device at the international level, so it was quickly introduced into routine use and to date it has been applied to over 28,000 patients with severe fractures in domestic and foreign clinics. Thereafter, the patent was recognized not only in our country but also as a European (European Patent Office) and a world patent (World Intellectual Property Organization). This patent was nominated for the best world patent by the Patent Office in 2012. The application of the patent, commercially termed "External Fixator According to Mitković" played a key role in rescuing about 5,000 wounded during the war on the territory of the former Yugoslavia and the subsequent bombing of Serbia. Another patent resulting from these and additional studies is "Self-Dynamizing Internal Fixator According to Mitković" (Figure 3), which is also routinely used and has already been applied to about 10,000 patients [9]. This implant is based on the original concept of spontaneous dynamization, i.e. it can spontaneously change its biomechanical characteristics depending on the stage of fracture healing, so its other name is "Intelligent Implant."

Ten years later, this knowledge began to influence the scientific thought in this narrow field of science at the international level, so that it has been accepted by most of the scientific institutes today.

**Figure 2.** External fixator according to Mitković

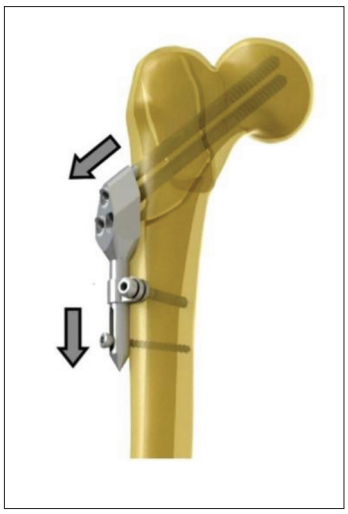

**Figure 3.** Self-dynamizing internal fixator according to Mitković

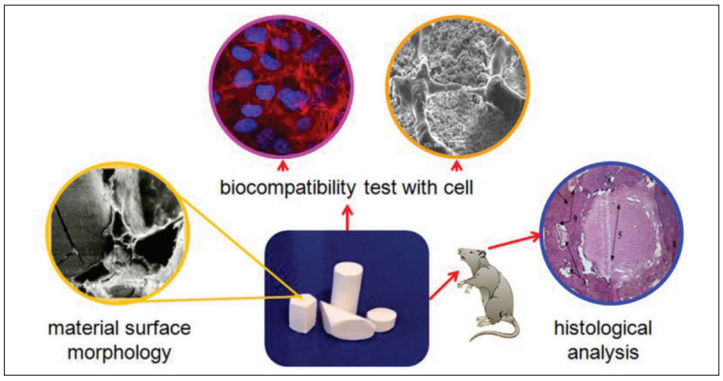


**Figure 4.** 3D scaffolds based on nano-hydroxyapatite (N-HAp) and a bioresorbable polymer in bone tissue engineering

In the 1990s, the same team at the University of Niš biomechanically tested a new original concept of artificial hip. It was thereafter produced by the Electronics Industry Niš and successfully applied to hundreds of patients. Many of them still walk today thanks to this endoprosthesis, the replacement of which was not deemed necessary even after 20 years of use. Progressive decay of the domestic producer and availability of prostheses of foreign companies in Serbia's market led to stopping the production of this hip endoprosthesis.

## Research in the field of biomaterials science

Within the BE field, and the discipline of biomaterials, significant results have been achieved in the team work of researchers of various educational profiles. All researchers (engineers and medical doctors) jointly participated in solving current problems in bone tissue engineering. Bone tissue is an especially interesting subject of scientific research, due to frequent osteoporosis, as well as potential frequent fractures. The mineral phase in natural bone is mostly composed of nano-crystals and nanoparticles of calcium phosphates. The research brought together several institutions across the country (Institute of Technical Sciences of the Serbian Academy of Sciences and Arts, Belgrade; University of Belgrade, University of Niš, and University of Novi Sad). Focusing on bone tissue engineering, the research included a complete research cycle, from synthesis and characterization of calcium phosphate-based materials (synthesized in the laboratory, but similar to human bone tissue), design at the molecular level, characterization, *in vitro* cell-based assays, *in vivo* studies on small and large animals, and pre-clinical tests, to patients, with the prior approval of the ethical committee. The concept underlying this biomaterial and resulting implants is innovative and different from all known concepts. To put it simply, after the implantation, this "smart material" becomes "alive," it attunes itself to the needs of the organism and its metabolism, and finally disappears (dies), replaced by a newly formed tissue. It is composed of a non-bioresorbable component (calcium phosphate-hydroxyapatite – HAp), needed by the organism, and of a bioresorbable polymer component. In the course of time, the polymer gets resorbed and disappears, whereas the final products of its degradation, water and carbon dioxide, are entirely harmless to the organism. The polymer disappears simultaneously with the formation of new organic tissue; at the end of the reparation process, the place of the polymer is taken by a new tissue generated by the organism. Thus, the implant allows tissue regeneration and growth [10, 11]. The first stage of the study included pre-clinical research carried out on cell cultures (Figure 4). The experimental study carried out on human cell cultures confirmed high potential for the application of these biomaterials as the cytotoxicity level was significantly below that observed for other biomaterials [12, 13].

Upon successful confirmation of the advantages of the application of these biomaterials in pre-clinical studies, the researchers proceeded to further studies in clinical conditions. The synthesized biomaterial was applied in the treatment of bone tissue deficiency caused by advanced resorption of alveolar bone in systemic osteoporosis. The results of the study have shown that the application of this composite biomaterial led to an increase in the density of the alveolar bone; the quality of prosthetic supporting tissue was satisfactory so that it could bear the load of inserted dentures. In short, the application of the biomaterial resulted in enhancement of the healing process in post-extraction wounds and a decreased need for additional interventions. It enables the creation of new bones, which are very similar to natural bone tissue, induces osteogenesis, and prevents resorption. Such reconstruction fully meets both aesthetic and phonetic requirements [14, 15].

The use of soft lasers (the so-called low-intensity or low-energy lasers) has made a significant contribution to modern medicine. The increasing evidence of the beneficial

effects in various therapeutic procedures is obvious. Low-energy lasers have powerful biostimulative effects reflected in more vigorous cell metabolism and microcirculation, resulting in increased mitosis of epithelial, connective tissue and bone cells. The results achieved so far confirm the use of lasers as stimulants in regenerative therapeutic methods and implantation of biomaterials based on HAp in bone defects. The use of lasers contributes to an increased bone tissue density, thus diminishing the possibility of undesirable processes [16].

Nanotechnology and nano-materials have enabled significant improvements in the application of the reconstructive materials based on HAp in bone tissue engineering. Composite biomaterials based on nanoparticulate bioresorbable polymer/HAp (particle size 1–100 nm) significantly accelerated the reconstruction of bone defects compared to the same material, but in micro-sizes (> 1 μm). Nano-particulate biomaterials in the form of fillers are highly convenient for practical applications; easily handled, they make a surgical intervention easier and simpler, whereas the final goal is achieved in a considerably shorter time [17].

Another example of the use of nanomaterials in biomedicine is in multifunctional wound dressings, which can contain, e.g., silver nanoparticles (AgNPs), inducing antibacterial activity by the controlled release of silver nanoparticles and/or ions [18, 19, 20]. Wound dressings based on AgNPs and alginate solutions and hydrogels enhanced healing of second-degree burns in rats [21].

Along with the development of novel products for medical and pharmaceutical applications, BE provides conception and introduction of new methodologies and protocols in preclinical and clinical studies as well as in the clinical practice. For instance, biomimetic bioreactors, which imitate physiological conditions in a certain tissue or organ, are primarily being developed for tissue engineering purposes. Still, these bioreactors also provide physiologically relevant studies of novel biomaterials and interactions of biomaterials with cells and tissues [22, 23, 24]. Thus, by this approach, it is possible to address the *in vitro – in vivo* gap, that is, discrepancies between results obtained in traditional monolayer cell cultures (in 2D environment) and those found in animal studies [25, 26, 27]. It was shown, for example, that nanocomposite alginate hydrogels with AgNPs had moderate to strong cytotoxic effects on chondrocytes in monolayers, while such effects were entirely absent in 3D bioreactor cultures of immobilized chondrocytes in alginate microbeads, as well as in cartilage tissue cultures in direct contact with the nanocomposite hydrogel [23, 28]. The results obtained from the bioreactor cultures were in agreement with the enhancement of wound healing without any adverse effects found in the treatment of second-degree burns in rats by the same nanocomposite hydrogels [21]. Mimicking the physiological conditions is necessary for a reliable prediction of biomaterial behavior upon implantation at a certain tissue site. Studies of novel macroporous, composite scaffolds based on gellan gum and bioactive glass particles have shown that the fluid flow in perfusion bioreactors significantly promoted the formation of a mineral phase as compared to static conditions, which could be related to scaffold implantation in vascularized tissues [24]. Finally, an integrative approach to optimization of biomaterial properties and operating conditions in biomimetic bioreactors may provide tools for evaluation of new drugs and treatment procedures on 3D models of human tissues, as well as for development of personalized medical therapies, and thus contribute to decrease the level of needed animal experimentation [29].

## Dentistry and engineering

Dentistry is a very dynamic scientific field, and its progress is a reflection of the development of basic, and, above all, technical sciences. Today, it is almost impossible to find an area in dentistry which has not been influenced by technical and technological achievements.

The third industrial revolution – the change from analog and electronic technology to the digital technology (1980s) made a breakthrough in clinical dentistry. The term 'digital dentistry' was coined, which was defined as "any dental technology or device that incorporates digital or computer-controlled components in contrast to that of mechanical or electrical alone" [30]. Digital dentistry opens up completely new areas, such as digital impressions, computer-assisted designed and computer-assisted manufactured (CAD/CAM) dentures technology, digital radiography and cone-beam computer topography (CBCT), digital face-bows and virtual articulators, tooth shade matching, computer-guided implant surgery, etc.

Building on the third revolution, in less than four decades, there is a new, remarkable turnaround – the fourth industrial revolution, which leads to automation and robotics in industry, but also in other areas. The dental profession and science, as well as the accompanying industry, have embraced at incredible speed the new concept of digital transformation called Dentistry 4.0. [31]. This new concept, as a synergy of doctors and engineers, has enabled almost all clinical and laboratory dentistry procedures to be supported by digital technologies. The progress in various fields of dentistry will only be enumerated here.

*Medical imaging* is the visualization of anatomical structures by computerized imaging techniques that provide a digital image as the first step in digital dentistry. Many diagnostic and therapeutic procedures were developed based on generation of digital images: digital tooth impression, cone-beam computed tomography, etc.

*Data processing* is now enabled by a large number of commercial software packages to support diagnostic and therapeutic procedures in dentistry. These include CAD-CAM software, digital face-bow and virtual articulators, tooth shade matching, digital smile design, computer-guided implant surgery, and computer-guided diagnostics and treatment in orthodontics [32, 33].

*Computer-aided production* is the application of software to control machine tools in the production of various 3D items. The most commonly used devices in dentistry are those for milling, sintering, rapid prototyping, and 3D printing different dental restorations [34].

*Biomaterials* used in dentistry are classified in the group of advanced materials and materials of the future (nanomaterials and smart materials) [35, 36].

*Education and science* – the introduction of digital technologies in the curricula of dentistry faculties has started globally and has reached different levels of application depending on local resources [37].

A commonly used method of numerical analysis in the dentistry research is the finite element method (FEM). Simplified, FEM considers the physical domain (tooth, dental implant, denture or bridge, temporomandibular joint, etc.) as a real continuum with infinitely many degrees of freedom of point movement and replaces it with a discrete (virtual) geometric model [35].

*Patient record management* – the first step in digitalization in the dental profession was the introduction of computers in clinics, health centers, and private practices. Today, the complete management of health care institutions has been digitized, which simplifies and speeds up all management activities.

## Cochlear implants – the most successful neural prosthesis

A cochlear implant provides an opportunity for patients with severe-to-profound hearing impairments to hear again, for children to learn to speak, to be involved in everyday life and regular schooling, and gain confidence to live a full life [38, 39, 40]. Cochlear implantation would not have been possible without the close collaboration of engineers, neurotologists, otorhinolaryngologists, and speech and hearing health professionals.

A cochlear implant is an electronic device, which bypasses damaged or destroyed receptor cells and transmits electrical stimulation directly to the fibers of the auditory nerve. A modern multi-channel cochlear implant, Nucleus® 24 Contour (Cochlear Americas, Lone Tree, CO, USA) was installed for the first time in Serbia, in Novi Sad, at the Clinical Center of Vojvodina, the Clinic for Ear, Throat and Nose Diseases on November 26, 2002, in a patient 40 years old with postlingual bilateral severe hearing impairment [40]. In May 2005, the Republic Health Insurance Institute made a decision on financing the purchase of cochlear implants in the Republic of Serbia, thus reviving the national program of cochlear implantation in our country. Four clinical centers in Serbia have been trained for cochlear implantation: the Clinical Center of Vojvodina, the Clinical Center of Serbia, the Zvezdara Clinical Hospital Center, Belgrade, and the Niš Clinical Center.

By the end of 2019, over 500 patients with the most severe forms of hearing impairment underwent surgical procedures in our country, primarily children, while the total number of cochlear implant recipients worldwide reached over 470,000. In the period from 2002 to 2020, a total of 147 patients underwent surgical procedures at the Clinic for Otorhinolaryngology and Head and Neck Surgery at the Clinical Center of Vojvodina.

Today, cochlear implantation is the standard treatment of severe-to-profound sensory-neural hearing loss in adults and children that enables patients to achieve good rehabilitation and a higher quality of life.

## IS BETTER COOPERATION BETWEEN MEDICAL DOCTORS AND ENGINEERS NEEDED?

Research in the field of engineering sciences has significantly contributed to the development of medicine by production of numerous devices for diagnostics and treatment of various diseases. Nevertheless, although every medical doctor uses modern technologies in practice, few of them have any training in technological development. There are few medical schools in the world with programs that link medicine and engineering. However, even such education is not sufficient, because it is necessary to achieve understanding between doctors, who use different technical tools, and engineers, who design them. Engineers do not have complete information on what clinicians need, while clinicians cannot express their suggestions accurately, since they do not know the technological possibilities [41, 42]. It is necessary to educate teams of doctors and engineers in order to achieve better results in the application of existing technical achievements. This requires continuous education of both doctors and engineers through joint seminars, workshops, conferences, etc. The leading role in healthcare innovation will be bestowed on those institutions that will find methods to train engineers, doctors, nurses and others to work effectively in teams and solve challenges together. AMS-SMS and AESS will strive to contribute to achieving this goal.

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# Интердисциплинарни приступ за брзи напредак – сарадња научника из области медицине и инжењерства с посебним осврtом на Србију

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**САЖЕТАК**

Напретку медицине су последњих деценија веома много допринели проналасци из различитих области инжењерства. Половином двадесетог века успоставља се нова научна област, биомедицинско инжењерство (БИ), које се до сада развило у веома сложену научну дисциплину која је захтевала и посебан образовни профил. На универзитетима широм света, као и на неколико универзитета у Србији установљени су различити програми из области биомедицинског инжењерства. Такође, у неколико научних институција у Србији спроводе се интензивна истраживања у овој области. У овом раду приказани су резултати истраживања неколико група научника из области инжењерства и медицине са циљем да се илуструје колико је широко поље истраживања у области биомедицинског инжењерства и какве су могућности њихове примене у дијагностици и лечењу различитих болести.

**Кључне речи:** биомедицинско инжењерство; истраживања; едукација; Србија

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REVIEW ARTICLE / ПРЕГЛЕД ЛИТЕРАТУРЕ

# Overcoming traps and challenges in child and adolescent psychiatry

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**SUMMARY**
Appropriate healthcare and psychological support for children and adolescents is essential for the successful development and good mental health. Unfortunately, this is often a neglected element in the healthcare systems around the world. It is known that approximately half of all adult psychiatric disorders start under the age of 14 and that the prevalence of child and adolescent-onset psychiatric conditions is increasing. The real reason for this increase remains unclear, but it demands our attention as does the care of affected children, adolescents, and their families. Transitions between different age groups need to be made easily navigable for the patients and their families.
Many challenges in child and adolescent psychiatry are present, especially in developing countries such as Serbia. A possible solution for overcoming these challenges is uniting the child and adolescent professional societies from all over the world. These societies should work together to develop unified strategies for diagnosis, treatment and support of children affected by psychiatric conditions. By working closely with pediatricians, family physicians, psychologists, nurses, and other professionals, child and adolescent psychiatry can use knowledge and skills to support practice while teaching other professionals how to optimize the utilization of child and adolescent psychiatry services.
**Keywords:** child and adolescent psychiatry; trends; child abuse and neglect

## INTRODUCTION

The early development of children and adolescents and their later transition into adulthood depends on the support and assistance of adults – their parents/caregivers, teachers, healthcare providers, and many others. Appropriate healthcare is essential to the successful development and good mental health. Unfortunately, this is often a neglected element in the healthcare systems around the world. The prevalence of child and adolescent-onset psychiatric conditions is increasing. The real reason for this change is unclear, but it demands our attention as does the care of affected children, adolescents and their families.

Many challenges are present; service capacity for children and adolescents is substantially limited by multiple barriers including the lack of resources, restrictive access criteria, distance from service providers, etc. As a result, some national studies, like a study conducted in the USA, estimate that approximately 49% of children do not receive professional mental health treatment or counseling (the prevalence ranged 29.5–72.2% in different states) [1]. The lack of trained clinicians, especially in child and adolescent psychiatry (CAP) is a global phenomenon.

CAP is a relatively new discipline. With the advancement of clinical knowledge, research, education and professional activities, this field is continually being enriched with a major commitment to the evidence-based practice. Additionally, with efforts of The European Union of Medical Specialists—Child and Adolescent Psychiatry (UEMS–CAP) along with the European Society of Child and Adolescent Psychiatry (ESCAP), the challenges of CAP are recognized widely. Currently there are continuous efforts to overcome them in every aspect of CAP – from enrollment in the training to the implementation of clinical practice [2, 3]. Some of the improvements of UEMS-CAP include the composition of the training manual and a logbook with comprehensive recommendations for the training with the standardized approach to CAP across Europe [3].

Studies have shown a high degree and type (i.e., homotypic and heterotypic) of continuity of psychopathology from childhood to middle adulthood [4, 5], and they underscore the need to study psychopathology through a developmental perspective. The progress in CAP is making it increasingly clear that psychiatrists must learn about healthy and pathological development for each individual because the development does not stop because an individual reaches the legal age of adulthood [3]. We are facing a public health crisis with a profoundly limited capacity to meet these significant health care needs of our youth. The best way to prove the need for better recognition of CAP is to analyze the epidemiology of the common mental health issue of children and adolescents.

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## EPIDEMIOLOGY

Young people under 25 years of age make up approximately 43% of the world's population [6]. The important health issues and risk factors for disease in later life emerge in those years, and their contribution to the global burden of disease are relevant [7]. Among the eight main causes of Disability Adjusted Life Years (DALYs) in these age groups, the majority were psychiatric and behavioral in nature. Furthermore, cross-sectional studies have shown that exposure to risk-factors (e.g., parental mental illness, child abuse, neglect) in childhood and adolescence increases the probability of developing mental health disorders in adulthood [8].

An increasing number of children that need psychiatric interventions and treatment have been the main interest to many researchers across the world during the last decade [9]. In terms of trends of psychiatric disorders in childhood, it has been believed that a trend of underdiagnosis and undertreatment constantly high in the last decades [10]. However, there is another side to the story. Some researchers believe that an increase in the prevalence of psychiatric disorders in childhood is mainly due to overdiagnosis and overtreatment [11].

There is gathered evidence that the trends of certain childhood psychiatric disorders tend to increase in adolescence such as depression, anxiety disorders (panic disorder and agoraphobia) and substance use disorder [12]. Some of these disorders (panic disorders, agoraphobia and substance use disorders) continue to increase into adulthood. On the other hand, some childhood disorders (attention-deficit hyperactivity disorder – ADHD, and separation anxiety disorders) tend to decrease across time [12]. These data again highlight the need for improved mental health care in this "transition to adult years."

## DEPRESSIVE DISORDERS

Depression in young children is a relatively rare disorder, but when it occurs at such a young age, it can cause severe symptomatology and possibly have fatal consequences. The prevalence rates of depression in youth range greatly in different communities. For example, according to the literature review conducted by Merikangas et al. [13], the rates of any depressive disorder (DD) ranges 0.9–3.4%; while for the major depressive disorder (MDD) ranges from 0.6% in Great Britain to 3% in Puerto Rico. Among preadolescents, researchers report no gender differences in rates of depression. However, from the beginning of adolescence, the prevalence of depression is greater among females than among males with differences persisting into the middle adulthood [13]. This gender difference is controversial in the literature with a variety of proposed mechanisms. It is mostly attributed to the impact of hormonal and neurodevelopmental patterns that occur during puberty [14] and greater exposure or sensitivity to psychosocial stress in adolescent girls [15]. A number of longitudinal studies from different countries noted that depression in children (MDD and DD) occurs between 11 and 14 years of age [13]. The results show that preschool-onset depression emerged as a robust predictor of Diagnostic and Statistical Manual of Mental Disorders, 5th Edition: (DSM-5) MDD in later childhood, even after accounting for the effect of maternal depression and other risk factors. Preschool-onset conduct disorder (CD) also predicted DSM-5 MDD in later childhood, but this association was partially mediated by maternal non-support, reducing the effect of preschool CD in predicting DSM depression by 21% [16]. Predictors of depressive symptoms may differ before and after the initial onset of major depression due to stress sensitization [17]. Every clinician should bear in mind that depression symptoms in young people can differ significantly in comparison to adults. For example, melancholic and psychotic symptoms occur less frequently in children [18]. The most common symptoms of depression in children of preschool age are behavioral problems and somatic complaints, while school-age children often present with increased irritability and impulsivity, a failure to properly function in school and among their peers, low self-esteem, guilt, increased boredom, etc. [19].

## BIPOLAR DISORDERS

Diagnosing pediatric bipolar disorder (PBD) is challenging due to the wide range of symptom expression, differences when compared to adults suffering from the same disorder, presence of comorbid disorders, and diagnostic criteria that may not be developmentally sensitive [20]. Until the last decade of the 20th century, PBD was rarely diagnosed. In that period researchers highlighted the fact that presentation of PBD differs significantly from adults. From that point on, PBD was much more frequently diagnosed; for example, the study conducted by Blader et al. [21] reported a significant linear increase of PBD diagnosis in discharged pediatric patients in the US from 1996 (1.3 per 10,000) to 2004 (7.3/10,000) [21]. This increase raised concerns that PBD is overdiagnosed.

The current reported prevalence of mania, hypomania, and bipolar disorder in population-based studies of youth range 1–3% in children and adolescents [22]. The results of most community surveys find nearly equal rates of bipolar disorder in boys and girls. The prospective studies found that the incidence of bipolar disorder peaks at the age of 14 in both males and females and decreases gradually thereafter [13].

## ANXIETY DISORDERS

Similar to the community studies of adults, anxiety disorders are also quite prevalent in the general population of children and adolescents. According to national-level survey conducted in the US, approximately 7.1% of children aged 3–17 years (about 4.4 million) have diagnosed anxiety [23].

Although there is substantial variation across studies, the literature review results reveal that the onset of any

anxiety disorder is usually in youth [24]. Further, specific subtypes of anxiety have differential peak periods of onset. The specific phobias and separation anxiety occur the earliest, in middle childhood, while other types (agoraphobia, panic disorders and generalized anxiety disorder) occur in adolescence with further incidence increase in early adulthood [24]. The review by Beesdo et al. [24] also points out that all anxiety disorders are more frequent in females with sex differences noticeable as early as childhood. Although this is a well-established difference confirmed by a number of studies, some researchers warn that this difference is often small or nonexistent [25].

## PSYCHOTIC SYMPTOMS AND DISORDERS

The population data suggests that the prevalence of subclinical psychotic symptoms in general population is 5% (many times greater than the actual diagnosis of a psychotic disorder) [26]. There is an increasing body of evidence that suggests that a large portion of young people experience psychotic symptoms and that these numbers can even be greater than that in adults; the rates vary 9–14% in interview-based research to the rates greater than 25% in some studies using self-report questionnaires [27]. These symptoms have been recognized and significant since a cohort study reported that adolescents who experience these phenomena have more frequent suicide attempts at follow ups [28].

The effective pharmacotherapy of these disorders in children remain the major obstacle for clinicians since this still remains the area of active research. A large portion of the antipsychotic treatment has been based on the research on adult population, however, there are finished and ongoing clinical trials that show promising results [27].

## ADHD

Attention-deficit/hyperactivity disorder (ADHD) is one of the most common psychiatric disorders that occur in childhood [29]. Although the point prevalence rates of ADHD have varied 5–12%, the median prevalence of ADHD in a recent meta-analysis conducted in China was 6.26% (5.36–7.22%) [30]. A higher prevalence of ADHD in boys has been well-established. Rates of ADHD consistently show a male preponderance of ADHD [29]. Researchers noted many risk factors related to the occurrence of ADHD. Among the most common factors are genetics, prematurity and exposure to lead, cigarettes and alcohol in pregnancy [31].

## CONDUCT AND OPPOSITIONAL DISORDER

Disruptive behavior disorders include two similar disorders: oppositional defiant disorder (ODD) and a CD. The estimated prevalence of CD is 3.5% (CI 2.7–4.7%), and the prevalence of ODD was estimated to be 2.8% (CI 2.1–3.7%) [32]. Similar to ADHD, CD is also more prevalent in boys than girls, while the male to female prevalence ratio for ODD was found to be 1.59:1 [33, 34]. Age of onset of disruptive behavior disorders appears to be an important predictor of outcome, those with earlier onset experience a longer duration of ODD and more negative outcomes [35]. The youth community studies have shown a high degree of co-occurrence of CD and ADHD, impulse control disorders, substance abuse, and major mood disorders (bipolar disorders); and a number of comorbid conditions predicted a slower recovery from these disorders and more negative outcomes [36, 37]. Likewise, there is also an association between the presence of disruptive behavior disorders with mood and anxiety disorders.

## EATING DISORDERS

A recent systematic review by Galmiche et al. [38] reported that the weighted ranges of lifetime prevalence for eating disorders (ED) were 8.4% (3.3–18.6%) for women and 2.2% (0.8–6.5%) for men, with the highest prevalence in America (4.6%), followed by Asia (3.5%) and Europe (2.2%). ED has a mortality rate of at least 5–6%, the highest mortality rate of any psychiatric illness [39]. The lifetime prevalence of bulimia nervosa is higher, between 0.9–3% [40], with an older age of onset of 16 to 17 years. Although female patients account for most ED diagnoses, males have accounted for 5–10% of ED cases over the past years, with some community-based research reporting up to 25% of cases being male [41].

## NEURODEVELOPMENTAL DISORDERS/AUTISM SPECTRUM DISORDER

The dramatical increase in the prevalence of neurodevelopmental disorders, especially autism spectrum disorders (ASD), is generally accepted. Studies in Asia, Europe, and North America have identified individuals with ASD with an average prevalence of 1–2% [42]. In other words, about 1 in 54 children has been identified with ASD according to estimates from Centre for Disease Control and Prevention Autism and Developmental Disabilities Monitoring Network [43]. Epidemiological data show that the incidence of ASD is higher among boys than girls with ratios around 4:1 [44]. Furthermore, it has been shown that sex doesn't moderate the intensity of the core ASD symptoms, although females tend to be more functional in everyday life [45]. The exact cause of ASD is still unknown, but literature data indicates that there is a strong genetic effect often with the impact of environmental factors (e.g., prenatal and postnatal factors) and their interactions in persons at risk [46].

Bosl et al. [47] recently provided initial evidence for a role of neural connectivity in early development. The authors contrasted electroencephalogram complexity in infants at risk for ASD with normal controls, revealing reduced complexity in infants-at-risk. Though this study

suggests the role of connectivity in early development is associated with ASD risk, the ultimate diagnostic outcome was not available for infants in this study, so it is unclear whether connectivity has any predictive validity for autism-risk [47]. Recent work has focused upon electroencephalogram complexity as a marker of connectivity in ASD [48].

## CHALLENGES AHEAD FOR CHILD AND ADOLESCENT PSYCHIATRY

### Number of child and adolescent psychiatrists

Among the world's seven billion population, almost half of the citizens are under 25 years of age (with 1.8 billion between the ages 10–24) [6]. With only about 20,000 child and adolescent psychiatrists worldwide this means that there is one child and adolescent psychiatrist for every 300,000 youth of which about 60,000 will have an active psychiatric disorder [49]. The geographic maldistribution of child and adolescent psychiatrists in favor of large urban areas leaves a great many youth unserved or at impossible distances from psychiatric care. As a consequence, if the clinicians are not there to identify psychiatric illness, then "it does not exist" and remains an invisible problem for the healthcare systems and policymakers [3]. Currently, in Serbia there are 33 CAP specialists (residents of the Republic of Serbia), mostly employed in the public sector. The current number of staff in CAP is not sufficient for the needs of children living in our country.

### What keeps child and adolescent psychiatry so small and, to a large extent, irrelevant?

1. Recruiting: CAP rarely plays a central role in medical education curriculum and policy. The same applies to healthcare planning and policy. Since there are so few child and adolescent psychiatrist, they rarely participate in the selection of students entering medical school and advanced training programs and they are generally not readily available to serve as "role models" and mentors for developing physicians.
2. Training: In the last two decades, there were a few Europe-based studies that focused on all aspects of CAP training. These studies had shown that the CAP training programs vary greatly across Europe [3]. This diversity was recognized by UEMS-CAP and ESCAP which lead to the development of programs, curricula, and guidebooks aiming to achieve harmonization and homogeneity in the training programs [2]. The effects of this initiative were presented in the recent findings from the study conducted in 2019, which revealed improvements and unexpected homogeneity in the training structure across countries but also recognized some of the challenges that need to be refined with in the future [3].
3. Practice: The practice of CAP is often isolated from the rest of medicine. Many practitioners are in private office practices and rarely have professional interactions with other physicians. Frequently colleagues from different specialties are replacing child and adolescent psychiatrists with social workers, psychologists, advanced practice nurses, pediatricians, neurologists, developmental pediatricians, and all manner of other "mental health professionals" who either meet their needs or allow them to say that they have "mental health services". Even when they are recognized as healthcare providers, their resources (e.g., medication availability) are often limited [50].
4. Research: CAP training programs give short shrift to research training, and there are few provisions for research career development for child and adolescent psychiatrists. As a result, there are remarkably few of them dedicated to full – or even half-time – research careers.
5. Economics: Because it takes long to prepare a child and adolescent psychiatrist for practice and the liabilities and responsibilities are so great, CAP services are more expensive than other "mental health" providers of care.

### Solutions to overcome problems in organization of child and adolescent psychiatry

The situation leads to two options, to continue down the same path, or to determine what can and will be done.

What is needed is to re-define discipline, to clearly distinguish CAP from the other "mental health providers." CAP are physicians who specialize in the medical specialty of CAP and who diagnose and treat real disorders of developmental psychopathology in real patients by using real evidence-based, scientific practice. CAP should be capable of helping patients and colleagues in emergencies; manage privacy concerns and communicate frequently and effectively with colleagues; use technology (telemedicine, computer/app-based interventions) without compromising quality standards; and seek feedback from colleagues about what helps and what does not.

## CONCLUSION

The CAP as a separate specialty is gaining recognition and establishment among other medical disciplines worldwide. In the South-Eastern Europe, the national and international CAP societies are working closely to develop unified strategies for training programs, research, and clinical practice in this field. These unified actions are already showing promising results in all aspects of CAP. On the other hand, by working closely with other professionals (e.g., pediatricians, family physicians, psychologists), CAP professionals can use the knowledge and skills to support practice while teaching other professionals how to optimize utilization of CAP services.

The CAP professionals should advocate for practice primary prevention in psychiatry, ranging from the provision of iodine to women of reproductive age to the education of parents about optimum child-rearing practices. They should make sure that they are keeping the general medicine knowledge to be able to deal with comorbid physical illness in their patients and at the same time to contribute

to the image of psychiatry as a medical discipline. CAP professionals can "see and be seen" through greater activity in professional organizations outside of CAP and by speaking loudly and clearly about the needs of children from a very unique developmental perspective.

One size does not fit all in the sense of cultural relativism, and in any service planning and delivery, we must take into account cultural variations. CAP professionals should not blindly follow one model, but modify it according to cultural norms and resources. Psychiatry is at a stage where a lot of exciting developments are emerging, and CAP is the best example of such a development.

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## Превазилажење изазова у дечјој и адолесцентној психијатрији

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**САЖЕТАК**

Одговарајућа здравствена и психолошка подршка деци и адолесцентима од суштинског је значаја за успешан психосоцијални развој, као и за очување менталног здравља. Нажалост, очување менталног здравља деце и младих је често занемарени домен у здравственим системима широм света. Познато је да половина свих психијатријских поремећаја код одраслих почиње пре 14. године и да се преваленција психијатријских стања код деце и адолесцената повећава. Прави разлог овог повећања и даље није потпуно разјашњен. Без обзира на нејасну етиологију, брига о оболелој деци, адолесцентима и њиховим породицама треба да представља приоритет у здравственим системима сваке државе. Глобално постоје многи изазови у дечјој и адолесцентној психијатрији, а посебно су наглашени у земљама у развоју, као што је Србија. Могуће решење за превазилажење ових изазова је удруживање професионалних друштава дечје и адолесцентне психијатрије из целог света. Та друштва би требало да раде заједно на развоју јединствених стратегија за дијагнозу, лечење и подршку деци која болују од развојних и психијатријских стања и поремећаја. Поред овога, кључна ствар за адекватно функционисање и напредовање дечје и адолесценте психијатрије је блиска интерсекторска сарадња са другим секторима који се баве децом и младима.

**Кључне речи:** дечја и адолесцентна психијатрија; трендови; злостављање и занемаривање деце

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CURRENT TOPIC / АКТУЕЛНА ТЕМА

# Emerging variants of novel coronavirus – myth and reality

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**SUMMARY**
The new coronavirus has crossed the species barrier leading to the pandemic of COVID-19. The lengthy circulation of the virus within the human population has enabled the development of many new viral variants, some of which are conducive to further pathogen spread. Notable variants are those that contain mutations within the S gene, particularly within the region that codes for the receptor-binding domain (RBD) that links to the hACE-2 receptor. These mutations are responsible for increased viral transmission and influence disease severity, reliability of clinical tests, as well as vaccine efficacy. At present, the variant first identified in the United Kingdom poses the greatest threat in Europe.
**Keywords:** coronavirus; COVID-19; variant; SARS-CoV-2; emerging virus

## INTRODUCTION

For all the queries that remain about the novel coronavirus (CoV) and the disease it causes, scientists have generated a vast amount of knowledge in a very short period of time. Some of them are answered, but some of them are still a mystery. Today, a year after the virus was discovered, the question still stands: what do we *really* know about SARS-CoV-2?

## GENERAL CHARACTERISTICS OF SARS-COV-2

*Coronaviridae* are enveloped, positive-sense single-stranded RNA viruses in the order of *Nidovirales*. The subfamily *Orthocoronavirinae* is further classified into four CoV genera: *Alfa-*, *Beta-*, *Gamma-* and *Deltacoronavirus* (Figure 1). They infect a variety of animals including birds and mammals. A broad range of coronaviruses are found in bats. They were identified as human pathogens since the 1960s and have been associated with 15–30% of annual respiratory tract infections [1]. Widespread human coronaviruses HCoV-OC43, HCoV-229E and HCoV-HKU1 cause common colds but also lower respiratory tract infections in the youngest and oldest age groups. The HCoV-NL63 is considered to be an important cause of pseudocroup and bronchiolitis in children.

In the last 20 years, two zoonotic coronaviruses have emerged: SARS-CoV and MERS-CoV, discovered in 2002 and 2012, respectively. Both have caused human outbreaks, the Severe Acute Respiratory Syndrome (SARS) and Middle East Respiratory Syndrome (MERS). In late 2019, a third novel coronavirus, initially related to a cluster of pneumonia cases in Wuhan (China), was identified and named SARS-CoV-2 [2].

The origin of SARS-CoV-2 is still a matter of debate. Bioinformatic studies revealed that is has a 96.2% identity with the coronavirus genome isolated from the feces of *Rhinolophus affinis* bats [3]. There are three possible mechanisms by which the bat virus has crossed the species barrier: by natural selection in an animal host before jumping into humans or natural selection in humans after the virus transferred into a human host. The third mechanism, which is the least likely one, is artificial manipulation with the bat virus in a laboratory [4].

The novel virus is closely related to SARS-CoV. However, SARS-CoV-2 genome sequence is more distant from SARS-CoV, with 79% of similarity throughout the whole genome, and more than 90% of the sequence identity for essential enzymes and structural proteins [5, 6]. The main difference lies in the receptor binding domain within the S gene [5, 6]. But characteristics of SARS-CoV-2 in the surface proteins lead to a higher viral load kinetics, enable virus to enhanced rate of transmission during the human infection.

## GENETIC VARIABILITY OF SARS-COV-2

The length of the SARS-CoV-2 genome is about 30,000 nucleotides – extremely long for an RNA virus. The replication error correction system with proofreading mechanism limits the mutation rate of the virus.

The first two thirds of the genome correspond to a single *ORF1ab* gene, coding for the polyprotein which is cleaved into 16 nonstructural viral proteins. The last third of the

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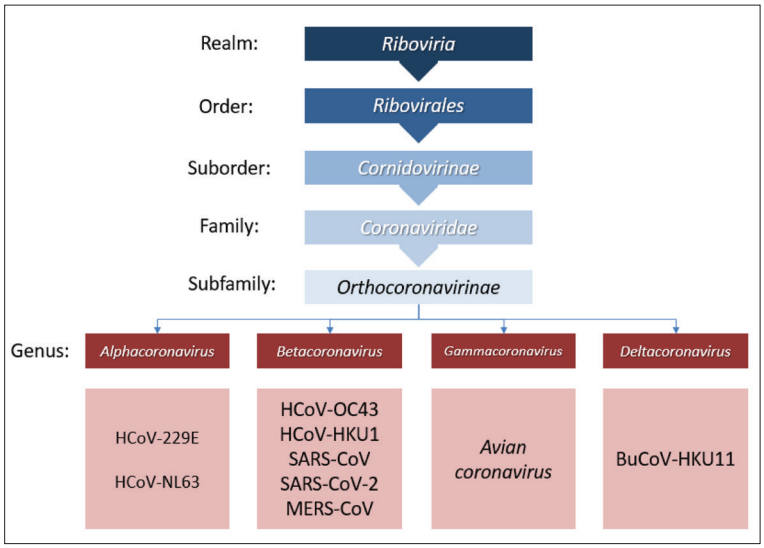


**Figure 1.** The novel SARS-CoV-2 position within the viral taxonomy

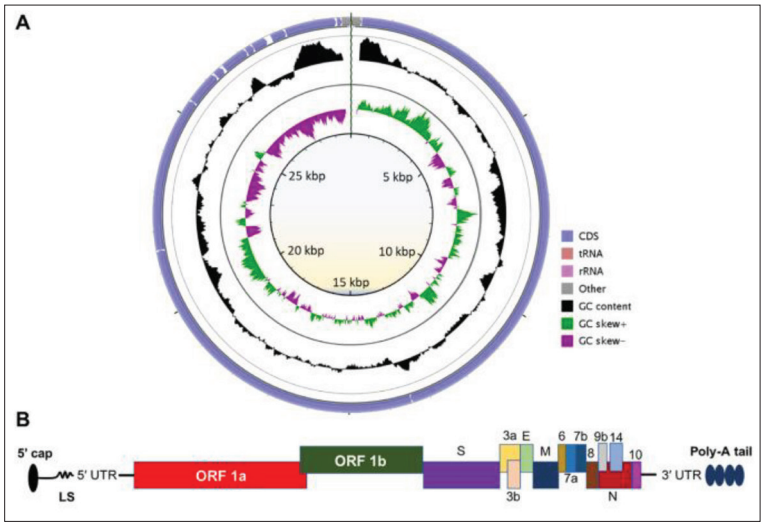


**Figure 2.** A: The organization of the SARS-CoV-2 reference genome; B: representation of the pathogen's single-stranded positive-sense RNA; source: Naqvi et al. [25]

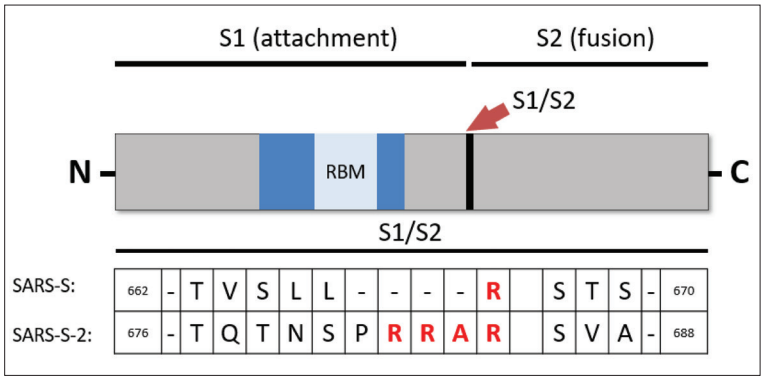


**Figure 3.** The insertion sequence of SARS-CoV-2 at the cleavage site

genome contains nine genes coding for structural proteins, with the most important being surface (spike, S), membrane (M), envelope (E), and nucleocapsid (N) proteins. Structural proteins aid in the assembly and release of new copies of the virus. The M and E proteins are involved in the formation of the viral envelope, while the N protein forms a helical ribonucleocapsid complex with the RNA and interacts with viral membrane proteins during pathogen assembly. Accessory genes (*3a*, *3b*, *6*, *7a*, *7b*, *8*, *9b*, *9c*, and *10*) present short bits of genome [7, 8]. They are clustered within the structural genes and help the virus to evade the immune system (Figure 2).

The life cycle of SARS-CoV-2 is a very dynamic process. After specific adsorption and membrane fusion, viral genome RNA is released into the cytoplasm, while uncoated RNA translates two polyproteins, pp1a and pp1b, which encode nonstructural proteins and form a replication–transcription complex (RTC) in the vesicle. The RTC continuously replicates and synthetizes a set of subgenomic mRNAs that encodes accessory and structural proteins. After the components of RNA and protein assemble, new viral particles are produced and then released into the extracellular space via exocytosis.

The polymerase, in addition to its canonical RNA-dependent RNA polymerase activity, is able to jump between the different RNA strands. This is a property that plays a key role in the recombination capacity of CoVs and promotes their evolution and host change in the case of dual or mix infection.

## THE MAIN ANTIGEN OF SARS-COV-2 – SPIKE PROTEIN

The S protein of the virus is a key factor involved in infection. Like other coronaviruses, the S protein of SARS-CoV-2 mediates receptor recognition, cell adsorption and fusion of viral envelope with plasma membrane – all events responsible for viral entry into the cell [9, 10, 11]. This means that S protein is a major player in the pathogeneses of viral infection, but is also involved in evolution of CoV and crossing of the species barrier [12]. The insertion of short sequences within the S gene is unique for SARS-CoV-2. These insertions add four amino-acids at the precise cleavage site of the protein, immediately upstream of the arginine, which creates a sequence RRAR, corresponding to the consensus recognition motif of the furin protease (Figure 3) [13].

The S protein is a trimer, composed of three identical units. It forms a characteristic crown-like halo surrounding the viral particle. The spikes are coated with polysaccharide molecules to camouflage them, evading surveillance of the host immune system during entry [14].

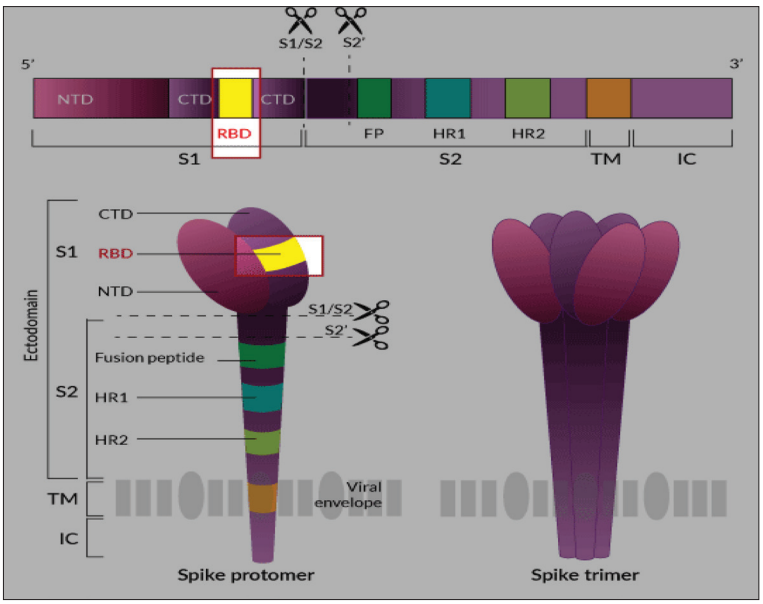


**Figure 4.** The S protein and its structure

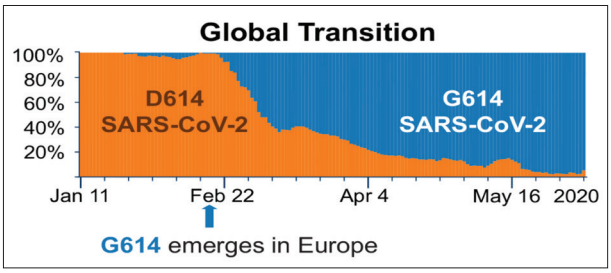


**Figure 5.** The D614G mutation and its frequency in regard to time

In its native state, S protein exists as an inactive precursor. During viral infection, target cell proteases activate the S protein and cleaved into two subunits (Figure 4). The first cleavage called "priming" generates the S1 and S2 subunits [15]. The second cleavage occurs within the S2 unit and releases the end of the fusion peptide located at the beginning of the S2 subunit [16]. These two proteolytic cleavages are catalyzed by furin and serine transmembrane proteases 2 – TMPRSS2 [15, 16, 17].

The S1 domain contains the receptor-binding domain, or RBD (319–541 residues), which is mainly responsible for binding of the virus to the receptor. The S2 domain mainly contains the heptapeptide repeat sequence, or the HR domain with HR1 (912–984 residues) and HR2 (1163–1213 residues) units, which is closely related to the virus fusion protein (FP – 788–806 residues) [12, 18]. Once the virus interacts with the host cell, extensive structural rearrangement of the S protein occurs, allowing the virus envelope to fuse with the host cell membrane and start replication in the cell.

## NEW EMERGING VARIANTS OF SARS-COV-2

Viruses constantly change through mutation. Scientists monitor changes in the virus by sequencing, trying to find the new variants of concern. New variants can change virulence and infectivity of viruses in the sense of how widely the new variants spread and how the disease differs from the disease caused by other variants. The most important question is how the new variants affect therapy, molecular diagnostic testing for COVID-19, and effectiveness of vaccination.

The first variant of SARS-CoV-2 occurred at the beginning of the pandemic, in late January or early February 2020. It was a D614G substitution (substitution of aspartic acid to glycine at position 614) in the gene encoding the spike protein, but outside of the RBD region [12, 19]. After several months, this specific D614G mutant replaced the initial SARS-CoV-2 strain identified in China and by June 2020 became the dominant form of the virus circulating globally (Figure 5) [19]. This strain has increased infectivity and transmission, but without the effects on clinical illness or effectiveness of commercial laboratory diagnostics tests.

The next new variant of SARS-CoV-2 identified in North Jutland, Denmark, in August and September 2020 has been linked to infection among farmed mink. This specific variant has a combination of mutations not previously observed. The impact of this mutant was that the infected mink transferred the infection to 12 humans, but it did not spread widely [20].

In the United Kingdom, a novel important variant has been identified as SARS-CoV-2 VOC 202012/01 (Variant of Concern, year 2020, month 12, variant 01) or 501Y.V1. In November 2020, a rapid increase in COVID-19 cases overall was associated with the emergence of this new variant in south east, east regions of the UK and London. Retrospectively, the first instance of VOC 202012/01 was identified in a case from September 20, 2020 in the UK [21]. This variant contains 23 nucleotide substitutions and is not phylogenetically related to the SARS-CoV-2 virus circulating in the United Kingdom at the time the variant was detected [21].

The three mutations that have the largest potential biological effect of the UK variants are N501Y, spike deletion 69/70 del and P687H [21, 22]. The N501Y mutation leads to an amino acid change from asparagine to tyrosine at position 501. It is one of six key contact residues within the RBD and has been identified with having increasing binding affinity to human ACE-2 [23].

The 69/70 del mutation is a deletion of six bases in the RNA, leading to the removal of two amino acids at position 69 and 70 of the spike protein. This spike deletion has been described in the context of evasion of the human immune response but has also occurred a number of times in association with other RBD changes [22, 23].

The P681H mutation led to a change from proline to histidine at position 681 [23]. This mutation is immediately adjacent to the furin cleavage site, a known location of biological significance.

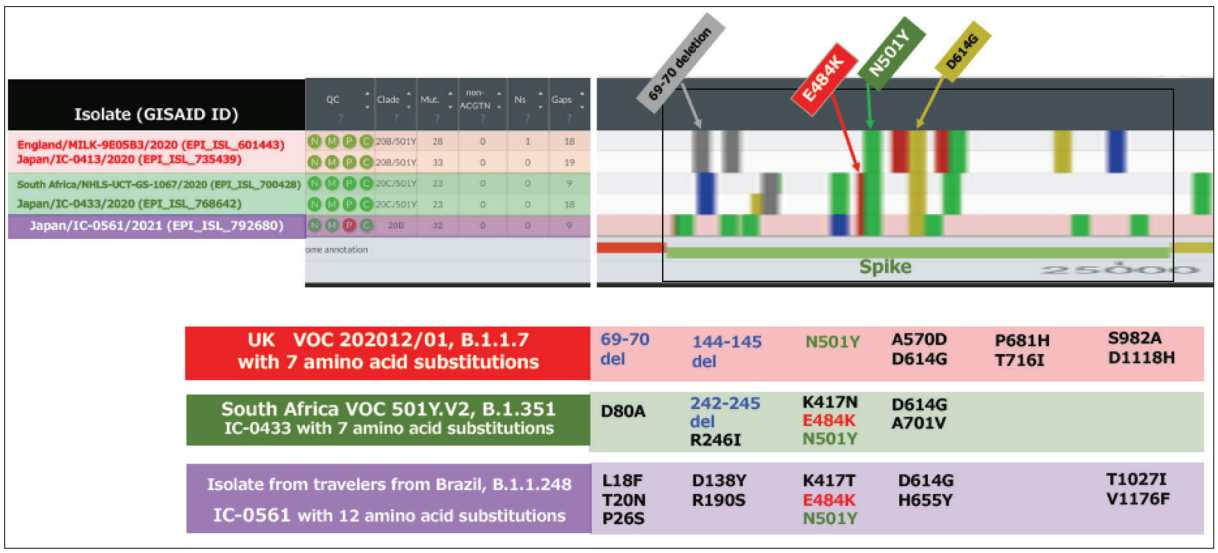


**Figure 6.** A vis-à-vis presentation of mutations in the UK, South African, and Brazilian variants; source: National Institute of Infectious Diseases, Japan [26]

Molecular virology, epidemiology, and phylogenetic analyses suggest that SARS-CoV-2 VOC 202012/01 has increased transmissibility. There is no evidence that this variant leads to changes in disease severity measured by length of hospitalization and 28-day case fatality, but the analyses have not been completed as yet [23]. This deletion was found to affect the performance of PCR assays with the S gene target. The deletion has no significant impact on the performance of antigen-based tests. Many countries all over the world have been reporting the presence of the British variant [22, 23].

A new variant rapidly spreading in South Africa named 501Y.V2 or the South Africa Variant was identified in December 2020. In addition to N501Y mutation, the new variant has another two mutations, K417N and E484K [23]. The combination of these three mutations results in the highest degree of conformation alteration of S RBD bound to hACE-2 compared to either E484E or N501Z alone. The 501Y.V2 has largely replaced other SARS-CoV-2 viruses circulating in the Eastern Cape, Western Cape, and KwaZulu-Natal provinces.

Retrospective analysis of whole-genome sequences from South Africa indicates that this variant emerged in early August 2020 [24]. Preliminary studies suggest that this variant is associated with a higher viral load, which may suggest a potential for increased transmissibility but no clear evidence of more severe outcomes of the disease [23, 24]. There is some evidence that one of the spike protein mutations, E484K, may affect neutralization by some polyclonal and monoclonal antibodies [23, 24].

Finally, the National Institute of Infectious Diseases in Japan, in January 2021, found a new variant with 17 unique amino acid changes and three deletions in four travelers from Brazil [23, 24]. This variant contains 17 unique amino acid changes with 10 in its S protein, including the three mutations in the spike protein receptor-binding domain: K417T, E484K, and N501Y. There is some evidence that the mutations in this variant may affect its transmissibility and antigenic profile, which may influence the ability of antibodies generated through a previous natural infection or through vaccination to recognize and neutralize the virus. A representation of mutations in current variants of concern in parallel to one another is presented in Figure 6.

## CONCLUSION

The SARS-CoV-2 genome has been fully sequenced. The function of the viral genes and their role in the virus–host interaction is elucidated. Scientists around the world follow the accumulation of mutations occurring in the pathogen's genome, with particular focus on the S gene coding for a protein important for the initial step in host infection. The change in the RBD region of the mentioned gene enabling infection of ACE-2-positive cells is especially significant. The longer the virus circulates within the general population, as well as immunocompromised persons, the number of mutations will increase and novel pathogen variants will arise. Mutations responsible for the faster transmission of SARS-CoV-2 have been identified in the variants of concern, namely the British, South African, and Brazilian variants. This highlights the importance of further sequencing efforts from patient samples, which aids in keeping track of viral characteristics linked to disease course and outcome, as well as in the application of epidemiological measures centered on the prevention of spread of variants of concern.

**Conflict of interest:** None declared.

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## Нове варијанте новог коронавируса – мит и реалност

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**САЖЕТАК**

Нови коронавирус прескочио је баријеру врсте и довео до пандемије инфекције *COVID*-19. Дуготрајна циркулација вируса у популацији омогућила је настанак бројних нових варијанти вируса, од којих су неке претеће за даље ширење епидемије. Од посебног су значаја оне варијанте код којих је дошло до мутација у оквиру гена *S*, посебно у региону за везивање вируса за рецептор *hACE*-2 (*RBD*). Мутације у овом гену одговорне су за повећану трансмисију вируса, тежину клиничке слике, поузданост дијагностичких тестова као и ефикасност вакцинације. За Европу највећу претњу тренутно представља британски сој вируса *SARS-CoV*-2.

**Кључне речи:** коронавирус, *COVID*-19; варијанта; *SARS-CoV*-2; нови вирус

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CURRENT TOPIC / АКТУЕЛНА ТЕМА

# Neurosarcoidosis – an ever-present clinical challenge

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**SUMMARY**
Sarcoidosis afflicts the central nervous system more frequently than previously believed. Neurological symptoms are present in roughly one-half of patients, and depend on the location in the central nervous system. The probability of spontaneous regression is significantly less when compared to other forms of sarcoidosis, which means that the proper diagnosis and treatment is paramount. Even when properly treated, functional defects are not uncommon. Majority of these patients require immunomodulating drugs and continuous follow-up. New immunomodulating drugs, especially biological agents, have shown to be significantly more effective, with fewer side effects, and are important when corticosteroids could not be applied. Less invasive methods, such as cerebrospinal analysis, help greatly in the diagnostics procedure, and require further research and improvement.
**Keywords:** sarcoidosis; neurosarcoidosis; cerebrospinal liquid; diagnostics; treatment

## INTRODUCTION

Even though it has been over a century since sarcoidosis was first discovered, it remains a disease of unknown etiology and course. Sarcoidosis is a systemic granulomatous disease which, most commonly, afflicts the lungs and hilar lymph nodes. The lungs are afflicted in 90–95% of all cases, and peripheral lymph nodes in 50–70%. In patients with systemic sarcoidosis, even though nervous system is rarely afflicted (5–15%), in those cases it can lead to serious complications and even death. The exact percentage is difficult to determine since there is a large number of subclinical cases, which are discovered only on autopsies. Neurological symptoms are present in roughly one-half of the patients. The characteristics vary greatly, depending on the distribution and inflammation of certain parts of the nervous system. The spontaneous regression of the disease is significantly less probable compared to the acute form of sarcoidosis, so the neurological symptoms are something that should always be checked for and treated [1, 2, 3].

## WHEN TO SUSPECT NEUROSARCOIDOSIS?

Sarcoidosis granuloma can be present in any part of the nervous system, which leads to a wide array of neurological symptoms. Possible manifestations of neurosarcoidosis are as follows: affliction of the cranial nerves, small fiber neuropathy, seizures, meningitis, lesions of the cranial tissue, tumor-like symptoms, disfunction of the hypothalamus and the pituitary gland, cerebellar ataxia, spinal cord lesions, skeletal muscles diseases, and psychiatric disorders.

The most common manifestation of neurosarcoidosis, present in 50–75% of all cases, is some form of cranial nerve disorder. Depending on which and how many of the cranial nerves are afflicted, the clinical presentation varies. The disfunction of the nerve can be caused by different pathological processes, such as granulomatous infiltration of the nuclei of the specific nerves, the increase of the intracranial pressure or by meningitis (damaging the subarachnoid part of the nerve). Sorting by the frequency of affliction, the unilateral affliction of the facial nerve is the most common presentation, followed by *n. opticus* with scotoma [4, 5]. Approximately 15% of patients with neurosarcoidosis have peripheral neuropathy, caused by the damage of either the large or small neural fibers. If large neural fibers are damaged, the patient will have mononeuritis, polyneuritis, Landry or Guillain–Barré syndrome. However, if small neural fibers are damaged, the patient can have restless legs syndrome or other disorders related to movement of the legs, as well as the loss of sensibility for pain or temperature, and autonomous disfunction [4]. Sudden onset of seizures in patients with sarcoidosis calls for a detailed examination of the central nervous system, and these patients, unfortunately, have

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poorer outcome with a fulminant course of the disease. Seizures, present in 5–10% of patients with neurosarcoidosis, show the severity, progression, and the relapses of the disease. Meningeal affliction is, according to the literature, a common location of neurosarcoidosis, and can be present in up to 25% of all the patients. Symptoms are similar to meningitis caused by other agents, and can include fever, headache, and stiffness of the neck. Lymphocytic pleocytosis can be found in cerebrospinal fluid (CSF), and the biochemical analysis shows the elevated values of proteins. Acute meningitis has a good response to corticosteroid treatment, but the chronic form requires a prolonged period of treatment and the outcome is difficult to predict. Roughly 50% of the patients can develop some form of brain lesions, such as encephalopathy, lesions of the gray mass, or lesions of the hypothalamus. The main mechanism of development of these lesions is the presence and fusion of multiple granulomas in the brain. Tumor lesions develop similarly; however, the fused granuloma are bigger, and clinically simulate any other tumor mass in the central nervous system [6]. Neurosarcoidosis has shown to have an affinity for the base of the brain, and 10–15% of all the patients develop neuro-endocrine symptoms due to the lesions of the hypothalamus and the pituitary gland, most commonly as a cause of infiltration in the third brain ventricle. One of the most frequent manifestations are polyuria with polydipsia, due to either diabetes insipidus or dysregulation of antidiuretic hormone. Hypovolemia, chronic hyponatremia, and unregulated thirst can also be present. Dysregulation of prolactin, with its elevation, can also be found in these patients, and can lead to galactorrhea and amenorrhea. Secondary hypogonadotropic amenorrhea with normal levels of prolactin has also been noted [7]. Cerebellum is rarely afflicted with sarcoidosis and, when afflicted, it is difficult to differentiate the symptoms from the symptoms caused by the lesions of the spinal cord. Spinal cord lesions are present in less than 10% of patients with neurosarcoidosis. Depending on the location of the granuloma (extradural, intradural, or intramedullary), the clinical presentation varies. It should be noted that it can be difficult to differentiate the granuloma from leptomeningeal tumors or infections. Cervical and thoracic parts of the spine are most commonly afflicted. The prognosis is unfavorable, and the symptoms at the beginning are muscular weakness and paresthesia. Skeletal muscles are afflicted in 1.4–2.3% of all patients with neurosarcoidosis; however, up to 80% of these patients have no clinical symptoms. The types of afflictions in these patients are acute, nodular, and chronic myopathy, which is the most common [8]. Up to 20% of patients with neurosarcoidosis develop cognitive and behavioral symptoms. The cause can be twofold – either by development of granuloma in the gray matter, or by psychological stress caused by having a chronic, relapsing, or progressive form of the disease. Psychiatric disorders present in these patients are hallucinations, refractory psychosis, paranoid psychosis, and delirium. Aphasia, amnesia, and dementia can also be present. In some rare cases, schizophrenia, depression, and bipolar disorders can develop [9].

## HOW TO DIAGNOSE NEUROSARCOIDOSIS

The biopsy of the central nervous system is the most precise, albeit not the most practical, way to definitively confirm the diagnosis. Zajicek has given the diagnostic criteria which are still being used [10]. The criteria are based on the levels of security of diagnosis, and the categories include the clinical presentation of neurosarcoidosis and exclude others. The criteria for definitive diagnosis: a positive biopsy of the nervous system. The criteria for possible neurosarcoidosis: clinical symptoms and diagnosis pointing to neurosarcoidosis; however, infections or malignancies are not excluded and the patient has histological conformation of sarcoidosis of other organ(s). The criteria for probable neurosarcoidosis: clinical symptoms and diagnostical evaluation pointing to neurosarcoidosis. The alternative diagnosis is excluded and there is a histological conformation of systemic sarcoidosis [10, 11].

Nuclear magnetic resonance (MRI) is the preferable method for radiological conformation of the disease. Any patient with a suspicion for neurosarcoidosis is suggested to perform the MRI scan of the endocranium. The normal finding does not exclude the diagnosis, especially if the patient is on corticosteroid treatment. Positron emission tomography (PET) scan can also be performed, although the interpretation is relatively difficult. Elevated metabolism is attributed to the inflammation in sarcoidosis, and the decreased metabolism is caused by the dysfunction of the neurons. Despite the limitations, PET scan can detect lesions in patients with no suspicion for neurosarcoidosis, or can be used to check the treatment response [12, 13].

## ANALYSIS OF THE CEREBROSPINAL FLUID

CSF analysis, which is considered a relatively noninvasive method, can provide a great deal of data to confirm the diagnosis. Lymphocytic pleocytosis, elevated protein levels, decreased levels of glucose, and elevated pressure are nonspecific signs of neurosarcoidosis. Elevated immunoglobulins, lysosomes, and $\beta_2$ microglobulin, as well as the ratio of CD4+/CD8+ over 5 can also be found in these patients. Elevated values of angiotensin converting enzyme (ACE) is something that can lead to the diagnosis of neurosarcoidosis. The publications so far have shown that over 60% of patients with neurosarcoidosis have elevated levels of ACE. However, it is not enough for a definitive diagnosis. Studies show that the chitotriosidase can be used as a new biomarker [14, 15]. A publication which analyzed the CSF in patients with neurosarcoidosis and multiple sclerosis (MS) has shown that the elevated values of IL-6 and CD4/CD8 ratio were statistically more significant in patients with neurosarcoidosis [16]. It was interesting to find that IL-6 in CSF was higher in patients with the active form of neurosarcoidosis compared with those with the inactive form, and that the patients with concentration of IL-6 above 50 pg/ml in CSF have shown to have a higher probability of reactivation or progression of the disease. The same publication has shown that the

concentration of IL-10 can also be elevated in neurosarcoidosis [16]. Another study tested the levels of IL-2 in CSF as a diagnostic and biomarker of activity in neurosarcoidosis [17]. In this study, the CSF was taken from patients with neurosarcoidosis, MS, neurotuberculosis, viral and bacterial meningitis, cerebral lymphoma, Guillain–Barré syndrome, and 115 patients with non-inflammatory neurological diseases as a control group. IL-2 concentration was related to the clinical activity of the disease, increased uptake of gadolinium, and the number of leucocytes in patients with neurosarcoidosis. It was discovered that IL-2 is elevated in patients with neurosarcoidosis; however, it was not specific enough. IL-2 in CSF can be used in order to differentiate between neurosarcoidosis and MS, and can be used in order to determine the activity of the disease [17].

## THE BEST TREATMENT?

Even though a great number of drugs have shown a positive response in treatment of neurosarcoidosis, corticosteroids, administered in a pulse dosage, still remain the golden standard. If remission is not achieved, or the clinical response on corticosteroids is not given, the application of another immunomodulator is the next treatment step – methotrexate, hydroxychloroquine, azathioprine, or cyclophosphamide. In severe forms of neurosarcoidosis, which are resistant to any and all pharmacological treatments, radio treatment, and even surgery, can be performed. Since tumor necrosis factor (TNF) is being produced within granuloma, anti-TNF drugs can be used in the treatment of sarcoidosis. The treatment with infliximab and adalimumab have shown promising results, and there are studies which test other monoclonal antibodies. However, there is still a great need for further clinical trials and experience with these treatments [18, 19].

## CONCLUSION

Neurosarcoidosis is an uncommon but significant clinical manifestation of sarcoidosis. There is a significant variation in clinical presentation of this form of the disease, depending on the location of the granuloma in the nervous system. The probability of spontaneous resolution is less than that in other forms of sarcoidosis, with functional deficits remaining long after remission is achieved. Due to previously noted characteristics, patients with neurosarcoidosis require immunosuppressive treatment and long-term follow-up. The variation in presentation, similarity to other diseases, and complexity of treatment are key points that require a multidisciplinary approach in diagnostics and treatment of this disease.

The development of less invasive methods, such as the analysis of CSF, can provide a quicker and easier way to the final diagnosis, and should be further developed.

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## Неуросаркоидоза – и даље велики клинички изазов

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**САЖЕТАК**

Саркоидоза захвата централни нерви систем чешће него што се раније сматрало. Неуролошки симптоми се манифестују код отприлике половине оних са неуросаркоидозом. Клиничке и сликовне карактеристике неуросаркоидозе варирају у великој мери, а пре свега зависе од анатомске дистрибуције болести. Сама вероватноћа да се неуросаркоидоза спонтано повуче много је нижа неко код акутних форми саркоидозе. Зато посебно треба обратити пажњу на неуролошке симптоме и по постављању дијагнозе болест одмах лечити. Вероватноћа да се спонтано разреши нижа је него код саркоидозе уопште, а заостали функционални дефицит није реткост. Стога су већини болесника са неуросаркоидозом потребни имуносупресивна терапија и континуирано праћење. Међутим, други имуносупресивни агенси, као и новији биолошки агенси, показали су се као ефикасна, добро подношљива терапија која је нарочито значајна у случајевима кад се кортикостероиди не могу примењивати. Развијање мање инвазивних метода, као што је анализа цереброспиналне течности код оболелих од неуросаркоидозе, свакако је нешто што умногоме може олакшати пут клиничару до дијагнозе, и треба да представља предмет даљих истраживања.

**Кључне речи:** саркоидоза; неуросаркоидоза; ликвор; дијагностика; лечење

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LETTER TO THE EDITOR / ПИСМО УРЕДНИКУ

# Psychological impacts of COVID-19

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Dear Editor,

Since the end of December 2019, the outbreak of a novel viral disease was reported in the city of Wuhan, China, which was caused by a novel coronavirus, and was officially named COVID-19 by the World Health Organization (WHO) [1].

The virus COVID-19 is a highly transmittable and pathogenic viral infection caused by severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), which emerged in Wuhan, China, and spread around the world. Genomic analysis revealed that SARS-CoV-2 is phylogenetically related to severe acute respiratory syndrome-like (SARS-like) bat viruses, therefore bats could be the possible primary reservoir. The intermediate source of origin and transfer to humans is unknown, however, the rapid human to human transmission has been widely confirmed [2].

There is no clinically approved antiviral drug or vaccine available to be used against COVID-19. However, few broad-spectrum antiviral drugs have been evaluated against COVID-19 in clinical trials, resulted in clinical recovery [3].

This disease will not only raise public health concerns but also cause several forms of psychological distress, including anxiety, fear, depression, stigmatization, avoidance behaviors, irritability, insomnia, and posttraumatic stress disorder. In this situation, the maintenance of the mental health of individuals is very important because people in different parts of the society may experience additional stressors during the COVID-19 pandemic. Individuals in different parts of a society may experience the psychological symptoms to COVID-19 during the rising phase of the outbreak, including patients of COVID-19, quarantine individuals, health care workers, and family members of medical staff, children, university students, pregnant women, and families. In this regard, there is strong evidence that the mental health status of these populations is vulnerable to psychological disorders [4].

In addition to public health problems and the financial burden on the people and governments, the coronavirus has forced governments to cancel travel and trade between countries and inflict heavy financial losses on governments. Also, with the closing of these industries, the staff of these centers face unemployment and financial problems, which are a psychological pressure and it puts a lot of stress on the individual and his family, as well as the community. Prolonged stay at home can also lead to depression. Also, there is a lot of stress among students due to the closure of educational centers [5].

From a psychological view, an experience that people percieve under the headings of fear, anxiety, and bad mood, we can consider it as stress and anxiety. These conditions are especially difficult to tolerate for people with mood and anxiety disorders. The following suggestions can be more or less helpful in dealing with this situation.

There are six skills that can help regulate the mental state and reduce boredom, anxiety, panic, obsession, and other psychological problems (critical thinking, use of perspective, precautionary skills, self-regulation, balance skills, and creativity skills) especially when coping with COVID-19 psychological consequences.

Critical thinking skills are ways to prevent disastrous COVID-19 effects for yourself and others. Using this skill, we focus on facts, not intimidating and false stories; those who think critically can look at the issue from different angles, and this can reduce their anxiety and fear.

Using perspective skills means having an overall picture in mind, the prospect does not mean that there is no need to worry, but this skill helps us take a step back and seen if something worse could happen to ourselves and those around us.

Precautionary skills for psychological confrontation with COVID-19, in this way, help a person prioritize caution and health standards, staying calm and collected, distinguishing rational and expert solutions in daily life.

In self-regulation skills we follow health regulations, create healthy habits, such as hand washing, but also manage temptations, such as traveling. We refrain from bad options, consider the long-term, and prioritize health.

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Another method for psychological coping with COVID-19 is balance skill. Although it is positive to stay informed, receiving too much information can fatigue, intimidate, and overstimulate. It is better to follow the overall news summary, and, in general, one has to dose receiving information and news.

Utilizing creativity as the sixth skill can be helpful in the crisis of the prevalence of COVID-19. If people are bored with staying home, they can use creativity. Playing creative games, performing group activities with family members, developing artistic skills, such as painting or music, and exercising at home are good ways to reduce anxiety, fear, and boredom.

It is recommended that psychologists and psychiatrists be consulted by telephone in situations where the person has been less able to use these skills.

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**Пре подношења рукописа Уредништву часописа „Српски архив за целокупно лекарство“ (СА) сви аутори треба да прочитају Упутство за ауторе (*Instructions for Authors*), где ће пронаћи све потребне информације о писању и припреми рада у складу са стандардима часописа. Веома је важно да аутори припреме рад према датим пропозицијама, јер уколико рукопис не буде усклађен с овим захтевима, Уредништво ће одложити или одбити његово публиковање. Радови објављени у СА се не хонорарашу. За чланке који ће се објавити у СА, самом понудом рада Српском архиву сви аутори рада преносе своја ауторска права на издавача часописа – Српско лекарско друштво.**

**ОПШТА УПУТСТВА.** СА објављује радове који до сада нису нигде објављени, у целости или делом, нити прихваћени за објављивање. СА објављује радове на енглеском и српском језику. Због боље доступности и веће цитираности препоручује се ауторима да радове свих облика предају на енглеском језику. У СА се објављују следеће категорије радова: уводници, оригинални радови, претходна и кратка саопштења, прикази болесника и случајева, видео-чланци, слике из клиничке медицине, прегледни радови, актуелне теме, радови за праксу, радови из историје медицине и језика медицине, медицинске етике, регулаторних стандарда у медицини, извештаји са конгреса и научних скупова, лични ставови**,** наручени коментари, писма уреднику, прикази књига, стручне вести, *In memoriam* и други прилози. Оригинални радови, претходна и кратка саопштења, прикази болесника и случајева, видео-чланци, слике из клиничке медицине, прегледни радови и актуелне теме, публикују се искључиво на енглеском језику, а остале врсте радова се могу публиковати и на српском језику само по одлуци Уредништва. Радови се увек достављају са сажетком на енглеском и српском језику (у склопу самог рукописа). Текст рада куцати у програму за обраду текста *Word*, фонтом *Times New Roman* и величином слова 12 тачака (12 *pt*). Све четири маргине подесити на 25 *mm*, величину странице на формат А4, а текст куцати с двоструким проредом, левим поравнањем и увлачењем сваког пасуса за 10 *mm*, без дељења речи (хифенације). Не користити табулаторе и узастопне празне карактере (спејсове) ради поравнања текста, већ алатке за контролу поравнања на лењиру и *Toolbars*. За прелазак на нову страну документа не користити низ „ентера“, већ искључиво опцију *Page Break*. После сваког знака интерпункције ставити само један празан карактер. Ако се у тексту користе специјални знаци (симболи), користити фонт *Symbol*. Подаци о коришћеној литератури у тексту означавају се арапским бројевима у угластим заградама – нпр. [1, 2], и то редоследом којим се појављују у тексту. Странице нумерисати редом у доњем десном углу, почев од насловне стране.

При писању текста на енглеском језику треба се придржавати језичког стандарда *American English* и користити кратке и јасне реченице. За називе лекова користити искључиво генеричка имена. Уређаји (апарати) се означавају фабричким називима, а име и место произвођача треба навести у облим заградама. Уколико се у тексту користе ознаке које су спој слова и бројева, прецизно написати број који се јавља у суперскрипту или супскрипту (нпр. $^{99}Tc$, *IL*-6, $O_2$, $Б_{12}$, *CD*8). Уколико се нешто уобичајено пише курзивом (*italic*), тако се и наводи, нпр. гени (*BRCA1*).

Уколико је рад део магистарске тезе, односно докторске дисертације, или је урађен у оквиру научног пројекта, то треба посебно назначити у Напомени на крају текста. Такође, уколико је рад претходно саопштен на неком стручном састанку, навести званичан назив скупа, место и време одржавања, да ли је рад и како публикован (нпр. исти или другачији наслов или сажетак).

**КЛИНИЧКА ИСТРАЖИВАЊА.** Клиничка истраживања се дефинишу као истраживања утицаја једног или више средстава или мера на исход здравља. Регистарски број истраживања се наводи у последњем реду сажетка.

**ЕТИЧКА САГЛАСНОСТ.** Рукописи о истраживањима на људима треба да садрже изјаву у виду писаног пристанка испитиваних особа у складу с Хелсиншком декларацијом и одобрење надлежног етичког одбора да се истраживање може извести и да је оно у складу с правним стандардима. Експериментална истраживања на хуманом материјалу и испитивања вршена на животињама треба да садрже изјаву етичког одбора установе и треба да су у сагласности с правним стандардима.

**ИЗЈАВА О СУКОБУ ИНТЕРЕСА.** Уз рукопис се прилаже потписана изјава у оквиру обрасца *Submission Letter* којом се аутори изјашњавају о сваком могућем сукобу интереса или његовом одсуству. За додатне информације о различитим врстама сукоба интереса посетити интернет-страницу Светског удружења уредника медицинских часописа (*World Association of Medical Editors – WAME; http://www.wame.org*) под називом „Политика изјаве о сукобу интереса“.

**АУТОРСТВО.** Све особе које су наведене као аутори рада треба да се квалификују за ауторство. Сваки аутор треба да је учествовао довољно у раду на рукопису како би могао да преузме одговорност за целокупан текст и резултате изнесене у раду. Ауторство се заснива само на: битном доприносу концепцији рада, добијању резултата или анализи и тумачењу резултата; планирању рукописа или његовој критичкој ревизији од знатног интелектуалног значаја; завршном дотеривању верзије рукописа који се припрема за штампање.

Аутори треба да приложе опис доприноса појединачно за сваког коаутора у оквиру обрасца *Submission Letter*. Финансирање, сакупљање података или генерално надгледање истраживачке групе сами по себи не могу

оправдати ауторство. Сви други који су допринели изради рада, а који нису аутори рукописа, требало би да буду наведени у Захвалници с описом њиховог доприноса раду, наравно, уз писани пристанак.

**ПЛАГИЈАРИЗАМ.** Од 1. јануара 2019. године сви рукописи подвргавају се провери на плагијаризам/аутоплагијаризам преко *SCIndeks Assistant* – Cross Check (iThenticate). Радови код којих се докаже плагијаризам/аутоплагијаризам биће одбијени, а аутори санкционисани.

**НАСЛОВНА СТРАНА.** На првој страници рукописа треба навести следеће: наслов рада без скраћеница; предлог кратког наслова рада, пуна имена и презимена аутора (без титула) индексирана бројевима; званичан назив установа у којима аутори раде, место и државу (редоследом који одговара индексираним бројевима аутора); на дну странице навести име и презиме, адресу за контакт, број телефона, факса и имејл адресу аутора задуженог за кореспонденцију.

**САЖЕТАК.** Уз оригинални рад, претходно и кратко саопштење, преглед литературе, приказ случаја (болесника), рад из историје медицине, актуелну тему, рад за рубрику језик медицине и рад за праксу, на другој по реду страници документа треба приложити сажетак рада обима 100–250 речи. За оригиналне радове, претходно и кратко саопштење сажетак треба да има следећу структуру: Увод/Циљ рада, Методе рада, Резултати, Закључак; сваки од наведених сегмената писати као посебан пасус који почиње болдованом речи. Навести најважније резултате (нумеричке вредности) статистичке анализе и ниво значајности. Закључак не сме бити уопштен, већ мора бити директно повезан са резултатима рада. За приказе болесника сажетак треба да има следеће делове: Увод (у последњој реченици навести циљ), Приказ болесника, Закључак; сегменте такође писати као посебан пасус који почиње болдованом речи. За остале типове радова сажетак нема посебну структуру.

**КЉУЧНЕ РЕЧИ.** Испод Сажетка навести од три до шест кључних речи или израза. Не треба да се понављају речи из наслова, а кључне речи треба да буду релевантне или описне. У избору кључних речи користити *Medical Subject Headings – MeSH (http://www.nlm.nih.gov/mesh)*.

**ПРЕВОД НА СРПСКИ ЈЕЗИК.** На трећој по реду страници документа приложити наслов рада на српском језику, пуна имена и презимена аутора (без титула) индексирана бројевима, званичан назив установа у којима аутори раде, место и државу. На следећој – четвртој по реду – страници документа приложити сажетак (100–250 речи) с кључним речима (3–6), и то за радове у којима је обавезан сажетак на енглеском језику. Превод појмова из стране литературе треба да буде у духу српског језика. Све стране речи или синтагме за које постоји одговарајуће име у нашем језику заменити тим називом. Уколико је рад у целости на српском језику, потребно је превести називе прилога (табела, графикона, слика, схема) уколико их има, целокупни текст у њима и легенду на енглески језик.

**СТРУКТУРА РАДА.** Сви поднаслови се пишу великим масним словима (болд). Оригинални рад и претходно и кратко саопштење обавезно треба да имају следеће поднаслове: Увод (Циљ рада навести као последњи пасус Увода), Методе рада, Резултати, Дискусија, Закључак, Литература. Преглед литературе и актуелну тему чине: Увод, одговарајући поднаслови, Закључак, Литература. Првоименовани аутор прегледног рада мора да наведе бар пет аутоцитата (као аутор или коаутор) радова публикованих у часописима с рецензијом. Коаутори, уколико их има, морају да наведу бар један аутоцитат радова такође публикованих у часописима с рецензијом. Приказ случаја или болесника чине: Увод (Циљ рада навести као последњи пасус Увода), Приказ болесника, Дискусија, Литература. Не треба користити имена болесника, иницијале, нити бројеве историја болести, нарочито у илустрацијама. Прикази болесника не смеју имати више од пет аутора.

Прилоге (табеле, графиконе, слике итд.) поставити на крај рукописа, а у самом телу текста јасно назначити место које се односи на дати прилог. Крајња позиција прилога биће одређена у току припреме рада за публиковање.

**СКРАЋЕНИЦЕ.** Користити само када је неопходно, и то за веома дугачке називе хемијских једињења, односно називе који су као скраћенице већ препознатљиви (стандардне скраћенице, као нпр. ДНК, сида, ХИВ, АТП). За сваку скраћеницу пун термин треба навести при првом навођењу у тексту, сем ако није стандардна јединица мере. Не користити скраћенице у наслову. Избегавати коришћење скраћеница у сажетку, али ако су неопходне, сваку скраћеницу објаснити при првом навођењу у тексту.

**ДЕЦИМАЛНИ БРОЈЕВИ.** У тексту рада на енглеском језику, у табелама, на графиконима и другим прилозима децималне бројеве писати са тачком (нпр. 12.5 ± 3.8), а у тексту на српском језику са зарезом (нпр. 12,5 ± 3,8). Кад год је то могуће, број заокружити на једну децималу.

**ЈЕДИНИЦЕ МЕРА.** Дужину, висину, тежину и запремину изражавати у метричким јединицама (метар – *m*, килограм (грам) – *kg* (*g*), литар – *l*) или њиховим деловима. Температуру изражавати у степенима Целзијуса (°*C*), количину супстанце у молима (*mol*), а притисак крви у милиметрима живиног стуба (*mm Hg*). Све резултате хематолошких, клиничких и биохемијских мерења наводити у метричком систему према Међународном систему јединица (*SI*).

**ОБИМ РАДОВА.** Целокупни рукопис рада који чине – насловна страна, сажетак, текст рада, списак литературе, сви прилози, односно потписи за њих и легенда (табеле, слике, графикони, схеме, цртежи), насловна страна и сажетак на српском језику – мора износити за оригинални рад, рад из историје медицине и преглед литературе до 5000 речи, а за претходно и кратко саопштење, приказ болесника, актуелну тему, рад за праксу, едукативни чланак и рад за рубрику „Језик медицине“ до 3000 речи; радови за остале рубрике могу имати највише 1500 речи.
Видео-радови могу трајати 5–7 минута и бити у формату *avi, mp4*(*flv*). У првом кадру филма мора се навести: у наднаслову Српски архив за целокупно лекарство, наслов рада, презимена и иницијали имена и средњег слова свих аутора рада (не филма), година израде. У другом кадру мора бити уснимљен текст рада у виду апстракта до 350 речи. У последњем кадру филма могу се навести имена техничког особља (режија, сниматељ, светло, тон, фотографија и сл.). Уз видео-радове доставити: посебно текст у виду апстракта (до 350 речи), једну фотографију као илустрацију приказа, изјаву потписану од свег техничког особља да се одричу ауторских права у корист аутора рада.

**ПРИЛОЗИ РАДУ** су табеле, слике (фотографије, цртежи, схеме, графикони) и видео-прилози.

**Свака табела** треба да буде сама по себи лако разумљива. Наслов треба откуцати изнад табеле, а објашњења испод ње. Табеле се означавају арапским бројевима према редоследу навођења у тексту. Табеле цртати искључиво у програму *Word*, кроз мени *Table–Insert–Table*, уз дефинисање тачног броја колона и редова који ће чинити мрежу табеле. Десним кликом на мишу – помоћу опција *Merge Cells* и *Split Cells* – спајати, односно делити ћелије. Куцати фонтом *Times New Roman*, величином слова 12 *pt*, с једноструким проредом и без увлачења текста. Коришћене скраћенице у табели треба објаснити у легенди испод табеле. Уколико је рукопис на српском језику, приложити називе табела и легенду на оба језика. Такође, у једну табелу, у оквиру исте ћелије, унети и текст на српском и текст на енглеском језику (никако не правити две табеле са два језика!).

**Слике су** сви облици графичких прилога и као „слике“ у CA се објављују фотографије, цртежи, схеме и графикони. Слике означавају се арапским бројевима према редоследу навођења у тексту. Примају се искључиво дигиталне фотографије (црно-беле или у боји) резолуције најмање 300 *dpi* и формата записа *tiff* или *jpg* (мале, мутне и слике лошег квалитета неће се прихватати за штампање!). Уколико аутори не поседују или нису у могућности да доставе дигиталне фотографије, онда оригиналне слике треба скенирати у резолуцији 300 *dpi* и у оригиналној величини. Уколико је рад неопходно илустровати са више слика, у раду ће их бити објављено неколико, а остале ће бити у е-верзији чланка као *PowerPoint* презентација (свака слика мора бити нумерисана и имати легенду).
Видео-прилози (илустрације рада) могу трајати 1–3 минута и бити у формату *avi, mp4*(*flv*). Уз видео доставити посебно слику која би била илустрација видеоприказа у *e*-издању и објављена у штампаном издању. Уколико је рукопис на српском језику, приложити називе слика и легенду на оба језика.

Слике се у свесци могу штампати у боји, али додатне трошкове штампе сносе аутори.

**Графикони** треба да буду урађени и достављени у програму *Excel*, да би се виделе пратеће вредности распоређене по ћелијама. Исте графиконе прекопирати и у *Word*-ов документ, где се графикони означавају арапским бројевима према редоследу навођења у тексту. Сви подаци на графикону куцају се у фонту *Times New Roman*. Коришћене скраћенице на графикону треба објаснити у легенди испод графикона. У штампаној верзији чланка вероватније је да графикон неће бити штампан у боји, те је боље избегавати коришћење боја у графиконима, или их користити различитог интензитета. Уколико је рукопис на српском језику, приложити називе графикона и легенду на оба језика.

**Цртежи и схеме** се достављају у *jpg* или *tiff* формату. Схеме се могу цртати и у програму *CorelDraw* или *Adobe Illustrator* (програми за рад са векторима, кривама). Сви подаци на схеми куцају се у фонту *Times New Roman*, величина слова 10 *pt*. Коришћене скраћенице на схеми треба објаснити у легенди испод схеме. Уколико је рукопис на српском језику, приложити називе схема и легенду на оба језика.

**ЗАХВАЛНИЦА.** Навести све сараднике који су допринели стварању рада а не испуњавају мерила за ауторство, као што су особе које обезбеђују техничку помоћ, помоћ у писању рада или руководе одељењем које обезбеђује општу подршку. Финансијска и материјална помоћ, у облику спонзорства, стипендија, поклона, опреме, лекова и друго, треба такође да буде наведена.

**ЛИТЕРАТУРА.** Списак референци је одговорност аутора, а цитирани чланци треба да буду лако приступачни читаоцима часописа. Стога уз сваку референцу обавезно треба навести *DOI* број чланка (јединствену ниску карактера која му је додељена) и *PMID* број уколико је чланак индексиран у бази *PubMed/MEDLINE*.

Референце нумерисати редним арапским бројевима према редоследу навођења у тексту. Број референци не би требало да буде већи од 30, осим у прегледу литературе, у којем је дозвољено да их буде до 50, и у метаанализи, где их је дозвољено до 100. Број цитираних оригиналних радова мора бити најмање 80% од укупног броја референци, односно број цитираних књига, поглавља у књигама и прегледних чланака мањи од 20%. Уколико се домаће монографске публи-

кације и чланци могу уврстити у референце, аутори су дужни да их цитирају. Већина цитираних научних чланака не би требало да буде старија од пет година. Није дозвољено цитирање апстраката. Уколико је битно коментарисати резултате који су публиковани само у виду апстракта, неопходно је то навести у самом тексту рада. Референце чланака који су прихваћени за штампу, али још нису објављени, треба означити са *in press* и приложити доказ о прихватању рада за објављивање.

Референце се цитирају према Ванкуверском стилу (униформисаним захтевима за рукописе који се предају биомедицинским часописима), који је успоставио Међународни комитет уредника медицинских часописа (*http://www.icmje.org*), чији формат користе *U.S. National Library of Medicine* и базе научних публикација. Примере навођења публикација (чланака, књига и других монографија, електронског, необјављеног и другог објављеног материјала) могу се пронаћи на интернет-страници *http://www.nlm.nih.gov/bsd/uniform_requirements.html*. Приликом навођења литературе веома је важно придржавати се поменутог стандарда, јер је то један од најбитнијих фактора за индексирање приликом класификације научних часописа.

**ПРОПРАТНО ПИСМО (*SUBMISSION LETTER*).** Уз рукопис обавезно приложити образац који су потписали сви аутори, а који садржи: 1) изјаву да рад претходно није публикован и да није истовремено поднет за објављивање у неком другом часопису, 2) изјаву да су рукопис прочитали и одобрили сви аутори који испуњавају мерила ауторства, и 3) контакт податке свих аутора у раду (адресе, имејл адресе, телефоне итд.). Бланко образац треба преузети са интернет-странице часописа (*http://www.srpskiarhiv.rs*).

Такође је потребно доставити копије свих дозвола за: репродуковање претходно објављеног материјала, употребу илустрација и објављивање информација о познатим људима или именовање људи који су допринели изради рада.

**ЧЛАНАРИНА, ПРЕТПЛАТА И НАКНАДА ЗА ОБРАДУ ЧЛАНКА.** Да би рад био објављен у часопису *Српски архив за целокупно лекарство*, сви аутори који су лекари или стоматолози из Србије морају бити чланови Српског лекарског друштва (у складу са чланом 6. Статута Друштва) и измирити накнаду за обраду чланака (*Article Processing Charge*) у износу од 3000 динара. Аутори и коаутори из иностранства су у обавези да плате накнаду за обраду чланака (*Article Processing Charge*) у износу од 35 евра. Уплата у једној календарској години обухвата и све наредне, евентуалне чланке, послате на разматрање у тој години. Сви аутори који плате ову накнаду могу, уколико то желе, да примају штампано издање часописа. Треба напоменути да ова уплата није гаранција да ће рад бити прихваћен и објављен у *Српском архиву за целокупно лекарство*. Обавеза плаћања накнаде за обраду чланка не односи се на студенте основних студија и на претплатнике на часопис.

Установе (правна лица) не могу преко своје претплате да испуне овај услов аутора (физичког лица). Уз рукопис рада треба доставити копије уплатница за чланарину и претплату / накнаду за обраду чланка, као доказ о уплатама, уколико издавач нема евиденцију о томе. Часопис прихвата донације од спонзора који сносе део трошкова или трошкове у целини оних аутора који нису у могућности да измире накнаду за обраду чланка (у таквим случајевима потребно је часопису ставити на увид оправданост таквог спонзорства).

**СЛАЊЕ РУКОПИСА.** Рукопис рада и сви прилози уз рад достављају се искључиво електронски преко система за пријављивање на интернет-страници часописа: *http://www.srpskiarhiv.rs*

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За све додатне информације, молимо да се обратите на доле наведене адресе и број телефона.

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The papers are always submitted with Summary in both English and Serbian, included in the manuscript file. The text of the manuscript should be typed in *MS Word* using the *Times New Roman* typeface, and font size 12 pt. The text should be prepared with margins set to 25 mm and onto A4 paper size, with double line spacing, aligned left and the initial lines of all paragraphs indented 10 mm, without hyphenation. Tabs and successive blank spaces are not to be used for text alignment; instead, ruler alignment control tool and *Toolbars* are suggested. In order to start a new page within the document, *Page Break* option should be used instead of consecutive enters. Only one space follows after any punctuation mark. If special signs (symbols) are used in the text, use the *Symbol* font. References cited in the text are numbered with Arabic numerals within parenthesis (for example: [1, 2]), in order of appearance in the text. Pages are numbered consecutively in the right bottom corner, beginning from the title page.

When writing text in English, linguistic standard American English should be observed. Write short and clear sentences. Generic names should be exclusively used for the names of drugs. Devices (apparatuses, instruments) are termed by trade names, while their name and place of production should be indicated in the brackets. If a letter-number combination is used, the number should be precisely designated in superscript or subscript (i.e., 99Tc, IL-6, O2, B12, CD8). If something is commonly written in italics, such as genes (e.g. BRCA1), it should be written in this manner in the paper as well.

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The authors should enclose the description of contribution to the article of every co-author individually (within the Submission Letter). Funding, collection of data or general supervision of the research group alone cannot justify authorship. All other individuals having contributed to the preparation of the article should be mentioned in the *Acknowledgment* section, with description of their contribution to the paper, with their written consent.

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**SUMMARY.** Along with the original article, preliminary and short communication, review article, case report, article on history of medicine, current topic article, article for language of medicine and article for practitioners, the summary not exceeding 100–250 words should be typed on the second page of the manuscript. In original articles, the summary should have the following structure: Introduction/Objective, Methods, Results, Conclusion. Each segment should be typed in a separate paragraph using boldface. The most significant results (numerical values), statistical analysis and level of significance are to be included. The conclusion must not be generalized, it needs to point directly to the results of the study. In case reports, the summary should consist of the following: Introduction (final sentence is to state the objective), Case Outline (Outline of Cases), Conclusion. Each segment should be typed in a separate paragraph using boldface. In other types of papers, the summary has no special outline.

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