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LETTER TO THE EDITOR / PISMO UREDNIKU



Yoshinori Ohsumi (February 9, 1945, Fukuoka, Japan), a professor of biology at the Tokyo Institute of Technology, has been awarded the 2016 Nobel Prize in Physiology or Medicine for his discoveries in autophagy – the process whereby a cell recycles part of its own contents.
These discoveries opened the path to understanding the fundamental importance of autophagy in many physiological processes, such as in the adaptation to starvation or response to infection. Mutations in autophagy genes can cause disease, because the autophagic process is involved in several conditions including cancer and neurodegenerative disorders.

Yoshinori Ohsumi (9. februar 1945, Fukuoka, Japan), profesor biologije na Tokijskom institutu za tehnologiju, ovogodišnji je dobitnik Nobelove nagrade za medicinu. Nagrada mu je dodeljena za otkrića u vezi sa autofagijom – procesom kojim ćelija reciklira deo sopstvenog sadržaja.
Ova otkrića otvorila su put za razumevanje fundamentalne važnosti autofagije u mnogim fiziološkim procesima, npr. u adaptaciji na gladovanje ili odgovoru na infekciju. Mutacije u genima za autofagiju mogu prouzrokovati bolest budući da je proces autofagije uključen u više stanja kao što su kancer i neurodegenerativne bolesti.

ORIGINAL ARTICLES

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# Alp Rose stem cells, olive oil squalene and a natural alkyl polyglucoside emulsifier: Are they appropriate ingredients of skin moisturizers – *in vivo* efficency on normal and sodium lauryl sulfate-irritated skin?

## Matične ćelije alpske ruže, skvalen maslinovog ulja i prirodni alkil-poliglukozidni emulgator: da li su odgovarajući sastojci kremova za vlaženje – *in vivo* efikasnost na zdravoj i koži iritiranoj natrijum lauril sulfatom?

**Mila Filipović*, Ana Gledović†, Milica Lukić†, Marija Tasić-Kostov‡, Tanja Isailović†, Ivana Pantelić†, Gordana Vuleta†, Snežana Savić†**

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**Abstract**

**Background/Aim.** Since skin moisturization may be achieved by both actives and chosen carrier, plant stem cells, squalene and natural alkyl polyglucoside emulsifier may be potential components of contemporary cosmetic products. The aim of the study was *in vivo* evaluation of the skin irritation potential and the efficacy of Alpine Rose stem cells incorporated into liposomes and olive oil squalene as ingredients of moisturizing creams, with respect to the novel emulsifier used for creams' stabilization. **Methods.** With the employment of noninvasive skin biophysical measurements, skin hydration (EC), transepidermal water loss (TEWL), erythema index (EI) and viscoelasticity were measured on 76 healthy volunteers. In the first phase, skin irritation after a 24-hour occlusion and the long-term efficacy of creams (a 21-day study) on healthy skin were evaluated. Phase II of the study focused on the cream efficacy assessment after a 6-day treatment of sodium lauryl sulfate-irritated skin. **Results.** After a 24-hour occlusion, there were no significant changes in the EI for any tested sample. In the second phase of the study, the EI was not significantly altered for the cream containing squalene, while the application of all active samples resulted in a significant reduction of TEWL. In both phases of the study an EC increase was recorded, especially for the squalene-containing cream. **Conclusion.** Due to the lack of skin irritation and skin barrier impairment along with the marked hydration effect, it could be said that the investigated actives incorporated into alkyl polyglucoside emulsifier-stabilized creams may be safely applied as ingredients for "tailor-made" cosmetic moisturizers intended for normal and dry skin care, whereas olive oil squalene could be used for the treatment of irritated or sensitive skin as well.

**Key words:**
**plant extracts; stem cells; cosmetics; skin; squalene; emulsifying agents; skin irritancy tests.**

**Apstrakt**

**Uvod/Cilj.** S obzirom na to da vlaženje kože može biti postignuto izborom kako aktivnih supstanci, tako i odgovarajućeg nosača, biljne matične ćelije, skvalen i prirodni alkil poliglukozidni emulgator mogu biti potencijalni sastojci savremenih kozmetičkih proizvoda. Cilj ovog rada bio je *in vivo* procena iritiranog potencijala i efikasnosti matičnih ćelija alpske ruže dodatih u liposome i skvalena maslinovog ulja, kao sastojaka vlažećih krema, imajući u vidu nov emulgator koji je korišćen za njihovu stabilizaciju. **Metode.** Upotrebom neinvazivnih metoda zasnovanih na biofizičkim merenjima na koži, kod 76 zdravih dobrovoljaca mereni su: hidratacija kože (EC), transepidermalni gubitak vlage (TEWL), eritema indeks (EI) i viskoelastičnost kože. U prvoj fazi studije vršena je procena iritacije kože nakon 24-h okluzije, kao i procena efikasnosti krema nakon primene na zdravoj koži dobrovoljaca u trajanju od 21 dana. Druga faza studije bila je bazirana na proceni efikasnosti krema nakon 6-dnevnog tretmana kože prethodno iritirane natrijum-lauril-sulfatom. **Rezultati**. Nakon 24-h okluzije nije bilo značajne promene vrednosti EI ni kod jednog ispitivanog krema. U drugoj fazi studije, krem sa skvalenom nije značajno uticao

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na promenu vrednosti EI, dok je primena svih aktivnih krema dovela do značajnog sniženja vrednosti TEWL. U obe faze studije zabeležen je porast EC, naročito nakon primene krema koji sadrži skvalen. **Zaključak**. Uzevši u obzir odsustvo nadraženosti kože i narušavanja kožne barijere, kao i porast hidratacije površinskog sloja kože, može se reći da se ispitivane aktivne supstance ubačene u kreme stabilizovane alkil-poliglukozidnim emulgatorom mogu bezbedno koristiti kao komponente tzv. „skrojenih" kozmetičkih ovlaživača namenjenih za negu zdrave i suve kože, dok se skvalen maslinovog ulja može koristiti i za negu iritirane i osetljive kože.

**Ključne reči:**
**ekstrakti, biljni; ćelije, matične; kozmetička sredstva; koža; skvalen; emulzije; koža, iritacija, testovi.**

## Introduction

Moisturizers play an important role in healthy skin care, as well as in treatment of dermatoses accompanied with dry skin. In addition to moisturizing and preventing dryness, they may also influence the structure and barrier function of both healthy and diseased skin [1, 2]. With long-term daily use, depending on their composition, moisturizers may either improve or deteriorate skin barrier function, and consequently increase penetration of ingredients in/through the skin and cause (further) skin dryness and irritation [1–3]. Therefore, with the appropriate formulation and ingredient selection it is possible to achieve the preferred effect of topical preparations on the structure and barrier function of healthy or diseased skin in terms of skin hydration, while also repairing the skin barrier and reducing the signs of its irritation.

Plant stem cells, though interesting, are not sufficiently investigated as cosmetic actives. They represent extracts of the whole cell culture containing all the important compounds–epigenetic factors and secondary metabolites significant for protection and maintaining of these factors [4]. According to the manufacturer, stem cells from the Alpine Rose, *Rhododendron ferrugineum*, possess the ability to increase the vitality of epidermal stem cells and protect them against UV-induced stress, due to the content of various polyphenols and proteins called dehydrins. Furthermore, Alpine Rose stem cells (ARSC) are claimed to protect the skin barrier, supposedly by making it more resistant to the combination of strong environmental stress factors (UV, cold, wind) during the winter season. Finally, the anti-wrinkle effect has also been attributed to ARSC [5].

Squalene is naturally present in the sebum and it is one of the main constituents of skin surface polyunsaturated lipids. The most abundant source of squalene is the shark liver oil, although nowadays, as a cosmetic active, squalene is exclusively obtained from plant-natural sources (*eg* olive oil). Squalene is used in cosmetics for its emollient, moisturizing and anti-irritant effects, as well as for the repair of skin barrier function. Also, it has been reported that squalene protects skin surface from lipid peroxidation caused by UV light and acts as a highly efficient quencher of singlet oxygen, which explains its antioxidant properties [6, 7].

Although the proper selection of emollients and active ingredients certainly is the most relevant step towards assuring the desired effect of moisturizing creams, the importance of the used emulsifier system should not be neglected. The applied emulsifier together with the oil phase (emollients) defines the system's structure, and, consequently, the cream's behaviour during and after the application, as well as its effect on the skin [8]. Additionally, certain safety aspects are based on the final choice of the emulsifier system [1, 2].

Alkyl polyglucosides (APGs) are non-ionic, polyethylene glycol-free surfactants, derived from natural, renewable sources. APGs show favorable dermatological properties, *ie* they are mild to the skin and therefore considered as skin friendly. Furthermore, they are highly valued for their biodegradability, and hence frequently labelled as environment friendly emulsifiers. The specific chemical structure, water holding capacity and ability to form certain lamellar structures similar to the *stratum corneum* (SC) structural organization, makes them interesting cosmetic and pharmaceutical raw materials [8–10]. A novel APG-mixed emulsifier, hydroxystearyl alcohol and hydroxystearyl glucoside (INCI), has improved sensory properties and due to the additional diol structure in hydrophilic sugar unit, provides significant, continuous hydration of the upper layers of the skin [11]. To the best of our knowledge, there is insufficient data available on this natural APG-mixed emulsifier as a potential stabilizer of moisturizing creams.

In accordance with the aforementioned, the aim of this study was to investigate *in vivo* skin irritation as a certain aspect of safety, and *in vivo* skin efficacy of Alp Rose stem cells incorporated into liposomes, olive oil squalene and a novel natural APG-mixed emulsifier, as ingredients of moisturizing creams. Efficacy was evaluated on both normal and experimentally irritated skin.

With that aim, we conducted the two phase study: the first phase was performed so as to assess the irritation potential of the samples after a 24-h occlusion, as well as the long-term effects of the samples' daily use in healthy skin care, while the second phase was carried out to evaluate the efficacy of the samples in the treatment of sodium lauryl sulfate (SLS) – irritated skin.

## Methods

### *Subjects*

The study was performed in accordance with the Declaration of Helsinki after obtaining written informed consent from the volunteers and permission from the Ethical Committee of the Faculty of Pharmacy, University of Belgrade, Serbia (the approval number: 1583/1). All measurements in this study were carried out in accordance with the relevant guidelines [12–15].

A panel of 76 healthy female volunteers without the history or clinical signs of dermatological disease and with

normal to moderate dry skin participated in the study. The type of the skin (normal to moderate dry skin) was evaluated thanks to the basal values of the measured parameters, primarily on the basis of transepidermal water loss (TEWL) values (TEWL values were lower than 12 g/m$^2$h) [1, 16].

The volunteers were instructed not to use any skin care products on their arms a week before and throughout the study, but were allowed to wash normally during the study.

According to the published guidelines, measurements were carried out under controlled conditions: temperature (21 ± 1°C) and relative humidity (40 ± 5%), after a 30-min acclimatization period of the participants [16].

*Materials*

A natural emulsifier of APG type, INCI (Simulgreen™ 18-2) was kindly provided by Seppic, France. Olive oil squalene with 96% purity (Olifeel® SQ) was kindly provided by Amedeo Brasca & C. SRL, Italy, and stem cells of Alpine Rose leaves encapsulated in liposomes (PhytoCellTec™ Alp Rose) were kindly provided by Mibelle AG Biochemistry, Switzerland. Olifeel® SQ is clear oily liquid with slight odour, yellow color and high purity which prevents degradation and coloring. According to the producer statement, it can be easily incorporated in all types of cosmetic products. PhytoCellTec™ Alp Rose is powder with specific odor, dissolvable up to 20% in water, which can be incorporated in all advanced "stem cell cosmetic" formulas, face and body care products and every weather formulations [5].

Other components were: caprylic/capric triglyceride (Levate, Italy), *Prunus amygdalus dulcis* (Sweet Almond) seed oil, isopropyl myristate (A&A Fratelli Parodi, Italy) and mineral oil (RA.M. Oil S.p.A., Italy). All samples were suitably preserved and also contained tocopheryl acetate (BASF, Germany) as an antioxidant, glycerin (BASF, Germany) as a humectant and Xanthan gum (Gum Technology, Arizona) as an additional stabilizer.

*Test samples*

In order to determine the optimal formulation, the number of oil in water (O/W) cream samples were prepared containing a multi-component oil phase, fixed emulsifier content (5%) and different types of the additional stabilizers applied. Also, in order to decrease the number of potential confounding factors, cream samples were formulated as simply as possible. Along with the placebo samples (creams without the active substances), the active samples were prepared accordingly.

Based on the conducted preliminary physical stability evaluation, the placebo sample F1p, the active sample F1a (cream with ARSC incorporated into liposomes, 0.4% w/w, since the recommended use level for this active is 0.4–1% w/w) and the active sample F1s (cream with olive oil squalene, 1% w/w) were singled out and prepared for the subsequent *in vivo* investigation.

The fourth sample (Fc) was a commercial cream from the market with ARSC incorporated into liposomes (the same concentration and the same manufacturer of the active), and the following composition: ingredients (INCI)/Aqua, propylheptyl caprylate, coco caprylate, dicaprylyl carbonate, hydrogenated vegetable glycerides, avocado oil, pentaerythrityl distearate, *Butyrospermum parkii*, ethylhexyl methoxycinnamate, diglycerine, imperata cylindrica root extract, glycerine, polyethylene glycol (PEG)-8, carbomer, tri-ceteareth-4 phosphate, ammonium acryloyldimethyl taurate/VP copolymer, phenoxyethanol, methylparaben, butylparaben, isobutylparaben, ethylparaben, propylparaben, tocopheryl acetate, butyl methoxydibenzoylmethane, parfum, polysorbate-20, *Rhododendron ferrugineum* leaf cell culture extract, isomalt, lecithin, sodium benzoate, lactic acid, tocopherol, ascorbyl palmitate, ascorbic acid, citric acid, disodium ethylenediaminetetraacetic (EDTA), linalool, limonene.

In the second phase of the study two new O/W cream samples were introduced instead of the commercial cream: the cream sample F1a1s containing the combination of ARSC (0.4% w/w) and 1% of olive oil squalene, and the cream sample F1a6s containing the same concentration of ARSC but 6% of squalene. The composition of all the prepared samples is given in Table 1.

*Preparation of the formulations*

The oil phase was heated in a closed vessel on the heating plate of a magnetic stirrer (IKA Combimag RCH, Germany) to 70°C. The aqueous phase containing glycerin

**Table 1**

**Composition of the investigated samples**

| Ingredients/INCI | Sample*, % (w/w) | | | | |
|---|---|---|---|---|---|
| | F1p | F1a | F1s | F1a1s | F1a6s |
| A (Oil phase) | | | | | |
| caprylic/capric triglyceride | 7.0 | 7.0 | 7.0 | 7.0 | 5.0 |
| mineral oil | 3.5 | 3.5 | 3.5 | 3.5 | 2.5 |
| isopropyl myristate | 4.5 | 4.5 | 4.5 | 4.5 | 3.25 |
| *Prunus amygdalus dulcis* (sweet almond) seed oil | 3.0 | 3.0 | 3.0 | 3.0 | 2.25 |
| squalene (Olifeel® SQ) | | | 1.0 | 1.0 | 6.0 |
| B (Water phase) | | | | | |
| INCI (SimulgreenTM 18–2) | 5.0 | 5.0 | 5.0 | 5.0 | 5.0 |
| glycerin | 2.0 | 2.0 | 2.0 | 2.0 | 2.0 |
| water | ad 100.0 | ad 100.0 | ad 100.0 | ad 100.0 | ad 100.0 |
| C | | | | | |
| xanthan gum | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |
| tocopheryl acetate | 0.3 | 0.3 | 0.3 | 0.3 | 0.3 |
| *Rhododendron ferrugineum* leaf cell culture extract (and) Isomalt (and) lecithin (and) sodium benzoate (and) lactic acid (and) aqua (PhytoCellTec™ Alp rose) | | 0.4 | | 0.4 | 0.4 |
| Preservatives blend | 0.5 | 0.5 | 0.5 | 0.5 | 0.5 |

**INCI – Hydroxystearyl alcohol and Hydroxystearyl glucoside; Alp – alpine.**
***For explanation see Methods (*Test samples*).**

was heated in a closed vessel to 75°C, when the emulsifier was added while stirring (propeller laboratory stirrer, Heidolph Instruments GmbH & Co, Kelheim, Germany) for 1 min at 1,500 rpm. At the same temperature, the oil phase was added to the aqueous phase followed by stirring at 1,500 rpm for 5 min. The cream was being gradually cooled for 3 min at 1,050 rpm, at 725 rpm for 5 min and further at 1,050 rpm to room temperature. Xanthan gum was added in the cooling phase of the cream at the temperature below 60°C, while the preservatives blend and tocopheryl acetate were incorporated at below 40°C. Alp Rose stem cells, previously dispersed in 5 g of water (this was taken into account after having determined the needed amount of the aqueous phase), were carefully added during the cooling phase of the cream (at a temperature below 60°C), after adding of the stabilizer.

The samples containing squalene did not change their appearance (namely color or smell) throughout the temperature stress tests, after having been stored at room temperature or when submitted to the application during the study, which indicated the absence of oxidation products of squalene [*eg* squalene monohydroperoxide (SQOOH), as a main photo-oxidation product and a primary UV oxidized lipid produced from squalene] [6]. Temperature stress tests were performed by 24-h preservation of samples at three different temperature conditions (4°C, room temperature and 40°C), during 6 cycles (18 days).

*Experimental design*

The study was randomized, double-blind and organized in two phases.

In the first phase of the study, 52 volunteers were randomly divided into two groups.

The first group of 16 volunteers (mean age 21.8 ± 3.6) participated in the evaluation of irritation potential of cream samples under a 24-h occlusion. Samples were applied on the volar aspects of the forearms using a precisely marked cardboard ruler with three empty rectangular spaces (each measuring 9 $cm^2$, 3 cm × 3 cm). Two samples *per* arm were applied, and a rectangle next to the wrist was left as an untreated control (UC) on each forearm. The untreated control on the left arm was occluded (untreated control occluded – UCO). After samples application, the treated sites were immediately covered with Parafilm® (Pechiney Plastic Packaging, Inc., Menasha, Wisconsin, USA) and cotton adhesive tape Sensifix® (Belgrade, Serbia). In this phase of the study the following parameters were measured: electrical capacitance (EC), TEWL and erythema index (EI), before samples application (baseline values) and 3 h following the occlusion removal.

The second group of 36 volunteers (mean age 20.5 ± 0.5) participated in the evaluation of the efficacy of the same samples during their long-term application on the healthy skin. The samples were applied on the volar aspects of forearms using the same cardboard ruler and in the same, previously described manner. The rectangle closest to the wrist on each forearm served as an untreated control. The samples were distinguished solely by the color of the packaging. The volunteers received clear instructions regarding the sample amount and application manner. They applied samples to the specific test sites twice a day (in the morning and in the evening). In this part of the study EC, TEWL and skin elasticity were monitored. The measurements were conducted before the application of the samples commenced (baseline values), after a 14-day and subsequently after a 3-week treatment. The volunteers were instructed to skip the application the morning before the measurement.

In the second phase of the study, 24 volunteers (mean age 29.9 ± 8.9) participated in the evaluation of the efficacy of the samples in the treatment of SLS-irritated skin. The experimental irritation with SLS under a 6-h occlusion was performed in accordance with the published guidelines [17] and in the previously reported manner [18]. A total of 100 μL of 10% aqueous solution of SLS (purity 99%, Merck, Germany) was placed on each of the six filter papers. The filter papers were subsequently placed on the test sites of the volar aspects of forearms using a cardboard ruler with four rectangular empty spaces in the following manner: three rectangular spaces from the left wrist up and three rectangular spaces from the right elbow down were covered with filter papers. Then, the filter papers were immediately covered with a Parafilm® and cotton adhesive tape Sensifix®. The test site next to the left wrist represented the induced UCO, and the rectangular empty space next to the right wrist was covered without induced irritation and served as an UC. The volunteers removed Sensifix®, Parafilm® and the filter papers after 6 h. After irritation, a 6-day treatment of the tested sites was conducted. Five samples were applied, two samples to the left and three samples to the right hand, twice a day (in the morning and in the evening). In this phase of the study the following cream samples were investigated: F1p, F1a, F1s, and instead of the commercial cream (Fc), two new samples were introduced (F1a1s and F1a6s). In this part of the study the following parameters were measured: EC, TEWL and EI, before the irritation test ("primary" baseline values), 24 h after the occlusion removal ("secondary" baseline values) and after 6 days of the treatment. The last sample application was performed 12 h prior to measurements.

Corneometer® CM825 was used for skin measurement EC as an indicator of skin hydration. TEWL was measured using Tewameter® TM210, EI using Mexameter® MX18 and the skin elasticity using Cutometer® MPA580 (all devices manufactured by Courage + Khazaka Electronic GmbH, Germany).

*Statistical analysis*

All data were presented as mean ± standard error of the mean (SEM). Data from different sites (treated with different samples including both controls) at different time points were analyzed using one-way ANOVA, followed by Tukey's *t*-test where appropriate. The differences were accepted as statistically significant at $p < 0.05$. Statistical analysis was performed with commercial statistical software package SPSS for Windows 17.0.

**Results**

In the first phase, as well as in the second one, all the volunteers completed the study and reported strict compliance with the given instructions.

*Phase I*

In this phase of the study, the following cream samples were tested: placebo cream (F1p), active cream containing 0.4% of ARSC (F1a), cream with 1% of squalene (F1s) and commercial cream (Fc) containing the same concentration of ARSC as the cream sample F1a.

*In vivo* evaluation of irritation potential

After the 24-h occlusion, there was no statistically significant change in EI for any tested cream sample, as well as the untreated controls. TEWL was significantly decreased for the cream samples F1p and F1a compared to the baseline. Also, EC was significantly increased for all active creams (F1a, F1s and Fc) compared to the baseline values.

The *in vivo* measured parameters (EC, TEWL and EI) were expressed as absolute changes (Δ values) compared to the baseline (Figure 1).

*In vivo* efficacy on normal skin (the long-term 21-day study)

The results of the long-term 21-day study for EC and TEWL are shown as relative percentage change compared to untreated control (Figures 2 and 3), while Table 2 shows the calculated parameters for viscoelasticity of the skin (R2, R5 and R7) as mean values ± SD.

In this part of the study, following positive testing for normality, parametric tests were used. Data involving values of the parameters measured in the forearm areas treated by different samples (F1p through F1s) at distinct time points, was analyzed by the one-way within-subjects (repeated measures) ANOVA, followed by Tukey's *t*-test, where appropriate. Differences between the values of the parameters obtai-

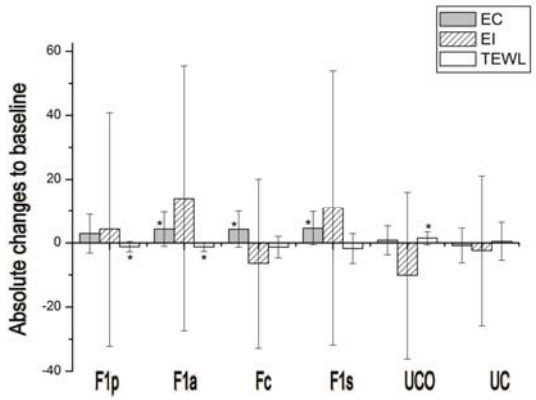


**Fig. 1 – *In vivo* irritation potential of the investigated samples† F1p, F1a, Fc, F1s *vs* the untreated controls – UC: without occlusion (UC) and occluded (UCO). The data from different sites was analyzed using one-way ANOVA, followed by Tukey's test where appropriate. Electrical capitance (EC), erythema index (EI) and transepidermal water loss (TEWL) for the same sample at various time points were compared using paired sample *t*-test (significant changes marked with*).**
**†For explanation see. Methods (*Test samples*).**

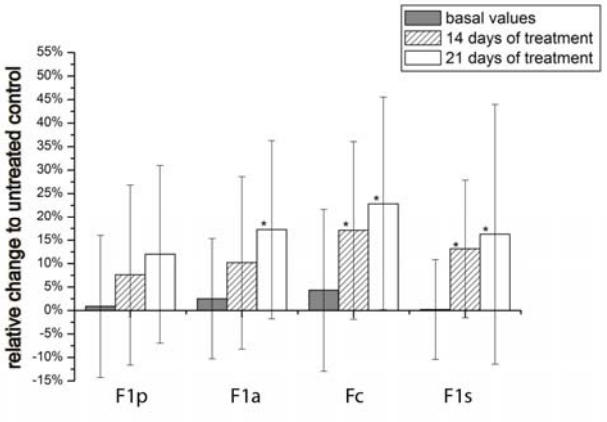


**Fig. 2 – The effect of the topical application of the samples† F1p, F1a, Fc and F1s on electrical capitance (EC), related to the untreated control (percentage change). Data involving the values of the parameters measured on the forearm skin areas treated by different samples was analyzed by the one-way within-subjects (repeated measures) ANOVA, followed by Tukey's *t*-test, where appropriate; differences between the values of the parameters obtained for the cream-treated skin and the corresponding untreated control were checked by Student's unpaired *t*-test; all significant differences ($p < 0.05$) being marked with (*).**
**†For explanation see Methods (*Test samples*).**

**Table 2**

**The effect of the investigated samples on selected viscoelastic parameters of the skin (the control values are also given)**

| Samples* | R2 | R5 | R7 | R2† | R5† | R7† |
|---|---|---|---|---|---|---|
| F1p | 0.843 ± 0.036 | 0.995 ± 0.101 | 0.649 ± 0.057 | 0.833 ± 0.041 | 0.950 ± 0.097 | 0.621 ± 0.056 |
| F1a | 0.828 ± 0.026 | 0.921 ± 0.078 | 0.606 ± 0.039 | 0.847 ± 0.033 | 0.955 ± 0.077 | 0.632 ± 0.046 |
| UC1 | 0.813 ± 0.041 | 0.847 ± 0.081 | 0.576 ± 0.051 | 0.806 ± 0.061 | 0.824 ± 0.094 | 0.563 ± 0.072 |
| Fc | 0.842 ± 0.044 | 0.972 ± 0.071 | 0.641 ± 0.055 | 0.856 ± 0.037 | 0.996 ± 0.093 | 0.650 ± 0.052 |
| F1s | 0.828 ± 0.030 | 0.924 ± 0.071 | 0.616 ± 0.041 | 0.820 ± 0.036 | 0.909 ± 0.065 | 0.599 ± 0.036 |
| UC | 0.808 ± 0.057 | 0.846 ± 0.092 | 0.601 ± 0.064 | 0.812 ± 0.042 | 0.831 ± 0.076 | 0.611 ± 0.050 |

**The results are shown as mean values ± standard errors measured initially (basal values) and after a 21-days application (†).**
**UC – untreated controls.**
***For explanation see Methods (*Test samples*).**
**†R2 – the gross – elasticity of the skin including the viscous deformation, and is represented by the ratio of the ability of redeformation of skin to final distension; R5 – the biological elasticity (the portion of elasticity compared to the final distension).**

ned for the cream-treated skin and the corresponding untreated control were checked by Student's unpaired *t*-test.

After 14 days of application, although all tested creams exhibited a rising trend, skin hydration (measured as the EC), it was significantly changed only for the creams F1s and Fc compared to the untreated control, whereas after 21 days the increase was significant for all active creams (F1a, F1s and Fc) (Figure 2).

The results for TEWL (Figure 3) showed that after 21 days of treatment, the TEWL values significantly decreased for the cream F1s compared to all the other tested samples. The investigated creams F1a and Fc did not alter TEWL significantly at any time point, whereas the cream F1p significantly increased TEWL after both 14 days and 21 days of application.

*Phase II*

For the second phase of the study, the following samples were selected for further investigation: placebo cream (F1p), active cream (F1a) with 0.4% of ARSC and the cream with 1% of squalene (F1s). In order to evaluate the joint effects of the investigated actives on previously SLS-irritated skin, as well as the concentration dependent impact of squalene on the tested skin parameters, two new samples were included: active cream with 1% of squalene (F1a1s) and the active cream with 6% of squalene (F1a6s) – both containing 0.4% of ARSC.

*In vivo* efficacy on SLS-irritated skin

The measured parameters were expressed as absolute changes as compared to baseline (Δ values) and presented in Figures 4–6. Data from different sites (treated with different samples and the controls) at different time points were analyzed using one-way ANOVA, followed by Tukey's *t*-test where appropriate. The values of the measured parameters after the irritation as well as after six days of the samples' application were compared to both the baseline values and between one another using paired sample *t*-test. The differences were accepted as statistically significant at $p < 0.05$.

After a 6-day treatment of the SLS-irritated skin, a significant decrease in TEWL for all tested creams (F1p, F1a, F1a6s, F1s and F1a1s) was seen (Figure 4) when compared to the values after irritation ("secondary" baseline values), whereas the creams F1a6s and F1s also significantly decreased TEWL related to the "primary" baseline values.

As regards skin hydration, measured as EC, after a 6-day treatment, the creams F1p, F1a6s and F1s significantly increased EC related to the values measured after the irritation ("secondary" baseline values) (Figure 5). A trend of EC increase, though insignificantly, could be noticed for the tested creams F1a and F1a1s (Figure 5).

Furthermore, after 6 days of application, there was no significant change in the EI for the creams F1p and F1s, whereas the active creams F1a, F1a6s and F1a1s significantly increased EI when compared to the "primary" baseline values (Figure 6).

**Discussion**

In the initial phase of the study, *in vivo* evaluation of the irritation potential and efficacy during the long-term application of the investigated creams was performed on healthy skin with two groups of volunteers.

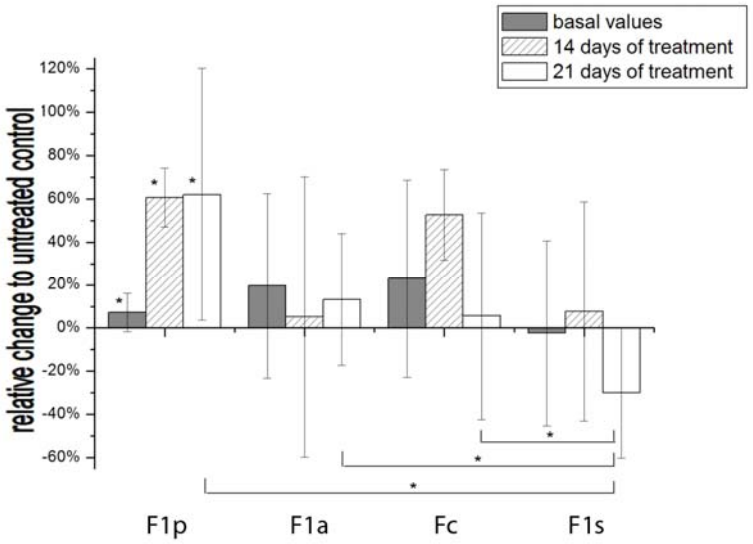


**Fig. 3 – The effect of the topical application of the samples† F1p, F1a, Fc and F1s on transepidermal water loss (TEWL), related to the untreated control (percentage change). Data involving the values of the parameters measured on the forearm areas treated by different samples was analyzed by the one-way within-subjects (repeated measures) ANOVA, followed by Tukey's *t*-test, where appropriate; differences between the values of the parameters obtained for the cream-treated skin and the corresponding untreated control were checked by Student's unpaired *t*-test; all significant differences ($p < 0.05$) being marked with (*).**
**†For explanation see Methods (*Test samples*).**

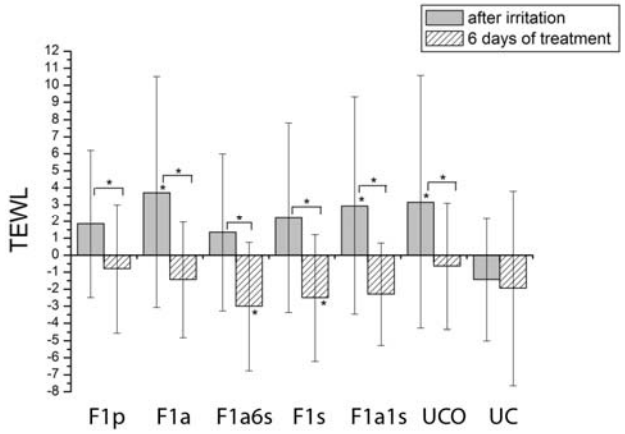


**Fig. 4 – The effects of the irritation and subsequent topical application of the samples† F1p, F1a, F1a6s, F1s and F1a1s to irritated skin on transepidermal water loss (TEWL) related to the "primary" baseline (absolute changes); both controls are also included occluded (UCO) and without occlusion (UC).**

**The values of the parameters after the irritation ("secondary" baseline values) as well as after six days of the samples' application were compared to the "primary" baseline values and between one another using paired sample *t*-test, significant differences ($p < 0.05$) being marked with (*).**

**†For explanation see Methods (*Test samples*).**

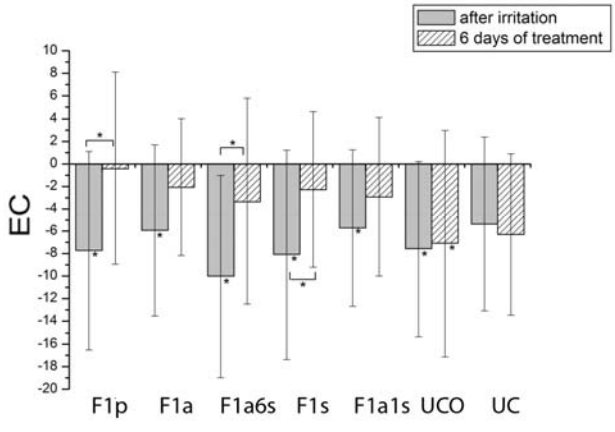


**Fig. 5 – The effects of the irritation and subsequent topical application of the samples† F1p, F1a, F1a6s, F1s and F1a1s to irritated skin on EC related to the "primary" baseline (absolute changes); both controls are also included occluded (UCO) and without occlusion (UC).**

**The values of the parameters after the irritation ("secondary" baseline values) as well as after six days of samples' application were compared to the "primary" baseline values and between one another using paired sample *t*-test, significant differences ($p < 0.05$) being marked with (*).**

**†For explanation see Methods (*Test samples*).**

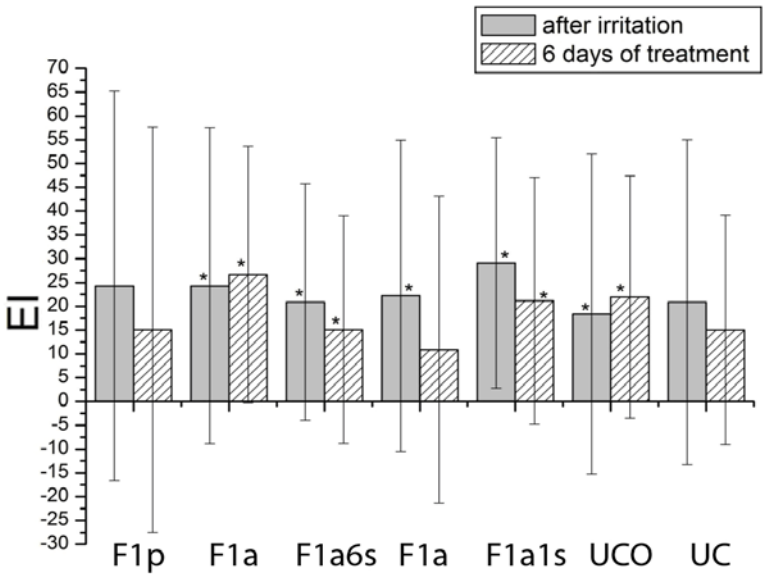


**Fig. 6 – The effects of the irritation and subsequent topical application of the samples† F1p, F1a, F1a6s, F1s and F1a1s to irritated skin on EI related to the "primary" baseline (absolute changes); both controls are also included occluded (UCO) and without occlusion (UC).**

**The values of the parameters after the irritation ("secondary" baseline values) as well as after six days of the samples' application were compared to the "primary" baseline values and between one another using paired sample *t*-test, significant differences ($p < 0.05$) being marked with (*).**

**†For explanation see Methods (*Test samples*).**

The evaluation of the irritation potential, as an important safety aspect of cream samples, was conducted under a 24-h occlusion on the first group of volunteers. In order to investigate the irritation potential, EI was monitored. Additionally, the potential skin barrier impairment was assessed *via* TEWL and EC of the skin.

It is known that occlusion itself induces barrier damage without skin dryness, increases penetration of some ingredients from topically applied products and may cause irritation [19, 20]. For that reason, to assess the overall irritation potential, as well as the potential skin barrier impairment of the tested samples, it was necessary to eliminate the influence of occlu-

sion. Hence, final measurement of the monitored parameters was performed 3 h after occlusion removal, when the effects of occlusion had subsided [19].

It was shown (Figure 1) that the EI, as an indicator of the skin irritation *via* the measurement of skin redness [14], was not significantly changed compared to the baseline values for any cream sample. Also, for the cream Fc and UCO, an insignificant reduction of the EI related to the baseline could be noticed. The rationale for this is probably a prolonged (24-h) occlusion and maceration of the skin caused by the increased water binding of SC (hyper-hydration of SC) [21, 22].

As irritants application is often accompanied by the increase of TEWL – a marker of skin barrier function and its structural changes – this measurement is commonly used in safety evaluation studies of cosmetic products [2, 3, 20]. Although, in our study, TEWL showed a falling trend, these results may be considered statistically significant only for the placebo cream F1p and the active sample F1a. Since there was no significant change in the TEWL values on the site reserved for UC, and due to the fact that this parameter was significantly increased 3 h after the 24-h occlusion on the site reserved for UCO, it could be assumed that the obtained results are caused by the effect of the investigated active (ARSC incorporated into liposomes). On the other hand, if one compares TEWL values for the placebo F1p and the active creams F1a and Fc, both containing the same active (the same concentration and manufacturer of the active), it could be speculated that these results can be attributed not only to the carrier itself (the cream composition given in Table 1) but also to the used emulsifier. The rational for the obtained TEWL results is probably the similarity of the liquid crystalline structure of the investigated samples (unpublished data) stabilized with the used emulsifier to the SC structural organization. This assumption is consistent with the already reported results concerning skin mildness and favourable dermatological properties of this type of emulsifiers [8–10] and the investigated emulsifier, as well [11].

As concerns the hydration of the skin (EC), it was increased in all the treated sites, however, only for the placebo F1p without statistical significance. Although occlusion can significantly increase SC hydration by blocking water loss from the skin surface [20], the obtained results cannot be attributed to the occlusion. Since there was no significant change in the EC for UCO and the skin hydration on the site reserved for UC was decreased (although without statistical significance), the obtained results can be attributed to the effect of the investigated actives, as well as the carrier and the used emulsifier, which is mainly responsible for the carrier's specific microstructure.

According to these results no alteration of the skin barrier function occurred.

The obtained results from this phase of the study generally indicated that the investigated cream samples had not irritated the skin. The absence of erythema and/or any impairment of the skin barrier function in a 24-h occlusion study may preliminarily imply the satisfying safety profile of the cream samples, as well as of the investigated cosmetic actives and the used emulsifier.

In order to evaluate the efficacy of the investigated samples, a long-term 21-day study was performed. After the initial baseline measurements, the second group of volunteers with normal/healthy skin was applying the tested samples twice a day following the written procedure. The first control measurements were performed after a 14-day treatment, and the final measurements were conducted a week afterwards.

As, it is well-known that the short-term application of moisturizers may increase hydration of the skin [1–3], EC measurements were conducted to investigate the potential of a long-term (21 days) use of the tested creams to hydrate the skin effectively. After 21 days of application, all tested active creams as well as the placebo sample F1p (although without significance) increased the skin hydration level compared to UC. It should be emphasized that the creams F1s and Fc, effectively changed the EC (a significant increase) as soon as after 14 days of application.

If one compares the active cream F1a and the commercial one Fc, both containing the same concentration of the active (ARSC incorporated into liposomes) but quite a different carrier, it could be assumed that the contribution of the investigated active to the moisturizing effect of the creams is as important as it was expected. Also, a similar assumption could be made by comparing the tested active cream F1a and the corresponding placebo F1p, both containing the same carrier. However, neither the exact mechanism of the moisturizing effect of ARSC leaves encapsulated in liposomes (PhytoCellTec™ Alp Rose) is fully understood, nor their ability to eventually penetrate the SC after the application to healthy skin. Bearing in mind that the investigated active is an extract of the whole cell culture incorporated into liposomes, it could be speculated that the observed hydration effect is probably attributed to some ingredients with water binding capacity which act like humectants, or to phospholipid-based liposomes (physiological lipids) themselves [23]. In future research, it would be interesting to investigate the mechanism of the observed hydration effect of ARSC incorporated into liposomes and their penetration ability, as well.

Secondly, according to the obtained results, the cream containing 1% of olive oil squalene, F1s, increased the EC significantly after 14 days, as well as after 21 days of application. Thus, if one considers the EC values for the placebo F1p and the cream F1s, it could be said that 1% of squalene significantly contributes to the cream hydration efficacy. Although, squalene is known as a good moisturizing agent [6], the obtained results indicate the possible achievement of effective skin hydration even at a low concentration of this active. On the other hand, there existed a rising trend of the EC compared to UC for placebo F1p, although without significance, and the lack of statistically significant difference in the EC values (skin hydration) between the placebo cream F1p and its counterparts – the active samples F1a and F1s. Since these samples are based on the same carrier system, the recorded results may indicate that for effective skin hydration, the carrier itself is beneficial and therefore, probably, the used emulsifier as well. This assumption is consistent with the previously reported results concerning the older generation of APG emulsifiers [8, 10, 24, 25], but also the emulsifier used in our study (INCI Simulgreen™ 18–2) [11].

It is well-known that due to their specific structure, water binding capacity and the formation of lamellar phases

APG emulsifiers can provide additional skin moisturization [8, 10, 24, 25]. It has recently been reported that creams stabilized with the used novel APG emulsifier may be attributed with even stronger water binding capacity in comparison with the previous generation of APG emulsifiers. Furthermore, water binding capacity and the formation of liquid crystals around oil droplets in creams stabilized with aforementioned emulsifier, depends on the nature of the used oil; thus the selection of some non-polar oils (*eg*, vegetable squalane) leads to the visible formation of these structures [11]. Since squalane is a saturated non-polar derivative of squalene, it could be assumed that squalene may also have certain impact on the specific colloidal structure of the investigated cream F1s. Squalene may contribute to both the formation of the lamellar phase in the tested cream sample and additional skin moisturization. Within the investigated cream samples, especially those containing squalene, anisotropic lamellar structure was recorded by polarization microscopy (unpublished data), performed during the formulation study and the preliminary physical stability investigation of the tested samples.

In the case of the impact of a long-term (21 days) use of the tested cream samples on the skin barrier, the TEWL measurement was conducted, as the most common method of evaluation of the skin barrier function [2]. The obtained values showed that after 21 days of application, the cream F1s significantly decreased TEWL compared to all other tested creams. Having compared the placebo F1p which significantly increased TEWL after 14 and 21 days of application and the cream F1s, it seems reasonable to attribute a favourable effect of the active sample to its cosmetic active substance (squalene). Taking into account that TEWL measurements are generally used for screening and objective, non-invasive perceiving of actives that may have a positive effect on the skin barrier function [13], it could be assumed that olive oil squalene may even improve the skin barrier function [2] after long-term application on healthy/normal skin. Furthermore, our findings imply that even a relatively low concentration of squalene (1%) significantly contributes to the moisturizing efficacy of the tested cosmetic cream (F1s).

The remaining tested creams F1a and Fc, both containing the same active (ARSC incorporated into liposomes) but a different carrier, did not alter TEWL significantly at any time point. The obtained results are consistent with the already reported, and indicate that repeated application of moisturizers on normal/healthy skin may increase skin hydration without affecting TEWL [1]. Therefore, it could be summarized that a 21-day application of the tested active creams, as well as of the investigated active substances (olive oil squalene and PhytoCellTec™ Alp Rose) have not resulted in the impairment of skin barrier function.

On the other hand, the obtained TEWL values for the placebo sample F1p are somewhat confusing. The TEWL values for this sample were elevated after 14, as well as after 21 days of application, suggesting a possible negative effect of the placebo sample on the skin barrier function during a long-term use. However, considering that TEWL did not increase in the case of samples with the same carrier (F1a and F1s), and due to the fact that the basal values on the particular site intended for the application of the sample had already been elevated (compared to the UC) with most volunteers, the obtained placebo affected TEWL findings could not be attributed to the carrier itself. Furthermore, it has been reported that TEWL on the dominant forearm might be significantly higher than on the non-dominant one, and that different sites on the same anatomical position might have significantly different TEWL values [26]. This assumption is consistent with our previously obtained results (the lack of impairment of the skin barrier function) in a 24-h occlusion study for the same sample – placebo F1p.

To evaluate further the impact of the 21-day application of the investigated creams on biomechanical characteristics of the skin, the measurement of skin viscoelastic properties was conducted, as well. It is widely acknowledged that the ageing process alters structural and mechanical properties of the skin through changes of the elastic and collagen fibers. As ageing is accompanied by a decrease of skin elasticity which begins in the early twenties, this measurement could be useful for the study of the influence of the investigated samples on biomechanical characteristics of the skin. Viscoelasticity of the skin was assessed with a calibrated Cutometer® MPA580, suction-based instrument equipped with a 2-mm-diameter probe. Applying the following settings: 400 mbar suction pressure, suction time 2 s, relaxation time 2 s, 3 repetitions, the skin deformation curve (a deformation *vs* time curve) was obtained. This curve offers two types of parameters: directly measured from the curve so-called *U*-parameters (total deformation recovery *Ua*, total extensibility *Uf*, immediate elastic deformation *Ue* and immediate elastic recovery *Ur*, which are the most commonly used *U*-parameters) and calculated so-called R parameters (R0 to R9 parameters). R-parameters were calculated using the Software Cutometer® MPA580, but for the evaluation of viscoelastic properties of the skin only a few were chosen: R2 (Ua/Uf), R5 (Ur/Ue) and R7 (Ur/Uf). The parameter R2 (gross elasticity) reflects the overall elasticity of the skin, R5 (net elasticity) reflects only the elastic component of the viscoelastic response of the skin (it is affected solely by the elastic fibers of the skin), while the parameter R7 represents the elastic recovery ratio and it can be affected by changes of elasticity and viscosity of the skin. These parameters were calculated as those unaffected by skin thickness of the volunteers and the experimental conditions of the study. Moreover, they were the most useful for this type of the study, especially the parameter R7 which is most closely related to the skin elasticity and has profound meaning for measuring skin ageing by evaluating its elasticity [27]. As it could be seen (Table 2) after 21 days of application, there was no significant change in the selected parameters. Hence, the obtained results indicate that the investigated creams do not have an impact on skin elasticity after a 21-day application to the skin of volunteers in their early twenties. This findings may suggest a few possible implications: selection of a proper group of human volunteers is of paramount significance to detect possible effects, if any, of the treatment that could tentatively be define as anti-age skin improvement; claims

indicating anti-age plant stem cells effect have to be supported by a set of adequate objective and subjective techniques/methods of evaluation; proper concentration range and the type of the vehicle/carrier system adjusted for the given active is always the most important task for a formulator attempting to achieve both a satisfying efficacy and an acceptable safety profile of a skin care product.

Overall, upon consideration of the obtained results from the first phase of our study, it was shown that the tested creams stabilized with a novel APG emulsifier have satisfying safety profiles, either with or without the incorporated active substances (1% of olive oil squalene (Olifeel® SQ) and 0.4% of ARSC leaves encapsulated in liposomes (PhytoCellTec™ Alp Rose)). In addition to this, after a long-term use (21 days) on healthy skin, these creams manage to increase SC hydration without compromising the skin barrier function. Finally, this study stage proved that the investigated actives, especially olive oil squalene, are efficient, indicating that they could be appropriate actives for cosmetic moisturizers intended for healthy skin care.

It is widely acknowledged that the application of the effective moisturizers – those with a proper composition and with adequate cosmetic actives in optimal contents, could have a positive impact on the renewal of the (SLS)-irritated skin [1, 11, 24], *ie* it may have a general favourable effect on the recovery of irritated skin. So, as to investigate the potential of the tested creams further, in the second phase of the study the samples were evaluated upon their application to the previously irritated skin, again employing non-invasive measurements of the predetermined skin parameters throughout the study. To perform this, an *in vivo* skin irritation test with 10% SLS aqueous solution under a 6-h occlusion was performed in the following manner. Initial basal values were taken before ("primary" basal values) and after ("secondary" basal values) the SLS solution was applied and the occlusion was placed on the investigated skin sites. In order to eliminate the influence of occlusion itself and determine the effects of SLS, the "secondary" basal values were measured 24 h after the irritation test and the occlusion removal [19]. TEWL values (in more than 50% of volunteers TEWL greater than 12 $g/m^2h^1$ was recorded), confirmed that skin dryness was successfully induced [16]. Final measurement was conducted after 6 days of treatment with the tested creams.

Non-invasive skin biophysical measurements are widely used in the assessment of skin irritation elicited with SLS as a model irritant. In addition to the TEWL measurement, which is a highly sensitive and precise method for the determination of SLS irritation effects on the skin, as well as the most valid measurement for assessment of low irritant skin reactions, it is also useful to monitor hydration of the skin (EC) and EI [17, 19].

After the 6-day treatment of the SLS-irritated skin, TEWL was significantly decreased for all investigated creams (F1p, F1a, F1a6s, F1s and F1a1s) related to the "secondary" basal values (values measured upon irritation) (Figure 4). Secondly, for the creams F1a6s (containing ARSC and 6% of squalene) and F1s (containing 1% of squalene), a significant decrease in TEWL could be noticed when compared to the "primary" baseline (normal skin prior to irritation). Although TEWL was significantly decreased for the induced UCO related to the "secondary" baseline, the results obtained at the treated sites could not be solely attributed to the physiological regeneration of the skin, especially regarding the creams F1a6s and F1s. For the creams F1a6s and F1s, the obtained TEWL values could also be addressed to squalene and the carrier itself. After the application of the aforementioned samples, squalene may remain on the skin surface and due to the skin occlusion it may decrease TEWL or even penetrate the skin and influence its barrier recovery [3]. On the other hand, stabilized with a novel APG emulsifier, the carrier itself may contribute to the skin barrier recovery after the application on SLS-irritated skin as well, which has already been reported for vehicles based on this type of emulsifiers [24]. Such results indicate that both the investigated creams and tested actives may have a positive effect on skin barrier integrity. This assumption is consistent with the reported results regarding moisturizers and their impact on the skin barrier recovery after exposure to SLS [3, 24].

When it comes to skin hydration, a significant change of the EC compared to the "secondary" basal values was detected (Figure 5) for the placebo sample F1p and the active creams F1a6s and F1s. Namely, the EC value for UCO after 6 days remained significantly lower compared to the "primary" baseline. Thus, the trend of the EC increase, however insignificant, caused by the skin treatment with both the F1a and F1a1s creams, might be interpreted as the contribution in hydration effect of the tested actives. Moreover, these results are in line both with those from the 21-day study and with our initial assumption that the carrier itself contributes to the skin hydration, and presumably together with the used emulsifier as well.

As concerns EI values, after the 6-day application of the tested creams there was no significant change in the EI for the placebo cream F1p or the active cream F1s in relation to the "primary" basal values, although the trend of the EI decrease could be observed compared to the "secondary" baseline (values measured upon irritation) (Figure 6). Secondly, for the cream sample F1a and the samples containing a combination of the actives F1a1s and F1a6s, the EI values were elevated compared to the "primary" baseline, whereas for the samples F1a1s and F1a6s the trend of the EI decrease compared to "secondary" basal values (values measured upon irritation) could be observed as well. These results may imply at least two possible scenarios: adverse effect of the tested active (ARSC incorporated into liposomes) on previously irritated skin; too short period left for skin regeneration even under the treatment. On the other hand, after 6 days of treatment, the EI value remained significantly increased for the induced UC (UCO) compared with the "primary" baseline, and the trend of the EI increase was noticed related to the "secondary" basal values. Regarding the results for UCO and the fact that the reduction of skin irritation without adverse effects has recently been ascribed to ARSC by some researchers [28], it can be speculated that the obtained results are somehow a consequence of SLS irritation and occlusion itself.

The results of the second phase of the study generally stand in good agreement with the results of the first phase. The tested creams and the incorporated actives *per se* exerted a positive effect on irritated skin and they proved to have the potential to renew certain skin damages. The fact that hydration of the skin was elevated after the application of all investigated samples (for F1a and F1a1s without any statistical significance) suggests the contribution of the investigated actives, the carrier and its specific colloidal structure, and it consequently implies that the used emulsifier contributed to the observed moisturizing effect. Finally, the tested actives proved to be efficient in the treatment of irritated, dry skin.

Additionally, regarding the creams containing a combination of the actives and different concentrations of squalene (F1a1s and F1a6s, containing 1% and 6% of squalene, respectively), the study shows a concentration-dependent impact of squalene on skin hydration. Although, squalene is considered to be a good emollient and moisturizing agent [6], the obtained results also imply a certain impact on the specific colloidal structure in the tested creams, probably due to the formation of the lamellar phase, resulting in the additional skin moisturization.

## Conclusion

With the use of non-invasive skin biophysical measurements, the performed study confirms that the investigated actives – Alp Rose stem cells incorporated into liposomes and olive oil squalene, as well as the used emulsifier – hydroxystearyl alcohol and hydroxystearyl glucoside, show no irritation potential, nor any negative influence on the skin barrier function during the application on healthy or irritated skin. In view of these results, it could be said that these ingredients can be safely applied in the formulation of cosmetic moisturizers. The present study also shows a good skin hydration potential of the tested actives used either on the healthy or irritated skin. Furthermore, our results imply that both actives can be used for the improvement of skin barrier function. In addition, considering the lack of impact on skin elasticity after the application of the investigated samples, our study failed to show the potential anti-age skin improvement effect of the tested actives, probably due to the inappropriate selection of young volunteers in their early twenties.

Therefore, Alp Rose stem cells incorporated into liposomes, olive oil squalene and hydroxystearyl alcohol and hydroxystearyl glucoside could be used both alone and combined in moisturizing formulations intended for normal and dry skin care, whereas olive oil squalene could be used for treatment of irritated or sensitive skin as, well.

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# Cataract, ocular surgery, aphakia, and the chromatic expression of the painter Jovan Bijelić

## Katarakta, operacija oka, afakija i hromatska ekspresija slikara Jovana Bijelića

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**Abstract**

**Background/Aim.** Approaching art from the standpoint of optics and the artist's eye pathology can sometimes explain the shift of the spectral colors in the work of some artists with cataract and aphakia. This may not be obvious in the paintings of other artists with the same eye pathology. The aim of this study was to create a timeline from the recently obtained details of the cataract surgery, his best corrected aphakic visual acuity, and the last paintings of the artist Jovan Bijelić. **Methods.** The research included primary and secondary source material: Bijelić's paintings from all stages of his career, interviews with Bijelić and his eye surgeon, art criticism, sources with the description of Bijelić's symptoms, hospital archives, discussion with art historians, comparison of his palette from different periods. **Results.** Jovan Bijelić was nearly blind from cataract in 1957. He underwent an unsuccessful cataract surgery in 1956, followed by enucleation of the operated eye. In 1958, 20/25–20/20 vision was regained, after the extracapsular cataract extraction and sector iridectomy in his right eye, with the posterior lens capsule discision afterwards. Xanthopsia and cyanopsia are not present in his art, which is not a representation of visualized objects. **Conclusion.** The response of Jovan Bijelić to cataract and aphakia was predominantly a change of his style.

**Key words:**
**cataract; ophthalmologic surgical procedures; postcataract aphakia; color vision; art; famous persons.**

**Apstrakt**

**Uvod/Cilj.** Pristup umetnosti sa stanovišta optike i patologije umetnikovog oka može ponekad da objasni skretanje spektralnih boja u radovima nekih slikara sa kataraktom i afakijom. Kod drugih umetnika sa istom patologijom ove promene ne moraju biti očigledne. Cilj ovog rada bio je da se istraži odgovor slikara Jovana Bijelića na kataraktu i afakiju upoređivanjem novih podataka o njegovoj katarakti, operaciji i najbolje korigovanoj afaknoj oštrini vida sa slikama nastalim u tom periodu. **Metode.** Podaci su prikupljeni pretraživanjem primarnih i sekundarnih izvora: Bijelićevih slika iz svih perioda njegovog rada, novinskih članaka i intervjua sa Bijelićem i njegovim hirurgom, umetničkim kritikama, izvorima sa opisima Bijelićevih simptoma, arhivima klinika, razgovorima sa istoričarem umetnosti i slikarem. **Rezultati.** Jovan Bijelić je bio gotovo slep 1957. Jedno oko mu je bilo neuspešno operisano od katarakte 1956, a kasnije i enukleisano. Posle ekstrakapsularne ekstrakcije sa iridektomijom i kasnijom discizijom kapsule, desnom oku je dve godine kasnije vraćena odlična oštrina vida od 0.9–1.0. Ksantopsija i cijanopsija nisu prisutne u Bijlićevom slikarstvu jer on nije prikazivao posmatrane, već imaginarne predmete, često menjajući stil. **Zaključak.** Odgovor slikara Jovana Bijelića na kataraktu i afakiju prevashodno se manifestovao u promeni stila.

**Ključne reči:**
**katarakta; hirurgija, oftalmološka, procedure; afakija, postkataraktna; vid, kolorni; umetnost; slavne ličnosti.**

### Introduction

The sclerotic nuclear cataract may induce myopia, monocular diplopia, glare, haloes, and a shift from the violet-blue to yellow-orange colors of the visible spectrum (xanthopsia). The world seen through an opacified brunescent lens is devoid of clear violets and blues, and becomes immerged in a yellowish atmosphere. To the contrary, the absence of the lens, aphakia, enables the near-ultrashort wave-lengths of light, invisible to a normal eye, to stimulate cones, and give a light-blue appearance to the white objects (cyanopsia).

Richard Liebreich [1], the chief ophthalmologist in St. Thomas' Hospital in London, was the first to recognize the possibility of a connection between cataract and art through his studies on Turner. As a graduate from L'Ecole des

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Beaux-Arts in Paris and a pupil of the inventor of ophthalmoscope, von Helmholz, he was the right person to analyze the influence of painters' eye disease upon their work. At an old age, Libriech examined the painter Claude Monet, who suffered from cataract but underwent surgery many years later. Monet's well-documented xanthopsia during the cataract aging, as well as his postoperative cyanopsia became a common knowledge [2] and the reason for a belief that both the disease and the state of aphakia produced by the lens extraction must have a profound effect on the pallete of the artist. Accepting Trevor Roper's [3] advice that one should be cautious in interpreting art using optics as the sole tool, we aimed at finding another example of a painter with cataract and postoperative aphakia, and examining his/her work.

The aim of this study was to investigate the response of the painter Jovan Bijelić to the symptoms caused by cataract and aphakia, by obtaining the unknown details of his cataract surgery, aphakic correction and visual acuity.

## Methods

This research included investigation of primary and secondary source material: Bijelić's paintings from all stages of his career, interviews with Bijelić and his eye surgeon, art criticism, sources with the description of Bijelić's symptoms by his friends; the time line of the palette used in his paintings and his eye disease; the hospital archives; discussion with an art historian and an artist; data on other artists' cataract; descriptions left by the doctors who underwent cataract surgery.

## Results

### *Biography*

Jovan Bijelić was born in a hamlet Revenik near Bosanski Petrovac on June 30, 1884 and died in Belgrade on March 12, 1964. He graduated from the Art Academy at Krakow. Further studies in Paris broadened his knowledge, but the main influence came from the German expressionist group "The Blue Rider", and from Vasily Kandinsky's abstract art. Yet, the most influential were his memories of the Bosnian mountains and their vivid colors, "kept within and carried wherever he went, lived and painted".

Bijelić was a serene optimist in spite of a chronic anxiety brought by the uncertain artist's income and poor dwelling conditions, and stressed by war, imprisonment and the tragic execution of his daughter.

His opus includes at least 981 paintings in oil or tempera exhibited at more than two hundred group shows, aside from a few of his own; countless drawings and watercolours, thirty historical compositions and dynastic portraits, numerous scenographies, almost a dozen pupils who became well-known artists, and a few novels [4]. His painting, influenced by Cezane at first, then cubistic, and shortly afterwards abstract, followed by neorealistic, then fauvistic, turned into expressionism until finally, after cataract surgery, he created a series of almost abstract paintings simultaneously with a few vividly coloured and sharply drawn Bosnian landscapes.

### *Art criticism*

The art critics praised Bijelić early as a completely modern painter, a colorist able to discard the unneccessary from his painting, even to disolve the form, in order to compose a brutal symphony of opposed colors [5]. It was not a simple decision, but a process of cleansing his palette and of changing the style until the circle was closed: from the first abstract painting in 1921 to its revival in 1960. In that year, Bijelić himself summarized his approach: "I am going to be more concise in my work, and I shall depict only what is essential in the motive... These paintings may appear abstract to someone, but they will stem from the real life [6]."

### *Recollections of the imminent blindness*

Passages from Smail Tihić's [7] "Jovan Bijelić: life and work" are almost the only available source of the artist's wrestling with the eye disease. He was painting even when he barely percieved colors at the palette, while his friends' opinion helped him to get an impression of what he had created [8] (Figure 1).

**Fig. 1 – Jovan Bijelić. "Tempest over Marinko's Pond" (Oil on canvas, 1955; Courtesy of Dušan Vukićević).**

His left eye was lost after cataract surgery in autumn 1956 [9]. Yet, his rich inner experiences helped him to combat the blindness [7].

At his retrospective exhibition in 1957, Bijelić could only imagine the paintings using his tactile sense and listening to the description of each of them [7]. Even in such a situation, he expressed his characteristic optimism [9].

*Ocular surgery*

Professor Olga Litričin, the doyen of our ophthalmologists, remembers well the days when Bijelić was hospitalized in 1956. Unfortunately, she did not get a chance to see him. The archive from that time does not exist at the University Eye Hospital in Belgrade, and no document was found to show the operative and postoperative course of his first cataract surgery.

The Archive of University Clinical Center, Ljubljana, keeps the documents on Bijelić's treatment in 1958 under the numebrs 11763 and 32640.

16.IV 58. *Enucleatio bulbi sin*. Op: prof As Dr Stergar

25.IV 58. Course normal. Right eye (RE): Redish colored cataract without red reflex from the periphery.

16.VI. 58. Visual acuity (VA) RE Light projection. Fundus invisible.

Left eye (LE) Anophthalmus. Right eye cataract surgery postponed because of an acute hepatitis.

29.X 58. Extr. cat. dex. extracaps. c. irid. tot.

/A large nucleus hardly passes through the round pupil, therefore total iridect. The vitreous pushes behind the lens, but retracts after the instillation of water/.

Op: Doc Stergar As. Doc Dr. Dereani

Cataract surgery, right eye (December 1958) #32640

6.XII 58. Discisio cat. sec.dex. Op. Doc Dr. Stergar As. Dr. Hrovatin

15.XII 58. The eye is quiet. The cornea transparent. The pupil in semimydriasis, without reaction (Mydrasis medic,). A total iris coloboma at 12. An arcuate remnant of the secondary cataract in the upper nasal part of the pupillary apperture. The central portion of the pupil clear.

Fundus: normal. Javal RE 2.0/180$^{o}$ VA RE +10.0 sph +1.50cyl/180$^{o}$ 5/6

25.XII 58 VA RE +10.0 = cyl +1.50/5$^{o}$ 5/6- 5/5

27.XII.58. Rp/ RE +10.0+1.50/5$^{o}$ LE +10.0

RE +13.0 = cyl +1.50/5$^{o}$ LE+13.0 PD 68/67

Demission: 27.XII 58. Dg. Cat. sen. dex. cured

On November 18, 1958, after cataract extraction, the daily newspaper "Borba" published the article "The painter Jovan Bijelić has regained sight" [10], quoting Dr. Stergar's remark that Jovan Bijelić had endured this more complicated surgery very well. The artist described his anxiety before the patch removal, his confidence and the first visual experience after many years. The article ends with the conclusion that the treatment was a full success and that the painter would soon discard the black glasses".

*Aphakia*

During the period of adaptation to aphakic glasses (a couple of photographs show Bijelić wearing them), Bijelić was missing the canvas with his brush while attempting at painting [11].

Two years after surgery, Bijelić exhibited a few landscapes (Figure 2) and a series of paintings in an almost abstract style (Figures 3 a and b).

A remark indicates that Bijelić did not see well at that time: "A change of palette, which is not as resonant and glowing as it used to be can be explained by a diminished visual ability, which dropped to 30% of the prior possibilities after surgery in Ljubljana" [12].

*Doctors as patients: descriptions of vision through cataract and through aphakic glasses*

Gaetan de Clerambault [13] gave a very detailed description of his symptoms of cataract, the experience of bilateral cataract surgery performed by Ignatio Barraquer, and of aphakic vision. Troublesome object distortions, haloes, and ocular fatigue were his dominant symptoms of cataract.

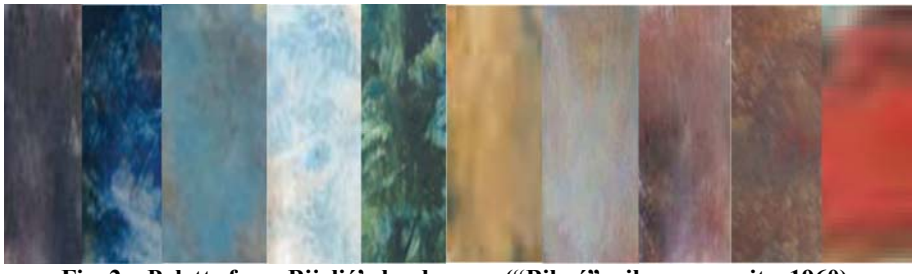

**Fig. 2 – Palette from Bijelić's landscapes ("Bihać", oil on masonite, 1960).**

**Fig. 3 – Palette from Bijelić's abstract art (1962).**

Barraquer's surgery with the use of vacuum was fast, gentle and highly successful, except for the iris prolapse in one eye due to the the pressure on the globe during postoperative period. Aphakic vision was dominated by blue and violet colors: white objects seemed to be bluish to one eye and slightly violet to the other and faces seemed to have blue or violet orbits. Allan C. Woods [14] experienced aphakia so profoundly that he wrote an editorial with a bottom line: "It can not be cured, it must be endured". Walter Stark has had an aphakic eye since an injury at the age od 10, and cataract surgery at the age of 12. Fascinated by his cyanopsia, he has investigated the ultrashort wave perception quite extensively [15].

*Other painters' experience with cataract surgery and aphakia*

Two well-known artists underwent cataract surgery at about the same time as Jovan Bijelić: Sir Mathew Smith, a British fauvist, in 1952, and Kay Sage, an American symbolist painter, in 1959 and 1960 [16].

Sir Mathew has never complained of either xanthopsia or cyanopsia. He continued to paint in the same style after surgery, feeling only that the colors seemed more vivid [16]. The palette used in one of his oil paintings created postoperatively does not show any signs of cyanopsia, but these signs can be traced in his pastels (Figure 4).

Kay Sage's palette during the development of cataract does show grey-yellowish and ochre hues (Figure 5). After a painful and partially successful bilateral cataract surgery, she stopped painting and created small sculptures made of wires, bullets and stones. She also wrote poetry. Finally, Kay Sage commited suicide, which she had already unsuccessfully attempted immediately after her husband's death in 1955 [17].

**Discussion**

In 1956, Bijelić was left with a low vision in his only functional eye. There are no hospital records to show why his left eye had lost sight and became atrophic after surgery. Most probably an expulsive hemorrhage or a massive vitreous prolapse with a subsequent retinal detachment must have happened. Bijelić's blood pressure values as measured in Ljubljana, at one point reached 190/90 mmHg. Such a spike during cataract extraction through a large incision could have triggered the expulsive hemorrhage. Two years later, the fear of the sympathetic ophthalmia originating from the blind, atrophic left eye was an indication for enucleation.

The right eye was operated by using extracapsular cataract extraction with sector iridectomy, and discision of the remaining posterior capsule at a later date. What was the reason for choosing this technique? The prevailing method at that time was intracapsular cataract extraction by grasping the capsule with the Arruga forceps and taking the whole lens out of the eye. A great skill was required for such a maneuver, which limited the number of able surgeons and uncomplicated operations. One of them was Leopold Ješe, who had performed intracapsular cataract extraction 22 years before Bijelić was hospitalized [18].

To overcome this difficult approach, some ocular surgeons accepted the Ignacio Barraquer's method of phacoerisis, using a vacuum device called erisiphake [19]. Its jovial inventor liked to picture the action of vacuum as the kiss of a beloved women compared to the grasp of the cat's claws exerted by the forceps. The vacuum extraction gained popularity after the introduction of enzymatic zonulolysis by

**Fig. 4 – Palette used by Matthew Smith after cataract surgery. The last stripe shows the approximate colors from his pastels.**

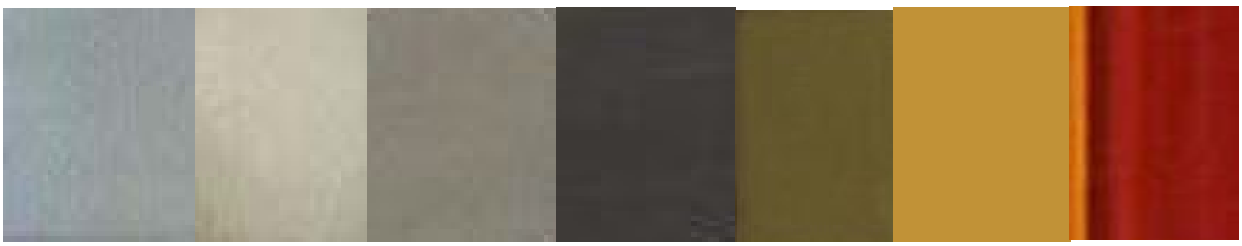

**Fig. 5 – Pallete used by Kay Sage in her last paintings before cataract surgery.**

Joaquin Barraquer [20] but was soon replaced by the safest method of all, Krwawitz's [21] intracapsular cataract kryoextraction. However, both the zonulolytic enzyme alpha-chymotrypsine and the kryoextractor were unavailable at the time of Bijelić's operation [22]. According to a paper presented at the anniversary celebration of the Maribor Hospital, yet another method was used by the ophthalmologists of this town in the vicinity of Ljubljana at the end of the fifties: a revived and modified colonel Smith's [23] tumbling techniue, called Hruby's cataract expression [24]. It worked well in the hands of colonel Smith, who had performed thousands of cataract extractions in India, but was not suited for testing in the only eye of Jovan Bijelić. Nor was the forceps intracapsular extraction with such dreaded complications as the vitreous prolapse or the rupture of the tense posterior capsule and the nucleus drop into the vitreous [25]. Instead, Dr. Stergar chose a method which could leave a barrier both to the expulsion of the ocular content, and to the movement of the nucleus backwards: extracapsular cataract extraction.

This method invariably lead to discison of the posterior capsule at a later date; but was the sector iridectomy neccessary? Again, without a viscoelastic to form the anterior chamber and to push the vitreous back, and a microscope to conmand a detailed view of the involved ocular structures, it was probably the safest choice for Dr. Stergar and for Bijelić, as it had been for Dr. Cutela and for Claude Monet. The modest comment of how well Bijelić had endured a somewhat complicated surgery reflects both the psychological pressure on the surgeon operating on a monocular patient, as well as the push of the vitreous behind the lens which had happened during extraction. The reward was an excellent best corrected vision of 20/25–20/20, with moderate two diopters of astigmatism.

Did this excellent visual acuity last or Bijelić became legally blind again, as Tihić [12] suggested? Was this the reason to paint his almost abstract "Compositions"? As the secondary cataract can not reobscure vision after discision, and the corneal edema from the late endothelial decompensation is conspicuous, the presumed loss of visual acuity could be caused either by macular or the optic nerve disease. It is impossible to prove or disprove the development of such diseses in Bijelić's eye during some 18 months after the final examination in Ljubljana, when a normal *fundus* appearance had been recorded. But, how could he paint those Bosnian landscapes with such a certainity of drawing with a visual acuity of 0.2–0.3 (20/60)? A careful analysis of the vocabulary used in the constatation that a diminished visual ability, which dropped to 30% of the prior possibilities after surgery in Ljubljana, reveals the use of the term visual 'ability' instead of the 'acuity'. It indicates that this constatation may be a misinterpretation of a medical report showing a 30% diminution of the working and visual 'ability', which used to be issued to all monocular patients, whose sound eye had a good visual acuity, in order to get a compensation from the state insurrance.

Adaptation to the aphakic correction with glasses is a process which involves the neural plasticity: it takes time, patience and even endurance, as suggested by Dr. Wood. The ability to adapt to a new perception of the world also depends on the character, temper and age.

One of the most readily noticed obstacles is the magnifying effect of the strong convex spactacle lenses intended to replace the refraction of the extracted crystalline lens. The objects seen through them do not seem to be larger, but they appear to be closer. That it why Bijelić was missing the canvas with the brush in his attempt to paint during the early postoperative course. His photographs with aphakic glasses indicate that he had finally succeeded to learn how to walk and work with them.

The abrupt change of color perception after cataract extraction is another obstacle. Provoked by Claude Monet's paintings of the same subject seen through his cataract or with his aphakic eye, and his letters with a detailed explanation of the troubles with perceiving colors have been an issue in hundreds of articles until the water lilies which turned from yellow-brown into blue-violet after cataract surgery became a common place [26]. Was every artist with cataracts and later aphakic spectacle correction doomed to paint in this color register?

The aging lens block the increasing amount of light of the short wavelengths; and yet, we do not notice a different color perception because of a cerebral adaptation to this slow process. Even with a sclerotic nuclear cataract this slow shift towards yellowish appearance of the world does not have to be conspicuous. Only after cataract extraction the quantity of light, especially of the shorter wavelengths that reaches the retina, changes abruptly creating a shift of color in the blue-violet direction. This shift can be enhanced by the aphakic spectacle lens chromatic aberation [27]. Some studies indicate that this barrier deprivation syndrome can last from six months to three years [28], while others, using achromatic settings, estimate that re-adaptation is likely to be a cortical process which takes three months [29]. Why then Monet needed three years to adapt, and how long did other painters need?

Most aphakic and pseudophaking patients, even some aphakic artists like Sir Matthew Smith, notice only that colors seem more vivid than before. Others, like Dr. Walter Stark, easily notice that what appears white to the phakic eye seems to be bluish to the aphakic eye. Further, some extremely sensitive persons, like Dr. Gaetan de Clerambault [13], notice even the difference between two aphakic eyes: one of them sees things bluish, the other – violet. Monet was one of these sensitive, impatient, old persons with monocular aphakia, who could see the difference and knew only too well how he wanted to perceive colors in order to create different appearances of the same visible objects. The incessant change of the spectacle correction, of the tinted and transparent glasses, and a deliberate closing of the aphakic eye, distracted him from the process of adaptation. In this state of interrupted chromatic mechanisms, his heroic attempts to catch the most elusive among the characteristics of an object, its color, created both the fluctuations of his mood and the axiom of the palette changes according to the presence of a cataract or aphakia.

As an expressionist, Bijelić was far away from Monet's colorist intentions. His colors were not a reflexion of light from the objects: they stemmed from the inner state of tension, opposing each other in the heights over his imaginary landscapes. A burst of blues, reds, greens and yellows in Figure 1 did not represent any object seen through his advanced cataracts; it was the

image of distorsion created by the conflict between the intruding outer world and the order of the inner world [30]. No eye disease can be suspected from this painting, as no style that Bijelić has ever used before can be recognized in it. This art is closest to *Art Informel*, which has just appeared in France.

Admittedly, Bijelić used plenty of blues in his Bosnian landscapes seen through the aphakic spactacle correction. But these blues are within the boundaries of a firm, almost scholarly drawing, matched by a very rich spectrum of other colors, and do not leave the river and the sky to spill over the red roofs of the light ochre houses, or the green trees (Figure 2).

His "Compositions", (oil on lesonite, 1962) had been created in a search of the essence in art, as Bijelić himself explained. The painting is characterized by a reduction of both form and color: burnt Sienna and yellow ochres stand out from a light-blue "background". The artist did not paint a white wall, appearing bluish to him – he simply matched the colors to get a painterly effect (Figure 3).

Similarly, Matthew Smith may seem to have cyanopsia when judged by the palette of his pastels, but one look at those prevailing reds and greens taken from his oil paintings after cataract extraction will assure us that he used the colors to paint a picture and not to represent an object (Figure 4).

Finally, the palette of Kay Sage's last paintings contains colors that may suggest xanthopsia, but these colors also seem to suit best the mood and atmosphere of her symbolist art (Figure 5).

## Conclusion

The monocular spectacle-corrected visual acuity of Jovan Bijelić, after an uneventful extracapsular cataract surgery and posterior capsule discision, was excellent. His artistic response to cataract and aphakia was predominantly a change of the style of painting.

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# The use of hyaluronic and aminocaproic acid in the treatment of alveolar osteitis

## Primena hijaluronske i aminokapronske kiseline u terapiji alveolitisa

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**Abstract**

**Background/Aim.** Alveolar osteitis (AO), also known as "dry socket", is relatively common post-extraction complication. It probably occurs due to excessive fibrinolytic activity in the coagulum and is characterized by intense pain sensations. The aim of this clinical study was to examine the role of hyaluronic acid and aminocaproic acid in the treatment of AO. **Methods.** The study included 60 patients with the clinical diagnosis of AO. All the patients were divided into two groups of 30 patients each according to the applied non-pharmacological measure: irrigation – irrigation of dry socket with sterile saline; curettage – careful curettage. Both of these groups were further divided into three subgroups regarding the applied treatment (hyaluronic acid; hyaluronic acid + aminocaproic acid; Alvogyl®, an anesthetic and antiseptic paste), each with 10 patients, according to the following protocol: 0.2 mL of hyaluronic acid in the form of a 0.8% gel; 2 mL of aminocaproic acid and hyaluronic acid; Alvogyl®. During each visit, scheduled for every two days until complete absence of painful sensations, the patients had the therapeutic method repeated as at the first examination. At each control visit the number of present symptoms and signs of AO was recorded, as well as the level of pain (measured with a visual analogue scale). **Results.** With the use of hyaluronic acid, with or without aminocaproic one, a statistically significantly faster reduction in pain sensations was achieved, along with the reduction in the number of symptoms and signs of AO compared to the use of Alvogyl®. **Conclusion.** Hyaluronic acid, applied alone or in combination with aminocaproic acid significantly reduces pain sensation, thus it can be successfully used in the treatment of AO.

**Key words:**
**tooth extraction; postoperative complications; dry socket; hyaluronic acid; aminocaproic acids; curettage.**

**Apstrakt**

**Uvod/Cilj.** Alveolitis je relativno česta postekstrakciona komplikacija. Nastaje, najverovatnije, usled izrazite fibrinolitičke aktivnosti u koagulumu, a karakteriše se pojavom intezivnog bola. Cilj ove kliničke studije bio je da se ispita mogućnost primene hijaluronske i aminokapronske kiseline u terapiji alveolitisa. **Metode.** Studija je uključila 60 pacijenata sa kliničkom dijagnozom alveolitisa. U odnosu na primenjenu nefarmakološku meru svi pacijenti su bili podeljeni u dve grupe sa po 30 pacijenata: ispiranje – ispiranje obolele alveole sterilnim fiziološkim rastvorom; kiretaža – pažljiva kiretaža. Obe ove grupe, u odnosu na primenjeni tretman [(hijaluronska kiselina, hijaluronska kiselina + aminokapronska kiselina, Alvogyl® (kombinacija anestetika i antiseptika u obliku paste)], bile su podeljene u tri podgrupe sa po 10 pacijenata po sledećem protokolu: 0,2 mL hijaluronske kiseline u obliku 0.8% gela; 2 mL aminokapronske kiseline i hijaluronske kiseline; Alvogyl®. Na kontrolnim pregledima, zakazanim na svaka dva dana do potpunog prestanka bolnih senzacija, pacijentima je ponavljana terapijska opcija sa prvog pregleda. Evidentiran je broj prisutnih simptoma i znakova alveolitisa kod pacijenata, kao i nivo bola (meren pomoću vizuelno-analogne skale). **Rezultati.** Primenom hijaluronske kiseline, sa ili bez aminokapronske kiseline, postignuto je statistički značajno brže sniženje bolnih senzacija kao i smanjenje broja prisutnih simptoma i znakova alveolitisa u odnosu na upotrebu Alvogyl®-a. **Zaključak.** Hijaluroska kiselina, samostalno ili u kombinaciji sa aminokapronskom kiselinom, značajno snižava bol, te se može uspešno primenjivati u terapiji alveolitisa.

**Ključne reči:**
**zub, ekstrakcija; postoperativne komplikacije; alveolitis, suvi; hijaluronska kiselina; aminokapronske kiseline; kiretaža.**

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## Introduction

Alveolar osteitis (AO) sometimes occurs after tooth extraction due to the disturbances in the healing processes. It is characterized by the appearance of postoperative pain in and around the extraction area, which increases from the first to the third post-extraction day, and is combined with partial or total destruction of a blood clot in the alveolus [1]. AO develops after 3–4% of all extractions, and even more often after surgical extraction of impacted lower third molars, up to around 30%. The incidence of AO is five times higher in women than in men and much higher in the lower jaw (10 times more often after the extraction of the lower molars than of the upper ones) [2–4]. AO is probably caused by excessive fibrinolytic activity in the post-extraction coagulum that leads to destruction of the fibrin matrix and blood clot degradation [5].

The first symptoms of AO usually appear 1–3 days after extraction [6, 7] in the form of severe pain, taste disorder and bad breath. All reported cases were diagnosed in up to 7 days following extraction. The symptoms of AO are present, depending on the aggressiveness of the disease, usually 5–10 days [1]. In the alveolus, there are either remains of a decomposed coagulum or the alveolus is empty, with exposed bone. The alveolus can be filled with food remains and the surrounding gingiva can be edematous and erythematous. Regional lymphadenopathy can be present [8].

There were various approaches to the treatment of AO that involved the use of a wide range of resources, rinsing solutions and procedures [2]. These procedures were proposed for suppressing the symptoms of AO. On the average, it takes 7–10 days for the exposed bone to become covered with young granulated tissue. During this time it is necessary to make efforts in order to reduce subjective discomforts of the patient [9]. Most commonly used for that purpose is Alvogyl® (Septodont, France), an anesthetic and antiseptic paste.

Hyaluronic acid has anti-inflammatory and anti-edematous potential which helps wound healing and increases the tissue elasticity [10–14]. A pilot study conducted on rats showed that treatment of a dry socket with a preparation that contains 1% of hyaluronic acid resulted in rapid formation of coagulum and accelerated healing of post-extraction wound [15].

Aminocaproic acid (epsilon-aminocaproic acid) is a potent antifibrinolytic agent. It competitively blocks high affinity lysine receptors on the plasminogen proenzyme and thus prevents formation of a ternary complex with tissue plasminogen activator (t-PA) and fibrin [16]. For many years, the aminocaproic acid has been used in various fields of surgery (oral surgery, cardiac surgery) [17].

The aim of this clinical study was to explore if hyaluronic and aminocaproic acid can be used successfully in the treatment of AO.

## Methods

This prospective randomized clinical study was carried out in the period from 2011 to 2014 at the Department of Oral Surgery of the Faculty of Medicine, University of Priš-tina, Kosovska Mitrovica, Serbia. All check-ups as well as surgical procedures were performed by one oral surgeon. The patients voluntarily participated in the study and were thoroughly familiarized with the principles of performing clinical research. The study was approved by the Ethic Committee of the Faculty of Medicine, University of Priš-tina, Kosovska Mitrovica.

The study included 60 patients of both sexes and of different age who, based on the anamnesis and clinical examination, were diagnosed with AO. The study included patients who had undergone tooth extraction and who came to check-up because of an onset of pain in and around the extraction region. The AO diagnosis was made in accordance with Blum's [1] definition, based on the following clinical signs and symptoms: onset of pain from the first to the third post-extraction day, the presence of a decomposed coagulum in the post-extraction alveolus or a bare alveolar bone. The study excluded patients who used antibiotics or analgesics after extraction, patients who previously received radiotherapy or chemotherapy, patients on anticoagulant therapy and patients with systemic diseases that influence the processes of healing of the post extraction wound (*eg*. diabetes mellitus, vascular or hematological disorders and other serious pathological lesions in the mouth).

All the patients were randomly divided into two groups *per* 30 patients each according to the applied non-pharmacological measure: irrigation – irrigation and medicament; curettage – curettage and medicament. Dry sockets of the patients from the irrigation group were irrigated with 20 mL of sterile saline (0.09% NaCl), which was enough to completely remove debris from the alveolus. Dry sockets of the patients from the curettage group were carefully curetted and a remotely healthy coagulum was preserved. Before curettage, local infiltration anesthesia, articaine with adrenaline (UbistesinTM forte, 3M ESPE AG, Germany), was administered to patients.

Furthermore, each of these two large groups was divided into three subgroups with 10 patients each regarding the used medicament: hyaluronic acid – irrigation (HA-I), hyaluronic acid + aminocaproic acid – irrigation (HA+AA-I) and Alvogyl® – irrigation (Alvogyl®-I); hyaluronic acid – curettage (HA-C), hyaluronic acid + aminocaproic acid – curettage (HA+AA-C) and Alvogyl® – curettage (Alvogyl®-C). Dry sockets of the patients from the subgroups were then treated according to the following protocol: HA-I and HA-C: 0.2 mL of hyaluronic acid in the form of a 0.8% gel (Gengigel® prof. - 0.8% hyaluronic acid, Ricerfarma SRL, Milan, Italy); HA+AA-I and HA+AA-C: 2 ml of aminocaproic acid (Amicar®, XANODYNE PHARMACEUTICALS, INC, USA, ampoule 250 mg/ml) and hyaluronic acid; Alvogyl®-I and Alvogyl®-C: Alvogyl® (Septodont, France). The patients were not given any other types of medicaments.

The patients had scheduled control visits for every two days until complete absence of painful sensations. At every control visit, the patients had the therapeutic method repeated as at the first examination.

During each control visit the number of present symptoms and signs of AO was recorded for every patient. Marked as the

symptoms and signs of AO were: pain, pain irradiation, swelling of the regional lymph nodes, redness of the gingiva around the extraction wound and halitosis. The presence of symptoms and signs was recorded in the "present" / "not present" manner. The level of pain was determined (measured with a visual analogue scale – VAS, graded in centimetres from 0 to 10, where 0 was the lowest notch marking the "absence of pain" while notch 10 marked "unbearable pain"). The total number of examinations was also recorded, to a complete cessation of painful sensations, for each individual patient.

The data primarily obtained were analyzed with descriptive statistical methods and methods for testing statistical hypotheses. From descriptive methods, measures of central tendency ($\bar{x}$; median), measures of variability (SD, variation interval) and the relative numbers (structure indicators) were used. For testing the hypotheses, the methods used were: $\chi^2$ test for testing the difference in frequency among the groups; Kruskal-Wallis and Mann-Whitney test for testing the differences in value of the characteristics among the groups, Friedman and Wilcoxon test for testing the changes in the value of the characteristics in time. The statistical hypotheses were tested at a significance level of 0.05.

**Results**

Of the total number of patients, 60% were male and 40% female. There was no significant difference in the frequency of gender in the groups examined ($\chi 2 = 0.278$; SS = 1; $p = 0.598$). The mean age of patients in the groups was 37.72 ± 10.91 (Table 1).

At the first visit following tooth extraction, of all the determined symptoms and sings of AO, only pain was present in all the patients. There was no difference in the average number of present symptoms and signs of AO between the examined groups at the first and the second visit ($p > 0.05$). A statistical significance existed among the subgroups of the Curettage group, where hyaluronic acid was applied, during the third, fourth and fifth visit in relation to the group Alvogyl-C (Tables 2 and 3).

Regardless of the treatment, the scores on the VAS of pain decreased during the follow-up period but there was a statistical difference in pain levels among the groups (Table 4). Among the subgroups where hyaluronic acid was applied, with or without aminocaproic one, in relation to the subgroups that were treated with Alvogyl®, a statistical difference

**Table 1**

**Age and gender distribution of the patients with alveolar osteitis**

| Age (years) | Gender, n (%) | | Treatment, n (%) | | Total n (%) |
|---|---|---|---|---|---|
| | male | female | irrigation | curettage | |
| > 30 | 8 (22.2) | 7 (29.2) | 7 (23.3) | 8 (26.7) | 15 (25) |
| 30–39 | 14 (38.9) | 9 (37.5) | 8 (26.7) | 15 (50) | 23 (38.3) |
| 40–49 | 8 (22.2) | 5 (20.8) | 9 (30) | 4 (13.3) | 13 (21.7) |
| 50–59 | 4 (11.1) | 2 (8.3) | 3 (10) | 3 (10) | 6 (10) |
| ≥ 60 | 2 (5.6) | 1 (4.2) | 3 (10) | 0 (0) | 3 (5) |
| Total | 36 (100) | 24 (100) | 30 (100) | 30 (100) | 60 (100) |

**Table 2**

**Clinical symptoms and signs of the study groups that underwent irrigation (I) during follow-up**

| Visit | Patients' group | Symptoms and signs of alveolar osteitis (AO) | | | | | |
|---|---|---|---|---|---|---|---|
| | | n | Pain* | Pain irradiation | Swelling of the regional lymph nodes | Redness of the gingiva | Halitosis |
| 1 | HA-I | 3.3 | 10 | 8 | 5 | 4 | 6 |
| | HA+AA-I | 3 | 10 | 7 | 4 | 5 | 4 |
| | Alvogyl®-I | 3.4 | 10 | 6 | 7 | 6 | 5 |
| 2 | HA-I | 2.6 | 10 | 5 | 4 | 3 | 4 |
| | HA+AA-I | 2.7 | 10 | 6 | 5 | 4 | 2 |
| | Alvogyl®-I | 3.4 | 10 | 6 | 7 | 6 | 5 |
| 3 | HA-I | 1.7 | 7 | 4 | 2 | 2 | 2 |
| | HA+AA-I | 1.1 | 7 | 1 | 1 | 2 | 0 |
| | Alvogyl®-I | 2 | 9 | 3 | 3 | 4 | 1 |
| 4 | HA-I | 0.7 | 4 | 2 | 0 | 1 | 0 |
| | HA+AA-I | 0.6 | 4 | 0 | 0 | 2 | 0 |
| | Alvogyl®-I | 1.3 | 7 | 2 | 1 | 3 | 0 |
| 5 | HA-I | 0.1 | 1 | 0 | 0 | 0 | 0 |
| | HA+AA-I | 0.2 | 2 | 0 | 0 | 0 | 0 |
| | Alvogyl®-I | 0.5 | 4 | 0 | 0 | 1 | 0 |

**HA – hyaluronic acid; AA – aminocaproic acid; n – average number of patients with symptoms and signs of AO.**
***Pain was estimated by visual analog scale (VAS): 0 – no pain; 10 – unbearable pain.**

**Table 3**

**Clinical symtoms and signs of the study groups that underwent curretage (C) during follow-up**

| Visit | Patients' group | Symptoms and signs of alveolar osteitis (AO) | | | | | |
|---|---|---|---|---|---|---|---|
| | | n | Pain* | Pain irradiation | Swelling of the regional lymph nodes | Redness of the gingiva | Halitosis |
| 1 | HA-C | 3.4 | 10 | 8 | 6 | 5 | 5 |
| | HA+AA-C | 3.2 | 10 | 8 | 4 | 5 | 5 |
| | Alvogyl-C | 3.4 | 10 | 7 | 6 | 7 | 4 |
| 2 | HA-C | 2 | 8 | 4 | 4 | 2 | 2 |
| | HA+AA-C | 2.1 | 8 | 5 | 2 | 3 | 3 |
| | Alvogyl-C | 2.9 | 9 | 6 | 5 | 6 | 3 |
| 3 | HA-C | 0.9† | 6 | 1 | 0 | 2 | 0 |
| | HA+AA-C | 0.9† | 7 | 0 | 0 | 0 | 2 |
| | Alvogyl-C | 2.3 | 9 | 5 | 4 | 3 | 2 |
| 4 | HA-C | 0.3† | 2 | 0 | 1 | 0 | 0 |
| | HA+AA-C | 0.4 | 3 | 0 | 1 | 0 | 0 |
| | Alvogyl-C | 1.1 | 7 | 2 | 1 | 1 | 0 |
| 5 | HA-C | 0† | 0 | 0 | 0 | 0 | 0 |
| | HA+AA-C | 0† | 0 | 0 | 0 | 0 | 0 |
| | Alvogyl-C | 0.3 | 3 | 0 | 0 | 0 | 0 |

**† – statistical significance compared to the Alvogyl® - C group ($p = 0.05$). For abbrevations see under Table 2.**
***Pain was estimated by visual analog scale (VAS): 0 – no pain; 10 – unbearable pain.**

**Table 4**

**Average level of pain estimated by visual analog scale (VAS)* in the patients with alveolar osteitis by the groups during follow-up visits**

| Visit | Irrigation ($\bar{x} \pm SD$) | | | Curettage ($\bar{x} \pm SD$) | | |
|---|---|---|---|---|---|---|
| | HA-I | HA+AA-I | Alvogyl®-I | HA-C | HA+AA-C | Alvogyl®-C |
| 1 | 7.3 ± 2.06 | 7.9 ± 1.66 | 7.4 ± 1.43 | 7.7 ± 1.49 | 7.9 ± 1.59 | 7.4 ± 1.65 |
| 2 | 5.1 ± 2.51 | 5.1 ± 2.51 | 7.2 ± 1.34 | 3.8 ± 2.7 | 3.5 ± 2.32 | 6.1 ± 2.6 |
| 3 | 2.4 ± 2.12† | 2.4 ± 2.12† | 5.1 ± 1.91 | 1.6 ± 1.5† | 1.8 ± 1.8† | 4.3 ± 2.5 |
| 4 | 0.7 ± 1.1† | 0.7 ± 1.1† | 2.9 ± 1.9 | 0.3 ± 0.68† | 0.6 ± 1.1† | 2.1 ± 1.91 |
| 5 | 0.2 ± 0.63 | 0.4 ± 0.84 | 0.8 ± 0.92 | 0† | 0† | 0.5 ± 0.85 |

***VAS: 0 – no pain; 10 – unbearable pain; † – statistical significance compared to Alvogyl® group ($p = 0.05$); $\bar{x}$ – mean; SD – standard deviation.**

was noticed during the third, fourth and fifth visit. The pain levels were the same between the groups where hyaluronic acid was used and those treated with aminocaproic acid, as well.

Comparison of the groups in relation to the average number of visits, up to a complete cessation of painful sensations, demonstrated similar results (Table 5). In the irrigation group, there was a statistically significant difference in the number of visits between the treatment subgroups HA-I and HA+AA-I compared to Alvogyl®-I, while in the curettage group, statistical significance existed between the treatment subgroups HA-C and HA+AA-C compared to Alvogyl®-C.

## Discussion

The therapy of AO is symptomatic. It includes irrigation or curettage of the dry socket and topical use of different pharmacological and non-pharmacological packaging [18]. By irrigation with saline, food debris and remains of decomposed blood cloth are eliminated and the number of bacteria from the

**Table 5**

**Average number of visits up to the complete cessation of painful sensations by the groups during follow-up**

| Group of patients | $\bar{x}$ | SD | Med | Min | Max |
|---|---|---|---|---|---|
| HA-I | 3.2† | 1.03 | 3 | 2 | 5 |
| HA+AA-I | 3.3† | 0.95 | 3 | 2 | 5 |
| Alvogyl®-I | 4.3 | 0.82 | 4.5 | 3 | 5 |
| HA-C | 2.6† | 1.07 | 3 | 1 | 4 |
| HA+AA-C | 2.8† | 1.13 | 3 | 1 | 4 |
| Alvogyl®-C | 3.9 | 1.1 | 4 | 1 | 5 |

**† – statistical significance compared to the Alvogyl® group ($p = 0.05$); $\bar{x}$ – mean; SD – standard deviation; Med – median; Min-max – minimal-maximal value. For abbrevations see under Tables 2 and 3.**

socket reduced. A similar effect is achieved with curettage, but it is possible to provoke bleeding from the alveolar bone, which could enable the creation of a new, healthy blood clot. After the irrigation, *ie* curettage, topical packaging should suppress subjective discomfort of patients, up to the moment when the bare bone is covered with young granulated tissue. The idea to use hyaluronic acid in the treatment of AO stemmed from its proven qualities: analgesic, reparative and regenerative potential, high elasticity, biocompatibility, biodegradability and low immunogenicity [11, 19].

The results of this study are consistent with the results obtained by McGregor [20] who states that AO usually occurs in the third and fourth decade of life. The results show that AO often occurs in the fifth decade of life, which coincides with the claims of some other authors [1]. While the study was being conducted, there were no any patients of younger age (< 20 years old), so it can be concluded that in this age AO occurs only exceptionally.

Even though some authors [21] state that AO occurs more frequently in the female population, no such results were obtained in this study.

Although the main goal in the treatment of AO is pain relief, it is very important to evaluate other, more objective, clinical parameters that will provide an unbiased comparison between the examined treatment methods. It is obvious that the promotion of wound healing will lead to the reduction of painful sensations [22]. The data obtained in this study indicate that hyaluronic acid significantly faster reduces the number of symptoms and signs of AO compared to relatively frequent use of Alvogyl®. The best results were gained within the group with curettage performed and followed by the use of hyaluronic acid.

The main and most discomforting symptom of AO is severe pain. All the treatment methods investigated in this study decreased the level of pain, but there was a statistically significant difference in pain level throughout the examination period between the patients treated with hyaluronic acid and those treated with Alvogyl®. In all the groups of patients treated with hyaluronic acid a faster reduction of pain level was achieved comparing to Alvogyl® groups.

With the introduction of aminocaproic acid, followed by hyaluronic acid in the treatment of AO in this study, the expected positive results were not obtained. There was no statistically significant difference in terms of the reduction of subjective discomfort of patients whose painful alveoli were, besides with hyaluronic acid, treated with aminocaproic acid, as well. This coincides with some previous studies in which there were attempts to reduce the incidence of AO with local application of tranexamic acid (TXA), a potent antifibrinolytic, but with little or no sucsess [23, 24].

All the results gained in this study prove that hyaluronic acid has a positive influence on the healing process of dry socket, but the exact mechanism of its actual role is yet to be investigated. Based on the results of this study and the available literature, it might be possible to suppose the following: when the dry socket is curetted and bleeding is provoked, a new blood clot may be created; hyaluronic acid placed into this new wound will act as a coagulum stabilizer, preventing its uneven and excessive degradation; during the first phase of wound healing, the fibrin threads form a web that represents a matrix for the platelet clot formation, followed by a production of hyaluronic acid (by simulating different inflammatory mediators, especially interleukin –β and platelet-derived growth factor); the molecules of hyaluronic acid penetrate the fibrin matrix and stimulate cell migration, particularly the fibroblast from the surrounding tissue, and the production of new collagen; [25–29] additional amount of hyaluronic acid in this way probably stabilizes coagulum and has a positive effect on the wound healing.

If the alveolus affected with AO is rinsed with saline until the complete removal of debris, and then hyaluronic acid is applied to it, other positive qualities of hyaluronic acid will come to the fore. Namely, anti-inflammatory, antiedematous and analgesic potential of hyaluronic acid will contribute to wound healing and, consequently, lower pain sensations. One of the advantages of local application of hyaluronic acid is reflected in its adhesive properties which allow its positive qualities to establish healthy coagulum for a longer period of time [30]. Covering completely the bare alveolar bone, hyaluronic acid represents a barrier to irritant influences of external substances.

## Conclusion

The obtained results confirm the hypothesis that hyaluronic acid can be successfully used in the treatment of alveolar osteitis in the manner in this study described. Hyaluronic acid application accelerates the reduction of painful sensations, and also reduces the number of symptoms and signs of alveolar osteitis compared to the use of Alvogyl® alone.

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# Prevalence of metabolic syndrome in Montenegrin patients with psoriasis

## Prevalencija metaboličkog sindroma kod bolesnika sa psorijazom u Crnoj Gori

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**Abstract**

**Background/Aim.** Increasing epidemiological studies suggest the association between psoriasis and metabolic syndrome. The aim of this study was to assess the association of metabolic syndrome and its components with psoriasis in a sample of patients from Montenegro, and to predict the factors that determine the metabolic syndrome. **Methods.** A case-control study was conducted at the Clinic of Dermatology and Venereology, Clinical Center of Montenegro, Podgorica, Montenegro, between January and December 2012. The study group included 101 patients with psoriasis (cases) and 126 patients with the diagnosis of dermatological disease other than psoriasis (controls) consecutively admitted to the same clinic. **Results.** Metabolic syndrome was more prevalent in the psoriasis patients than in the controls (48.5% *vs* 20.6%; OR = 2.99). In addition, the psoriasis patients were significantly more likely to be smokers (OR = 2.16) and were less physically active (OR = 0.58). **Conclusion.** The results of this study demonstrate a strong association between psoriasis and metabolic syndrome independent of psoriasis severity. Patients with psoriasis should be routinely screened for metabolic syndrome and its components.

**Key words:**
**metabolic syndrome x; psoriasis; comorbidity; prevalence; risk factors; montenegro.**

**Apstrakt**

**Uvod/Cilj.** Sve veći broj epidemioloških studija ukazuje na povezanost psorijaze sa metaboličkim sindromom. Cilj ove studije bio je da se proceni veza između metaboličkog sindroma i njegovih komponenti i psorijaze u uzorku bolesnika sa ovom bolešću iz Crne Gore, kao i da se predvide faktori koji determinišu metabolički sindrom. **Metode.** Studija slučajeva i kontrola sprovedena je na Klinici za dermatologiju i venerologiju Kliničkog centra Crne Gore, Podgorica, Crna Gora, u periodu januar–decembar, 2012. godine. U grupi obolelih bio je 101 bolesnik sa dijagnozom psorijaze, dok su kontrolnu grupu činili konsekutivni pacijenti iste klinike koji nisu imali psorijazu (126), već neku drugu kožnu bolest. **Rezultati.** Prevalencija metaboličkog sindroma bila je veća kod bolesnika sa psorijazom u poređenju sa bolesnicima iz kontrolne grupe [48,5% naspram 20,6%; unakrsni odnos (UO) = 2,99]. Bolesnici sa psorijazom bili su statistički značajno češće pušači (UO = 2,16) i manje fizički aktivni (UO = 0,58). **Zaključak.** Rezultati ove studije pokazuju jaku povezanost između psorijaze i metaboličkog sindroma, nezavisno od težine kliničke slike. Bolesnike sa psorijazom treba podvrgnuti redovnom pregledu na prisustvo metaboličkog sindroma i njegovih komponenti.

**Ključne reči:**
**metabolički sindrom x; psorijaza; komorbiditet; prevalenca; faktori rizika; crna gora.**

### Introduction

Psoriasis is one of the most common chronic inflammatory skin diseases associated with a significant morbidity and substantial economic costs to health care systems and patients worldwide [1]. It has a significant impact on patients's quality of life [2–5]. This may lead to unhealthy lifestyle choices, which in turn, increases the risk of several diseases including the metabolic syndrome.

Metabolic syndrome is a clustering of risk factors which include central obesity, hypertension, dyslipidemia [raised triglycerides and lowered high-density lipoprotein (HDL) cholesterol], and raised fasting glucose. This syndrome is a strong predictor of cardiovascular diseases and type 2 diabetes mellitus [6–9].

Increasing epidemiological evidence suggests the association between psoriasis and metabolic syndrome [10–16].

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The aim of this study was to investigate the association of metabolic syndrome and its components with psoriasis, and to predict the factors that determine metabolic syndrome in psoriatic patients from Montenegro.

## Methods

### *Study design and participants*

A case-control study was conducted at the Clinic of Dermatology and Venereology, Clinical Center of Montenegro (CCM), Podgorica, Montenegro, between January and December 2012. The study group included 101 patients with psoriasis (cases) and 126 patients without psoriasis (controls) consecutively admitted to the same clinic.

The inclusion criteria for the cases were age 18 years or older, clinical diagnosis of chronic plaque psoriasis, disease duration of at least six months and not receiving any systemic treatment for psoriasis for at least one month before enrolment.

The control group consisted of patients aged 18 years and more with the diagnosis of dermatological disease other than psoriasis and any autoimmune or chronic inflammatory disease (such as basal cell carcinoma, eczema, vitiligo and infective skin diseases).

The study was approved by the Ethics committee of the CCM. Written informed consent was obtained from all the patients.

### *Study variables*

A standardized questionnaire was used to collect the main characteristics of the patients (age, sex, education, smoking habits, physical activity and family history of psoriasis), and clinical characteristics of psoriasis such as disease onset and duration of the disease.

Education levels were categorized as low (no schooling, incomplete primary school and primary school), middle (three or four years of secondary school), and high (college and university education). Smoking status was categorized as never, former and current. Physical activity in this study was measured with a question: "In your leisure time, how often do you do physical exercise for at least 30 minutes which makes you at least mildly short of breath or perspire?" Those who participated in physical activity four times or more a week were categorized as active, those who exercised less than four times a week but at least 2–3 times a months were categorized as moderately (in)active and those who exercised several times a year or did not exercise at all were categorized as inactive.

The Psoriasis Area and Severity Index (PASI) was used in evaluating the disease severity by physicians. The PASI is a composite index providing an area-weighted assessment of the severity of psoriasis. It can vary from 0 (the lowest score) to 72 (the highest score), with higher scores indicating greater severity [17]. Usually, a PASI score of 10 is used as a cut-off point for mild and moderate/severe psoriasis in daily clinical practice. However, most severe forms of psoriasis are treated at the CCM as a tertiary care hospital and center of excellence for dermatology; therefore, we set a higher criterion of clinical severity and defined psoriasis as mild (PASI $\leq 20$), moderate (PASI 21–29), or severe (PASI $\geq 30$).

Anthropometric measures recorded were weight, height and waist circumference. Measures of weight (kilograms) and height (meters) were assessed using a standard physician's scale and a stadiometer, respectively. Waist circumference (centimetres) was measured at the minimum circumference between the iliac crest and the rib cage. Body mass index (BMI) was calculated as weight in kilograms divided by squared height in meters ($kg/m^2$). According to World Health Organization [18] overweight was defined as BMI $\geq 25$ $kg/m^2$ and $< 30$ $kg/m^2$, and obese as BMI $\geq 30$ $kg/m^2$.

Sitting blood pressure (BP) was measured three times after a 5 min rest. The average of the last two measurements was used for analysis.

Blood was collected following 8 or more hours of fasting for measurement of plasma lipids (triglycerides, total cholesterol and low HDL cholesterol) and glucose.

### *Assessment of metabolic syndrome*

Metabolic syndrome was diagnosed according to the American Heart Association/National Heart, Lung and Blood Institute [modified Adult Treatment Panel III (ATPIII)] criteria [19]. At least 3 of the following conditions had to be present: abdominal obesity presented as large waist circumference (men: $\geq 102$ cm; women: $\geq 88$ cm), elevated triglyceride level ($\geq 1.7$ mmol/L), low HDL cholesterol levels (men: $< 1.0$ mmol/L; women: $< 1.3$ mmol/L), hypertension ($\geq 130/85$ mm Hg or treated hypertension) or elevated fasting glucose level ($\geq 5.6$ mmol/L or treated diabetes).

### *Statistical analysis*

Continuous variables were described by the means and SD, while categorical ones with frequencies and percentages.

The comparison of the groups with/without psoriasis and with/without metabolic syndrome was performed by bivariate analysis, taking $\chi^2$ test and Student's *t*-test where appropriate. Multivariate logistic regression analysis was performed to examine the relationship between psoriasis and potential risk factors. The dependent variable was belonging to cases (psoriatic patients)/controls (non-psoriatic patients).

All statistical analyses were performed using SPSS version 20.0 software (SPSS Inc., Chicago, IL, USA). Statistical significance was set at 2-sided $p < 0.05$.

## Results

The study included 101 patients with the diagnosis of psoriasis (cases) and 126 patients with diagnosis of skin disease other than psoriasis (controls). Table 1 shows the description of the study population. The psoriatic patients were older, and compared with the controls reported more frequently cigarette smoking, physical inactivity and family history of psoriasis. They also had higher means of BMI, blood pressure, and glucose and lower mean of HDL-cholesterol.

Comparing the presence of metabolic syndrome and all its components in the cases and controls (Table 2), we observed that metabolic syndrome, low HDL-cholesterol, elevated blood pressure, high glucose level, and BMI > 25 kg/m$^2$ were more prevalent in the psoriatic patients.

The comparison of the subjects with psoriasis alone with those with psoriasis and metabolic syndrome is shown in Table 3. The psoriatic patients with metabolic syndrome were older, had longer disease duration and a higher BMI. No relationship was observed regarding gender, the prevalence of smoking, disease onset or psoriasis severity (Table 3).

The results of multivariate logistic regression showed that psoriasis patients compared to controls were older, more frequently were smokers and more frequently had metabolic syndrome, while less frequently were physically active (Table 4).

**Table 1**

**Characteristics of the study patients according to the presence of psoriasis**

| Parameters | Psoriasis (n = 101) | No psoriasis (n = 126) | *p* |
|---|---|---|---|
| Age (years), mean ± SD | 50.00 ± 14.39 | 43.70 ± 14.62 | 0.001* |
| Sex, n (%) | | | |
| male | 51 (50.5) | 49 (38.9) | |
| female | 50 (49.5) | 77 (61.1) | 0.080† |
| Education, n (%) | | | |
| low | 8 (7.9) | 15 (11.9) | |
| middle | 57 (56.4) | 59 (46.8) | |
| high | 36 (35.7) | 52 (41.3) | 0.416† |
| Cigarette smoking, n (%) | | | |
| never smoker | 47 (46.5) | 70 (55.6) | |
| former smoker | 12 (11.9) | 25 (19.8) | |
| current smoker | 42 (41.6) | 31 (24.6) | 0.017† |
| Physical activity, n (%) | | | |
| inactive | 64 (63.4) | 50 (39.7) | |
| intermediate active | 32 (31.7) | 52 (41.3) | |
| active | 5 (5.0) | 24 (19.0) | 0.000† |
| Family history of psoriasis, n (%) | 36 (35.6) | 2 (1.6) | 0.000† |
| Body mass index (kg/m$^2$), mean ± SD | 26.81 ± 3.17 | 25.45 ± 5.12 | 0.020* |
| Waist circumference (cm), mean ± SD | 88.98 ± 13.01 | 85.63 ± 13.70 | 0.062* |
| Systolic blood pressure (mmHg), mean ± SD | 146.19 ± 18.88 | 119.02 ± 11.01 | 0.000* |
| Diastolic blood pressure (mmHg), mean ± SD | 93.56 ± 9.96 | 75.29 ± 8.40 | 0.000* |
| Triglycerides (mmol/L ), mean ± SD | 1.31 ± 0.54 | 1.49 ± 1.04 | 0.116* |
| HDL-cholesterol (mmol/L), mean ± SD | 1.03 ± 0.41 | 1.34 ± 0.44 | 0.000* |
| Glucose (mmol/L), mean ± SD | 5.40 ± 1.20 | 4.79 ± 0.83 | 0.000* |

**SD – standard deviation; HDL – high-density lipoprotein; **t*-test; †$\chi^2$ test.**

**Table 2**

**Prevalence of metabolic syndrome and its components in the study patients according to the presence of psoriasis**

| Parameters | Psoriasis (n = 101) | No psoriasis (n = 126) | *p* |
|---|---|---|---|
| Waist circumference ≥ 102 cm (men) and ≥ 88 cm (women), n (%) | 34 (33.7) | 33 (26.2) | 0.220* |
| Body mass index ≥ 25 kg/m$^2$ (overweight + obesity), n (%) | 75 (74.3) | 57 (45.3) | 0.000* |
| Triglycerides ≥ 1.7 mmol/L, n (%) | 20 (19.8) | 31 (24.6) | 0.389* |
| HDL-cholesterol < 1.0 mmol/L (men) and < 1.3 mmol/L (women), n (%) | 74 (73.3) | 49 (38.9) | 0.000* |
| Blood pressure (systolic ≥ 130 mmHg or diastolic ≥ 85 mmHg), n (%) | 82 (81.2) | 33 (26.2) | 0.000* |
| Diabetes mellitus type 2 or glucose ≥ 5.6 mmol/L, n (%) | 44 (43.6) | 17 (13.5) | 0.000* |
| Metabolic syndrome, n (%) | 49 (48.5) | 26 (20.6) | 0.000* |
| Components of metabolic syndrome, mean ± SD | 2.15 ± 0.96 | 1.29 ± 1.30 | 0.000† |

**SD – standard deviation; HDL – high-density lipoprotein; *χ2 test; †*t*-test.**

**Table 3**

**Characteristics of the psoriatic patients according to the presence of metabolic syndrome (MetS)**

| Parameters | Psoriatic patients with MetS (n = 49) | Psoriatic patients without MetS (n = 52) | *p* |
|---|---|---|---|
| Age (years), mean ± SD | 55.22 ± 11.83 | 46.13 ± 15.27 | 0.001* |
| Sex (males/females) n (%) | 24 (49.00)/25 (51.00) | 27 (51.90)/25 (48.10) | 0.767† |
| Age of onset (years), mean ± SD | 42.20 ± 9.36 | 38.06 ± 11.45 | 0.050* |
| Duration of psoriasis (years), mean ± SD | 12.88 ± 7.69 | 8.09 ± 8.05 | 0.003* |
| Smoking (current), n (%) | 20 (40.8) | 22 (42.9) | 0.767† |
| BMI, mean ± SD | 28.39 ± 2.60 | 25.33± 2.95 | 0.000* |
| PASI, mean ± SD | 28.31 ±9.47 | 27.59 ± 13.66 | 0.761* |
| PASI ≤ 20, n (%) | 10 (20.4) | 17 (32.7) | |
| PASI 21–29, n (%) | 17 (34.7) | 14 (26.9) | |
| PASI ≥ 30, n (%) | 22 (44.9) | 21 (40.4) | 0.360† |

**BMI – body mass index; PASI – psoriasis area and severity index; **t*-test; †$\chi^2$ test.**

**Table 4**

**Odds ratios for risk factors in the psoriatic patients *vs* the non-psoriatic patients – results of multivariate logistic regression analysis**

| Variable | Odds ratio | 95% Confidence intervals | *p* |
|---|---|---|---|
| Age | 1.03 | 1.01–1.06 | 0.005 |
| Sex | 0.64 | 0.35–1.19 | 0.161 |
| Smoking | 2.16 | 1.11–4.20 | 0.023 |
| Physical activity | 0.58 | 0.36–0.94 | 0.027 |
| Metabolic syndrome | 2.99 | 1.59–5.62 | 0.001 |

## Discussion

To our knowledge, this is the first study in Montenegro undertaken to assess the association between psoriasis and metabolic syndrome.

Our finding that the prevalence of metabolic syndrome was higher in psoriatic patients than in controls (OR = 2.99) is in agreement with a number of recently published studies [10, 11, 14, 20–24]. However, some studies found no statistical difference in metabolic syndrome between patients with psoriasis and controls [25–27]. It may be attributed to different criteria by which metabolic syndrome was assessed (WHO, NCEP – National Cholesterol Education Program, ATP III), and different population studied [23, 25].

Like in several previous studies [14, 21, 25, 26, 28] we found that metabolic syndrome in psoriatic patients was associated with older age. It was expected knowing that the individual components of metabolic syndrome are more common in the elderly. Older age of patients with metabolic syndrome directly correlates to disease duration: psoriatic patients with metabolic syndrome had a longer disease duration compared with psoriatic patients without this syndrome.

We did not find gender difference in the prevalence of metabolic syndrome in our study that is in accordance with several other studies [14, 26, 29]. However, some authors found that metabolic syndrome was significantly more common in female psoriatic patients [25, 27, 30]. Recently Danielsen et al. [24], in a large population based study, found the strongest association between metabolic syndrome and psoriasis in young women who had up to fourfold increased odds of metabolic syndrome. In contrast, according to Cohen et al. [28] the association between psoriasis and the metabolic syndrome was pronounced in men.

We found no difference in the severity of psoriasis between psoriatic patients with and without metabolic syndrome. No relationship was observed with either the PASI score or when the subjects were classified as having mild, moderate and severe psoriasis. Our finding is in agreement with several other studies [14, 25, 27, 30]. However, Langan et al. [10] demonstrated a strong association between psoriasis and metabolic syndrome, with increasing psoriasis severity being associated with increasing odds of metabolic syndrome. Kim et al. [26] also found that metabolic syndrome was associated with severe forms of psoriasis.

Our study shows that cigarette smoking is an independent risk factor for developing psoriasis (OR = 2.16). Smoking has been linked with psoriasis in numerous studies [31–33]. Patients with psoriasis are more likely to be current or former smokers [33]. According to Naldi et al. [34] the risk of psoriasis was higher in former smokers (OR = 1.9) and current smokers (OR = 1.7) than in never smokers.

Like in previous studies [35, 36] our psoriatic patients had positive family history of psoriasis more frequently than controls.

Concerning the individual components of the metabolic syndrome we found that they were more prevalent in the psoriatic patients compared to the controls, with the exception of hypertriglyceridemia.

Substantial evidence indicates an association between increased BMI and psoriasis suggesting that psoriasis patients are more frequently overweight or obese than the general population [37, 38]. In our study the psoriasis patients were more frequently overweight and obese (75%) compared with the controls (57%) that is in agreement with a number of case control studies [28, 34, 39]. We also found higher BMI in psoriatic patients with metabolic syndrome compared to those with psoriasis alone. This is logical given that BMI and abdominal (central) obesity strongly correlated. However, controversy still exists as to whether obesity causes or is a consequence of psoriasis [31, 40].

Like in several other studies [41–43] we observed that physical activity is negatively affected by psoriasis. Frankel et al. [44] found that vigorous physical activity was independently associated with a reduced risk of incident psoriasis in women. However, another study found no difference in mean physical activity between women with and without psoriasis [45].

Reduced physical activity contributes to increased adiposity, inflammation, oxidative stress, lipids and adhesion molecules, which are physiologically linked to psoriasis and its cardiometabolic co-morbidities [46].

Most of the studies performed showed that psoriasis is associated with atherogenic dyslipidemia [28, 37, 38]. Because psoriasis is associated with obesity and the excess adipose tissue might contribute to dyslipidemia, the exact relation of dyslipidemia in psoriasis is not clear [38]. According to Cohen et al. [28] it is possible that psoriasis is an inflammatory disease that is associated with atherosclerosis, similar to the association of atherosclerosis and some other diseases (e.g., lupus erythematosus and rheumatoid arthritis).

In the present study the psoriasis patients had more frequently low HDL cholesterol, compared to the controls without psoriasis, while we failed to find any statistically significant difference between the two groups in triglyceride levels.

The majority of studies have reported the prevalence of hypertension in psoriasis. Psoriasis patients showed several times higher prevalence of hypertension compared with other dermatological disease patients [21, 38]. A recent meta-analysis

concluded, that the OR for hypertension among patients with mild psoriasis was 1.30 [95% confidence interval (CI) 1.15–1.47] and 1.49 (95% CI 1.20–1.86) in patients with severe psoriasis compared with healthy controls [47].

A number of studies, including a large systematic review and meta-analysis, [6–9, 48] found the increased prevalence of diabetes mellitus type 2 in psoriatic patients, what is in accordance with our finding.

Some limitations within our study design should be considered. Firstly, the data are cross-sectional and do not allow us to determine which developed first, psoriasis or the metabolic syndrome. Secondly, factors which have not been evaluated in this study, including diet, alcohol, mental health or genetic predisposition, may be confounders or effect-modifiers in this relationship. Thirdly, the study was conducted in the tertiary care center and therefore the patients were biased toward having more severe psoriasis.

## Conclusion

This study confirms the association between psoriasis and metabolic syndrome. This finding supports regular screening for metabolic syndrome and its components among all psoriatic patients, regardless of age and disease severity, in order to reduce their risk of serious complications associated with metabolic syndrome. Moreover, the clinicians should be aware of the importance of recommending the patient lifestyle modifications.

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# Correlation of local and systemic expression of survivin with histopathological parameters of cutaneous melanoma

## Korelacija lokalne i sistemske ekspresije survivina sa patohistološkim parametrima melanoma kože

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**Abstract**

**Background/Aim.** Survivin is a multifunctional protein abundantly expressed in tumors of various types, including melanoma. There are still sparse data regarding relationship of melanoma cell survivin expression with accepted histopathological characteristics as well as serum concentration. The aim of this study was to investigate the association of local tumor survivin expression (primary tumor and metastatic lesions) and serum concentration with clinical and histopathological parameters in melanoma patients. **Methods.** The level of survivin expression was determined immunocytochemically in tumor tissue and with ELISA test in the serum of 84 melanoma patients diagnosed from 2009 to 2013 at the Institute for Pathology and Forensic Medicine and Institute for Medical Research at Military Medical Academy, Belgrade, Serbia. **Results.** The intensity of survivin expression was significantly higher in the patients whose tumor had ulceration, higher mitotic index, higher Clark and Breslow stage, that made vascular invasion or spread through lymphatic vessels in primary tumor, and was significantly higher in the patients with metastatic disease. Survivin expression and the number of survivin positive cells in metastatic lesions were significantly associated with the duration of disease free interval (DFI). The patients with high expression score had almost double shorter DFI comparing to those with weak local survivin expression and a small number of survivin+ cells (9 ± 7 *vs* 19 ± 13 months, respectively). The degree of tumor infiltrating lymphocytes presence in tumor tissue was significantly associated with serum survivin concentration, with lowest average level detected in samples of patients with the highest degree of infiltration. Serum survivin concentrations were highest in samples of melanoma patients with IA American Joint Commission on Cancer (AJCC) clinical stage, pT1a histological stage, patients whose tumors were still in horizontal growth phase, without signs of lympho-hematological disease spreading, with the highest number of mitoses and the smallest Clark index. **Conclusion.** Survivin expression in tumor tissue and its serum concetration significantly correlate with clinical and histopathological parameters. Serum levels could be important in initial follow-up as indicators of those patients that would have aggressive local tumor growth and spreading. Survivin determination in tumor tissue is of great significance in estimation of DFI.

**Key words:**
**neoplasm proteins; biological markers; melanoma; histology; immunohistochemistry; sensitivity and specificity.**

**Apstrakt**

**Uvod/Cilj.** Survivin je multifunkcionalni protein bogato ispoljen u tumorima različite vrste, uključujući i melanom. Retki su radovi koji opisuju odnos ispoljavanja survivina u melanomskim ćelijama sa njegovom serumskom koncentracijom kao i sa histopatološkim karakteristikama melanoma. Cilj rada bio je da se ispita udruženost lokalne ekspresije survivina u tumoru (primarni tumor i metastatske promene) i serumske koncentracije sa kliničkim i histopatološkim parametrima kod bolesnika sa melanomom. **Metode.** Nivo ekspresije survivina određivan je imunocitohistohemijski u

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tumorskom tkivu i ELISA testom u serumu 84 bolesnika sa melanomom, dijagnostikovanih u periodu od 2009. do 2013. na Institutu za patologiju i sudsku medicinu i Institutu za medicinska istraživanja na Vojnomedicinskoj akademiji, Beograd, Srbija. **Rezultati.** Intezitet ekspresije survivina bio je značajno veći kod bolesnika čiji su tumori bili ulcerisani, sa visokim mitotskim indeksom, visokim Clark i Breslow indeksom, sa prisutnom vaskularnom i limfnom invazijom, kao i kod onih sa metastatskom bolesti. Ispoljavanje survivina i broj survivin pozitivnih ćelija u metastatskim lezijama bio je značajno udružen sa trajanjem intervala bez bolesti (*disease free interval* – DFI). Bolesnici sa visokim skorom ekspresije imali su skoro dvostruko kraći DFI u odnosu na one sa slabom lokalnom ekspresijom survivina i malim brojem survivin pozitivnih ćelija ($9 \pm 7$ *vs* $19 \pm 13$ meseci). Stepen prisustva tumor infiltrišućih limfocita u tumorskom tkivu bio je značajno udružen sa koncetracijom survivina u serumu, sa najnižim prosečnim vrednostima detektovanim u uzorcima bolesnika sa najvećim stepenom infiltracije. Serumske koncentracije survivina bile su najveće u uzorcima bolesnika sa melanomom IA kliničkog stadijuma *American Joint Commission on Cancer* (AJCC), pT1a histološkog stadijuma, bolesnika čiji su tumori bili u horizontalnoj fazi rasta, bez znakova širenja limfohematogenim putem, sa najvećim brojem mitoza i koji su imali najmanji Clark indeks. **Zaključak.** Ekspresija survivina u tumorskom tkivu i njegova serumska koncentracija značajno korelišu sa kliničkim i histopatološkim parametrima melanoma. Serumski nivo može biti važan kao inicijalni indikator kod onih bolesnika koji bi mogli imati agresivan lokalni tumorski rast i širenje. Određivanje survivina u tumorskom tkivu, kako u primarnom tumoru tako i u metastazama, od velikog je značaja u utvrđivanju trajanja DFI.

**Ključne reči:**
**proteini, onkogeni; biološki pokazatelji; melanom; histologija; imunohistohemija; osetljivost i specifičnost.**

## Introduction

Melanoma is the deadliest form of skin cancer. It is recognized that in humans, the malignant transformation of normal melanocytes into melanoma cells is due to specific genetic predisposition and the influence of environmental factors [1]. Recent studies of the role of survivin in the pathogenesis of malignant tumors were extensive and primarily directed into its role as a biomarker. The latest publications suggest that survivin might have an important role in melanomas.

Survivin is a multifunctional protein with an important role in the inhibition of apoptosis, regulation of mitotic activity and angiogenesis. External and intrinsic pathways of apoptotic signals are interrelated at the levels of effector enzymes called caspases. Caspases 3 and 7 are targets for suppression by a family of endogenous inhibitors of apoptotic proteins (IAPs) that in humans is composed of 8 proteins such as X-IAP, cIAP1, cIAP2, ML-IAP (Livin; K-IAP), Naip, ILP2 (TS-IAP), Apollon/Bruce and survivin) [2]. Survivin is the inhibitor of apoptosis through its effect on various caspases, through binding and inhibition of mitochondrial protein SMAC/Diablo and stabilization of XIAP proteins by blockade of ubiquitination and degradation of proteasome activity.

Under normal physiological conditions, expression of survivin is regulated by the cell cycle and connected to the G2M phase. Survivin is a part of mitotic spindle in connection with tubulin and is important regulator of mitosis. Malignant tumor cells and human fetal cells have increased expression of survivin while it is absent in the mature and well-differentiated human tissues. The results of the most recent investigations show that survivin correlates well with progression and with outcome of various types of solid tumors and hematological malignancies. It has been shown that high concentrations of survivin in malignant tumors induce resistance of tumor cells on chemotherapy and ionizing radiation.

Immunocytochemical studies show that increased expression of survivin is not just a sign of increased mitotic rate in tumor but that its increase is independent from tumor mitotic rate [3]. Furthermore, in the vast majority of tumors, survivin is increased not only during cell mitosis but in all phases of the cell cycle [4–6]. Out of many acquired genetic alterations in melanoma cells, the best described are mutations of BRAF, HRAS or NRAS, increased telomerase activity, as well as defects in signaling cascade and retinoblastoma gene and p53 [1, 4]. Although the mechanisms responsible for increased expression of survivin in the transformation of normal melanocytes into malignant melanoma cells are unknown, the epigenetic, genetic and post-translational mechanisms for regulation of survivin gene are described in other types of cells. The role of survivin in cancerogenesis is not limited only on the inhibition of apoptosis and subsequently chemoresistance of malignant cells but survivin is also important for neoangiogenesis.

In the animal model of melanoma it has been shown that expression of survivin is increased in melanoma cells in comparison with normal melanocytes and that survivin is necessary for viability of melanoma cells and that in these animals exposure of melanocytes to UV light leads into malignant transformation of melanoma cells and their metastatic potential [3].

Increased expression of survivin has been demonstrated in invasive and metastatic melanoma and it is believed that this is the consequence of dysregulation of apoptosis, mitosis and angiogenesis [3]. DNA microarray analysis has shown that survivin gene is one out of four most important genes with increased expression in melanoma. Immunocytochemical studies performed on melanocytic lesions and melanoma cases show different results in survivin expression in relation to the phase of this malignant disease and variation of survival localization in different cell compartments such as cytoplasm, nucleus or in both, nucleus and cytoplasm simultaneously [5–7].

The aim of this study was to assess the values of localized and systemic expression of survivin in melanoma patients as well as the correlation between expression of survivin and disease progression and histopathological parameters [clinical stage, histological stage, growth phase, mitotic rate of the tumor, tumor infiltrating lymphocytes (TIL), Clark's

level, Breslow's thickness, tumor ulceration, histological subtype and tumor regression].

## Methods

The tumor survivin expression was determined immunohistochemically in tissue samples of 84 patients, 48 male, 36 female, aged from 25 to 78 years, diagnosed in the Institute for Pathology and Forensic Medicine, Military Medical Academy (MMA), Belgrade, Serbia in a time interval from 2009 to 2013. Serum survivin concentration was determined by commercial ELISA (R&D Systems, USA) in samples of the same patients, at the Institute for Medical Research, MMA, Belgrade, Serbia.

The level of survivin expression was determined immunocytochemically in tumor tissue and with ELISA test in the serum of 84 melanoma patients of which 48 were male and 36 female, aged from 25 to 78 years, diagnosed at the Institute for Pathology and Forensic Medicine of the MMA, Belgrade, Serbia. All the patients were stratified according to the American Joint Commission on Cancer (AJCC) clinical stage in the following groups: stage I 23 patients, stage II 17 patients, stage III 28 patients and stage IV 12 patients. The control group was composed of 20 patients with dysplastic and 20 patients with classic naevi; for testing of survivin level in the serum, control group was composed of 20 healthy persons without melanoma.

as follows: 0 for no staining, 1+ for weak staining, 2+ for moderate staining and 3+ for strong staining and according to the percentage of positive tumor cells results were evaluated as: 0 – (< od 5%); 1 – (5–25%); 2 – (25–50%); 3 – (50–75%) i 4 – (> 75%) [8].

Blood samples were left to completely coagulate, serum samples were centrifuged (1000 × g) for 15 min and stored at -70°C until tested for human survivin using Human Survivin Immunoassay, R&D ELISA Quantikine USA, cat. no. DSV00.

Statistical analysis of our data was performed with GraphPad Prism software using ANOVA test (with Bonferroni post testing), Mann-Whitney test and Wilcoxon test.

## Results

Tumor tissue samples from melanoma patients showed a significantly higher average survivin expression in comparison with the samples of dysplastic naevi and benign melanocytic lesions of the control group ($p < 0.0001$) (Table 1). Analysis of survivin tissue expression in patients samples according to the AJCC clinical staging showed that even the patients with stage Ia had significant local tumor production. Comparison of survivin expression between the patients with different clinical stages showed the lowest values in stage IA and highest in stages IIIA and IIIC (Table 1). Survivin tissue expression in the melanoma patients stage IIA was

**Table 1**

**Survivin tissue expression according to the investigated parameters ($\bar{x}$ ± SD of intensity score)**

| Clinical stage | Histological grade | Mitoses | Clark | Breslow | Ulceration | Vascular invasion | Spreading | Patients |
|---|---|---|---|---|---|---|---|---|
| IA 1.1 ± 1.5 | pT1a 1.1 ± 1.4 | 0 1.6 ± 1.1 | I nd | < 1 1.3 ± 1.3 | - 1.6 ± 1.1 | - 1.7 ± 1.0 | none 1.5 ± 1.0 | CN 0.3±0.5 |
| IB 1.6 ± 1.1 | pT1b 1.8 ± 1.0 | 1 1.9 ± 0.8 | II 1.0 ± 1.0 | 2 1.6 ± 1.1 | + 2.1 ± 0.8 | + 2.2 ± 0.8 | L 2.0 ± 1.0 | DN 0.4±0.5 |
| IIA 1.3 ± 0.5 | pT2a 1.5 ± 1.2 | 2 1.3 ± 1.2 | III 1.5 ± 1.0 | 3 1.4 ± 0.6 | | | L+H 1.6 ± 1.0 | MP 2.2±1.0 |
| IIB 1.6 ± 0.6 | pT2b 1.0 ± 0.8 | 3 2.5 ± 0.6 | IV 1.9 ± 1.0 | 4 1.7 ± 0.8 | | | | |
| IIC 1.8 ± 0.9 | pT3a 1.3 ± 0.5 | 4 2.5 ± 1.0 | V 2.0 ± 0.9 | 5 2.4 ± 0.5 | | | | |
| IIIA 2.0 ± 0.9 | pT3b 1.5 ± 0.6 | 6 1.0 ± 0.7 | | > 5 1.9 ± 0.9 | | | | |
| IIIB 2.0 ± 0.8 | pT4a 2.4 ± 0.7 | > 6 2.3 ± 0.5 | | | | | | |
| IIIC 2.2 ± 0.7 | pT4b 2.2 ± 0.8 | | | | | | | |
| IV 1.8 ± 0.8 | | | | | | | | |
| IA /IIB * | pT1a /pT4a * | 3 / > 6 * | II /IV * | < 1 5 * | - /+ * | - /+ * | none /L * | CN /MP *** |
| IIA /IIIA * | pT1a /pT4b * | 6 / > 6 * | II /V ** | 3 5 ** | | | | DN /MP *** |
| IIA /IIIC * | pT2b /pT4a * | | | | | | | |
| | pT2b /pT4b * | | | | | | | |
| | pT3a /pT4a * | | | | | | | |
| | pT3b /pT4b * | | | | | | | |

**Spreading (L – lymphatic, L+H – lympho-hematological); Patients (CN – control naevus, DN – dysplastic naevus, MP – melanoma patients); (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.0001$, Mann Whitney test).**

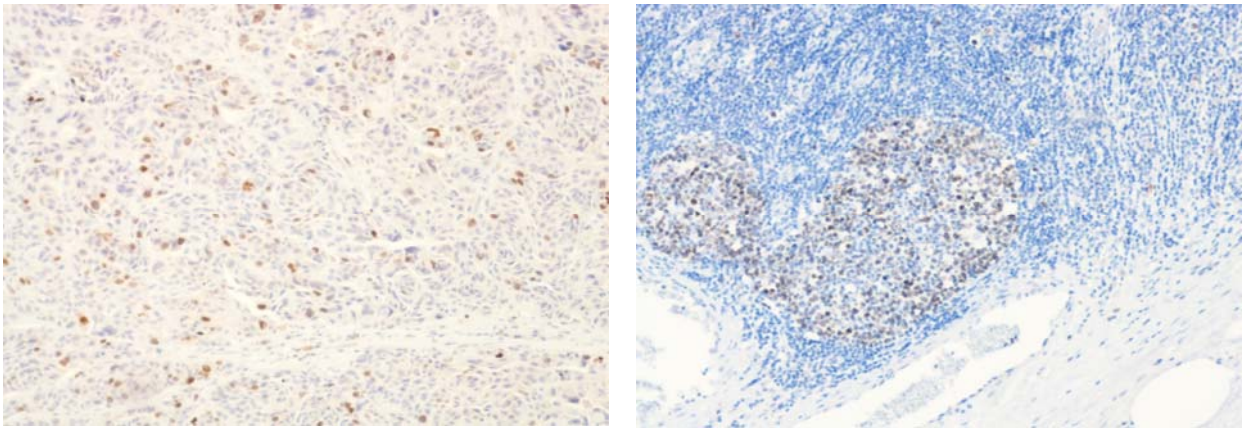
Tissue samples from patients' melanomas were fixed in 4% buffered formalin, dehydrated, cleared in xylen and paraffin impregnated in a Leica ASP300 tissue processor, and paraffin embedded tissue was sliced in 4μ thin tissue sections. The DAKO anti-survivin mouse monoclonal primary antibody 1:100 was used after microwave antigen retrieval in the DAKO retrieval solution pH 6.0. The CSA II DAKO labelling system was also used. DAKO mouse anti IgG2a antibody was used for negative control in the same 1:100 dilution as the primary antibody. The intensity of staining for survivin was determined using the semi-quantitative method

statistically lower than in tumor tissue in the patients in stages IIIA i IIIC ($p = 0.0371$; $p = 0.0428$). We found a graduate and constant increase of survivin tissue expression level following the disease progression reflected in advancing clinical stages. The correlation of values of survivin tissue expression with histological stage of melanoma showed similar results. The lowest average values of survivin expression score were detected in the samples of tumor tissue from the patients with pT1a stage, significantly lower than values found in the patients stage pT4a ($p = 0.0486$) and pT4b ($p = 0.0286$), that had the highest expression of survi-

vin in tumor cells (Table 1). Average survivin expression score in the melanoma tissue of the patients in pT4a and pT4b was significantly higher comparing to the patients in stage pT2a ($p = 0.0276$; $p = 0.0256$) and pT3a ($p = 0.0321$; $p = 0.0418$). Comparing expression of survivin in melanoma tissue with the Clark levels we found that the intensity of survivin expression in Clark level II was significantly lower than in Clark level IV and V ($p = 0.0180$; $p = 0.0102$) (Table 1). Analysis according to the Breslow score showed similar findings with the highest average values of survivin found in the tissue samples of the patients with the Breslow thickness 4–5mm and lowest in the patients with the Breslow thickness < 1 mm. The patients with the highest Breslow score had significantly higher survivin tissue expression in their tumors comparing to the patients that had Breslow less than 1 mm ($p = 0.0353$) and the patients that had Breslow 2–3 mm thick melanomas ($p = 0.0087$) (Table 1). Ulcerated melanomas showed increased survivin expression comparing to non ulcerated ones ($p = 0.0476$) (Table 1). The intensity of expression of survivin was statistically significantly higher in melanomas with the highest mitotic activity (> 6 mitosis *per* $mm^2$) than in melanomas with 3 mitosis ($p = 0.0334$) and melanomas with 6 mitoses ($p = 0.0371$) (Table 1). We found the highest values of survivin expression in melanomas without intratumoral lymphocytic infiltration (TIL) and the lowest values in melanomas with the brisk tumor infiltrating lymphocytes (TILs). This difference was not significant.

There was no significant difference between the intensity of survivin staining and vertical or horizontal phases of melanoma growth, histological type of tumor and the presence or the absence of tumor regression. Positive immunohistochemical staining to survivin in tumor cells of primary and metastatic melanoma is shown in Figures 1a and b.

Analysis of survivin expression and the number of survivin positive cells in metastatic lesions of stage IV patients showed a significant association with the duration of disease free interval (DFI) (Figure 2). The patients with high expression score had almost double shorter DFI comparing to those with weak local survivin expression and a small number of survivin+ melanoma cells (9 ± 7 *vs* 19 ± 13 months).



**a)** **b)**

**Fig. 1 – Survivin positive tumor cells in primary melanoma (a), and metastatic melanoma (b) (survivin original magnification ×100 catalized system amplification – CSA II).**

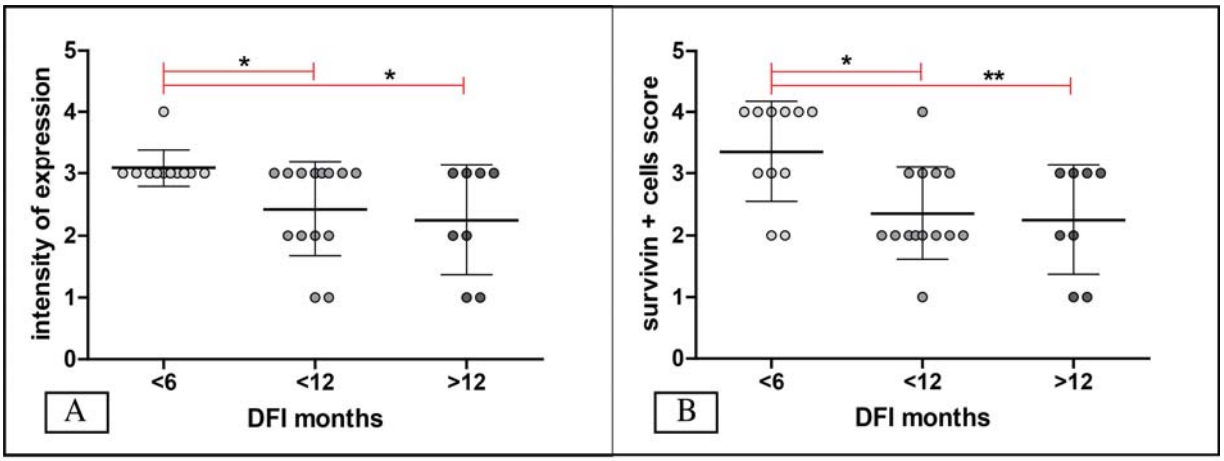



**Fig. 2 – Association of disease free interval (DFI) duration with intensity of survivin expression and the number of survivin positive cells in metastatic melanoma lesions. A – Survivin expression in metastatic tumor lesions according to DFI duration; B – Number of survivin positive tumor cells in metastatic lesions according to DFI duration; ($\bar{x} \pm SD$, intensity score, *$p < 0.05$, ** $p < 0.01$, Mann Whitney test).**

The average survivin serum concentration was significantly increased in melanoma patient samples comparing to samples of examinants with dysplastic naevi, benign pigmented skin shanges and control healthy persons. Analysis of survivin concentration according to clinical stages showed that the patients in IA stage had the highest average value, significantly higher than the patients in stage IB ($p = 0.0363$) (Figure 3A). The melanoma patients in IIIC clinical stage had the lowest average survivin concentration, significantly lower comparing to the patients of IA ($p = 0.0495$), IIB ($p = 0.0286$) and IIIA stage ($p = 0.0286$). Histological staging of primary tumors showed that the patients with pT1a stage had the highest average serum survivin values, similarly to data found when the patients where classified according to the AJCC staging system. The patients with melanomas classified as pT2b had the lowest average survivin concentration, significantly less than those patients whose tumors were pT1a ($p = 0.0424$), pT1b ($p = 0.0294$), pT3b ($p = 0.0286$), pT4a ($p = 0.0159$) and pT4b ($p = 0.0120$) (Figure 3B). Lymphocyte infiltration of primary tumor was significantly associated with survivin concentration. The patients with the highest degree of tumor infiltration by lymphocytes had a significantly decreased average survivin level comparing to the patients with no detectable infiltrating lymphocytes ($p = 0.0114$), or with mild ($p = 0.0098$) or moderate ($p = 0.0036$) degree of infiltration (Figure 3C). The highest survivin concentration was detected in samples of the patients with tumor with the highest mitotic activity, significantly higher comparing to the patients with 1 mitosis/mm$^3$ of tissue ($p = 0.0276$) (Figure 3D). The patients in horizontal growth tumor phase had significantly more survivin values than those in vertical growth phase ($p = 0.0211$) (Figure 3E). Aggressive melanoma spreading according to Clark level was associated with lower survivin serum concentration. The patients with melanomas qualified as Clark II had a significantly increased survivin concentration comparing to those with Clark IV ($p = 0.0290$) or Clark V ($p = 0.0285$) tumors (Figure 3F). Anatomical localization of primary tumor was significantly associated with survivin concentration (Figure 3G). The patients with melanoma localized at the foot had a significantly decreased survivin concentration comparing to the patients with melanoma locali-

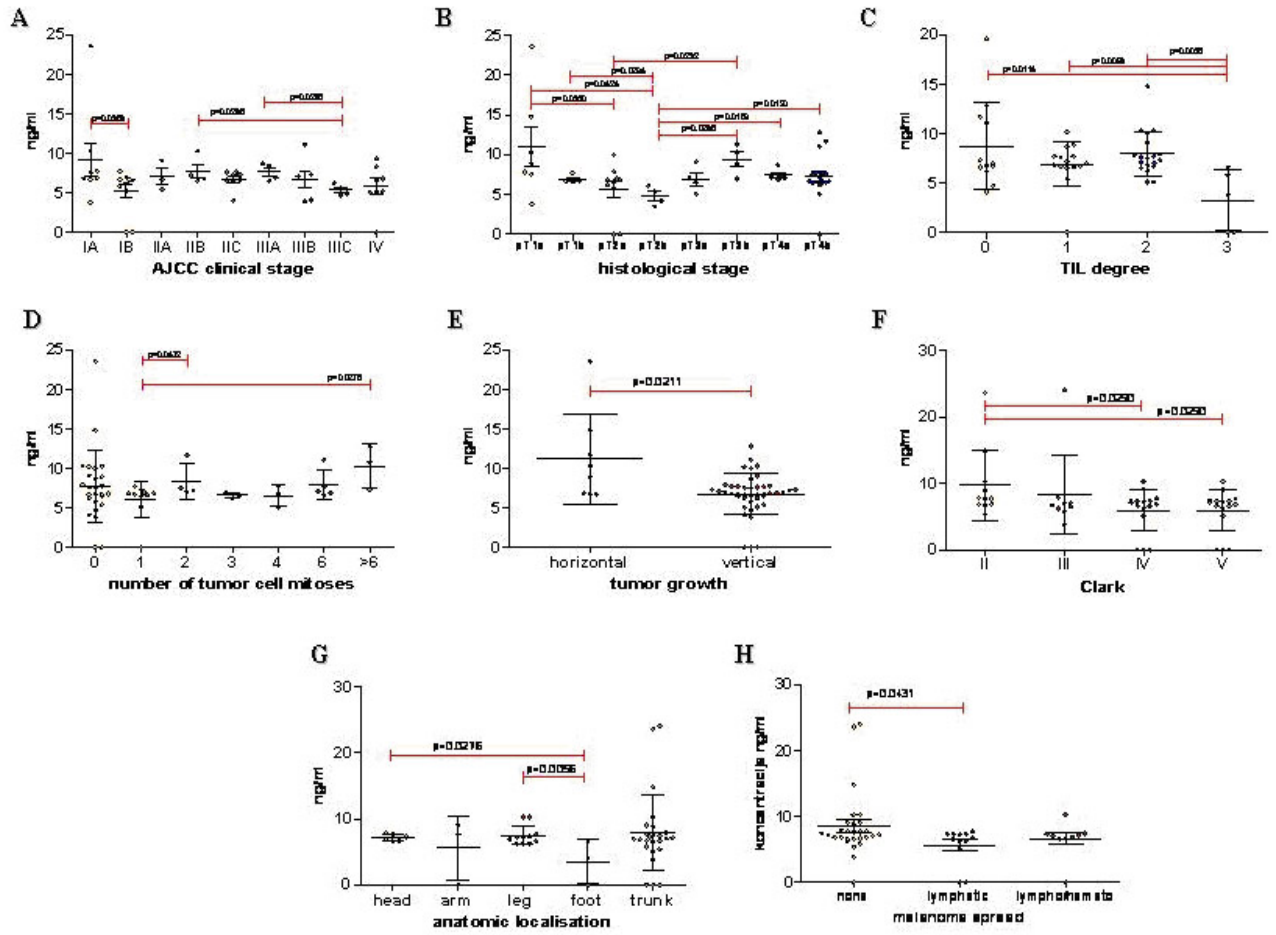



**Fig. 3 – Survivin serum concentration ($\bar{x} \pm SD$ ng/mL) in melanoma patients samples. A – clinical stage of melanoma patients (AJCC); B – histological grade of primary tumor; C – tumor infiltration (lymphocytes degree in primary tumor); D – number of mitoses estimated in tumor cells; E – phase of primary tumor growth; F – Clark score; G – anatomic localization of primary tumor; H – direction of melanoma spread.**

zed at the head ($p$ = 0.0276). Finally, the type of spreading was significantly related to serum survivin values. The patients without histological evidence of tumor spreading through lymphatic or blood vessels had a significantly increased average survivin concentration comparing to the patients with lympho and/or hematological spread tumor ($p$ = 0.0431) (Figure 3H).

Survivin concentration did not differ significantly in the patients with different histological type of tumor with different Breslow score, the presence or the absence of tumor regression, ulceration or metastases. Also, survivin concentration did not differ significantly in the patients that had stable disease or clinical progression, and the patients who survived or died.

Finally, when we analyzed survivin expression *vs* serum concentration of the same patient, we found that the patients with lowest intensity of tissue expression had a significantly higher serum level than those with intensive local tissue expression ($p$ = 0.0153) and also that the patients with the smallest number of survivin+ cells had the highest value of survivin serum concentration (Figures 4A and B).

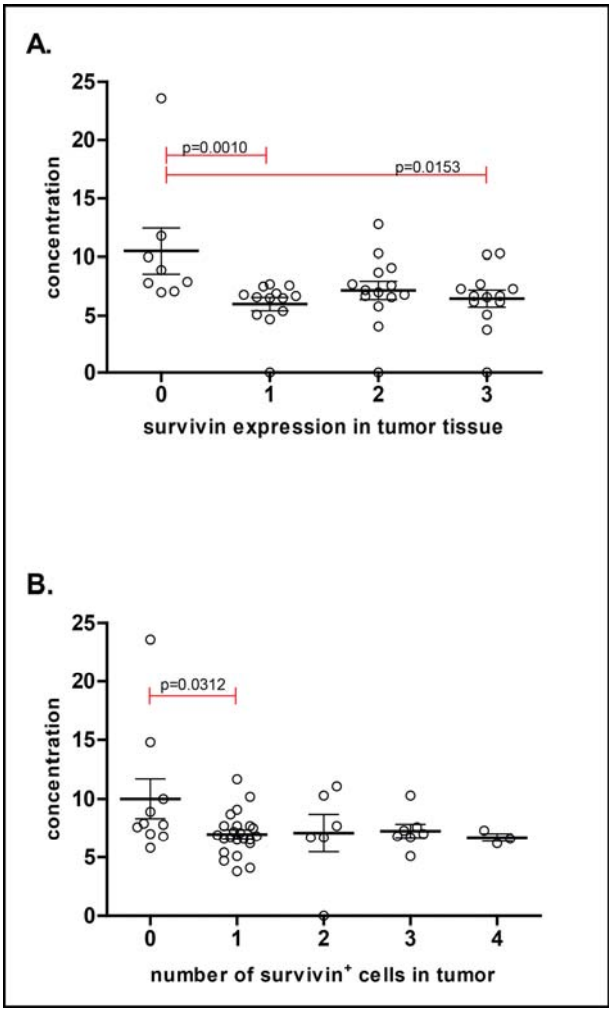



**Fig. 4 – Survivin serum concentration ($\bar{x}$ ± SD ng/mL) in relation to survivin expression in tumor tissue. A – average survivin serum concentration according to the degree of survivin expression in primary tumor tissue; B – average survivin serum concentration according to the degree of survivin positive tumor cells. (Mann Whitney test).**

## Discussion

Survivin is the only member of IAP family that is able to interact with the mitotic apparatus and has several functions, serving as mitotic regulator, cell death inhibitor and a regulator of cell migration/metastasis [9, 10]. It has been proposed that multiple function of survivin are associated with its structure modifications and distinct localization in different cell compartments. Nuclear localization seems to represent survivin potential to control the cell cycle, while cytoplasm/mitochondrial localization are associated with inhibition of the programmed cell death process [11]. Therefore, survivin performs both, cycle-dependent and non-cycle-dependent functions, important in response to hematopoietic and vascular remodeling cytokines. Additionally, there is a mode that cell use to transport survivin inside out, which is stress-induced and executed by exosome extracellular release. This extracellular survivin retains both antiapoptotic and proliferative functions [12–14].

Both clinical and experimental data showed survivin is excessively expressed in human melanoma samples [15], making it the important initial factor for melanoma growth [16], having significant influence on melanoma cell migratory potential [17]. Finally, comprehensive bioinformatic analysis of immunohistochemical and gene array studies of expressed genes and proteins, in order to define melanoma prognosis biomarkers, selected proliferating cell nuclear antigen (PCNA) and survivin among 254 others as priorities for further melanoma biomarkers examination [18].

Our results showed that detectability and average score of survivin expression were lowest in the patients in IA AJCC stage, constantly increasing towards advanced disease stages, with a significantly highest value in the patients that were in stages IIIA and IIIC. These findings are consistent with several other studies. Piras et al. [19] showed that survivin detection increase in melanoma samples with disease progression from 67% in initial stage I to over 90% in patients in stage II. Survivin expression was mainly localized to nuclear compartment and that nuclear expression was significantly associated with melanoma thickness. Samples from primary melanomas of our patients showed predominantly nuclear localization (> 91%), with weak cytoplasmic staining. Analysis of metastatic lesions showed increment of cytoplasmic localization of survivin from 9% to 25%, comparing to primary tumors of the same persons, which implicate different regulation of local survivin production, at least in some melanoma metastases.

Survivin expression in metastatic lesions from stage IV melanoma patients documented both by immunohistochemistry (IHC) and mRNA level was significantly associated with survival [20]. Although stage IV melanoma patients represent a very inhomogeneous population respective to their disease progression and more important, to response to adjuvant therapy, mRNA survivin was detected in almost all (98%) of samples. But there was a significant distinction between numbers of survivin mRNA copies in metastatic lesions. These melanoma patients whose lesions had low survivin mRNA load showed double longer median survival interval than those with high number of

survivin mRNA copy number (24 *vs* 11 months). Immunohistochemical findings correlated well with molecular data, characterized by intense survivin staining in tumors with high mRNA survivin load and *vice versa*. There was no significant correlation between clinicopathology factors and survivin mRNA copy number of tumors from 63 patients.

In the group of the stage IV patients the differences of survivin expression in metastatic lesions were not significantly associated with the differences in survival interval. But, the intensity of survivin expression and the number of survivin positive cells in metastatic lesions were significantly associated with DFI. The patients with a high expression score had almost double shorter DFI comparing to those with weak local survivin expression and a small number of survivin+ melanoma cells ($9 \pm 7$ *vs* $19 \pm 13$ months).

Analysis of survivin expression according to histological grade showed similar results as seen in different clinical stages. In 50% of our patients with pT1a stage survivin was absent and other half had weak local expression detected in a small number of tumor cells. Contrary, all the patients with pT4a and pT4b tumors had strong local expression in numerous tumor cells, significantly higher than other histological stages. A significant correlation of survivin expression with histologic grade and stage was reported not only in melanoma [21] but also in tumor samples of the patients with endometrial carcinoma [22], ovarian carcinoma [23], hepatocellular carcinoma [24] and breast cancer [25, 26].

The intensity of survivin expression was significantly higher in the patients whose tumor had ulceration, higher mitotic index, higher Clark and Breslow stage, that made vascular invasion or spread through lymphatic vessels in primary tumor, and was significantly higher in the patients with metastatic disease. Local survivin expression in tumor tissue was directly associated with the presence of ulceration, at least in experimental condition [27], which was explained by the mutually exclusive mechanism which regulates expression of caspase 3 and survivin. Takeushi et al. [20] showed that high level of survivin expression in metastatic melanoma lesions were associated with shorter median survival interval.

The presence of TIL is considered as independent prognostic factor for melanoma patients. It is reasonable to assume that some TIL is specific for survivin overexpression in tumor tissue. There are several lines of evidence supporting this viewpoint. Patients suffering from cancers of different origin frequently show spontaneous anti-survivin response mediated by specific T lymphocytes [28, 29]. In a study on TIL of melanoma patients, Hadrup et al. [30] identified CD8+107a+ cytotoxic lymphocytes specific for MAGE 1,3,4 NY-ESO-1 antigens. Although strong in tumor expression of survivin, they failed to demonstrate survivin specific T cells. But, when they monitored specific response in a melanoma patient with long term complete remission after interleukin (IL-2) therapy, they identified population of T lymphocytes specific for survivin (HLA-A11 restricted) [31]. These T lymphocytes were detectable during remission period, 7 years. Ellebaek et al. [32] further confirmed that TIL from melanoma patients contain significant distinct populations of CD8+ CD107a+ cells that were cytotoxic to autologous target cells expressing survivin (SUR53-62) in context of HLA-A3+/A11+, and also showed significant potential to lyse autologous tumor cells [32–34]. We did not find any significant difference in tumor tissue survivin expression whether there was no infiltrating lymphocytes or most intensive lymphocyte infiltration. But, the degree of TIL presence in tumor tissue was significantly associated with serum survivin concentration, with lowest average level detected in samples of patients with the highest degree of infiltration.

Serum survivin concentrations were highest in samples of melanoma patients with IA AJCC clinical stage, pT1a histological stage, patients whose tumors were still in horizontal growth phase, without signs of lymphoid hematopoietic disease spreading, with the highest number of mitoses and that had the smallest Clark index. All these indicate that melanoma in the initial phase have abundant local survivin production, underlying the importance of exosome survivin compartment at the disease beginning. Experimental data showed that extracellular survivin is essential in stimulating melanoma cell motility through upregulation of α5 integrin function [35], implicating that significant survivin production could enable early melanoma cells spread, both local and systemic.

In a study on serum anti-apoptotic markers Tas et al. [36] investigated survivin and BCL2 concentration in serum samples of 45 melanoma patients. They did not find any significant difference in survivin values between control subjects and patients, nor between patients according to standard prognostic parameters. But they did find a significantly higher survivin concentration in patients that had lymph node involvement and in patients that had metastatic disease and underwent dacarbazine (DTIC)-based chemotherapy. Contrary, in patients with early stage breast cancer survivin concentration significantly correlated with Ki67 and p53 concentration, histological and nuclear grade of tumor [37].

Finally, when we analyzed survivin expression *vs* serum concentration of the same patients, we found that the patients with the lowest intensity of tissue expression and the smallest number of survivin+ cells had significantly higher serum level than those with intensive local tissue expression. Those differences could be addressed to methods sensitivity, with s 100 EIA kit being more sensitive than immunohistochemistry. But, again, these findings underline that even smallest melanoma lesion, without signs of local survivin expression had significant capacity to secrete survivin, probably in exosome form, and to mediate all tumor biological functions that are important for further growth and disease spreading.

## Conclusion

According to the obtained results we could conclude that local survivin expression in tumor tissue (primary tumor, metastatic tissue) and its serum concentration significantly correlate with clinical and histopathological parameters of melanoma. Serum levels could be important in initial follow up as indicators of those patients that would have aggressive local tumor growth and spreading. Survivin determination in tumor tissue, both in primary tumors and metastases, is of great significance in estimation of disease free interval.

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# The effects of industrial noise of higher spectrum on workers' auditory perception abilities

## Uticaj industrijske buke povišenog spektra na sposobnost slušne percepcije kod radnika

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**Abstract**

**Background/Aim.** Results of previous studies gave support to the idea that machines in power plants produce noise of different levels of loudness and frequency, and that it could cause deterioration of the hearing ability of workers. As a matter of fact, noise-induced hearing loss is the most widespread occupational disease nowadays. As noise is a complex acoustic phenomenon, more factors have to be considered when studying it, such as frequency, intensity and the period of exposure. The aim of this study was to find if there are differences in the absolute threshold of hearing between workers in the factory production lines that are constantly exposed to the industrial noise of higher spectrum and those exposed to the noise of standard spectrum at different frequencies of sound. **Methods.** In the research plan, there were 308 workers employed in the production line of the Factory "Knjaz Miloš", Aranđelovac. A total of 205 of them were working in the conditions of higher spectrum noise (4,000 Hz – 8,000 Hz) and 103 workers were exposed to standard noise spectrum (31.5 Hz – 2,000.0 Hz). The objective measures of noise (frequency and amplitude) were acquired by phonometer, and measures of absolute threshold of hearing for both ears were obtained by audiometer by exposure to nine sound frequency levels. Data were statistically analyzed by establishing the significance of differences between absolute thresholds of hearing for both groups and for all nine frequency levels. **Results.** It was found that the absolute threshold of hearing is significantly higher for the group exposed to high-frequency noise at the 4,000 Hz and 8,000 Hz levels of frequency. **Conclusion.** Reduction of hearing sensitivity is evident for those exposed to higher spectrum noise, which is particularly evident at the higher frequency levels. Employees are often unaware of its effects because they are the results of prolonged exposure. Therefore, working in those conditions requires preventive measures and regular testing of the hearing ability.

**Key words:**
**noice, occupational; occupational exposure; serbia; hearing disorders; auditory perceptual disorders.**

**Apstrakt**

**Uvod/Cilj.** Rezultati ranijih studija govore u prilog ideji da mašine koje u proizvodnim halama prave buku različite frekvencije i jačine, mogu prouzrokovati slabljenje slušne sposobnosti radnika. Gubitak sluha prouzrokovan bukom jedno je od najrasprostranjenijih profesionalnih oboljenja. Buka je složeni fenomen, te je stoga potrebno u razmatranje uzeti više faktora, kao što su frekvencija, jačina i dužina izloženosti. Cilj istraživanja bio je da se proveri da li postoje razlike u apsolutnom slušnom pragu između zaposlenih koji su dugotrajno izloženi buci visokog spektra i onih koji su izloženi buci umerenog spektra, pri različitim frekvencijama zvuka. **Metode.** Istraživanje je obuhvatilo 308 zaposlenih u proizvodnom pogonu preduzeća „Knjaz Miloš" u Aranđelovcu. Ukupno 205 zaposlenih radilo je u uslovima buke povišenog (4 000–8 000 Hz), dok je njih 103 radilo u uslovima buke standardnog spektra (31,5–2 000,0 Hz). Objektivne mere buke (frekvencije i amplitude) dobijene su pomoću fonometra, a mere apsolutnog slušnog praga za oba uha pomoću audiometra, pri izlaganju zvuku na devet nivoa frekvencija. Podaci su analizirani proverom statističke značajnosti razlika u pogledu apsolutnog praga draži za obe grupe, na svih devet frekventnih nivoa. **Rezultati.** Rezultati su pokazali da je apsolutni slušni prag značajno viši kod ispitanika izloženih buci višeg spektra i to na frekventnim nivoima od 4 000 i 8 000 Hz. **Zaključak.** Smanjenje čulne osetljivosti primetno je kod onih radnika koji su izloženi buci višeg spektra, što je naročito vidljivo pri merenjima na višim frekventnim opsezima. Zaposleni su često nesvesni ovih efekata jer su oni posledica dugotrajne izloženosti. Stoga se preporučuju preventivne mere i redovno audiometrijsko testiranje čula sluha.

**Ključne reči:**
**buka na radnom mestu; profesionalna izloženost; srbija; sluh, poremećaji; slušna percepcija, poremećaji.**

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## Introduction

Noise is one of the most frequent environmental, as well as workplace pollution factors [1]. Noise pollution is the third in scope, following air and water pollution [2]. There is no doubt that noise is pervasive and affects more than a billion people. It is also one of the threats to human well-being and work ability [3, 4]. The presence of noise is the result of mass usage of different production appliances, technological processes and audiovisual and explosive devices [5].

Psychologically, noise is unwanted sound and sound is created by rapidly changing air pressure and provoking vibrations in the eardrum [6]. There are two main physical parameters to consider while analyzing the sound. First, there is the question of frequency (of waves) which is perceived as a higher or lower pitch, and the question of the height (amplitude) of waves that we experience as loudness [6]. Besides its intensity and durability, it also has a quality of dynamics in appearance, tone, harmony and resonance [5].

Although we often use physical measures of sound, we are really more interested in the brain`s interpretation of sound stimuli. Interpretation of the sound as a noise depends on physical and psychological factors [1]. It is a complex acoustic phenomenon often manifested as diffuse collection of sounds evoking unpleasantness and annoyance among those exposed to them. More precisely, noise is defined by the sounds in amplitude of frequencies between 16 and 20 000 Hz, with the intensity of 5 to 100/120 dB [7]. In terms of physical quality, sound is defined as the prevalent frequency wavelength of the source of sound. Human auditory sense has a different sensitivity and different threshold for different frequencies [1]. So, on some levels it is considerably higher than on the others. The general rule is that the sensitivity is amplified at medium levels of frequency in the area of human speech and slightly above it, and that it is lower at the beginnings and ends of the intervals of human hearing [7].

Alberti [8] claimed that noise-induced hearing loss happens to be the most widespread occupational disease in industrialized countries. Also, previous studies show that there are some occupations that are particularly hazardous due to exposure to higher levels of noise and that the noise-induced hearing loss is a common symptom [9]. Occupational noise is one of the physical characteristics of the work setting to which the workers have to adapt. Their capacity for adaptation depends on different parameters of noise, its quality and meaning. Also, in office and industrial settings the nature of work tasks and activities are relevant [1]. Further, the consequences of noise exposure are connected with its controllability [10], duration[4], personal characteristics [11] and current state of organism [12]. In cases of chronic noise exposure, which are frequent in industry, there is a decrease in performance combined with a wide spectrum of different behavioral aftereffects [13].

Finally, noise is seen as an environmental stressor [1] that has serious effects on the workers mood [14], attention [15], job satisfaction [16], psychological well-being [5, 7], stress [17] and performance [18]. Nevertheless, researches did not find direct effects of noise on productivity in industry [7]. Bell et al. [1] believe that the influence is indirectly mediated by other psychological consequences of noise. In the work protection practice and in a few studies [4, 9, 19, 20] the focus is on the intensity and duration of sound that generates noise. Despite the humans` huge potential to adapt to noise and to carry on performing quite well [7], it has its costs and consequences on health. Among other negative effects (on the functioning of the autonomic nervous system [1, 5] and on mental health [1, 5]), prolonged work in the conditions of industrial noise of higher spectrum has adverse effects on auditory perception abilities of workers [21]. More than a few researchers confirmed that exposure to noise of high intensity may cause momentary or even permanent hearing damage [9, 21]. This effect is more serious than the effects of noise of standard spectrum, especially when the exposure to noise is recurring [2, 4, 8, 22, 23].

Following the given trend of speculation, our research goal was to find if there were differences between long-time effects of industrial noise of high and moderate intensity at different frequency levels, on audio perceptual abilities of exposed workers performing relatively simple tasks at the production line in the liquid production industry. There was an implicit idea to put the focus on detecting the characteristics of effects of higher spectrum noise (4,000 Hz – 8,000 Hz).

The impairment of the workers` hearing abilities would be detected and inferred based on measuring of baseline amplitude thresholds at different (nine) noise frequencies. It is a well-known fact that, "when a hearing loss occurs at the given frequency, it requires more than the normal amplitude (in dB) for a person to hear that frequency" [1]. It means that amplitude threshold would be greater in the situations of hearing decline. Subsequently, the hypothesis emerged and we expected that there would be significant differences in threshold levels between workers exposed to high and moderate intensity noise with the greater hearing amplitude threshold for subjects exposed to high intensity noise. That should be especially true for higher spectrum noises (from 4,000 Hz up to 8,000 Hz). So, there was an assumption that higher thresholds would be assessed among workers in the group exposed to high-frequency noise with the stronger effect obtained on measures at the high level of frequencies than on standard frequency levels.

## Methods

This study is a part of the longitudinal research with the same procedure administered in 1985, 1995, 2005, and, finally, data presented here were obtained in 2012 at an industrial plant (bottling plants of 1 and 2 factory lines) of the "Knjaz Miloš" corporation at Aranđelovac, Serbia. During the years, some technological innovations were introduced. In the last decade improvements of the working conditions included the reconstruction of the old halls and better sound isolation of the ceiling and walls. Also, as sources of noise, machines were protected with acoustic planking, and the main technological improvement was achieved by providing the gradual slowdown of the transporting conveyor in order to prevent bottles from striking one another and generating unwanted sound. A remarkable fact in the context of health

protection of workers is the availability of silicone antiphons for each worker. Yet, as we unofficially found out, a great percentage of them avoid using this kind of sound protection. Therefore, the problem of noise remains.

In order to test the hypothesis that there are differences in the hearing ability of subjects continually exposed to noise of standard and high intensity, two instruments were used at the different levels of frequency range. Measuring of the objective presence of noise was carried out within the periodical (semi-annual) control of microclimate conditions with phonometer (sound level meter) and analyzer (Iskra-Kranj). Assessment was conducted at the workplace during the working hours. The instrument was positioned at seven working locations. The first measures were obtained in the first production line at the stationary for washing bottles. The second was conducted in the middle of the hall. Subsequently, the measures of noise were obtained from the second production line at the stationary for washing bottles and then at the bottling stationary. At these locations a high-frequency noise was recorded. On the other hand, measures at the compressor station, accumulation plant and on carpenters‘ were of low frequency. The measures of noise intensity are collected in the context of different noise frequencies.

Noise amplitude, subjectively perceived as loudness, is given in dB although it is objectively measured. As a matter of fact, the size of the amplitude wave expresses the energy or pressure in the sound wave, where the greater the pressure, the louder the sound. So, the objective measure of the noise intensity is given in microbars. Nevertheless, decibels (dB) are taken as the basic units of sound being the logarithmic function of microbars [1]. It is also given as a measurement unit in instructions for the instrument. Due to the fact that we use decibels as a measure unit, we bear in mind that the human ear is differently sensitive to sounds at different frequencies and we made some comparisons between these cases.

In this research, we limited our interests to measuring the absolute hearing threshold for different noise frequencies. The human ear can register frequencies between 20 and 20,000 Hz and the absolute threshold is the minimal quantity of energy (physical and chemical) sufficient to produce the first barely recordable sensation [5]. This measure is often used for establishing perception abilities. The inability to hear pure tones below 25 dB indicates hearing problems [7]. The measuring procedure in the study was determined by technical possibilities for measuring and common practice. The procedure was completely safe and not harmful for subjects. The absolute threshold of hearing among workers was determined using the test apparatus audiometer (Siemens RA2000) that registers atlas-audiogram. The minimum volume required to hear each tone was graphed. The audiogram was used for both ears. During the procedure of measuring one ear, the other was blocked and pure tones of controlled intensity were delivered to one ear at a time. The subjects were expected to indicate that they heard the sound by raising their hand.

The measures were taken at frequencies of 31.5; 63; 125; 250; 500; 1,000; 2,000; 4,000 and 8,000 Hz. We conducted 9 assessments for the left and 9 for the right ear *per* worker. So, it took 18 assessments *per* subject. If there were (which was rare) differences in measures for the left and right ear, we took the average value for the hearing threshold parameter. Considering that the measuring procedure takes about 10–15 min for 9 frequency levels, we spent approximately 20 min *per* each subject, which implies that we ideally needed about 100 h for conducting the research. In reality, we conducted the field study for over a month. Data was collected during the month of December. The reason for choosing that period is that it is the month with the most intense production. So, the field study was conducted during the working hours throughout the month of December 2012.

The whole sample consisted of 308 respondents with the exposure period to noise from one to 28 years. They were working in two shifts with a half-hour break. The sample was convenient, but comprising about 90% of all employees in the production line of the “Knjaz Miloš” industrial plant. There were no overt objections to participating in the study, but those 10% of workers not included in our sample were absent from the workplace. So, this relatively low rate of rejection is a consequence of the procedure. As a matter of fact, our study was conducted as part of the regular systematic examination of the workers` health condition. Further, the sample might be representative of the workers who are exposed to noise in the glass production line with similar technological method due to the fact that this is the largest bottler plant with glass packaging in our country. Nevertheless, we could not extrapolate data on other employees in the plant engaged on different jobs and in different work conditions.

According to the results of the measuring of loudness and frequency, workers were surrounded with the constant source of sound, and we agreed that their workplace physical conditions were defined by constant occupational noise. Nevertheless, their working tasks were not cognitively demanding, which is an important factor due to the fact that noise might diminish cognitive performance [7]. The background noise may also interfere with relevant communication among employees. The work process was organized in such a way that the tempo was dictated by the machine (production line) and the communication among workers was not required.

The research sample was divided into the group of 205 workers exposed long-term to noise in the production hall (washing and bottling machines) and a group of 103 workers exposed to noise while working at three locations outside the production line (compressor and accumulator station, machine, electro and carpenters studios). So, we could conclude that the working conditions varied according to which production line they were assigned to and due to the nature of their working tasks, they were exposed to lower or higher levels of noise intensity.

Both groups were approximately equal in age: in the first group the average age was $\bar{x} = 33.2$ and in the second group it was $\bar{x} = 35.4$. Further, the average age of workers in the conditions of acoustic pressure for the first group was $\bar{x} = 10.4$ and for the second group $\bar{x} = 11.4$. Although previously conducted researches on the topic did not find si-

gnificant differences between genders [5], it might be informative to say that 41% of the sample included female workers and 59% were male. A total of 42% of females were working in louder conditions, while 58% of the female sample worked in the conditions of moderate frequency noise. Some of these parameters are given in Table 1.

Statistical analysis of the obtained data was conducted with appropriate techniques. The hypothesis was tested by establishing the existence of statistically significant difference between the means using *t*-test for independent sample.

**Results**

The results of the comparison of auditory perception abilities of the two groups of workers exposed to industrial noise of high and moderate noise levels might be better understood in the context of exposure duration over the years (Table 1). Accordingly, we could see a similar trend in the distribution of workers in the high and moderate intensity noise exposure groups. Nevertheless, the highest percentage of workers in the production hall was in the category of those working from 9 to 12 years (37.1%), compared to the group of workers in the other subsample (working outside the production hall) working from 17 to 20 years were prevalent (48.54%).

Also, the number of female and male workers in both groups was approximately equal and, what is more important, there were no significant differences in the average absolute threshold of hearing (ATH). For female workers at the frequencies of f = 4,000 Hz it was ATH $\bar{x}$ = 39.01 dB, and for male workers ATH $\bar{x}$ = 40.59 dB. For those working in the moderate noise conditions, at the same frequency, it was found that the average ATH was $\bar{x}$ = 35.72 dB for females and $\bar{x}$ = 35.92 dB for males. At f = 8,000 Hz, the difference was almost similar, although values for thresholds were lower. For female workers working in the conditions of higher noise average ATH was $\bar{x}$ = 29 dB, and for males ATH was $\bar{x}$ = 27.23 dB. In moderate noise conditions average ATH was $\bar{x}$ = 28.93 dB for female workers and $\bar{x}$ = 27.55 dB for males (Table 1).

According to the objective parameters obtained by the phonometer for the group exposed to high-frequency noise, noise level near the machines for washing bottles at the production line 1 was 99 dB; noise level in the middle of the production hall was 94 dB; noise level near the machines for washing bottles at the production line 2 was 96 dB; noise level near the machines for bottling at the production line 1 was 96 dB; noise level near the machines for bottling at the production line 2 was 98 dB.

Octave analysis of sound in this hall (production lines 1 and 2) gave results shown in Table 2.

Table 2 shows that noise exceeds the acceptable level in frequency area between 4,000 and 8,000 Hz.

On additional premises placed outside the production hall, the acoustics was analyzed in several positions: noise level in compressor station was 96.5 dB; noise level in accumulation plant was 81.5 dB; in carpenter station 91.5 dB. Octave analysis of noise in these locations is given in Table 3.

Verification of the hypothesis that prolonged work in conditions of industrial noise of higher spectrum (4,000 –

**Table 1**

**Distribution of noise exposure time (in years) among the employees working in the production hall compared with the employees working outside the production hall**

| Exposition time (years) | Work location, n (%) | |
|---|---|---|
| | Production hall | Outside production hall |
| ≤ 4 | 5 (0.29) | 6 (4.85) |
| 5–8 | 60 (29.23) | 18 (17.46) |
| 9–12 | 74 (37.10) | 18 (17.46) |
| 13–16 | 20 (9.76) | 16 (15.53) |
| 17–20 | 17 (8.29) | 20 (48.54) |
| 21–24 | 17 (8.29) | 14 (13.59) |
| ≥ 25 | 12 (5.85) | 11 (10.68) |
| Total | 205 (100.00) | 103 (100.00) |

**Table 2**

**Noise intensity measures for different noise frequencies at three locations outside the production hall**

| Average values of noise frequency amplitude (area) (Hz) | Noise intensity (dB) | | | |
|---|---|---|---|---|
| | Washing mashine – production line 1 | In the middle of the hall | Washing mashine – production line 2 | Bottling machine – production line 2 |
| 31.5 | 32.0 | 31.5 | 34.5 | 30.0 |
| 63.0 | 37.0 | 32.5 | 38.0 | 35.5 |
| 125.0 | 39.5 | 35.0 | 39.5 | 38.0 |
| 250.0 | 43.5 | 42.0 | 45.0 | 41.0 |
| 500.0 | 44.0 | 44.0 | 47.0 | 42.5 |
| 1000.0 | 50.0 | 48.0 | 52.0 | 48.5 |
| 2000.0 | 62.5 | 60.0 | 63.0 | 62.0 |
| 4000.0 | 90.0 | 89.5 | 91.0 | 88.5 |
| 8000.0 | 91.0 | 99.5 | 92.5 | 99.5 |

**Table 3**

**Noise intensity measures for different noise frequencies at three locations outside the production hall**

| Average values of noise frequency (Hz) | Noise intensity (dB) | | |
|---|---|---|---|
| | Compressor station | Accumulation plant | Carpenters` |
| 31.5 | 73.5 | 71.0 | 71.0 |
| 63.0 | 76.0 | 73.0 | 73.0 |
| 125.0 | 78.5 | 78.5 | 77.0 |
| 250.0 | 81.5 | 84.0 | 80.0 |
| 500.0 | 93.0 | 94.5 | 93.5 |
| 1,000.0 | 87.0 | 88.0 | 87.5 |
| 2,000.0 | 72.0 | 74.0 | 71.0 |
| 4,000.0 | 57.5 | 52.5 | 49.0 |
| 8,000.0 | 30.0 | 39.0 | 39.5 |

8,000 Hz) has stronger effect on the deterioration of perception abilities in the domain of the hearing sense, than work in the conditions of noise of standard spectrum (31.5 – 2,000 Hz), when those two categories of noise have approximately equal intensity and duration of exposure chracteristics, was accomplished by analyzing the differences between means obtained for the two groups. Specifically, the effects of noise on perception abilities of workers were verified by testing the significance of differences between the means of absolute threshold of audio perception of the group working in the production hall (where noise intensity exceeded the acceptable level in higher levels of frequency) and group working outside the production hall. Differences were analyzed on each and every nine levels of frequency and the results are given in Table 4.

**Table 4**

**Differences between the means of the absolute thresholds of hearing on each frequency level, for the group exposed to high intensity noise and the group exposed to moderate intensity noise (working outside and in the production hall)**

| Frequency level (Hz) | Mean difference | *p* | df |
|---|---|---|---|
| 125 | 1.17 | n.s. | 307 |
| 250 | 0.15 | n.s. | 307 |
| 500 | 1.20 | n.s. | 307 |
| 1,000 | 0.11 | n.s. | 307 |
| 2,000 | 0.89 | n.s. | 307 |
| 3,000 | 2.08 | n.s. | 307 |
| 4,000 | 7.55 | < 0.01 | 307 |
| 6,000 | 1.80 | n.s. | 307 |
| 8,000 | 11.89 | < 0.01 | 307 |

Data given in Table 4 show that differences in the ATH between the employees working in the conditions that exceed the noise intensity at some frequency levels (in the production hall), and those working in conditions of moderate intensity noise (outside the production hall) are detectable only at the particular frequency levels. As a matter of fact, we could imply that the absolute threshold for the high frequency tones continues to increase as the level of frequency approaches extreme levels (2,000 Hz). So, with the frequency increasing, the difference between absolute thresholds of the two groups rises. Nevertheless, we could notice that the differences of means for ATH hearing were statistically significant at the level of $p < 0.01$ only at the frequency areas of 4,000 Hz and 8,000 Hz. No statistically significant differences between the means were found on other measured levels of frequency. The results of the study gave us support for the main hypothesis that threshold levels of hearing abilities are higher among those industrial workers who are continually exposed to higher noise intensity levels at the higher-frequency spectrum with one limitation (at the frequency areas of 6,000 Hz).

**Discussion**

When comparing general hearing capabilities of the employees working in conditions of different levels of noise intensity at the workplace, there were no data enough to conclude unambiguously that noise intensity itself increases the ATH. Moreover, we could not be sure, if the difference occurs, whether it is temporary or a noise-induced permanent threshold shift. Only by observing thresholds at different levels of noise frequency we can gain better understanding of the phenomenon.

Accordingly, we found that there were tendencies of the ATH to increase with the increment in frequency level. This inclination had some oscillations on lower levels of

frequencies and at the frequency of 6,000 Hz for both groups. So, data give evidence that a decline in hearing sensitivity was obvious in the high tones area and it could also be registered in working conditions of moderate intensity of noise. As a matter of fact, some studies imply that moderate levels of low frequency noise could also have adverse effects (annoyance [24] or sleepiness [25]), but there were no proofs for establishing the link with the physiological reactions and hearing impairment [25]. Analysis of the amplifying trend of the absolute threshold suggest that the difference between the groups will increase toward the higher tones, which was corroborated in this study. Also, the difference between workers exposed to sounds from the medium level of the audibility intensity could be explained in similar manner.

The fact that the main effects of noise found at frequency levels higher than 4,000 Hz could be explained, as in some previous researches, and in accordance with the results of those studies [9, 26, 27]. For example, according to the study of Pražić [21] initial acoustic trauma develops at the level of 4,096 Hz, as well as 4,186 Hz. At those frequencies, audiometrics showed a lowering of the ATH for about 30 db. Nevertheless, some contemporary studies do not support those points of initial trauma. Current researches stands on a position that the critical diapason of frequencies is between 3,500 and 5,000 Hz, depending on noise intensity and other sound characteristics. Also, Rachiotis et al. [9] found that 44% of electro production workers had hearing loss located mainly at 4,000 Hz. Interestingly, they found that smoking might be associated with the prevalence of hearing impairment among workers. Finally, the differences were found among workers exposed to noise of diverse intensity and frequencies spectrum posted in our hypothesis. The hypothesis that the ATH would be different for workers exposed to a variety of noise intensity in two diverse working conditions were proven for the two levels of frequency (4,000 Hz and 8,000 Hz). Sudden increase in absolute threshold in sound perception at the frequency of 4,000 Hz was rather expected, but not in so evident manner, which is especially distinct for the group working in conditions considered as being of higher intensity noise. Possible explanations could be found in the facts connected with the structure of sound as a stimulus. By octave analysis of sound in the production plant, the most intensive level was established at the amplitude between 2,000 and 8,000 Hz. These differences might also be explained in the context of similar studies considering the threshold effects of noise of different spectrum [8, 9, 19, 21–23, 26].

First of all, the perception of the loudness of sound is different at different frequency levels due to the fact that it depends on it [6]. Further, diminishing of the hearing sensitivity at a particular frequency level is detectable by the variation of baseline of amplitude thresholds. When more than usual (normal) amplitude is required for a person to hear particular frequencies (higher amplitude threshold), hearing problems occur. As a measure of declined acoustic sensitivity, scholars often use index of hearing loss, that indicates the number of decibels above the normal threshold required for reaching the new threshold [1]. At last, it is believed that high sound stimulation gradually leads toward the contraction of the cochlear blood vessels to cause hypoxia that slows down the metabolism of sensor cells and their functional processes [21]. Initial hearing loss, first found in the area of frequencies around 4,000 Hz, could be explained by the fact that the sensitivity for sound of this quality is localized in the part of the cochlea with the least blood supply. Constant exposition to the effects of sound affects more and more sensor cells, more and more regions of the cochlea [1, 21].

Alternatively, the results might be seen in the light of the sample characteristics. In our sample, workers working between 4 and 10 years were prevalent (72.59%). Among the subjects with this period of exposure to intensive noise, it was expected to find the effect of "initial acoustic trauma" that is particularly evident in the range of frequencies mentioned above [5]. According to the available, but not sufficiently verified data from the literature [2, 4, 8, 21, 23, 26] the first indices of professional hearing loss arrives later, with initial permanent impairment of auricular sensitivity. In the beginning, augmentation is subtle and the subject does not notice any changes and does not experience any defect. In this particular period, localization of the damage affects the area of high tones that are above the speech zone. The insensitivity of auricular could be found in frequency area between 3,000 and 6,000 Hz. Obvious hearing loss occurs while the hearing sensitivity broadens toward frequencies of 2,000 Hz and further toward 10,000 Hz and more. Further exposure to the hazardous conditions continues toward the more severe damage of auricular function [3, 19, 23, 28].

Consequently, traumatic changes in hearing might be irreversible and for the moment there is no curative. A single action that could be undertaken is to remove the victim from the acoustically perilous situation, so that the damage would not be total. This is the main reason why the more regular periodical audiometrical measuring of the hearing function of workers in risky occupations and jobs is extremely important [5]. This is particularly significant because workers are rarely aware of causality of their deafness [20]. It emerges rather in slow pace and progressively. Although studies continuously demonstrate that occupational noise appears to be the strongest predictor of hearing loss among workers (before aging and other factors), relatively modest attention is dedicated to this problem [9]. Some of the reasons are certainly its complexity and obscurity and some shortcomings of our studies are connected with this fact. We only tackled the issue by trying to keep work characteristics constant and to control the years of exposure, age and gender. The research design of our study is rather simplified due to the idea to prove working condition differences in noise intensity on hearing threshold levels along the spectrum of noise frequencies. Nevertheless, the effects of noise are not only hard to recognize, but they are complex. They cover a wide range of neurological and psychological symptoms. Measuring other physiological parameters along with the hearing threshold, as some studies did [14, 26], might give us a more precise perception of the deteriorating impact of high frequency and intensity noise. Also, the effect of potential stress related to the noise exposure is not controlled. Stress

reactions and stress related disorders are tightly related to prolonged noise exposure [19]. As a matter of fact, stress might interfere between noise and other functions, producing the well known side effects of unwanted sounds [29, 30]. Further, Leather et al. [17], found that lower levels of occupational noise could mitigate the negative impacts of job stress on health and job satisfaction variables. Also, the effects of noise might be modified by different personal and situational variables [12]. Studies often found the mediating effect of the individual characteristics of workers exposed to noise [11] as well as the effect of contextual variables (work characteristics) [14, 23, 25]. For example, in a study of Bjelojevic et al. [11] the lack of concentration and fatigue symptoms related to noise conditions occurred only in the group of introvert subjects, compared with extroverts who also performed faster in noise conditions. Some studies introduced the psychological parameters of depression, hostility, tension [14] with many different indicators of workers' well-being. Gopinath et al. [28] dealt with cardiovascular problems and even death.

On the other hand, while Eleftheriou [23] conducted the research to analyze a wide range of different occupational domains, Fernandez et al. [20] covered a variety of different tasks in the construction process, and there are studies that combine different microclimate features with noise pollution (vibration [29] and other atmospheric parameters [3]). Our research was focused only on the variety of conditions in one plant.

Previous researches show that prolonged noise of high intensity might cause serious health problems, even death [28]. Starting with the possibility to permanently damage the peripheral auditory organ, it can also cause changes in cortical responses to sounds even if there are no external indicies [18]. Therefore, further studies should go into two directions. First, there is an attempt to deal with individual differences that might interfere with noisness working conditions combined with the mental load of the activity undertaken. Second, different effects of noise should be taken into consideration, such as psychological and physiological health issues, as well as the performance. Further, although our study gave similar results as Rachiotis et al. [9] got in their research, they found the potential effect of smoking on hearing loss, the variable that we did not incorporate in our analysis.

Severity, reversibility, delicacy and pervasiveness of the phenomenon are the motives for authors to search for a palliative measures. Some authors propose modification of the workplace setting and equipment, usage of hearing protection devices and others try to find some other preventive solutions. We propose to establish and stick to the practice of regular control of hearing ability changes, as it is the only method of prevention of permanent hearing loss.

## Conclusion

The results of this study support the idea that the noise of high-frequency spectrum, especially in the range 4,000 – 8,000 Hz, causes the augmentation of the absolute threshold of hearing, which is higher for the workers that constantly work in the conditions of intensive noise, compared to those working in the less intensive noise environment. These findings are consistent with the great body of research on the topic of industrial noise and effects of occupational noise. Yet, the possibilities for generalization of our study were limited by the characteristics of the sample and research procedure.

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# Disturbance of oxidative balance in the first trimester of spontaneous abortions

## Poremećaj redoks ravnoteže kod žena sa spotanim pobačajima u prvom trimestru trudnoće

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**Abstract**

**Background/Aim.** Pregnancy is defined as a condition of increased oxidative stress. The aim of this research was to determine the intensity of pro-oxidative processes and the content of GSH, as well as antioxidative enzymes: superoxide dismutase (SOD), catalase (CAT), gluthatione peroxidase (GSH-Px), and the total antioxidative status (TAS) in patients with spontaneous abortions. **Methods.** A total of 120 patients were involved in the research (70 spontaneous abortions and 50 healthy pregnancies). The patients were divided into groups: 35 patients with incomplete and complete spontaneous abortion (group S), 35 patients with missed abortion (group M) and a control group of 50 healthy pregnancies (group N), all of them being in the first trimester of pregnancy. The intensity of lipid proxidation (LPx) was determined with a modified thyobarbituric acid method. The GSH content in erythrocytes was determined by the method based on the amount of non-protein sulfhydryl residues using the Ellman's reagens. The following antioxidative parameters in the blood were measured: SOD – by the method with xanthine oxidase-using commercial RANSOD sets; CAT – by the method of Aebi (the enzyme activity was measured by monitoring the decomposition of $H_2O_2$ at 240 nm); GSH-Px was determined using hydrogen peroxide as a substrate. The TAS was determined using the ferric reducing autioxidant potential (FRAP) method. **Results.** The highest average value of LPx was recorded in the spontaneous abortion group (48.03 pmoL/mg Hgb), and the lowest value was recorded in the control group (26.06 pmoL/mg Hgb). A statistically significant positive correlation between LPx and CAT in the group of patients with missed abortion was also noted ($p < 0.05$, $r = 0.37$). There was a statistically highly significant difference ($p < 0.001$) in SOD and in CAT activitices between the examined patients (groups S and N) and the control group (Student's *t*-test and ANOVA). The highest average value of TAS was recorded in the group S (710.39 μmol/L), while the value in the group M was 277.66 μmol/L. The average value of TAS in the control group was 452.12 μmol/L. Student's *t*-test showed a statistically highly significant difference in the values of TAS between the examined patients (groups S and M) and the control group. **Conclusion.** Determination of the value of pro-oxidative and antioxidative parameters in patients with spontaneous abortion can be the indicator of condition of fetoplacental unit and these analyses can be included in the protocol of the rutine perinatal diagnostics.

**Key words:**
**oxidative stress; abortion, spontaneous; lipid peroxidation; superoxide dismutase; catalase; glutathione; glutathione peroxidase; gravidity.**

**Apstrakt**

**Uvod/Cilj.** Trudnoća se definiše kao stanje povišenog oksidativnog stresa. Cilj rada bio je da se odredi intenzitet prooksidativnih procesa i sadržaja glutationa (GSH), kao i antioksidativnih enzima: superoksid dismotaze (SOD), katalaze (CAT), glutation peroksidaze (GSH-Px) i totalnog antioksidativnog statusa (TAS) kod žena sa spontanim pobačajem. **Metode.** U istraživanje je bilo uključeno 120 žena (70 sa spontanim pobačajem i 50 zdravih trudnica). Žene su bile podeljene u grupe: 35 žena sa nekompletnim i kompletnim spontanim pobačajem (grupa S), 35 žena sa *missed* abortusom (grupa M) i kontrolna grupa od 50 zdravih trudnica (grupa N) u prvom trimestru trudnoće. Intenzitet lipidne peroksidacije (Lpx) određivan je modifikovanom metodom sa tiobarbiturnom kiselinom. Sadržaj GSH u eritrocitima određivan je na osnovu količine neproteinskih sulfhidrilnih ostataka pomoću Ellman-ovog reagensa. Od antioksidativnih parametara u krvi vršeno je određivanje: SOD – metodom sa ksantin oksidazom, komercijalnim setovima RANSOD; CAT – Aebi-jevom metodom (aktivnost enzima merila se praćenjem razlaganja $H_2O_2$ na 240 nm); aktivnost GSH-Px određivana je pomoću vodonik-peroksida kao supstrata. TAS je određivan sa *ferric reducing*

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*antioxidant potential* (FRAP) metodom. **Resultati.** Najveća prosečna vrednost LPx zabeležena u grupi žena sa spontanim pobačajem (grupa S), 48,03 pmoL/mg Hgb, a najniža vrednost zabeležena je u kontrolnoj grupi (26,06 pmoL/ mg Hgb). Takođe, zapaženo je postojanje pozitivne korelacije između LPx i CAT ($p < 0,05$, $r = 0,37$) u grupi žena sa *missed* abortusom (grupa M). Postoji statistički visokoznačajna razlika ($p < 0,001$) u vrednostima SOD i CAT između žena u grupama S i M i kontrolne grupe (*t*-test i ANOVA). Najviša prosečna vrednost TAS zabeležena je u grupi S (710,39 μmol/L), dok je vrednost u grupi M bila 277,66 μmol/L. Vrednosti TAS u kontrolnoj grupi zdravih trudnica iznosila je 452,12 μmol/L. Vrednosti *t*-testa i ANOVA pokazuju da postoji statistički visokoznačajna razlika u vrednostima TAS između žena sa abortusom (grupe S i M) i kontrolne grupe. **Zaključak.** Određivanje vrednosti prooksidativnih i antioksidativnih parametara kod žena sa spontanim pobačajem može biti pokazatelj stanja fetoplacentarne jedinice i ove analize u budućnosti mogu biti uvrštene u protokol rutinske prenatalne dijagnostike.

**Ključne reči:**
**stres, oksidativni; abortus, spontani; lipidi, peroksidacija; peroksid dismutaza; katalaza; glutation; glutation peroksidaza; trudnoća.**

## Introduction

Human reproduction is nowadays no longer considered to be a highly efficient biological process. Before the end of the first trimester of pregnancy, 30–50% of conceptions result in spontaneous abortions. A greater number of conceptions are lost during implantation, while 15–20% of clinically proven pregnancies result in spontaneous abortions [1]. Recurrent, spontaneous abortions occur in 0.5–3% of women in the reproductive period of their life, and 50–60% are idiopathic [2].

Pregnancy is defined as a condition of increased oxidative stress. In normal pregnancy, during the earliest stages, the development of the embryo occurs in a low $O_2$ level environment. This physiological hypoxia in the early gestational sac protects the fetus from teratogenic effects of oxygen free radicals. Once the embryogenesis has been complete, the maternal intervillous circulation is completely established, and the intraplacental concentration of $O_2$ rises [3].

In spontaneous abortions, the development of placental decidual basis is highly disrupted. Some studies show that the systemic and placental oxidative stress have a role in pathophysiological mechanism of spontaneous and recurrent miscarriage occurence [3]. The oxidants induce damage to the endothelium, damage to the placental vascularisation, immune malfunctions, and have a major role in the pathophysiology of idiopathic recurrent miscarriages. In a physiological pregnancy the antioxidative mechanisms compensate for the intensified pro-oxidative processes. The spontaneous abortion is coupled with a heavy disruption of the pro-oxidative-antioxidative homeostasis, *ie* the disruption of the antioxidative defense system. According to the literary data lipid peroxidation and antioxidative protection components are significantly changed during pregnancy as compared to the condition without pregnancy [4, 5]. Most of the authors have established an increase in lipid peroxidation during pregnancy [6]. The study of Burton and Jauniaux [7] indicates an increase in lipid peroxidation intensity in the placenta as well as in the uterus of the pregnant woman.

Some controversial data have been found in the literature concerning the changes of certain antioxidative parameters in pregnancy [8]. The review of all previous research literature data has confirmed that the antioxidative response in pregnancy of healthy women is present to a degree that provides protection from the increased oxidative risk and is a part of physiology of the pregnancy itself [9–11].

The attempts to understand the etiopathogenesis of spontaneous abortions at the molecular level have been under way throughout the world for decades and this research has led to partial explanations of this problem, yet the cause behind most spontaneous abortions remains unknown [12, 13]. By diagnosing the values of antioxidative enzymes, in our study cases of spontaneous abortions, we can acquire additional parameters for diagnosing the idiopathic and recurrent spontaneous abortions.

The aim of this work was to determine the intensity of pro-oxidative processes: the values of lipid peroxidation (LPx) and the contents of the reduced glutathione (GSH), as well as the antioxidative enzymes: superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px), and the total antioxidative status (TAS) in patients with spontaneous abortions.

## Methods

This randomized, prospective, comparative study was conducted on a sample of pregnant women with spontaneous abortions at the Clinical Center of Vojvodina, Department of Obstetrics and Gynecology, in Novi Sad during the 2011–2012 period.

Measuring of lipid peroxidation and antioxidative enzymes in the samples was carried out in the laboratory of the Clinical Center of Vojvodina, Department of Obstetrics and Gynecology in Novi Sad, and the Faculty of Medicine, Department of Pharmacy, University of Novi Sad. Measuring of the glutathione contents and the total antioxidative status was carried out at the Faculty of Medicine, Institute of Pharmacology, Toxicology and Clinical Pharmacology, University of Novi Sad. The prospective research included 120 patients, 70 of which had spontaneous abortions in the first trimester of pregnancy and 50 of which had healthy pregnancies of the same age of gestation. The examinees were divided into two groups: the first groups comprised of 35 patients with incomplete and complete spontaneous abortions (the group S) and the second group comprised of 35 patients with missed abortions (the group M), as well as the control group of 50 healthy pregnancies (group N), all of them being in the first trimester of pregnancy.

The samples of erythrocyte and serum hemolysates from women with spontaneous abortions and healthy pregnancies in the first trimester of pregnancy were collected, prepared and stored. By puncturing the cubital vein, 5 mL of blood was collected into the vacutainer lined with ethylendiamintetra aetic acid (EDTA). The preparation of hemolysates was performed, the serum was separated and frozen. Within the time intervals of 3 months, the collected samples were thawed and smaller series of all of the examined analyses were performed. All of the samples, including the control samples, were analyzed at the same time.

The intensity of LPx was determined by a modified method using thiobarbituric acid [14]. The GSH contents in erythrocytes was determined by method based on the amount of non-protein sulfhydryl remnants using the Ellman's reagent, by colorimetric analysis according to Kapetanović and Mieyal [15]. The following antioxidative parameters in the blood were measured: SOD, using the xanthine oxidase method – the commercial RANSOD kits [16]; CAT using the Aebi's method [17], (enzyme activity was measured by monitoring the decomposition of $H_2O_2$ at 240 nm); the GSH-Px activity was measured using the hydrogen-peroxide as a substrate, by a modified method using cumene hydroperoxide [18]. The total antioxidative status (TAS) was measured using the ferric reducing antioxidant potential (FRAP) method according to Benzie and Strain [19].

This research was approved by the Ethics Committee of the Clinical Center of Vojvodina and Faculty of Medicine, University of Novi Sad. All the patients signed a consent form.

The collected data were presented in the form of median values and relative numbers (the mesure of variability). The statistical significance of noted differences was tested using the parametric (Student's *t*-test) and ANOVA-MANOVA test. Statistical data processing was performed using the programs Excel and PSS.

**Results**

All of the patients participating in our research were uniform according to their age, body weight (BW), body height (BH) and the body mass index (BMI) (Table 1).

The median values of LPx in the examined patients (groups S and M) and the control group (N) were within the range of 26.06–48.03 pmoL/mg Hgb (Table 2). The minimum value of LPx in the patients of all the tree examined groups was 8 pmoL/mg Hgb, and the maximum value was 89 pmoL/mg Hgb. Analyzing the acquired values from the *t*-test, were found a statistically significant difference ($p < 0.001$) in the LPx values between the patients in the examined groups and the control group (N : M; N : S). The analysis of the ANOVA-test results proved the existance of a statistically significant difference in LPx values between the groups M : N; S : N.

The median values of GSH in the exemined patients (groups S, M) and the control group (N) were within the range of 2.68–3.10 μmol/mL Er (Table 3). The minimum value of GSH in the patients of all the three examined groups was 2.48 μmol/mL Er, and the maximum 3.49 μmol/mL Er. Analyzing the acquired values of the *t*-test, we found a statistically significant difference ($p < 0.001$) in the GSH values between the groups S and the control group. Analysis of the results of ANOVA-test proved a statistically significant difference in GSH values between the groups S : N, and S : M.

The median SOD values in the examined patients (groups S, M) and the control group (group N) were within the range of 1116.36–1313.23 IU/g Hgb (Table 4). The minimum value of SOD in the patients of all the three examined groups was 728 IU/g Hgb, and the maximum value was 1811 IU/g Hgb. Analyzing the acquired values of the *t*-test, we found a statistically significant difference ($p < 0.001$) in SOD values

**Table 1**

**The distribution of the examined patients (groups S, M) and the control group (group N) according to the body weight (BW), body height (BH) and body mass index (BMI)**

| Characteristics of the patients | Group S (35) $\bar{x} \pm SD$ | Group M (35) $\bar{x} \pm SD$ | Group N (n = 50) $\bar{x} \pm SD$ |
|---|---|---|---|
| Age (years) | 28.09 ± 6.3 | 29.83 ± 6.1 | 27.46 ± 5.1 |
| BM (kg) | 61.23 ± 11.9 | 65.77 ± 10.7 | 62.55 ± 11.1 |
| BH (cm) | 168.20 ± 5.8 | 167.71 ± 5.6 | 167.78 ± 7.0 |
| BMI (kg/m$^2$) | 21.71 ± 4.7 | 23.42 ± 4.0 | 22.16 ± 3.2 |

**Group S – women with incomplete and complete spontaneous abortion;**
**Group M – women with missed abortion.**

**Table 2**

**The lipid peroxidation (LPx) values in the examined patients (groups S and M) and the control group (group N)**

| Groups | LPx (pmoL/mg Hgb) $\bar{x} \pm SD$ |
|---|---|
| S (n = 35) | 48.03 ± 19.61* |
| M (n = 35) | 44.57 ± 16.48* |
| N (n = 50) | 26.06 ± 14.78 |

**Hgb – hemoglobin;**
***$p < 0.001$.**
**For explanation see under Table 1.**

**Table 3**

**Glutathione contents (GSH) in the examined patients (groups S, M) and the control group (group N)**

| Groups | GSH (μmol/mL Er) $\bar{x} \pm SD$ |
|---|---|
| S (n = 35) | 2.68 ± 0.16* |
| M (n = 35) | 3.10 ± 0.10 |
| N (n = 50) | 3.07 ± 0.14 |

**Er – erythrocytes;**
***$p < 0.001$.**
**For explanation see under Table 1.**

between the group S and the control group (N : S). The analysis of the ANOVA test proved the existance of a statistically significant difference in SOD values between the groups M : N; S : N, and S : M.

The median CAT values in the examined patients (S, M) and the control group (group N) were within the range of 20.40–30.94 nmoL/mg Hgb (Table 4). The minimum value of CAT in the patients of all the three examined groups was 8 nmoL/mg Hgb, and the maximum value was 96 nmoL/mg Hgb. Analyzing the acquired values of the *t*-test, we found a statistically significant difference ($p < 0.001$) in the CAT values between the examined patients and the control group (N : M; N : S). The analysis of the results of ANOVA test proved the existance of a statistically significant difference in the CAT values between the groups M : N, and S : N.

The median GSH-Px values in the examined patients (groups S, M) and the control group (group N) were within the range of 952.89–1291.38 nmoL/mg Hgb (Table 4). The minimum value of GSH-Px in the patients of all the three examined groups was 310 nmoL/mg Hgb, and the maximum value was 3,020 nmoL/mg Hgb. Analyzing the acquired values of the *t*-test, we found a statistically significant difference ($p < 0.05$) in the GSH-Px values between the examined patients and the control group (N : S). The analysis of the ANOVA test proved the existance of a statistically significant difference in GSH-Px values between the groups M : N; S : N.

The median TAS values in the examined patients (groups S, M) and the control group (group N) were within the range of 277.66–710.39 μmol/L (Table 5). The minimum value of total antioxidative stress in the patients of all the three examined groups was 183.31 μmol/L, and the maximum value was 850.84 μmol/L. Analyzing the acquired values of the *t*-test, we found a statistically significant difference ($p < 0.001$) in the TAS values between the group S and the control group (N : M; N : S). The analysis of results of the ANOVA test proved the existance of a statistically significant difference in the TAS values between the groups M : N; S : N; and S : M.

There was a statistically positive correlation between LPx and CAT values ($p < 0.05$, r = 0.37) in the group M (Figure 1).

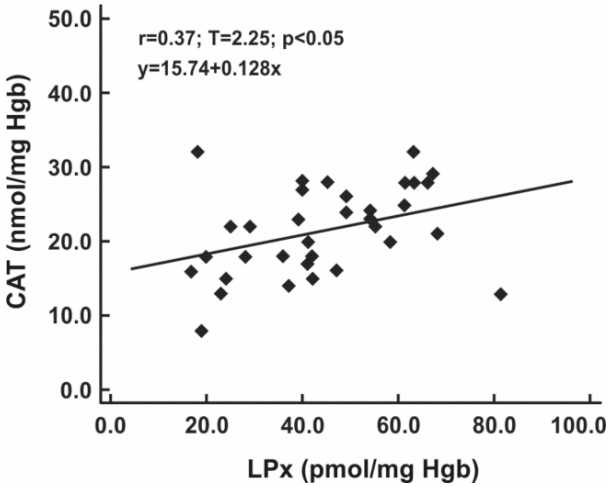


**Fig. 1 – Correlation between lipid peroxidation (LPx) (pmoL/mg Hgb) and catalase (CAT) (nmoL/mg Hgb) in the group M (women with missed abortion). Hgb – hemoglobin.**

## Discussion

The groups of pregnant women included in this study were homogenous in the age structure, body weight and height as well as in the gestation age of the pregnancy (Table 1). These parameters were not a risk factor in the occurence of spontaneous abortions.

Lipid peroxidation is an oxidative damage the lipids, *ie* lipoproteins [20]. Lipid peroxidation exists in the human population both in the early stages of pregnancy and in later gestation within the process of peroxidation [21, 22]. Elevated levels of lipid peroxidation as well as the degradation products can be found in the blood of pregnant women during normal pregnancy, especially in the state of preeclampsia. Analysis of LPx values

**Table 4**

**The values of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GSH-Px) in the examined patients (groups S, M) and the control group (group N)**

| Groups | SOD (IU/g Hgb) | CAT (nmoL/mg Hgb) | GSH-Px (nmoL/mg Hgb) |
|---|---|---|---|
| | $\bar{x} \pm SD$ | $\bar{x} \pm SD$ | $\bar{x} \pm SD$ |
| S (n = 35) | 1313.23 ± 198.74*** | 20.40 ± 9.06*** | 952.89 ± 625.12* |
| M (n = 35) | 1211.66 ± 246.76 | 21.46 ± 5.79*** | 1091.57 ± 849.78 |
| N (n = 50) | 1116.36 ± 175.96 | 30.94 ± 17.71 | 1291.38 ± 813.58 |

**Hgb – hemoglobin; *$p < 0.05$; **$p < 0.01$; ***$p < 0.001$.**
**For explanation see under Table 1.**

**Table 5**

**Total antioxydative status (TAS) values in the examined patients (groups S, M) and the control group (group N)**

| Groups | TAS (μmol/L) |
|---|---|
| | $\bar{x} \pm SD$ |
| S (n = 35) | 710.39 ± 78.34* |
| M (n = 35) | 277.66 ± 58.38* |
| N (n = 50) | 452.12 ± 74.40 |

***$p < 0.001$.**
**For explanation see under Table 1.**

in our study showed the highest median values of LPx, found in the group of patients with spontaneous abortions (the group S – 48.03 pmoL/mg Hgb), while the control group of healthy pregnancies had the lowest median values of LPx (the group N – 26.06 pmoL/mg Hgb) (Table 2). A research conducted by Ozakaya et al. [23] as well as by Simşek et al. [24] yielded the same results. The increase of lipid peroxidation levels immediately before the onset of a spontaneous abortion and significantly lower values following the abortion were measured in the research by Sane et al. [25].

Our research showed a statistically important ($p < 0.05$, $r = 0.37$) positive correlation between lipid peroxidation and an antioxidative enzyme, catalase, in the group of patients with missed abortions (group M) (Figure 1). The increase in lipid peroxidation value was followed by the increase in catalase value.

SOD is a metalloenzyme which catalyzes the dismutation reaction of $O_2^-$ to $H_2O_2$ with the change in the metal ion redox status ($Cu^{2+}$ or $Mn^{2+}$) in the active center [16]. Mammals have two types of this enzyme: cytosolic homodimer CuZnSOD and mitochondrial homotetramere MnSOD. CAT "translates" high concentrations of $H_2O_2$ into the molecular oxygen and water quickly and can be found in peroxisomes.

Glutathione (γ-L-glutamyl-L-cysteinylglycine – GSH) is an a-amino acid as well as a tripeptide, which originates from glutamine, cysteine and glycine [26]. Glutathione has a great number of functions such as metabolic, catalytic, transport and cell protection from oxidation and takes part in the transmembrane transport of amino acids, especially cysteine (and glutamine) which is necessary for protein synthesis [27, 28]. GSH-Px catalyzes the reduction of $H_2O_2$ and other organic hydroperoxides by using glutathione as a reductant, performing the function of protecting cell membrane lipids from oxidative stress [29]. It has also been shown that selenium is an integral part of the enzyme glutathione peroxidase [29].

The highest median SOD value in our study was found in the group of patients with spontaneous abortions (the group S – 1,313.23 IU/g Hgb), while each of the two examined groups had equal median CAT values, and the control group of healthy pregnancies had the highest values (the group N – 30.94 nmoL/mg Hgb). The highest median GSH values in our study was found in the group of patients with missed abortions (the group M – 3.10 μmoL/ml Er), while the control group of patients had the highest median GSH-Px values (the group N – 1291.38 nmoL/mg Hgb) (Table 3).

Biri et al. [30] have examined the antioxidative enzyme values in the placental tissue of patients with normal pregnancies and patients with missed abortions. Their study showed significantly low SOD values, high CAT and GSH-Px values in the patients with missed abortions than in the control group. Some authors claim that there is a correlation between the occurence of spontaneous abortions and low values of selenium in the plasma, as well as low GSH-Px activity. Similar results were found in a research by Ozkaya et al. [23], Prokopenko et al. [31] and Sugino et al. [32].

The Zachara et al. [33] examined the concentrations of Se-GSH and GSH-Px in the blood of patients with spontaneous abortions. The research included patients divided into three groups: the group I of 40 patients with the first trimester spontaneous abortions, the group II of 36 patients with the first trimester normal pregnancies, and the group III of 28 non-pregnant patients. Selenium concentrations in the full blood and plasma of the patients with spontaneous abortions and normal pregnancies were equal, while the selenium concentrations in non-pregnant patients were lower. Glutathione values were significantly higher in the patients with spontaneous abortions than in the patients with normal pregnancies and non-pregnant patients. GSH-Px values were significantly lower in the patients with spontaneous abortions than in the patients with normal pregnancies and non-pregnant patients. The same authors concluded that a decreased activity of antioxidative anzymes and GSH-Px may be a major etiological factor in spontaneous abortion occurrence.

The total antioxidative capacity represents the total capacity of the organism to protect itself from unwanted effects of physiological metabolism. The highest median TAS value in our study was found in the group of patients with spontaneous abortions (the group S – 710.39 μmol/L) (Table 5), while the *t*-test values indicated a statistically highly significant difference in TAS values between the examined patients and the control group. Aksoy et al. [34] found a statistically significant decrease in total antioxidative status in patients with missed abortions in contrast to the patients with normal pregnancies. The decrease in total antioxidative status values in the patients with missed abortions may indicate that the damage of the total antioxidative system in the organism occurs [34]. Panzan et al. [35] researched the antioxidative defense system in the patients with recurrent spontaneous abortions and found a decrease in nonenzymatic antioxidants in the plasma, as well as increased level of oxidative stress and damaged antioxidative defense system.

According to the literature data, the oxidative stress may influence the complete reproductive system of a woman, even in the period of menopause [36–38].

## Conclusion

There was a statistically significant difference in the values of LPx and GSH as well as in the values of antioxidative enzymes activity: SOD, CAT and GSH-Px between the patients with spontaneous aobrtions and the control group.

The highest average value of the total antioxidative status (TAS) was recorded in the group of patients with spontaneous abortion. There was a statistically highly significant difference in the values of TAS between the examined patients and the control group.

Determining the values of pro-oxidative and antioxidative parameters in patients with sponteneous abortions may indicate the condition of the fetoplacental unit, and in the future these analyses could be included in the routine prenatal diagnostic protocol.

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# Can probiotics improve efficiency and safety profile of triple *Helicobacter pylori* eradication therapy? A prospective randomized study

## Mogu li probiotici poboljšati efikasnost i bezbednosni profil trostruke eradikacione terapije za *Helicobacter pylori*? Prospektivna randomizirana studija

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**Abstract**

**Background/Aim.** Some studies suggest the benefit of applying different probiotic strains in combination with antibiotics in the eradication of *Helicobacter pylori* (*H. pylori*) infection. The aim of this study was to evaluate the effect of coadministration of multiple probiotic strains with triple *H. pylori* eradication therapy. **Methods.** This prospective study included 167 patients with dyspeptic symptoms and chronic gastritis who were diagnosed with *H. pylori* infection and randomized into two groups. The group I of 77 patients underwent triple eradication therapy, for 7 days, with lansoprazole, 2 × 30 mg half an hour before the meal, amoxicillin 2 × 1.000 mg *per* 12 hours and clarithromycin 2 × 500 mg *per* 12 hours. After the 7th day of the therapy, lansoprazole continued at a dose of 30 mg for half an hour before breakfast for 4 weeks. The group II of 90 patients received the same treatment as the patients of the group I, with the addition of the probiotic cultures in the form of a capsule comprising *Lactobacillus Rosell-52*, *Lactobacillus Rosell-11*, *Bifidobacterium Rosell-1755* and *Saccharomyces boulardii*, since the beginning of eradication for 4 weeks. Eradication of *H. pylori* infection control was performed 8 weeks after the therapy by rapid urease test and histopathologic evaluation of endoscopic biopsies or by stool antigen test for *H. pylori*. **Results.** Eradication of *H. pylori* infection was achieved in 93.3% of the patients who received probiotics with eradication therapy and in 81.8% of patients who were only on eradication therapy without probiotics. The difference in eradication success was statistically significant, ($p < 0.05$). The incidence of adverse effects of eradication therapy was higher in the group of patients who were not on probiotic (28.6%) than in the group that received probiotic (17.7%), but the difference was not statistically significant. **Conclusion.** Multiple probiotic strains addition to triple eradication therapy of *H. pylori* achieves a significantly better eradication success, with fewer side effects of antibiotics.

**Key words:**
**helicobacter pylori; helicobacter infection; disease eradication; clinical protocols; probiotics; treatment outcome.**

**Apstrakt**

**Uvod/Cilj.** Pojedine studije ukazuju na dobrobit primene različitih probiotskih sojeva u kombinaciji sa antibioticima u eradikaciji infekcije prouzrokovane bakterijom *Helicobacter pylori* (*H. pylori*). Cilj ove studije bio je da se proceni efekat koadministracije multiplih probiotskih sojeva i trostruke eradikacione terapije za *H. pylori*. **Metode.** U ovu prospektivnu studiju bilo je uključeno 167 bolesnika sa dispeptičkim simptomima i hroničnim gastritisom kod kojih je dijagnostikovana *H. pylori* infekcija i koji su randomizirani u dve grupe. Grupa I, od 77 bolesnika, podvrgnuta je trostrukoj eradikacionoj terapiji u trajanju od 7 dana, sa lansoprazolom 2 × 30 mg pola sata pre obroka, amoksicilinom 2 × 1 000 mg na 12 sati i klaritromicinom 2 × 500 mg na 12 sati. Posle 7. dana nastavljena je terapija lansoprazolom u dozi od 30 mg pola sata pre doručka još 4 nedelje. Grupa II, sastavljena od 90 bolesnika, podvrgnuta je istoj terapiji kao i bolesnici grupe I, uz dodatak kulture probiotika u vidu jedne kapsule, koja je sadržala *Lactobacillus Rosell-52*, *Lactobacillus Rosell-11*, *Bifidobacterium Rosell-1755* i *Saccharomyces boulardii*, od početka eradikacione terapije, u trajanju od 4 nedelje. Kontrola eradikacije *H. pylori* infekcije izvršena je 8 nedelja nakon terapije brzim ureaza testom i patohistološkom procenom endoskopskih biopsija ili testom antigena u stolici na *H. pylori*. **Rezultati.** Eradikacija *H. pylori* infekcije postignuta je kod 93,3% bolesnika koji su dobijali probiotik uz eradikacionu terapiju i kod 81,8% bolesnika koji su bili samo na eradikacionoj

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terapiji bez probiotika. Razlika u uspehu eradikacije *H. pylori* bila je statistički značajna, ($p < 0,05$). Učestalost ispoljavanja neželjenih efekata eradikacione terapije bila je veća u grupi I bolesnika koji nisu bili na probiotiku (28,6%), nego u grupi II koja je dobijala probiotik (17,7%), ali razlika nije bila statistički značajna. **Zaključak.** Dodatkom multiplih probiotiskih kultura trostrukoj eradikacionoj terapiji *H. pylori* infekcije postiže se značajno bolji stepen eracikacije sa manje neželjenih efekata primene antibiotika.

**Ključne reči:**
**helicobacter pylori; infekcija, helicobacter; bolest, eradikacija; protokoli, klinički; probiotici; lečenje, ishod.**

## Introduction

*Helicobacter pylori* (*H. pylori*) is a Gram-negative, microaerophilic bacterium that colonizes the gastric mucosa. Since the discovery of *H. pylori* in 1983, numerous studies have shown that this bacterium is a major risk factor in the development of peptic ulcer, chronic gastritis, gastric cancer and mucosa associated lymphoid tissue (MALT) lymphoma. The prevalence of infection in developed countries is 20–50%, while in developing countries it reaches up to 80% [1]. However, the majority of infected people, despite the existence of chronic gastritis has no symptoms, while 10–20% obtain peptic ulcer. In 1–2% of infected persons there is the risk of developing gastric cancer during the lifetime, and in less than 1% the risk of developing gastric lymphoma. Therefore, elimination of infection is a good strategy for the prevention of gastric malignancy. In addition, indications for eradication of *H. pylori* infection are certain extragastric diseases, such as idiopathic thrombocytopenic purpura, vitamin B12 deficiency and unclear iron deficient anemia [2–4].

Standard triple eradication therapy with a proton pump inhibitor (PPI) and two antibiotics (clarithromycin, amoxicillin or metronidazole) is still the most frequent first line therapy. The rising resistance to clarithromycin requires the introduction of sequential or concomitant therapy as the first option, especially for the areas with high resistance to clarithromycin. Levofloxacin in combination with different antibiotics showed a good therapeutic effect as the first, second or third line therapy but arriving problem is the emergence of resistance to fluoroquinolones [5, 6]. In a study from Japan newer fluoroquinolones sitafloxacin, which shows the lowest minimum inhibitory concentration for *H. pylori*, proved to be effective in combination with PPI, amoxicillin and metronidazole as third line therapy [7]. Quadruple therapy with bismuth, like fluoroquinolones, has proved effective as a first line therapy or as rescue therapy. The main reason for the increased resistance to antibiotics is point mutations which accumulate in the *H. pylori* DNA [5].

The main principle of treating *H. pylori* infection is based on the introduction of newer therapeutic regimes which would achieve better therapeutic effects and reduce side effects of antibiotics. A number of studies suggest that lactic acid bacteria, such as *Lactobacillus* and *Bifidobacterium*, increase the effect of eradication of *H. pylori* and reduce side effects when combined with antibiotics. These bacteria inhibit the growth of *H. pylori* by means of the secretion of protein components, or organic acids, reduce the capacity of adherence of *H. pylori* on the gastric epithelial cells, reduce the mucosal inflammation, and stabilize the gastric barrier [8, 9]. Many preparations of probiotics in addition to the strains of *Lactobacillus* and *Bifidobacterium* contain probiotic yeast, such as *Saccharomyces boulardii* [10]. Unlike the studies that support the co-administration of probiotics with the standard therapy, sequential therapy and therapy based on levofloxacin [11, 12], other studies do not support co-administration of probiotics [13, 14]. The literature has evaluated the use of individual probiotic strains (usually *Lactobacillus spp*, *Saccharomyces spp*, *Bifidobacterium spp* and *Bacillus clausii*) and multiple strains. The reason for conflicting results of particular studies is the lack of placebo-controlled trials, a significant heterogeneity in probiotics treatment duration and the time of administration of probiotics with respect to the use of antibiotics and the use of different probiotic strains [2].

The aim of this prospective randomized study was to evaluate the effect of co-administration of multiple probiotic strains (*Lactobacillus spp*, *Bifidobacterium spp* and *Saccharomyces spp*) and triple *H. pylori* eradication therapy.

## Methods

This prospective randomized study included a total of 167 patients with endoscopic and histological findings of chronic gastritis (41.3% or 69 males and 98 or 58.7% females), diagnosed with *H. pylori* infection in the period of one year (during 2014). The patients had symptoms of upper dyspepsia (nausea, epigastric pain, postprandial bloating, belching, heartburn), without alarming symptoms (bleeding, anemia, weight loss). The criteria for exclusion of patients from the study were: younger than 18 years, the use of antibiotics, proton pump inhibitors (PPI) and H2 receptor antagonists in the last two weeks (according to Maastricht IV consensus report [4]), allergy to penicillin and any other administered drugs, previous eradication of *H. pylori*, pregnancy, lactation, previous gastric surgery, gastric malignancy, peptic ulcer, peptic pyloric stenosis, reflux esophagitis and significant comorbidity with the presence of malignant disease and/or bad general condition.

Patients were randomized into two groups. The group I of 77 patients (27 or 35.1% males and 50 or 64.9% females), average age 56.2 ± 14.8 years (range 21 to 80 years) were treated with triple eradication therapy of *H. pylori* infection, within 7 days, with a proton pump inhibitor lansoprazole 2 × 30 mg half an hour before a meal, amoxycillin 2 × 1.000 mg at 12 hours and clarithromycin 2 × 500 mg *per* 12 hours. After the 7th day of the therapy, lansoprazole was continued in a dose of 30 mg for half an hour before breakfast for 4 weeks. The group II of 90 patients (42 or 46.7% males and

48 or 53.3% females), average age 56.3 ± 14.8 years (range 20 to 82 years) underwent the same therapy as well as the patients in the group I (lansoprazole, amoxicillin and clarithromycin in the same dose and duration), with the addition of probiotic cultures in the form of a capsule during the lunch, from the beginning of eradication therapy within 4 weeks. One capsule contains 5 billion live probiotic lyophilized microorganisms: *Saccharomyces boulardii*, *Lactobacillus Rosell*-52, *Lactobacillus Rosell*-11 and *Bifidobacterium Rosell*-175. The lyophilization process makes that the viability of probiotic microorganisms in the composition is maintained. Upon release from the capsule microorganisms become active again in the intestine where their effect is required.

The initial diagnosis of *H. pylori* infection was done by biopsy tests during upper gastrointestinal (GI) endoscopy (rapid urease test and histopathological examination). Upper GI endoscopy was performed with videoendoscopy OLYMPUS EXERA CV-165 and OLYMPUS EXERA CLV-180. Standard forceps was used for taking endoscopic biopsies from the antrum and corpus of the stomach. The result of rapid urease test was read after 1 h, 3 h and 24 h. The test was considered positive if the substrate color changed from yellow to red. For identification and semiquantitative assessment of *H. pylori* by histological examination, endoscopic biopsy specimens were fixed in a standard solution of 10% formalin for 24 hours. Subsequently, the tissue was routinely processed, embedded in paraffin, cut at a microtome to the thickness of 4 microns. After dewaxing and processing in graduated alcohol, selected sections were stained with hematoxylin-eosin (HE) and the Giemsa method.

*H. pylori* infection eradication control was done 8 weeks after the treatment with upper GI endoscopy, rapid urease test, and histopathologic evaluation of staining by Giemsa. Eradication of *H. pylori* infection was considered successful in case of negativity of both tests to *H. pylori* (urease test and histological examination). In a small number of patients who did not agree with the second endoscopy (5 or 6.5% of the patients of the group I, and 13 or 14.4% of the patients of the group II) assessment of the success of eradication was carried out by stool antigen test for *H. pylori*. Stool antigen test is a qualitative immunochromatography test type CER-TEST BIOTEC SL. The test relies on the presence of nitrocellulose membranes coated with mouse monoclonal antibodies against *H. pylori* in the test line, in the field of results, and with rabbit polyclonal antibodies to a specific protein, in the control line. Anti-*H. pylori* antibodies present on the membrane (test line) bind dye conjugate and the red line used to read the results becomes visible.

Statistical analysis of the results was carried out with the help of tests for the arithmetic mean, standard deviation, Student's *t*-test, Fisher's exact test and $\chi^2$ test. Differences between individual parameters were considered significant at *p* values less than 0.05.

## Results

Among the groups of patients there were no statistically significant differences found in gender, age, smoking status, use of nonsteroidal anti-inflammatory drugs (NSAIDs) and acetyl salicylic acid (ASA), as well as in terms of comorbidity (Table 1). Regarding the comorbidity in the group I there was hypertension (14 or 18.2%), diabetes mellitus 3 (3.9%), hypothyroidism (3 or 3.9%), hyperthyroidism (1 or 1.3%) and chronic obstructive pulmonary disease (3 or 3.9%). Within the group II of the patients there was hypertension (22 or 20%), diabetes mellitus (8 or 8.9%), hypothyroidism (2 or 2.2%) and chronic obstructive pulmonary disease (COPD) (1 1.1%).

*H. pylori* infection eradication was achieved in 63 out of 77 (81.8%) patients of the group I and in 84 out of 90 (93.3%) patients of the group II. The difference in the success of eradication between the groups of patients was statistically significant, $\chi^2 = 5.16 > \chi^2$ (1 and 0.05) = 3.84, $p < 0.05$, adds ratio (OR) = 3.1 (1.04 < OR < 9.63), confidence interval (CI) 95%; relative risk (RR) = 1.14 (1.01 < RR < 1.28), CI 95% (Table 2).

**Table 1**

**Basic characteristics of the examined groups of patients**

| Parameter | Group I (n = 77) | Group II (n = 90) | *p* |
|---|---|---|---|
| Gender (male/female), n (%) | 27 (35.1)/50 (64.9) | 42 (46.7)/48 (53.3) | ns |
| Age, $\bar{x}$ ± SD | 56.2 ± 14.8 | 56.3 ± 14.8 | ns |
| Smokers, n (%) | 10 (13) | 13 (14.4) | ns |
| NSAID users, n (%) | 24 (31.2) | 26 (28.9) | ns |
| Comorbidity, n (%) | 24 (31.2) | 31 (34.5) | ns |

**NSAID – non-steroidal anti-inflammatory drugs;**
**Group I – patients treated with triple eradication therapy of *Helicobacter pylori* infection (lansoprazole, amoxycillin, clarithromycin); Group II – patients treated with the same triple eradication therapy as patients of the Group I + probiotic cultures; n – number of patients; *p* – significance ($\chi^2$–test).**

**Table 2**

**Comparison of success of eradication of *Helicobacter pylori* (*H. pylori*) infection between the examined groups of patients**

| Group | Eradicated, n (%) | Not eradicated, n (%) | Total, n (%) |
|---|---|---|---|
| Group I | 63 (81.8) | 14 (18.2) | 77 (46.1) |
| Group II | 84 (93.3)* | 6 (6.7)* | 90 (53.9) |
| Total | 147 (88) | 20 (12) | 167 (100) |

**For explanations see under Table 1. *$p < 0.05$ ($\chi^2$ test).**

Comparing the success of eradication between the genders in the group I, infection eradication was achieved in 23/27 (85.2%) women and in 40/50 (80%) men. The difference in the success of eradication by gender was not statistically significant in the group I of patients, $p < 0.05$, OR = 1.44 (0.35 < OR < 6.21) CI 95%; RR = 1.06 (0.86 < RR < 1.31) 95% CI. In the group II *H. pylori* infection eradication was achieved in 39/42 (92.8%) males and 45/48 (93.7%) women. The difference in the success of eradication of *H. pylori* by gender was not statistically significant in the group II, $p < 0.05$, OR = 0.89 (0.13 < OR < 5.94), CI 95%; RR = 0.99 (0.89 < OR < 1.11), 95% CI.

While taking eradication therapy in the patients of the group I there were nausea (in 9 11.6% the patients), metallic taste in the mouth (5 or 6.5%), headache (3 or 3.9%), diarrhea (3% or 3.9) and epigastric pain (3 or 3.9%). Also, in the patients of the group II during eradication therapy there were nausea (7 or 7.7%), metallic taste in the mouth (2 or 2.2%), headache (2 or 2.2%), diarrhea (1 or 1.1%) and epigastric pain (3 or 3.3%). With individual comparative analysis of the incidence of adverse effects there was no statistically significant difference observed between the group I and the group II. In the total of 22 (28.5%) patients of the group I and in 15 (16.7%) patients of the group II adverse effects of the therapy occurred. The total difference in respect of adverse effects of eradication therapy was also not statistically significant between the groups of patients, $\chi^2 = 3.35 < \chi^2$ (1 and 0.05) = 3.84, $p > 0.05$, OR = 1.99 (0.89 < OR < 4.46), CI 95%, RR = 1.16 (0.98 < RR < 1.37), 95% CI (Table 3). In both groups of the patients adverse effects in any case did not lead to discontinuation of the therapy.

**Table 3**

**Analysis of the incidence of adverse effects in the examined groups of patients**

| Adverse effect | Group I (n = 77) | Group II (n = 90) | *p* |
|---|---|---|---|
| | n (%) | n (%) | |
| Nausea | 9 (11.7) | 7 (7.7) | ns |
| Metallic taste | 5 (6.5) | 2 (2.2) | ns |
| Headache | 3 (3.9) | 2 (2.2) | ns |
| Diarrhea | 3 (3.9) | 1 (1.1) | ns |
| Epigastric pain | 3 (3.9) | 3 (3.3) | ns |
| Total | 23 (29.9) | 15 (16.7) | ns |

**For explanations see under Table 1.**

We compared the frequency of lagging dyspeptic symptoms after eradication therapy. Dyspeptic symptoms maintained in 19 (24.6%) of the patients of the group I and in 21 (23.3%) of the patients of the group II. The difference was not statistically significant, $\chi^2 = 0.04 < \chi^2$ (1 and 0.05) = 3.84, $p > 0.05$, OR = 0.93 (0.43 < OR < 2.01) CI 95%, RR = 0.95 (0.55 < RR < 1.63), CI 95%.

## Discussion

In recent years, many alternative treatments of *H. pylori* infection have been studied because of the phenomena of resistance to antibiotics and occurence of adverse effects of the application of several antibiotics simultaneously, as in case of concomitant therapy. One attempt to solve this problem is the use of probiotic cultures. Certain initial studies had promising results, but many issues remained unresolved. Namely, we do not know the exact mechanism of probiotics' action. Different probiotic strains can cause different host responses, depending on the immune status of the host. Studies on animal models suggest that probiotic bacteria establish immune regulation by controlling the balance of proinflammatory and anti-inflammatory cytokines, which can lead to reduction of the activity of inflammation in the stomach. Previous studies had shown that *Lactobacillus salivarius* inhibits the secretion of gastric epithelial cells stimulated by *H. pylori* over the interleukin-8. It also leads to increased production of secretory IgA in the intestinal epithelium, which enhances the mucosal barrier. The non-immunological mechanisms of probiotics action are the product of antimicrobial substances, competition with *H. pylori* to adhesion receptors, stimulation of mucus production and stabilization of the mucosal barrier. Some strains, such as *Lactobacillus plantarum 299V* and *Lactobacillus rhamnosus GG* induce mucin gene expression. Certain strains of *Bifidobacterium* release antimicrobial protein substances that act against *H. pylori* [15].

Latest studies show that the strain of *Lactobacillus pentosus LPS16* through the production of lactic acid achieves the bactericidal effect against *H. pylori* and that the bactericidal effect is identical to the antibiotics sensitive and resistant strains of *H. pylori*. It has been shown that lactic acid have a higher bactericidal activity than the acetic acid and hydrochloric acid. Therefore, the application of *Lactobacillus pentosus LPS16* proved to be useful in prevention and in the treatment of *H. pylori* infection, especially in cases of *H. pylori* resistance to many antibiotics [9].

In most studies on animal and human models different strains of *Lactobacillus* were tested (*L. Jahnsonii La1*, *L. rhamnosus GG*, *L. casei*, *L. acidophilus*, *L. brevis*, *L. gasseri OLL2716*, *L. reuteri*), *Bifidobacterium* strains (*B. lactis*, *B. animalis*, *B. breve*) and probiotic yeast *Saccharomyces boulardii*. The diagnosis of *H. pylori* infection and the effect of probiotics on *H. pylori* gastritis is usually assessed by serological tests, rapid urease test, urea breath test, stool antigen test and histological examination of gastric biopsies [16, 17].

In our study we examined the effect of probiotic cultures to *H. pylori* infection, which contained *Lactobacillus Rosell-52*, *Lactobacillus Rosell-11*, *Bifidobacterium Rosell-175*

and *Saccharomyces boulardii*. For the initial diagnosis of *H. pylori* infection, we used rapid urease test and histological examination of gastric biopsies. In order to assess the eradication success we used rapid urease test, histological examination of gastric biopsies and stool antigen test for *H. pylori*. Qualitative immunochromatography test antigen in stool, which we used, has high sensitivity, over 94%, and specificity over 99%.

Our study showed a significantly higher success in infection eradication in the group of patients treated with triple eradication therapy combined with probiotic cultures than in the group without probiotics (93.3% *vs* 81.8%), $p < 0.05$. The success of eradication by gender was not statistically significantly different in the examined groups of patients. Despite the fact that in a small number of patients in the group with probiotic there were side effects of eradication therapy manifested (17.8%) compared to the group without probiotics (28.6%), the difference was not statistically significant ($p > 0.05$).

One of the first clinical studies on the effects of probiotics on *H. pylori* eradication is a study by Canducci et al. [18], which shows that *Lactobacillus acidophilus LB* significantly enhances the effect of eradication, but does not diminish the adverse effects of antibiotic therapy. The open and uncontrolled study by Sheu et al. [19] shows that *Lactobacillus acidophilus La5* and *Bifidobacterium lactis Bb12* increase the effect of eradication and decrease adverse effects of triple eradication *H. pylori* therapy. Studies of some authors show that the application of *Lactobacillus acidophilus La5* together with *Bifidobacterium lactis Bb12* before quadruple secondary line therapy after failure of triple eradication therapy enhances the effect of eradication. The benefit of the application of probiotic strains seems not to depend on the type of applied strains [1].

A recent meta-analysis of 14 randomized controlled trials has shown that the addition of probiotics to standard triple therapy improves eradication effect, established by both intention-to-treat (ITT) analysis (OR = 1.67, 95% CI: 1 : 38 to 2 : 02) and per-protocol (PP) analysis (OR = 1.68, 95% CI: 1 : 35 to 2 : 08). Also, most studies have proved a positive effect of probiotic supplementation on the adverse effects of antibiotics. In particular, the frequency of diarrhea caused by antibiotics was reduced with the correction of intestinal dysbiosis by probiotics. However, the authors conclude that the results should be interpreted with caution because the studies are designed differently with different antibiotics and different probiotic strains being used [20]. Padoł et al. [21] suggest that when assessing eradication effect of triple therapy, one should take into account the distribution of CYP2C19 polymorphism, which may be of greater importance in relation to the manner of use of probiotics (single or combined strains) and in relation to the length of triple therapy (7 or more days).

After completing the triple eradication therapy, maintenance of dyspeptic symptoms was slightly more common in the group without probiotics (24.7%) than in the group with probiotics (23.3%) but the difference was not statistically significant. Maintenance of dyspepsia after termination of antibiotic therapy could be linked to the success of eradication but also with the application of probiotics. It would be expected from probiotics to have positive effect on reducing of dyspeptic symptoms, while causing dyspeptic symptoms by probiotics would be less likely. In fact, studies have shown no adverse effects of probiotics, considering that some strains of probiotics such as *Lactobacilli* and *Bifidobacteria* are part of a normal gastrointestinal microbiota [22–24].

## Conclusion

Our study shows that triple *H. pylori* eradication therapy achieves a statistically significantly better eradication success combined with probiotic strains *Lactobacillus Rosell-52*, *Lactobacillus Rosell-11*, *Bifidobacterium Rosell-175* and *Saccharomyces Boulardii*. Also, there are fewer adverse effects of antibiotic therapy by using probiotics although the difference is not statistically significant. To come to more accurate conclusions about the effects of probiotic strains in the treatment of *H. pylori* infection further studies and standardization of studies in terms of the type of the applied probiotic strain is required, as well as the number of probiotic strains (single use of a single strain or multiple strains), and the length and time of administration of probiotics in relation to antibiotic therapy.

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# The influence of type 2 diabetes mellitus on the frequency and complexity of ventricular arrhythmias and heart rate variability in patients after myocardial infarction

## Uticaj šećerne bolesti tipa 2 na učestalost i kompleksnost ventrikularnih aritmija i varijabilnost frekvencije srčanog rada kod bolesnika nakon infarkta miokarda

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**Abstract**

**Background/Aim.** After myocardial infarction arrhythmic cardiac deaths are significantly more frequent compared to non-arrhythmic ones. The aim of the study was to investigate the influence of type 2 diabetes mellitus (T2DM) on the frequency and complexity of ventricular arrhythmias after myocardial infarction. **Methods.** The study included 293 patients, mean age 59.5 ± 9.21 years, who were at least six months after acute myocardial infarction with the sinus rhythm, without atrioventricular blocks and branch blocks. In the clinical group 95 (32.42%) patients were with T2DM, while 198 (67.57%) patients were without diabetes. All of the patients were subjected to the following procedures: standard ECG according to which the corrected QT dispersion (QTdc) was calculated, exercise stress test, and 24-hour holter monitoring according to which, the four parameters of time domain of heart rate variability (HRV) were analyzed: standard deviation of all normal RR intervals during 24 hours (SDNN), standard deviation of the averages of normal RR intervals in all five-minute segments during 24 hours (SDANN), the square root of the mean of the sum of the squares of differences between adjacent normal (RMS-SD), and percentage of consequtive RR intervals which differed for more than 50 ms during 24 hours (NN > 50 ms). **Results.** In patients after myocardial infarction, patients with T2DM had significantly higher percentage of frequent and complex ventricular arrhythmias compared to the patients without diabetes ($p < 0.001$). The patients with T2DM had significantly higher percentage of residual ischemia ($p < 0.001$), and arterial hypertension ($p < 0.001$), compared to patients without diabetes. The patients with T2DM had significantly lower values of HRV parameters: SDNN ($p < 0.001$); SDANN ($p < 0.001$); RMS-SD ($p < 0.001$), and NN > 50 ms ($p < 0.001$), and significantly higher values of QTdc ($p < 0.001$) compared to the patients without diabetes. **Conclusion.** The study showed that type 2 diabetes mellitus has significant influence on ventricular arrhythmias, HRV parameters and QT dispersion in patients after myocardial infarction.

**Key words:**
**diabetes melitus type 2; arrhythmias, cardiac; myocardial infarction.**

**Apstrakt**

**Uvod/Cilj.** Nakon infarkta miokarda srčana smrt je, usled razvoja kompleksnih ventrikularnih aritmija, značajno češća od nearitmijske. Cilj studije bio je da se ispita uticaj dijabetesa melitusa tipa 2 (T2DM) na učestalost i kompleksnost ventrikularnih aritmija nakon infarkta miokarda. **Metode.** Studija je obuhvatila 293 bolesnika, prosečne starosti 59,5 ± 9.21 godina, u periodu od najmanje šest meseci nakon akutnog infarkta miokarda. Svi su bili u sinusnom ritmu bez atrioventrikularnih blokova i blokova grana. Sa T2DM bilo je 95 (32,42%) bolesnika, dok je 198 (67,57%) bolesnika bilo bez dijabetesa. Ispitanicima je iz standardnog EKG izračunavana korigovana QT disperzija (QTdc), rađen test fizičkim opterećenjem, ehokardiografski pregled i 24-časovno holter-praćenje, iz koga su analizirana četiri parametra vremenske analize varijabilnosti frekvencije srčanog rada (HRV): standarna devijacija svih normalnih RR intervala registrovanih u toku 24 sata (SDNN), standarna devijacija prosečnih vrednosti svih petominutnih RR intervala u toku 24 sata (SDANN), kvadratni koren prosečne vrednosti kvadriranih

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razlika uzastopnih RR intervala u toku 24 sata (RMS-SD) i procenat uzastopnih RR intervala koji su se razlikovali za više od 50 ms u toku 24 sata (NN > 50 ms). **Rezultati.** Bolesnici sa T2DM imali su učestale i kompleksne ventrikularne aritmije u značajno većem procentu od onih bez dijabetesa ($p < 0,001$). Bolesnici sa T2DM imali su u značajno većem procentu rezidualnu ishemiju ($p < 0,001$) i arterijsku hipertenziju ($p < 0,001$) od bolesnika bez dijabetesa. Bolesnici sa T2DM imali su značajno niže vrednosti parametara varijabilnosti frekvencije srčanog rada: SDNN ($p < 0,001$); SDANN ($p < 0,001$); RMS-SD ($p < 0,001$) i NN > 50 ms ($p < 0,001$) i značajno više vrednosti QTdc ($p < 0,001$) od onih bez dijabetesa. **Zaključak.** Ova studija je pokazala da kod bolesnika nakon infarkta miokarda, dijabetes melitus tipa 2 ima značajan uticaj na pojavu ventrikularnih aritmija, parametre varijabilnosti frekvencije srčanog rada i QT disperziju.

**Ključne reči:**
**dijabetes melitus, insulin nezavisni; aritmija; infarkt miokarda.**

## Introduction

Patients with diabetes mellitus (DM) are at a high risk for cardiovascular morbidity and mortality. Patients with type 2 DM (T2DM) are 2–3 times more likely to develop cardiovascular diseases than the ones without this type of diabetes [1]. During post infarction period, patients with T2DM have two times higher mortality rate than non-diabetic ones [2].

Ventricular arrhythmias are the most common cause of death in coronary patients. During the first two years after recovering from myocardial infarction, cardiac deaths caused by arrhythmias are more frequent than non-arrhythmic deaths [3]. It was documented that 55% of total mortality in patients during post infarction period has been caused by malignant ventricular arrhythmias [4].

Many studies report that approximately 8.1% of the world population are patients with DM [5], and among patients with acute coronary syndromes, there are about 20–37% of patients with DM [6].

Considering the fact that in the post-infarction period the mortality rate in T2DM patients is much higher than in non-diabetic ones and that arrhythmic cardiac death is significantly more frequent than non-arrhythmic, the aim of the study was to investigate the influence of T2DM on the frequency and complexity of ventricular arrhythmias, and heart rate variability in patients six months after acute myocardial infarction.

## Methods

The patients (n = 293), at least six months after acute myocardial infarction, were recruited from the Clinic for Cardiovascular Diseases, between 2009 and 2013, at the Institute for Treatment and Rehabilitation "Niška Banja" in Niška Banja. There were 91 females and 202 males, mean age 59.5 ± 9.21 years. The clinical group was divided into two subgroups, according to the presence of T2DM. Among the patients, 95 (32.42%) of them were with 2TDM, mean age 60.1 ± 8.2, while 198 (67.57%) patients were without DM, mean age 59.5 ± 8.9 (they were observed as the control group). In the first clinical subgroup of the patients with T2DM, 50 (52%) patients were smokers and 56 (58.9%) of them had hypertension (or had an antihypertensive therapy); in the group of the patients without diabetes, 53 (26.8%) of them were smokers, and 86 (43.4%) patients had hypertension.

A fasting blood sample was collected from each participant and biochemical measurements were obtained using standard clinical laboratory methods. All analyses were performed on a Human Star 600, Germany.

The patients after myocardial infarction were monitored during the period of at least six months from the diagnosed acute myocardial infarction. All of them had sinus rhythm without atrioventricular and branch blocks.

All the patients were subjected to the procedures as follows: standard ECG according to which QT intervals were calculated, exercise testing, that was used to assess residual ischemia, which was performed on treadmill according to Bruce protocol [7], and 24-hour ambulatory ECG monitoring.

QT interval was determined according to ECG, from the starting point of the Q- or R-peak to the end of the T-wave where the down-slope of the T-wave merged with isoelectric line. The QT interval was determined in each offset from three consecutive sinus cycles as mean value. The values of QT intervals were corrected for the frequency of heart rate according to Bazett's formula [8]. QT dispersion was determined as the difference derived from maximal and minimal value of QT interval found in any of the 12 offsets. Out of the corrected value of QT interval, where the minimal value was subtracted from the maximum value found in any of the ECG offsets, the corrected value for QT dispersion (QT dc) was obtained.

The 24-hour ambulatory ECG monitoring was performed with the system Del Mar Avionics model 5268-505 MPA/R-ACQ: 2.15; Irvine, California, USA. The system included analysis of classic monitoring and determining heart rate variability (HRV). Analysis included the total number of ventricular premature complexes (VPCs) during 24 hours, the number of couples, triplets, bigeminies, the number of multiform VPCs, and the number of non-sustained and sustained ventricular tachycardia (VT). Further evaluation of ventricular arrhythmias was performed according to Lown and Wolf's classification [9]. Ambulatory monitoring results were used for software HRV analysis. The following four parameters of HRV analysis were analyzed: standard deviation of all normal RR intervals during 24 hours (SDNN); standard deviation of the averages of normal RR intervals in all five-minute segments during 24 hours (SDANN); the square root of the mean of the sum of the squares of differences between adjacent normal RR intervals during 24 hours (RMS-SD); percentage of consequtive RR intervals which differed for more than 50 ms during 24 hours (NN > 50 ms).

Characteristics of the study and the control group were expressed as mean ± SD (continuous variables), with the

numbers and percentages in brackets (categorical variables). We compared clinical and biochemical data of patients and control group using Student's *t*-test for normally distributed data (expressed as mean ± SD). All analyses were performed with SPSS statistical analysis software, version 10.0 (SPSS, Chicago, IL, United States) at the level of significance set at $p < 0.05$.

## Results

Clinical and biochemical data of the clinical group are presented in Table 1. Significant differences were found between the patients with T2DM and without T2DM in all the evaluated parameters: glycemia ($p = 0.001$), total cholesterol ($p = 0.001$), HDL cholesterol ($p = 0.02$), LDL cholesterol ($p = 0.001$), and triglycerides ($p = 0,001$), all presented in Table 1. The gender of patients with T2DM after myocardial infarction did not significantly influence the frequency and complexity of ventricular arrhythmias. The patients with T2DM, 83 (87.4%) of them, had significantly higher rate of residual ischemia compared to the patients without diabetes, 112 (56.6%), ($p = 0.001$).

Among the patients after myocardial infarction, the ones with T2DM had significantly higher rate of frequent and complex ventricular arrhythmias, classified by Lown and Wolf, compared to patients without diabetes mellitus: Lown 0 ($p = 0.001$), Lown I ($p = 0.001$), Lown II ($p = 0.025$), Lown III ($p = 0.001$), Lown IVa ($p = 0.005$), and Lown IVb ($p = 0.05$), all data presented in Table 2. Analyzing data on ventricular arrhythmias classified as Lown IVb, in the patients with T2DM there were 5 (5.3%) patients with triplets of ventricular extrasystoles, and 3 (3.2%) had non sustained ventricular tachycardia, while in the patients without T2DM there were 3 (1.5%) patients with triplets of ventricular extrasystoles, and one patient (0.5%) had non sustained ventricular tachycardia. Comparing the basic electrocardiographic parameters between these groups of patients we found differences in all evaluated parameters: SDNN (ms) ($p = 0.001$), SDANN (ms) ($p = 0.001$), RMS-SD (ms) ($p = 0.001$), NN > 50 (ms) ($p = 0.001$), RR (ms) ($p = 0.001$), and QTdc (ms) ($p = 0.001$), all data presented in Table 3.

## Discussion

The results of our study show that among the patients six months after myocardial infarction, those with T2DM had significantly higher rate of frequent and complex arrhythmias, compared to the patients without diabetes. It is documented that beside metabolic disorders and scar tissue originating from myocardial infarction, the patients with T2DM have fibrous changes in the interstitium included in the pathogenesis of the diabetic cardiomyopathy, as well as the damages of small blood vessels [10]. Those fibrous changes of the myocardium create anatomical substrate for the occurrence of macro- and micro-reentry ventricular tachycardia [11]. Furthermore, patients without DM, have scar tissue origina-

**Table 1**

**Comparison of biochemical and clinical parameters of the patients after myocardial infarction with and without type 2 diabetes mellitus (T2DM)**

| Parameter | Patients with T2DM | Patients without T2DM | *p* |
|---|---|---|---|
| Number (%) of patient | 95 (32.42) | 198 (67.57) | - |
| Age (years), $\bar{x} \pm SD$ | 60.1 ± 8.2 | 59.5 ± 8.9 | - |
| Glycemia (mmol/L), $\bar{x} \pm SD$ | 10.9 ± 3.9 | 5.5 ± 0.7 | 0.001 |
| Cholesterol ( mmol/L), $\bar{x} \pm SD$ | 6.8 ± 1.2 | 6.0 ± 1.3 | 0.001 |
| Tryglicerides (mmol/L), $\bar{x} \pm SD$ | 2.6 ± 1.9 | 1.9 ± 0.9 | 0.001 |
| HDL cholesterol (mmol/L), $\bar{x} \pm SD$ | 0.9 ± 0.2 | 1.1 ± 0.8 | 0.02 |
| LDL cholesterol (mmol/L), $\bar{x} \pm SD$ | 4.3 ± 0.4 | 4.1 ± 0.7 | 0.005 |
| Hypertension (mm/Hg), n (%) | 56 (58.94) | 86 (43.43) | 0.001 |
| Smoking, n (%) | 50 (52.63) | 53 (26.76) | 0.001 |

**Results compared with Student's-*t* test; LDL – low density lipoprotein; HDL – high density lipoprotein.**

**Table 2**

**Comparison of ventricular arrhythmias, classified by Lown and Wolf in the patients after myocardial infarction with and without type 2 diabetes mellitus (T2DM)**

| Parameter | Patients with T2DM | Patients without T2DM | *t* | *p* |
|---|---|---|---|---|
| Number of patients, n (%) | 95 (32.42) | 198 (67.57) | - | - |
| Age (years), $\bar{x} \pm SD$ | 60.1 ± 8.2 | 59.5 ± 8.9 | - | - |
| Lown 0, n (%) | 9 (9.5) | 43 (21.7) | 4.46 | 0.001 |
| Lown I, n (%) | 21 (22.1) | 109 (55.1) | 4.46 | 0.001 |
| Lown II, n (%) | 15 (15.8) | 11 (5.6) | 2.90 | 0.025 |
| Lown III, n (%) | 28 (29.5) | 25 (12.6) | 4.92 | 0.001 |
| Lown IVa, n (%) | 11 (11.6) | 4 (2.0) | 3.33 | 0.005 |
| Lown IVb, n (%) | 8 (8.4) | 4 (2.0) | 2.12 | 0.05 |
| Lown V, n (%) | 3 (3.2) | 2 (1.0) | 0.53 | NS |

**Results compared with Student's *t*-test; NS – non significant.**

**Table 3**

**Comparison of basic electrocardiographic parameters in the patients after myocardial infarction with and without type 2 diabetes mellitus (T2DM)**

| Parameters | Patients with T2DM | Patients without T2DM | *p* |
|---|---|---|---|
| Number of patiens | 95 | 198 | - |
| SDNN (ms), $\bar{x} \pm SD$ | 79.0 ± 20.5 | 101.5 ± 30.9 | 0.001 |
| SDANN (ms), $\bar{x} \pm SD$ | 68.0 ± 18.7 | 85.3 ± 29.5 | 0.001 |
| RMS-SD (ms), $\bar{x} \pm SD$ | 25.1 ± 10.5 | 35.3 ± 15.2 | 0.001 |
| NN>50 (ms), $\bar{x} \pm SD$ | 5.9 ± 5.7 | 12.0 ± 10.4 | 0.001 |
| RR (ms), $\bar{x} \pm SD$ | 0.78 ± 0.11 | 0.90 ± 0.10 | 0.001 |
| QTdc (ms), $\bar{x} \pm SD$ | 88.0 ± 22.7 | 66.2 ± 26.5 | 0.001 |

**Results compared with Student's *t*-test.**
**SDNN – standard deviation of all normal RR intervals during 24 hours;**
**SDANN – standard deviation of the averages of normal RR intervals in all five-minute segments during 24 hours; RMS-SD – the square root of the mean of the sum of the squares of differences between adjacent normal RR intervals during 24 hour); NN > 50 ms, percentage of consequtive RR intervals which differed for more than 50 ms during 24 hours; RR – RR interval; QTdc – corrected QT dispersion.**

ting from myocardial infarction, represented only as one fibrous island. Contrary to that, in patients with T2DM, beside the scar tissue, there are also diffuse fibrous changes in the interstitium, so there is a greater possibility for the occurrence of ventricular arrhythmias, what was confirmed in our study. These changes in the myocardium of the left ventricle lead to its dilatation, and therefore sustain further development of ventricular arrhythmias [12, 13].

It has been demonstrated that during dilatation of the left ventricle impulse conduction becomes slower, total block occurs, effective refractive period shortens, dispersion refractivity increases and the difference in refractivity between subepicardial and subendocardial layers also increases [11, 14]. Extension of the left ventricle walls leads to the changes of membrane resting potential and refractive period, which alleviates the occurrence of ventricular arrhythmias [15–17]. When the ventricle myocardium is damaged and electrophysiological remodeling occurs, activation of channels alters, which leads to reduced dispersion of repolarization.[14]

Increased QTd values also appear due to fibrous changes and myocardial ischemia [18–20]. Increased QTd value reflects regional difference in repolarization of the myocardium and is a potential predictor for cardiac mortality.[21,22] Among our patients early after myocardial infarction, those with T2DM had significantly higher percentage of frequent and complex ventricular arrhythmias and significantly higher QTd values, compared to patients without T2DM. Increased QTd value is a marker for electric instability of the ventricular myocardium and is a predictor for complex ventricular arrhythmias, including ventricular tachycardia,[21,23] also found in our patients. According to QTd values, we can identify patients with heart failure, who are at a higher risk for cardiac death.[24]

Most of our patients with T2DM had residual ischemia, which certainly contributed to the occurrence of arrhythmias [25]. In experimental studies, before the occurrence of ischemia, ventricular fibrillation (VF) could not be caused even by premature stimuli, while during ischemia, VF was caused by a single premature stimulus, between the 17th and 32nd minute from the beginning of the ischemia.[26] Ischemia causes intracellular and extracellular acidosis and damages cell membrane, causing potassium to exit and calcium to enter the cell, which leads to decreased membrane resting potential and creates conditions for trigger activity.[11, 27] Slowing or complete blocking of conduction is also caused by increased resistance of gap connection due to acidosis. Besides that, the decrease of action potential creates conditions for the occurrence of arrhythmias based on automatism.[25] These changes are heterogenic in the ischemic region, depending on the level of ischemia.[25] All our patients with T2DM and residual ischemia, had frequent and complex ventricular arrhythmias.

A correlation between increased level of fasting plasma glucose and cardiovascular diseases is well-known [28, 29]. Hyperglycemia causes coronary dysfunction by creating reactive oxygen species, which inactivates nitric oxide produced in the endothelium, and activate protein kinase C, which induces the production of vasoconstrictor prostanoids.[26] It is considered that hyperglycemia is one of the most important causes of the occurrence of myocardial fibrosis, it causes local production of angiotensin II in myocytes, leading to their apoptosis.[30] According to Framingham study, hyperglycemia, including mild hyperglycemia, causes reduced values of HRV parameters. Similar results were recorded in the ARIC study.[5]

Parameters of heart rate variability as markers of the autonomic nervous system state are used for evaluation of the influence of both, sympathetic and parasympathetic effect on the heart function, and for the identification of patients who are at higher risk for cardiovascular events [31–33]. It has been recorded that there is a significantly higher mortality rate in patients with reduced SDNN values [34]. Many studies have shown that HRV is an independent predictor for sudden cardiac death in patients after myocardial infarction [4, 31, 33, 35, 36]. It has also been documented that in patients with T2DM after myocardial infarction, reduced HRV values are predictors for cardiac death and sudden cardiac death [5].

Cardiovascular autonomic neuropathy (CAN) is significantly associated with subsequent mortality in people with DM (T1DM and T2DM) in meta-analysis of 15 studies. Probably, in our patients with T2DM, CAN also contributed to significantly higher percentage of registered frequent and

complex ventricular arrhythmias, compared to the patients without T2DM. Patients with T2DM had significantly lower values of HRV parameters, which maintained the vagal tone, as well as RMS – SD and NN > 50 ms, [37, 38] and RR interval values, compared to the patients without T2DM, which was also found in our patients. The presence of tachycardia at rest is an early sign of CAN in patients with T2DM, [39] and we recorded sinus tachycardia which occurred at rest or developed easily at slight effort [30]. Early detection of CAN may help us to detect earlier development of atherosclerosis in patients with T2DM to prevent unfavorable outcomes [39, 40].

Regulation of glycemia, hypertension, myocardial ischemia, increased sympathetic activity and dysfunction of the left ventricle would lead to reduced vulnerability of the myocardium and reduced arrhythmic deaths in patients with T2DM after myocardial infarction. Regulation of these pathological states would reduce arrhythmic deaths and thus improve the prognosis for these patients after myocardial infarction.

## Conclusion

This study showed that in patients after myocardial infarction, type 2 diabetes mellitus has a significant impact on ventricular arrhythmias, heart rate variability, and QT dispersion. We also showed that these patients had a significantly higher percentage of frequent and complex ventricular arrhythmias, compared to non-diabetic patients. Finally, heart rate variability and QT dispersion, may be an easy and helpful tool for easier identification of patients with type 2 diabetes mellitus who are at greater risk for further cardiovascular events.

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SHORT COMMUNICATION

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# The relation between nonspecific hyperreactivity of the airways and atopic constitution in asthmatics

## Odnos između nespecifične hiperreaktivnosti disajnih puteva i atopijske konstitucije kod astmatičara

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**Abstract**

**Background/Aim.** Hyperreactivity of the airways caused by inflammation in asthmatics is the most important pathophysiological change. It represents a suitable ground that in the presence of risk factors and the drivers of asthma, asthmatic attack occurs. Atopic constitution is one of the most important risk factors for the development and expression of asthma. The aim of our study was to investigate the relationship between nonspecific airway hyperreactivity and atopic constituton in asthmatics. **Methods.** This retrospective analysis was conducted considering the results of nonspecific bronchoprovocative test with histamine, skin tests to inhalant allergens and total IgE levels in the serum of asthmatic patients with controlled bronchial asthma. The sample consisted of 162 asthmatics examined during one-year period. **Results.** The examinees were male asthmatic patients, aged between 18 and 30 years. We found that the examinees with a pronounced non-specific hyperreactivity had more significant skin reaction to inhaled allergens and higher levels of total IgE in serum. **Conclusion.** The results of our study show that the intensity of airway hyperresponsiveness to histamine in asthmatics is directly related to atopic constitution.

**Key words:**
**asthma; respiratory hypersensitivity; immunoglobulin e; hypersensitivity, immediate; histamine; skin test.**

**Apstrakt**

**Uvod/Cilj.** Hiperreaktivnost disajnih puteva uzrokovana inflamacijom kod astmatičara je najznačajnija patofiziološka promena. Ona predstavlja pogodan teren da se u prisustvu faktora rizika i pokretača astme ispolji astmatični napad. Atopijska konstitucija je jedan od najznačajnijih faktora rizika od nastanka i ispoljavanja astme. Cilj našeg rada bio je da se ispita odnos između nespecifične hiperreaktivnosti disajnih puteva i atopijske konstitucije kod astmatičara. **Metode.** Izvršena je retrospektivna analiza rezultata nespecifičnog bronhoprovokacijskog testa sa histaminom, kožnih proba na inhalacione alergene i nivoa ukupnih IgE u serumu bolesnika sa kontrolisanom bronhijalnom astmom. U analizu su bila uključena 162 astmatičara, ispitivana tokom jednogodišnjeg perioda. **Rezultati.** Svi bolesnici su bili muškarci, starosti od 18 do 30 godina. Utvrđeno je da su ispitanici sa izraženijom nespecifičnom hiperreaktivnošću imali izraženiju kožnu reakciju na inhalacione alergena i viši nivo ukupnih IgE u serumu. **Zaključak.** Rezultati našeg istraživanja ukazuju na to da je intenzitet hiperreaktivnosti disajnih puteva na histamin kod astmatičara u direktnoj vezi sa atopijskom konstitucijom.

**Ključne reči:**
**astma; hipersenzibilnost, respiratorna; ige; hipersenzibilnost, rana; histamin; koža, testovi.**

### Introduction

As a consequence of chronic inflammation, enhanced bronchoconstriction occurs in bronchial asthma as a response of the airways to many endogenous and exogenous factors. That amplified response of the airways to a variety of stimuli that elicit the bronchoconstriction represents the bronchial hyperreactivity (BHR) [1].

BHR is variable and dependent upon the nature of the inflammatory processes whose intensity varies with asthma and is influenced by numerous factors such as allergens, respiratory infection, and certain medications [2]. Considering that a certain degree of reactivity of the airways in asthmatic patients is always present, BHR can be referred to as variable or constant phenomenon. Strict division of the mentioned components is not possible because the processes that influ-

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ence BHR are interdependent. Such is the case with inflammation in chronic form which can lead to cell changes that can cause permanent BHR [3].

BHR can also be divided to the direct or indirect depending on the mechanisms of action and the stimuli which caused it. At the same time, some of them influence more permanent and the others variable components. Histamine acts directly on the receptor in the airway smooth muscle leading to their contractions and bronchoconstriction.

On the other hand, indirect stimuli, which include physical exertion, hyperventilation, hypertonic solution, cause the release of mediators (prostaglandins, leukotrienes and histamine), which act on receptors in the smooth muscle of the respiratory tract, leading to the same effect – bronchospasm [4].

Histamine with its direct influence amplifies the effect of permanent BHR. However, histamine achieves the effect of histamine on the reflex mechanisms of bronchoconstriction and contributes to the manifestation of the effects of indirect stimulus to the airways. Although the mechanism of effects of histamine is not clear enough, nor investigated in detail, it is known that it depends on the preexisting inflammation and other bronchoconstrictor stimuli [1, 3].

Viewed from the aspect of pathophysiology, hyperreactivity is a complex process associated with a series of immunological, neurological, and structural changes that lead to bronchial obstruction which is manifested by characteristic symptoms and signs of an asthma attack.

The occurrence and expression of bronchial asthma is affected by multiple risk factors for worsening of preexisting asthma or asthma exacerbation factors, provocative factors or triggers of asthma. Among these the most common are allergens, respiratory infections, physical exertion, hyperventilation, emotional stress, respiratory irritants that directly or indirectly trigger bronchoconstriction receptors in the airways.

The existence of hyperreactivity of the airways can be determined and measured by laboratory tests that involve controlled exposure to direct or indirect stimuli. These tests contribute significantly to the precise diagnosis of asthma or reduce the risk of wrong diagnosis of the disease based only on asthma symptoms [5, 6].

Atopic constitution is the tendency of the body to react to a contact with allergens with increasing production of immunoglobulin E (IgE). Atopic constitution and bronchial hyperresponsiveness are tightly linked characteristics of bronchial asthma [7]. Atopy affects the hyperreactivity of the airways in a manner that it induces and promotes inflammation in the airways and thereby increases BHR [8].

Although there are various indicators of the degree of atopy, there is no complete consensus in the scientific community concerning this issue. The most frequently used are total IgE, specific IgE, the number of positive intracutaneous tests or sum of several indicators [7].

Recent evidences suggest that the increase of IgE serum levels to dust mites is a reliable indicator of worsening asthma [9].

The aim of this study was to invesigate the relationship between nonspecific airway hyperreativity and atopic constitution in asthmatics.

## Methods

We retrospectively analyzed the results of bronchoprovocation test with histamine on 162 asthmatics with controlled bronchial asthma who were examined during the one-year period in the functional diagnostics of the lung in the Military Medical Academy in Belgrade, Serbia. The examinees were male, aged between 18 and 30 years. They all underwent bronchoprovocation test with histamine. The assay started with a minimal concentration of 0.03 mg/mL and each next concentration was twice the previous. They all had a positive bronchoprovocation histamine test. All of them had a decrease in forced expiratory volume in the first second (FEV1) compared to baseline value of at least 20% with inhaled histamine concentrations up to 4 mg/mL. In addition, all the subjects underwent skin prick-tests with inhaled allergens. The diameter of changes on the skin at the site of application of allergens was measured in mm. Test was considered positive if there was the appearance of skin lesions of the diameter at least 50% of the diameter of the skin change on the site of histamine administration, which was used as a positive control. The level of total IgE in the serum was determined in all patients using nephelometric method. The examinees were divided into two equal experimental groups *per* 81 patients each. The first group consisted of the patient with positive histamine test at the concentration of histamine up to 2 mg/mL and the second group of the patients had positive histamine test at the concentration of 2–4 mg/mL.

### *Statistical analysis*

Complete statistical analysis was performed using a commercial statistical software SPSS Statistics 17. A certain number of variables was incorporated in the form of the frequency of certain features (categories) and the statistical significance of differences between the groups was evaluated using the $\chi^2$ test.

In the case of continuous variables, the data were presented as the mean ± standard deviation (SD). Normal distribution of the data was checked using the Kolmogorov-Smirnov test. In case of fulfillment of the conditions of normality, statistical significance within and between the groups was checked using the *t*-test for dependent and independent features. Otherwise, for the estimation of significance between the groups, the Mann-Whitney U-test was used. A statistically significant difference was estimated at the minimum level of $p < 0.05$.

## Results

All the participants were male, asthmatics. There was no significant difference in age or in the baseline FEV1 between two groups of patients (Table 1).

The examination of atopic constitution showed some characteristics. All the subjects had positive skin tests to most standard inhaled allergens. However, the intensity of skin reactions to certain allergens, expressed as atopic index,

**Table 1**

**Characteristics of the patients (n = 62) with asthma according to the groups**

| Characteristics | Group I (n = 81) | Group II (n = 81) |
|---|---|---|
| Age (years), $\bar{x} \pm SD$ | 23.98 ± 0.22 | 23.62 ± 0.30 |
| Men/women, n | 81/0 | 81/0 |
| FEV1 (l) basal (%), $\bar{x} \pm SD$ | 4310 ± 1.50 | 4653 ± 1.20 |
| Histamine PC20 (mg/mL), $\bar{x} \pm SD$ | 0.60 ± 0.07 | 1.25 ± 0.10 |

**FEV1 – forced expiratory volume in 1 s; Histamine PC20 – the concentration causing a 20% fall of the FEV1.**
**Group I: positive histamine test at a concentration of histamine up to 2 mg/mL;**
**Group II: positive histamine test at a concentration of histamine 2–4 mg/mL.**

which represents the mean maximum diameter of skin lesions, was not the same by the groups. The patients from the first group had a more intense, statistically important, skin reaction to dermatophagus and grass pollen (Figure 1). By analysing the levels of total IgE in the serum as another indicator of atopic constitution, we obtained the results that confirmed the previous ones, related to skin tests. Respectively, the first group of our examinees with more intense skin reaction, also had significantly higher levels of total IgE in the serum (Table 2).

When examining of nonspecific airway hyperresponsiveness to histamine it was found more pronounced hypersensitivity and airway hyperresponsiveness in the subjects of the first group. They reacted to a much lower concentration of histamine, on the average of about 0.6 mg/mL (Table 2).

With regard to hyperreactivity, or the degree of FEV 1 decrease after histamine test, subjects of the first group also showed a higher degree of reduction of this parameter. The patients in the first group had an average decline in FEV1 of 33.5%, whereas the respondents in the second group had a decline in FEV1 of 29.8% (Table 2).

## Discussion

BHR and atopy are two characteristic features of bronchial asthma. Their association contributes to complex pathophysiological events and clinical manifestations of bronchial asthma.

In our study, we aimed to investigate the relationship between nonspecific airway hyperreactivity and the atopic constitution of asthmatics.

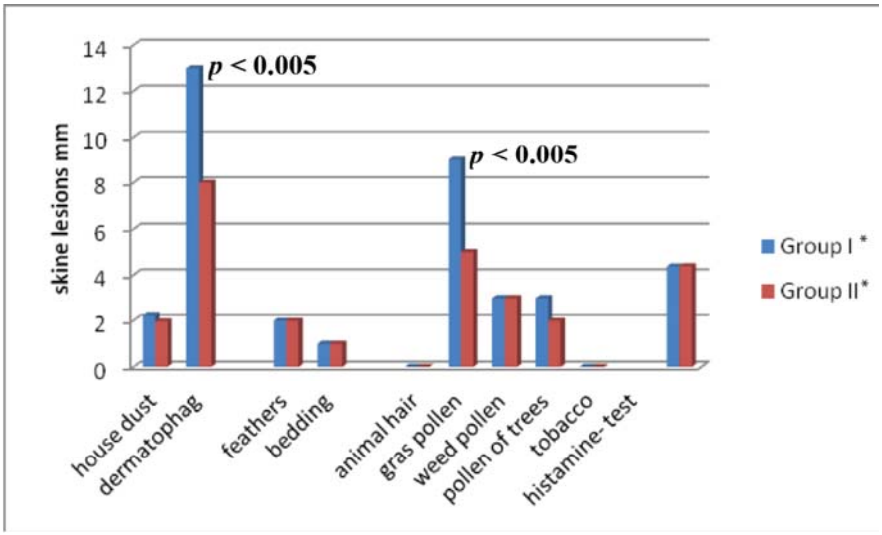


**Fig. 1 – Skin reactions to inhaled allergens according to the groups of the patients with asthma (*For explanation see under Table 1).**

**Table 2**

**Average values of the serum level of IgE, concentration of histamine causing the decrease in FEV1 of 20% and percentage of decrease in FEV1 after histamine test in asthmatic patients**

| Parameters | Group I* | Group II* | *p* |
|---|---|---|---|
| Average level of IgE in the serum (U/mL) | 354 | 225.87 | < 0.05 |
| Average concentration of histamine causing the decline in FEV1 of 20% (mg/mL) | 0.6 | 1.3 | < 0.05 |
| Average decrease in FEV1 after histamine test (%) | 33.5 | 29.8 | < 0.05 |

**FEV1 – forced expiratory volume in 1 s.**
**(*For explanation see under Table 1).**

Although all the respondents were sensitive to most of inhaled allergens, we observed a significantly higher response to dermatophagus and grass pollen consistent with the climate they lived in. The intensity of skin reactions to inhaled allergens is also positively correlated with the level of total IgE in the serum as another indicator of atopic constitution. Backer et al. [10] got similar results in the Australian study conducted on a population of 527 children and adolescents, where they had proven that atopic index and BHR are closely related.

Results of other studies confirm that atopy promotes otherwise already present chronic inflammation in the airways of asthmatics, which leads to increased BHR [8].

The association of allergy and atopic constitution and bronchial asthma is characteristic of allergic asthma, generally among younger population of asthmatics.

However, the effect of atopy on asthma is significant and in later life to which, among others, pointed Plaschke et al. [11] in their research.

The specificity of our research was in the fact that we tested the older population than it was the case with other authors and that all of our respondents had clearly manifested bronchial asthma. This research confirms the previously stated fact that atopic constitution remains an important factor not only in adolescents but also in adults.

Kim et al. [7], have demonstrate that the relation between atopy and asthma is positive, *ie* BHR increases with increasing levels of total IgE in serum.

The results that we obtained, lead us to a similar conclusion. A group of our patients with pronounced BHR also had a pronounced atopic constitution or higher levels of total IgE in the serum and intense skin reaction to inhaled allergens.

## Conclusion

Based on the results of this study, we can conclude that the asthmatic's bronchial hyperreactivity depends on atopic constitution.

Atopic constitution represents the most important event in the development of bronchial hyperreactivity in asthmatics. The intensity of bronchial hyperreativity in asthmatics is directly related to the intensity of atopic constitution manifested by the increased formation of IgE in the serum and skin hypersensitivity to inhaled allergens.

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C A S E R E P O R T S

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# Pulmonary sequestration mimicking lung cancer – A case report

## Plućni sekvestar koji imitira karcinom pluća

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**Abstract**

**Introduction.** Pulmonary sequestration is a rare congenital anomaly and most intralobar sequestrations were located in lower lobes. **Case report.** We reported an unusual 28-year-old female patient with intralobar pulmonary sequestration on the left lower lobe, successfully treated with lobectomy. Computed tomography (CT) of the chest with intravenous contrast revealed multiple clustered cystic lesions in the left lower lobe with aberrant artery from descedenting aorta. Additional aortography showed an aberrant artery (3 mm in diameter) arising from the abdominal aorta and flowing into the lesion. **Conclusion.** Standard therapy regimen for pulmonary sequestration includes surgery. CT scan of thorax with intravenous contrast and aortography represent the gold standard for its diagnosis. Tumor-like shadows seen on the chest radiography or CT scans should not be always suspected on malignant lesions.

**Key words:**
**bronchopulmonary sequestration; diagnostic techniques and procedures; diagnosis, differential; thoracic surgical procedures.**

**Apstrakt**

**Uvod.** Plućni sekvestar predstavlja retku kongenitalnu anomaliju, najčešće zastupljenu intralobarno u donjim režnjevima pluća. **Prikaz bolesnika.** Prikazali smo redak slučaj intralobarnog plućnog sekvestra kod bolesnice, stare 28 godina, uspešno operisane. Kompjuterska tomografija grudnog koša sa intravenskim kontrastom ukazala je na multiple cistične promene u levom donjem režnju, sa aberantnom arterijom iz descedentne aorte. Dodatno načinjena aortografija dokazala je aberentnu arteriju (3 mm u prečniku) iz abdominalne aorte koja se uliva u ledirano pluće. **Zaključak.** Standardni terapijski pristup za plućni sekvestar podrazumeva hirurško lečenje. Skener grudnog koša sa intravenskim kontrastom i arteriografija predstavljaju zlatne dijagnostičke standarde. Tumorske senke uočene na radiogramima grudnog koša ili na kompjuterizovanoj tomografiji ne moraju uvek da budu sumnjive na maligne promene.

**Ključne reči:**
**sekvestracija, bronhopulmonalna; dijagnostičke tehnike i procedure; dijagnoza, diferencijalna; hirurgija, torakalna, procedure.**

### Introduction

Pulmonary sequestration is a rare congenital malformation of lung characterized by abnormal pulmonary parenchyma without tracheobronchial airway connection and with blood supply from a systemic artery [1]. Definitive treatment includes surgical excision, usually lobectomy, followed by division of the anomalous artery *via* standard thoracotomy.

### Case report

A 28-year-old female was hospitalized due to chest pain and high temperature. Chest radiography showed a tumor-like shadow in the left lower lobe (Figure 1). Laboratory test findings showed elevated sedimentation rate – 94 mm/h, C-reactive protein (CRP) – 198 mg/mL, blood cell count – 17.3 × $10^3$/$mm^3$ and D-dimer – 4.39 mg/L fibrinogen equivalent units (FEU). Electrocardiogram (ECG), heart ultrasound, lung functions tests and blood gas analysis were normal. A bronchoscopy finding was normal. The computed tomography (CT) scan of the chest with intravenous contrast revealed multiple clustered cystic lesions in the left lower lobe with the aberrant artery from the descedenting aorta (Figure 2). Aortography showed the aberrant artery (3 mm in diameter) arising from the abdominal aorta and flowing into the lesion (Figure 3). Left lower lobectomy was done. The intraoperative finding showed the abberant artery coming

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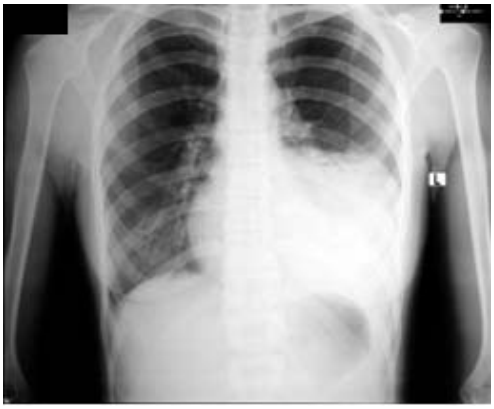
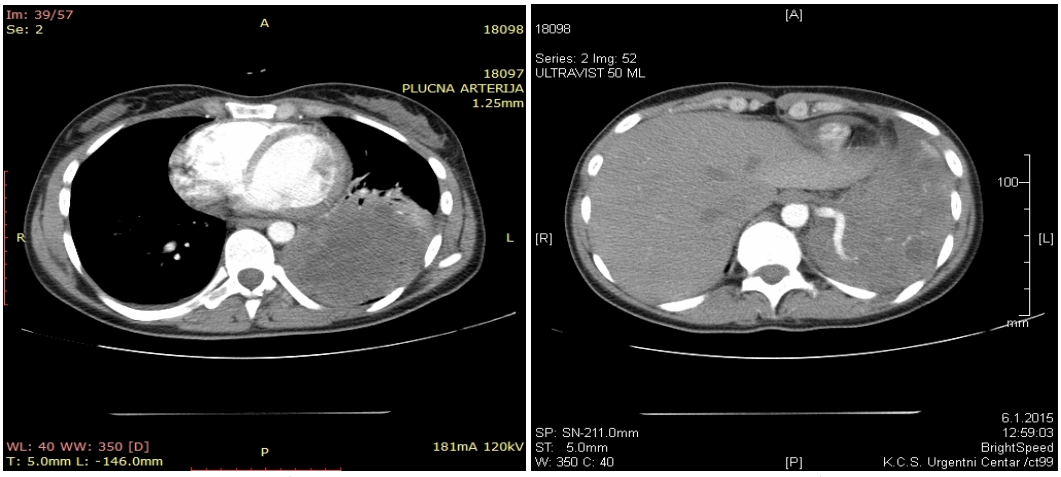


**Fig. 1 – Chest radiography: tumor-like shadow in the left lower lobe.**




**a)** **b)**

**Fig. 2 – a) Thorax computed tomography (CT) scan: multicystic mass in the left lower lobe; b) Thorax CT scan: aberrant artery arises from the descedenting thoracic aorta.**

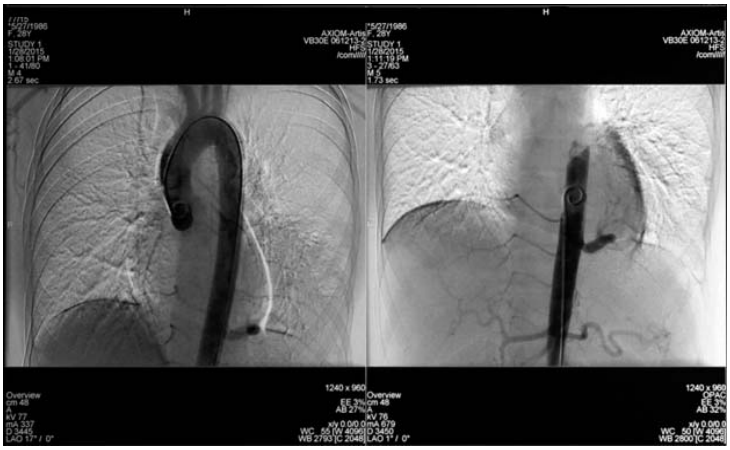


**Fig. 3 – Aortography showing the aberrant artery.**

through the diaphragm into the mass (Figure 4). The artery was carefully dissected from the surrounding tissue. A pathological finding revealed intralobar pulmonary sequestration (Figure 5).

## Discussion

Pulmonary sequestration is a rare congenital lesion of the lung parenchyma of unknown etiology without normal connection with the tracheobronchial tree and with the blood supply directly from the descending aorta [2]. It is a rare developmental abnormality which account for 0.15–6.4% of all congenital lung anomalies [3]. There are two morphological types: extralobar (25% of all cases), which have their own pleura, and intralobar (75% of all cases), which are surrounded by visceral pleura [1, 2]. Patients were presented in younger age with symptoms of recurrent bronchopulmonary infections and if they are recognized they should be operated on. If

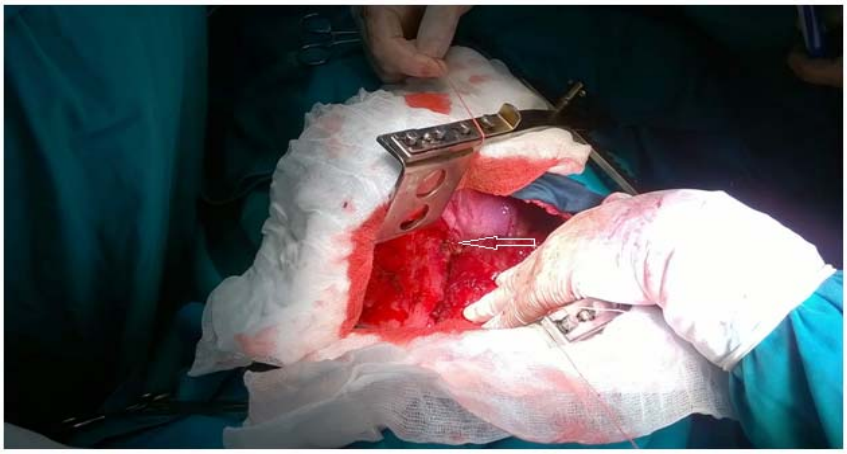


**Fig. 4 – Intraoperative photo showing the aberrant artery.**

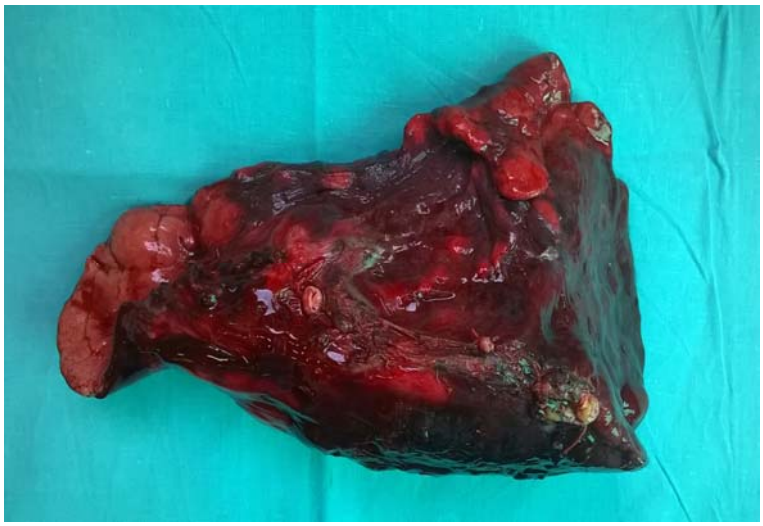


**Fig. 5 – The left lower lobe with multicystic changes.**

the symptoms of patients with pulmonary sequestration are not serious, intervention should be prolonged for several years. In the cases of repeated episodes of pneumonia, after physical investigation, diagnostic methods should include usual laboratory analysis, chest radiography and thorax CT scan with intravenous contrast. In our patient thorax CT scan with intravenous contrast revealed the aberrant artery from the descedenting aorta. Examination was completed with digital subtraction arteriography. Pulmonary sequestration is often misdiagnosed as congenital pulmonary cysts of bronchiectasis complicated with infection, benign lung tumor, diaphragmatic hernia and lung cancer [4]. Surgical resection is a definitive treatment in patients with pulmonary sequestration. Standard posterolateral thoracotomy has been used for a years. Because of its benign etiology partial lung resection for pulmonary sequestration should be more appropriate than lobectomy. Video assisted thoracoscopic surgery (VATS) is a better alternative to standard thoracotomy for pulmonary sequestration because of minimal surgical trauma, postoperative pain and duration of hospitalization [5, 6]. In the presented patient VATS was not done because of deficient technical equipment. Potential barriers to VATS include intrapleural adhesion due to the inflammatory process and dissection and dividing of the feeding artery, but rarely reported in case series [6, 7]. There are a few reports on successful management of asymptomatic pulmonary sequestration with angiographic embolization [8].

## Conclusion

In a young patient tumor-like shadow on chest radiography should be suspected on a benign lesion. Detailed radiological investigations, including thorax CT scan, followed with arteriography, are mandatory.

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CASE REPORT

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# Hyperparathyroidism as a cause of recurrent acute pancreatitis – A case report

## Hiperparatireoidizam kao uzrok recidivantnog akutnog pankreatitisa

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**Abstract**

**Introduction.** One of the more uncommon etiological factors responsible for the development of acute pancreatitis (AP) is hypercalcemia. Hyperparathyroidism (HPT), as a cause of hypercalcemia, is responsible for 1.5–13% of AP according to a number of studies. A mechanism of the development of AP in hyperparathyroidism is still unclear. **Case report.** We presented a 47-year-old female patient, who had five episodes of AP in total before the etiological factors were finally determined. The patient had certain comorbidities which were considered to be potential causes of AP. She had chronic renal insufficiency (she was on a regular hemodialysis program), systemic lupus erythematosus and mioma uteri. She used to regularly take an antiepileptic drug (combination of sodium valproate and valproic acid). During the fifth episode of AP, the serum calcium level was for the first time elevated to twice the normal value. Level of parathyroid hormone was several times higher. A static scintigraphy found hyperplasia or hyperfunctional adenoma of the right inferior and superior parathyroid glands. Abdominal multislice computed tomography (MSCT) scan verified the enlargement of the entire pancreas, as well as the presence of heterogeneous structures with diffuse amorphous calcifications. The lytic lesions in the pelvic bones could be seen in both sides. Parathyroidectomy was being postponed by an endocrine surgeon because of the poor overall condition of the patient. In the next period the patient had five more episodes of AP. The condition was significantly contributed by increasingly more frequent and longer episodes of metrorrhagia. Despite all therapeutic measures that were taken, systemic inflammatory response syndrome (SIRS) developed, and fatal outcome occurred. **Conclusion.** In case of recurrent pancreatitis, hyperparathyroidism is to be considered even if a significant elevation of serum calcium is not present. This is especially the case for patients with chronic renal insufficiency or impaired vitamin D metabolism, who have a higher risk of secondary hyperthyroidism.

**Key words:**
**pancreatitis; hyperparathyroidism; comorbidity; diagnosis, differential; kidney failure, chronic.**

**Apstrakt**

**Uvod.** Jedan od ređih etioloških faktora odgovornih za razvoj akutnog pankreatitisa (AP) jeste hiperkalcemija. Hiperparatireoidizam (HPT), kao uzrok hiperkalcemije, odgovoran je za 1,5–13% AP prema različitim studijama. Mehanizam nastanka AP kod hiperparatireoidizma još je uvek nedovoljno jasan. **Prikaz bolesnika.** Predstavili smo 47-godišnju bolesnicu, koja je imala pet epizoda akutnog pankreatitisa, pre nego što je konačno utvrđen etiološki faktor. Bolesnica je imala i određene komorbiditete koji su razmatrani kao potencijalni uzročnici AP. Imala je hroničnu bubrežnu insuficijenciju (bila je na hroničnom programu hemodijalize), sistemski eritemski lupus i miom materice. Redovno je uzimala antiepileptik (kombinacija natrijum valproata i volproinske kiseline). Tokom pete epizode AP vrednosti kalcijuma u serumu prvi put su bile dvostruko veće od normalnih vrednosti. Nivo paratirodnog hormona bio je višestruko povećan. Statičkom scintigrafijom nađena je hiperplazija ili hiperfunkcionalni adenom gornje i donje desne paratireoidne žlezde. Na ponovljenoj multislajsnoj kompjuterskoj tomografiji (MSCT) trbuha verifikovan je u celini uvećan pankreas heterogene strukture sa difuznim amorfnim kalcifikatima. Takođe, pri ovom pregledu uočene su li-

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tične lezije u karličnim kostima obostrano. Paratireoidektomija je odložena na predlog endokrinog hirurga zbog lošeg opšteg stanja bolesnice. U narednom periodu bolesnica je imala još pet epizoda AP. Stanje je značajno pogoršano sve češćim i dužim epizodama metroragije. Uprkos svim terapijskim merama, došlo je do razvoja sindroma sistemskog inflamatornog odgovora (SIRS) i fatalnog ishoda. **Zaključak.** Kod recidivantnog AP treba razmišljati o hiperparatireoidizmu i onda kada nema značajnog porasta serumskog kalcijuma. Ovo se naročito odnosi na bolesnike sa hroničnom bubrežnom insuficijencijom ili poremećenim metabolizmom vitamina D, što uvećava rizik od razvoja sekundarnog hiperparatireoidizma.

**Ključne reči:**
**pankreatitis; hiperparatireoidizam; komorbiditet; dijagnoza, diferencijalna; bubreg, hronična isuficijencija.**

## Introduction

Timely detection of the etiological factor responsible for the development of acute pancreatitis (AP) is important for designing an optimal therapeutic treatment plan and prevention of AP. Numerous etiological factors are responsible for the development of AP. AP of alcoholic or biliary origin is most commonly seen in practice. Idiopathic AP (10% of the diseased) comes in the third place by frequency [1]. Such a significant percentage of idiopathic AP is probably a consequence of limited diagnostic possibilities or nonspecificity of manifestation of certain etiological forms of pancreatitis.

One of the more uncommon etiological factors responsible for the development of AP is hypercalcemia. Hyperparathyroidism (HPT), as a cause of hypercalcemia, is responsible, according to a number of studies, for 1.5–13% of AP [2, 3]. A mechanism of the development of AP in hyperparathyroidism is still unclear [3–5]. Although the correlation between AP and HPT is controversial, HPT is recognized as an etiological factor responsible for the development of AP [5–7].

We presented a patient with recurrent AP which used to be defined as idiopathic until the fifth episode. The patient had certain comorbidities which were considered to be potential causes of AP.

## Case report

A 47-years-old woman had the first episode of AP in January, 2012, when she was clinically examined and treated at the Clinic for Gastroenterology and Hepatology in Niš, Serbia. In the medical history, the patient had a record of several surgical procedures in her childhood because of benign brain tumors and she used to regularly take an antiepileptic drug (combination sodium valproate and valproic acid). In 2006 the patient was diagnosed with systemic lupus (SLE) and chronic renal insufficiency (she refused a kidney biopsy). From 2010 the patient was on the regular hemodialysis program, three times *per* week, for four hours each time, and dialysis had a good depuration effect. The phosphorus-calcium ratio was maintained regardless of regular intake of phosphate binders. From 2011 the patient had leiomyoma of uterus, and since then frequently suffered from metrorrhagia.

During the first episode of AP, biochemical analyses showed elevated levels of serum amylase (1,604 U/L), lipase (1,676 U/L) and C-reactive protein (CRP) (18.3 mg/L); the levels of bilirubin, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase (ALP), gamma glutamyl transferase (GGT), cholesterol and triglycerides were within reference values. The level of Ca in the serum was at the upper reference limit (2.65 mmol/L). An abdominal ultrasound of liver, bile ducts, gallbladder and pancreas showed normal findings. Spleen had an interpolar diameter of approximately 140 mm and a homogenous structure. The kidneys were reduced in size and had an altered structure. After consulting a neurologist, on antiepileptic drug (combination sodium valproate and valproic acid), as a possible cause of AP, was replaced with a different antiepileptic drug. The patient was released home in a satisfactory overall condition. Pancreatic enzyme supplements were recommended.

Three months later, a new episode of AP occurred with similar clinical, laboratory and ultrasound findings. After consultations with rheumatologists, the possibility of active SLE was ruled out. As a part of immunological analyses, IgG4 antibodies were also tested and since they turned out to be negative, the possibility of autoimmune AP was excluded. The patient was released home in satisfactory overall condition. The continuation of pancreatic enzymes supplementation was recommended.

Three months later, a new AP episode occurred. Biochemical analyses showed elevated values of amylase, lipase, ALT, GGT, and Ca (slightly over the upper reference limit value). An abdominal ultrasound showed edematous pancreas and the presence of peripancreatic fluid collections in smaller amount. Multislice computed tomography (MSCT) finding was not significantly different from the ultrasound finding. Magnetic resonance cholangiopancreatography (MRCP) scan results came with the conclusion that the findings are normal. Because of the still unclear etiology of AP, endoscopic retrograde cholangiopancreatography (ERCP) with sphincterotomy was performed. Color Doppler of abdominal arteries and veins was within normal limits. During hospitalization, the patient developed metrorrhagia, because of which she was examined by a gynecologist on several occasions.

By the end of 2012, the patient had three more episodes of AP with a milder clinical picture and several episodes of metrorrhagia. During her last hospitalization in December 2012 pain in muscles of all extremities was present. Laboratory analyses showed serum calcium elevated to twice the normal value. Parathyroid hormone was several times

higher (1,165.0 pmol/L) than normal (1.6–6.9 pmol/L). A static scintigraphy with a radiopharmaceutical, technetium ($^{99m}$Tc) 2-metoxy-isobutyl-isonitrile ($^{99m}$Tc-MIBI) was performed. The finding corresponded to scintigraphic presentation of hyperplasia or hyperfunctional adenoma of the right inferior and superior parathyroid glands (Figure 1).

During the following period, until the end of 2013, the patient had five more episodes of AP. The endocrine surgeon postponed parathyroidectomy because of the poor overall condition of the patient. The condition was significantly contributed by increasingly more frequent and longer episodes of metrorrhagia, because of which the patient was examined

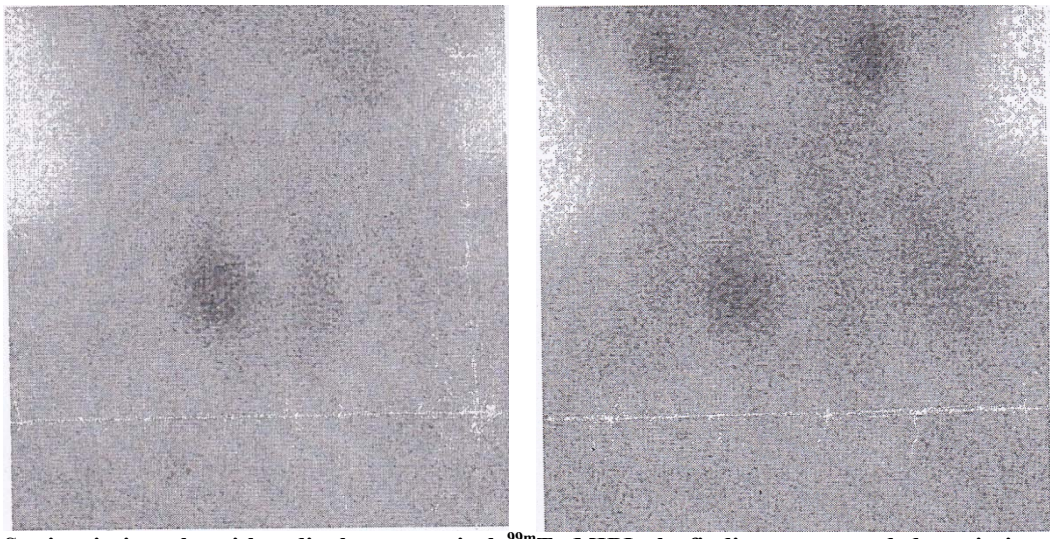

**Fig. 1 – Static scintigraphy with radiopharmaceutical, $^{99m}$Tc-MIBI: the finding corresponded to scintigraphic presentation of hyperplasia or hyperfunctional adenoma of the right inferior and superior parathyroid glands. $^{99m}$Tc-MIBI – $^{99m}$Tc 2-metoxy-isobutyl-isonitrile.**

Parathyroidectomy was indicated. However, soon after the ongoing AP episode was taken care of, a new AP episode occurred during the preparations for the parathyroid surgery. A repeated abdominal MSCT scan verified the enlargement of the entire pancreas, as well as the presence of heterogeneous structures with diffuse amorphous calcifications. The pancreatic duct was dilated in the tail of pancreas. There was a smaller amount of peripancreatic free fluid collections. Enlarged lymph nodes could be seen in retroperitoneum, some up to 10 mm. Also, during this examination, lytic lesions in the pelvic bones could be seen on both sides (Figure 2).

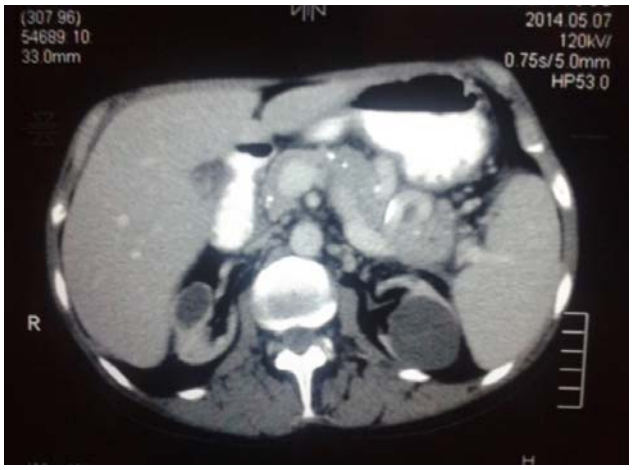


**Fig. 2 – Abdominal multislice computed tomography (MSCT) scan verified the enlargement of the entire pancreas, as well as the presence of heterogeneous structures with diffuse amorphous calcifications.**

and treated at the Gynecology and Obstetrics Clinic, where palliative interventions were performed in order to stop the bleeding. Shortly, a significant deterioration of a subjective and objective condition occurred along with the development of systemic inflammatory response syndrome (SIRS) without AP. Despite all therapeutic measures that were taken, a fatal outcome occurred three weeks later.

## Discussion

Hypercalcemia as a cause of AP is strongly connected with the presence of hyperparathyroidism. Pathophysiological mechanism of the development of AP in patients with hyperparathyroidism has not been precisely determined. It is believed that in cases of hypercalcemia, calcium stimulates exocrine secretion of pancreas, induces activation of trypsinogen with consequential autodigestion and it accumulates in pancreatic ducts, causing their obstruction [8–10]. Data from studies indicate that a mutation of certain genes (SPINK1 – serine protease inhibitor Kazal-type 1 and CFTR – cystic fibrosis transmembrane conductance regulator) can increase the risk of AP development in patients with HPT [11]. Also, a mutation of CaSR (calcium-sensing receptor) is mentioned which occurs in hypercalcemic conditions. Functional significance of the mutations of these genes in the context of pancreatitis needs further studies [12, 13].

The concentration of serum calcium greater than three times the reference value is a predisposition for the development of AP [10]. In the presented patient, the values of serum calcium were at the upper limit until the moment of establishing the diagnosis, when the value was doubled.

The risk of developing AP is thirty times greater in patients with primary hyperparathyroidism than in the general population [10]. The prevalence of AP in primary HPT is different. In a study conducted in India, authors diagnosed AP in 6.8% of patients with primary HPT; AP was the initial clinical manifestation of HPT in 5 of 6 patients, which was the case with the presented patient, as well. All patients with AP had two or more episodes before diagnosing with hyperparathyroidism. In some cases, there was a delay in the diagnosis up to 12 months [12]. Our patient had had five episodes of AP in total before the etiological factors were finally determined, 15 months after the first episode. Since the values of serum calcium were at the upper reference limit during the AP episodes, HPT was not suspected until the values of serum calcium doubled. Besides, imaging methods (ultrasound and MSCT) of the abdomen did not verify calcifications earlier. Acute pancreatitis is usually correlated with the decrease in serum calcium values. According to Ranson's criteria, lower values of calcium in serum have prognostic significance [14, 15]. Therefore, the detection of hypercalcemia in patients with severe AP should always draw attention of doctors to potential hyperparathyroidism or malignancy [16, 17]. Values of parathyroid hormones are to be determined and examination of parathyroid glands performed. Taught by our own experience, but not by the experience of other authors, in patients with recurrent AP, when other common factors are excluded, values of parathyroid hormones are to be tested even if the values of serum calcium are normal [3, 4, 11, 18]. Since the presented patient was on the regular hemodialysis program, there was a significant possibility that this was a case of tertiary hyperparathyroidism, which develops from the secondary after a long period of its duration, when parathyroid glands lose their ability to produce a regulatory response for a concentration of calcium that causes continuously increased secretion of parathyroid hormone. The consequence is an impaired phosphate and calcium metabolism, characterized by hyperphosphatemia and hypercalcemia (in people with previously lower or normal level of calcium in serum). Measuring PTH by C-fragments is unreliable, because of the accumulation of C-fragments in the organism and because of reduced clearance in renal insufficiency [19]. Parathyroid adenomas in HPT are larger in patients who during the evolution of HPT suffer from AP, as well [1].

## Conclusion

In case of recurrent pancreatitis, hyperparathyroidism is to be considered even if a significant elevation of serum calcium is not present. This is especially the case in patients with chronic renal insufficiency or impaired vitamin D metabolism, who have a higher risk of secondary hyperthyroidism.

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# Massive fetomaternal hemorrhage as a cause of severe fetal anemia

## Opsežna fetomaternalna hemoragija kao uzrok teške anemije fetusa

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**Abstract**

**Introduction.** Fetomaternal hemorrhage (FMH) is a transfusion of fetal blood into the maternal circulation. A volume of transfused fetal blood required to cause severe, life-threatening fetal anemia, is not clearly defined. Some authors suggest volumes of 80 mL and 150 mL as a threshold which defines massive FMH. Therefore, a rate of massive FMH is 1 : 1,000 and 1 : 5,000 births, respectively. Fetal and neonatal anemia is one of the most serious complications of the FMH. Clinical manifestations of FMH are nonspecific, and mostly it presented as reduced fetal movements and changes in cardiotocography (CTG). The standard for diagnosing FMH is Kleihaurer-Betke test. **Case report.** A 34-year-old gravida (G) 1, para (P) 1 was hospitalized due to uterine contractions at 39 weeks of gestation. CTG monitoring revealed sinusoidal fetal heart rate and clinical examination showed complete cervical dilatation. Immediately after admission, the women delivered vaginally. Apgar scores were 1 and 2 at the first and fifth minute, respectively. Immediately baby was intubated and mechanical ventilation started. Initial analysis revealed pronounced acidosis and severe anemia. The patient received intravenous fluid therapy with sodium-bicarbonate as well as red cell transfusion. With all measures, the condition of the baby improved with normalization of hemoglobin level and blood pH. Kleihaurer-Betke test revealed the presence of fetal red cells in maternal circulation, equivalent to 531 mL blood loss. The level of maternal fetal hemoglobin (HbF) and elevated alpha fetoprotein also confirmed the diagnosis of massive FMH. **Conclusion.** For the successful diagnosis and management of FMH direct communication between the obstetrician and the pediatrician is necessary as presented in this report.

**Key words:**
**fetomaternal transfusion; anemia; fetus; newborn; apgar score; diagnosis; intensive care, neonatal; treatment outcome.**

**Apstrakt**

**Uvod.** Fetomaternalna hemoragija (FMH) se definiše kao prelazak krvi ploda u cirkulaciju majke. Volumen fetalne krvi koji je neophodan da pređe u cirkulaciju majke i izazove tešku fetalnu anemiju nije precizno definisan. Većina autora sugeriše masivnu fetomaternalnu transfuziju pri volumenu od 80 mL odnosno 150 mL fetalne krvi, te je stopa FMH 1 : 1 000, odnosno 1 : 5 000 porođaja. Fetalna i neonatalna anemija je jedna od najozbiljnijih komplikacija FMH. Kliničke karakteristike FMH su nespecifične i najčešće se manifestuju redukcijom fetalnih pokreta i promenama u kardiotokografskom (CTG) zapisu. Dijagnostički standard FMH je Kleihaurer-Betke test. **Prikaz bolesnika.** Trudnica, stara 34 godine, primljena je na kliniku radi porođaja. CTG zapis bio je sinusoidalnog tipa dok je akušerskim pregledom konstatovana kompletna cervikalna dilatacija. Neposredno nakon prijema trudnica se vaginalno porodila. Apgar skor u prvom i petom minutu iznosio je 1 i 2. Odmah je sprovedena reanimacija, intubacija i mehanička ventilacija. Inicijalne gasne analize ukazivale su na to da se radi o teškoj acidozi i anemiji. Uz sve primenjene mere stanje novorođenčeta se stabilizovalo, uz normalizaciju vrednosti hemoglobina i pH vrednosti krvi. Kleihaurer-Betke testom ustanovljena je FMH u vrednosti od 531 mL. Povišene vrednosti fetalnog hemoglobina (HbF) kao i alfa fetoproteina u majčinoj krvi potvrdile su da se radilo o FMH. **Zaključak.** Za uspešnu dijagnozu i lečenje FMH neophodna je i direktna komunikacija između akušera i pedijatra kao što je prokazano u ovom slučaju.

**Ključne reči:**
**transfuzija, fetomaternalna; anemija; fetus; novorođenče; apgar skala; dijagnoza; intenzivna nega, neonatalna; lečenje, ishod.**

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## Introduction

Fetomaternal hemorrhage (FMH) is a transfusion of fetal blood into the maternal circulation. It is well-known that placenta enables communication between mother and fetus in both directions, but in almost all pregnancies a small amount of fetal blood enters into the maternal circulation. Normal volume of fetal blood detected in maternal circulation is under the 0.1 mL [1]. A volume of fetal blood that requires to be transfused into the maternal circulation and that causes severe, life-threatening anemia in a fetus, i.e. newborn, is not clearly defined. Therefore, various criteria are used to define massive FMH. Many authors suggest volumes of 80 mL and 150 mL as a threshold to define massive FMH, which is estimated to occur in 1 in 1,000 and 1 in 5,000 deliveries, respectively [2]. Etiology is idiopathic, but some conditions may predispose to FMH like some obstetrical procedures, and placental abruption. Clinical presentation of FMH during pregnancy is nonspecific, mostly presented as reduced fetal movements and changes in cardiotocography (CTG) [3]. Diagnosis of FMH may be established by the Kleihauer-Betke test, the standard method for detection and quantification of fetal blood in the maternal circulation. A prompt and appropriate treatment increases the survival rate, while a long-term prognosis is uncertain.

In this report, we presented a case of massive FMH in term pregnancy, resulted in severe neonatal anemia and asphyxia and confirmed by Kleihauer-Betke (KB) test.

## Case report

A 34-years-old gravida (G) 1, para (P) 1 was hospitalized due to uterine contractions at 39 weeks of gestation. Pregnancy was uneventful until two days before delivery, when the patient noticed diminished fetal movements, general weakness with mild fever and discrete joint pains. The patient was admitted to the hospital isolation unit with the diagnosis of viral upper respiratory tract infection. The patient did not suffer from any chronic illness, and never had surgical procedures. CTG monitoring revealed sinusoidal fetal heart rate (Figure 1) and clinical examination showed intact membranes and complete cervical dilatation with the head in occipital anterior presentation with a small fontanel at +1 cm in relation to the interspinal line. After amniotomy, amniotic fluid was clear and immediately after admission the women delivered vaginally a female newborn weighted 2,900 g. Apgar scores were 1 and 2 at the first and fifth minute, respectively. The infant was very pale, flaccid, without respiratory effort, bradycardic heart rate 40 beats *per* minute (bpm). Resuscitation started immediately, the baby was intubated and mechanical ventilation started. Initial blood gas analysis revealed pronounced acidosis with pH 6.8, $pO_2$ 5.3 kPa, $pCO_2$ 7.7 kPa, lactate level of 19.4 mmol/L and non-measureable base deficit. Complete blood count analysis showed severe anemia with the hemoglobin level of 5.7 g/dL, red blood cell number $1.47 \times 10^6/mm^3$, hematocrit 16% and marked reticulocytosis of 17.4%. The infant and the mother had the same blood O group, Rh- negative, and negative Coombs test. There were no signs of hydrops and hyperbilirubinemia. Intravenous fluid therapy with sodium-bicarbonate started as well as empirical antibiotic therapy (ampicillin and amikacin). On the first hospital day, the patient received two packed red cell transfusion. The infant received the third packed red cell transfusion on the second day of hospitalization. Sepsis screen was normal, as well as ultrasonography of the brain and the abdomen. With all these measures, the condition of the baby improved with normalization of the level of hemoglobin and blood pH. Mechanical ventilation was stopped on the third day of hospitalization. Electroencefalography showed normal activity, without specific changes. The infant was discharge from hospital on the 9th day in good general condition. Considering that hemolytic disease of the newborn and other most common causes of neonatal anemia were excluded, Kleihaurer-Betke test was performed due to suspicion of FMH. This analysis revealed the presence of fetal red cells in maternal circulation, equivalent to 531 mL blood loss. The level of maternal fetal hemoglobin (HbF) was 4.85% (normal less than 2%), and elevated alpha fetoprotein 4,214 IJ/ml also confirmed the diagnosis of massive FMH.

## Discussion

Fetomaternal hemorrhage is still a poorly understood condition which can result in severe fetal anemia leading to life-threatening newborn illness with high mortality and significant morbidity [4]. There are different standpoints regar-

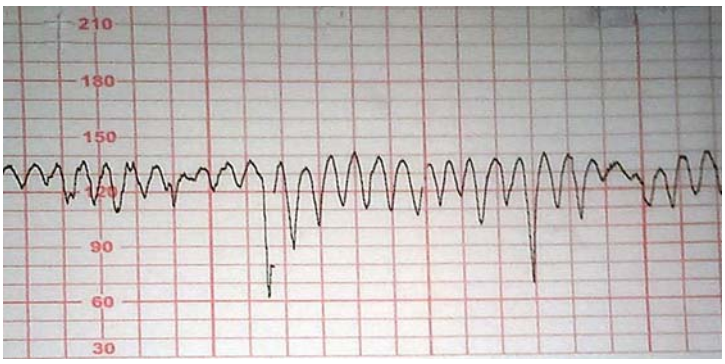


**Fig. 1 – Sinusoidal cardiotocography (CTG) of the patient with oscillation amplitude which is slightly higher than a typical one (about 25–30 bytes *per* minute).**

ding the volume of transfused fetal blood which defines massive FMH. Most frequently it is 80 mL and 150 mL of fetal blood in the maternal circulation, estimated to occur with the incidence of 1 : 1,000 to 1 : 5,000 deliveries, respectively [2, 5]. The volume of transfused blood is not the only factor influencing fetal or newborn clinical condition. Numerous factors can contribute to final outcome and long-term prognosis. The most significant among them are chronicity, gestational age, blood group compatibility and possible abdominal trauma. Animal studies revealed that the loss of 30% of total fetal blood volume is better tolerated if it happens within two hours than within 10 minutes [6]. Some authors report no statistically significant difference in long-term outcomes regardless if hemoglobin is higher or less than 60 g/L as well as the volume of transfused fetal blood into maternal circulation is more or less than 200 mL [7]. The chronicity of transfusion process could be the explanation of this finding. Chronic forms of FMH have more benign course than acute, even though the volume of transfused blood is higher, while poor prognosis is expected in acute blood loss. Considering gestational age, preterm infants have a higher risk for adverse outcome [8]. The main reason for this is certainly low adaptation capability to stress in preterm infants caused by FMH. In the context of ABO incompatibility, the maternal coagulation system can be activated which limits hemorrhage. However, if mother and fetus are ABO compatible, there is less possibility that coagulation system would be activated, so more massive FMH could be expected [7]. In the presented case, maternal and fetal blood types were compatible, actually they were both blood type O, Rh-negative, and Coombs test was negative, which according to previous assumption resulted in massive FMH. Other factors associated with FMH are placental abruption, umbilical cord anomalies, amniocentesis and some obstetrical procedures (external cephalic version, manual removal of the placenta) [8].

The clinical presentation of FMH is nonspecific and the literature reports that the most frequent symptoms are reduced or absent fetal movements which were present in the presented case, too [3]. CTG ranges from a sinusoid type over reduced variability with or without later decelerations [9]. The sinusoidal type of the CTG monitoring is typical for fetal anemia or acidosis, and the criteria required for the diagnosis were established by Modanlou and Freeman [10]. Some authors classify sinusoidal type of CTG into three subtypes depending on the oscillation amplitude: minor with the amplitude of 5 to 15 bpm, intermediate with amplitude 16–24 bpm and major with the ampilitude 25 or more bpm, in order to quantify fetal risk [11, 12]. In this case, CTG revealed sinusoidal type with slightly higher oscillation amplitudes of 25 bpm, referring to higher risk of adverse fetal outcome. In cases of massive FMH, transfusion reactions presented as nausea, fever, shiver etc. were described [13, 14]. In our case, the mother was admitted to the hospital isolation unit according to suspicion of viral infection presented with general weakness, mild fever and discrete joint pains, which also correspond to transfusion reaction.

The prenatal diagnostic of FMH is difficult and unreliable, and in most cases occurs in previously normal pregnancies [14]. Mari et al. [15] reported that both mild and severe fetal anemia could be diagnosed by Doppler ultrasound, i.e. by evaluating blood flow velocity through middle cerebral artery (MCA). When fetal anemia occurs, cardiac output and blood velocity increase which can be established by measuring blood flow velocity through MCA. There is an inverted correlation between fatal anemia and the highest flow velocity during systole (peak systolic velocity – PSV) through MCA. Doppler ultrasound evaluation of MCA-PSV is an effective non-invasive method for evaluation of fetal anemia which can be also helpful in making treatment decision: fetal transfusion or delivery, depending on gestational age. Cosmi et al. [16] show that measurement of MCA-PSV is useful in diagnosing fetal anemia caused by chronic fetomaternal hemorrhage. In cases of chronic FMH and sinusoidal fetal heart rate pattern MCA-PSV values greater than 1.5 multiples of the median were observed, while in cases of acute bleeding MCA-PSV were normal. Ultrasound examination can detect changes of biophysical profile manifested with reduction of fetal movements with adequate amount of amniotic fluid [17].

Treatment of choice in proven FMH is immediate delivery in term pregnancy or in a period when adequate maturity of a fetus can be expected. Cesarean section is a desirable mode of delivery, because fetoplacental circulation may be additionally compromised in vaginal delivery. However, in this case, the mother was admitted in the maternity with regular contractions in the expulsion stage with clinical finding refering to the possibility to finish vaginal labor soon. The most frequently applied test for detection of fetal blood in to the maternal circulation is KB test. The estimated volume of transfused fetal blood into maternal circulation was 531 mL in our case. Similar values of 410 mL and 710 mL were reported in other studies too [18, 19]. Such high values, higher than the total fetal blood volume, could be explained with chronic FMT. High reticulocytes count may support the diagnosis of chronic FMT as a sign of compensatory activation of fetal hematopoietic system.

We presented severe FMH in term pregnancy completed soon after admission in the maternity ward with vaginal delivery according to obstetrician finding. The immediately established diagnosis and appropriate and timely therapy resulted in stabilization of infant general condition and good recovery. The newborn was discharged on the day 5 of life without complications. Long-term outcome in such cases with severe acidosis and low hemoglobin level are difficult to predict. Some cases may result in poor outcome, especially if signs of damage were presented on the brain imaging [19]. Magnetic resonance of the brain and the outcome at the age of 6 mouths in the presented patient was normal despite massive FMH and severe anemia and asphyxia at birth.

Regarding the difficulties in the diagnosis of FMH more physician awareness of this condition is of crucial importance [20].

## Conclusion

For the successful diagnosis and management of FMH direct communication between the obstetrician and the pediatrician is necessary as presented in this report.

## Disclosure

The authors report no conflicts of interest.

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CASE REPORT

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# Synchronous mantle cell lymphoma and prostate adenocarcinoma – is it just a coincidence?

## Istovremena pojava *mantle* ćelijskog limfoma i adenokarcinoma prostate – samo slučajnost ili ne

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**Abstract**

**Introduction.** Synchronous occurrence of lymphomas and other cancers, mostly carcinomas are well established. The most of cases describe chronic lymphocytic leukemia as the leading lymphoproliferative disease with the tendency towards secondary malignancies development. Mantle cell lymphoma (MCL) has been described in only 2 cases to co-occur with prostate adenocarcinoma (PAC). There are scarce data about the connection between MCL and urology cancers. We presented the first case of synchronous occurrence of MCL and PAC in the same patient in Serbia. **Case report.** A 64-year-old male initially presented with fatigue, splenomegaly, and bicytopenia. The bone marrow biopsy specimen revealed extensive infiltration with MCL. During lymphoma staging procedure prostate enlargement (57 mm) was accidentally found by multislice-computed tomography (MSCT). The serum prostate specific antigen (PSA) was elevated (52 ng/mL; normal values ≤ 4 ng/mL). Transrectal ultrasound biopsy revealed PAC. High Gleason score determined high-risk locally advanced PAC. The patient underwent treatment with chemotherapy and hormone therapy due to the existence of double malignancies. Cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP) was applied for MCL, and luteinizing hormone-releasing hormone (LHRH) agonist, triptorelin, for PAC. Partial response was obtained for MCL, and stable disease for PAC. In a 1.5-year observation period the patient was still disease progression free for both of malignancies. **Conclusion.** This case points aut that elderly males are in need for careful observation during the staging procedure for lymphoma. The literature data suggest that MCL patients are in increased risk for urologic malignancies development. However, the etiologic connection between these two entities, except male gender and older age, remains unclear.

**Key words:**
**lymphoma, mantle-cell; prostatic neoplasms; adenocarcinoma; diagnosis; comorbidity; risk assessment.**

**Apstrakt**

**Uvod.** Istovremena pojava limfoma i drugih kancera, pre svega karcinoma, dobro je poznata. Najčešće se opisuje hronična limfocitna leukemija kao vodeća limfoproliferativna bolest sa tendencijom ka razvoju sekundarnih maligniteta. Opisana su samo dva bolesnika kod kojih se *mantle* ćelijski limfom (MĆL) pojavljivao istovremeno sa adenokarcinomom prostate (PAC). Postoji veoma malo podataka o povezanosti između MĆL i uroloških maligniteta. Prikazali smo prvi slučaj istovremene pojave MĆL i PAC kod istog bolesnika u Srbiji. **Prikaz bolesnika.** Muškarac, star 64 godine, najpre je došao zbog pojave malaksalosti, splenomegalije i bicitopenije. Uzorak koštane srži pokazao je ekstenzivnu infiltraciju ćelijama MĆL. U toku procedure stadiranja limfoma slučajno je, primenom multislajsne kompjuterizovane tomografije (MSCT) otkrivena uvećana prostata (57 mm). Prostata-specifični antigen (PSA) u serumu bio je povišen – 52 ng/mL (normalna vrednost ≤ 4 ng/mL). Transrektalna ultrazvučna biopsija pokazala je PAC. Visok Gleason skor ukazao je na visokorizični lokalno uznapredovali PAC. Bolesnik je lečen i hemioterapijom i hormonoterapijom zbog postojanja dualnog maligniteta. Protokol ciklofosfamid, doksorubicin, vinkristin i prednizon (CHOP) primenjen je za MĆL, a luteinizirajući hormon oslobađajući hormon (LH/RH) agonist triptorelin za PAC. Parcijalna remisija postignuta je za MĆL, a za PAC stabilna bolest. U periodu od 1,5-godišnjeg praćenja bolesnik nije imao progresiju ovih bolesti. **Zaključak.** Ovaj prikaz ukazuje na to da stariji bolesnici zahtevaju pažljivu opservaciju u toku postupka stadiranja limfoma. Podaci iz literature sugerišu da bolesnici sa MĆL imaju povišen rizik od razvoja uroloških maligniteta. Ipak, etiološka veza između ova dva entiteta, osim muškog pola i starijeg životnog doba, ostaje nejasna.

**Ključne reči:**
**limfom, mantle-ćelijski; prostata, neoplazme; adenokarcinom; dijagnoza; komorbiditet; rizik, procena.**

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## Introduction

Mantle cell lymphoma (MCL) accounts for 2–10% of all non-Hodgkin lymphomas (NHL), with male predominance 2.3–2.5 : 1, and a median age at presentation close to 70 years [1]. The stage is usually advanced, adenopathy typically non-bulky, with frequent extranodal involvement (bone marrow, leukemic presentation, liver, spleen, or Waldeyer ring). Gastrointestinal involvement in the form of multiple lymphomatous polyposis may be one of the possible presentation [2]. The blastoid variant is rare but highly aggressive. MCL is characterized by the chromosomal translocation t(11; 14) (q13; q32), resulting in constitutional overexpression of cyclin D1 and cell cycle dysregulation in virtually all cases [3]. Cyclin D1 is detected by immunohistochemistry in 98% of MCL, although in remaining cases it may lack [4]. The SOX11 is highly expressed in both Cyclin D1 negative and positive MCL suggesting this biomarker as an important factor in the pathogenesis of MCL [5].

Based on the results of Surveillance, Epidemiology, and End Results (SEER), for the period of 2008–2012 year, prostate cancer was fairly common with the incidence rate of 137.9/100,000 *per* year in all races [6]. The same data source indicates a 5-year survival rate above 98% for the disease. The risk of clinically significant prostate cancer is related to age, ethnicity, family history, prostate specific antigen (PSA) level, free/total PSA ratio and findings on digital rectal examination (DRE) [7]. PSA, although not highly specific, combined with DRE are the most commonly used clinical tools for prostate cancer early detection. The plasma PSA level, Gleason score and tumor-node-metastasis (TNM) classification are used for risk assessment of localized disease [8]. The prostate cancer antigen 3 (PCA-3) has higher specificity, as well, positive and negative predictive values over PSA, although its sensitivity is slightly weaker. Therefore, it has stronger power in predicting patients who will benefit from prostate biopsy [9]. Transrectal ultrasound (TRUS) biopsy of prostate with the minimum of 10–12 cores obtained is one of the diagnostic standards [8].

The association between MCL and urologic cancers was documented by some authors [10]. Both malignancies are related to the patient age and gender. However, every other connection between these two conditions remains unknown or unexplored. We presented the first published case of synchronous occurrence of MCL and PAC in the same patient in Serbia.

## Case report

A 64–year–old male had complaints of fatigue and feeling pressure under the left rib cage. Those were the only symptoms he had. Basic clinical examinations revealed splenomegaly and generalized lymphadenopathy. The routine blood picture showed marked bicytopenia [white blood cells – WBC 2.9 × $10^9$/L, normal range (nr) 4–9 × $10^9$/L; absolute neutrophil count (ANC) 0.6 × $10^9$/L (nr 1.7–7.7 × $10^9$/L); platelet (PLT) 55× $10^9$/L, (nr 120–380 × $10^9$/L)]. The diagnostic trephine biopsy was performed and revealed extensive MCL infiltration of the bone marrow. Immunohistochemistry was typical: CD79α+, CD5+, CD20+, CyclinD1+, CD23-, CD43-/+, CD3- (Figures 1a–d). Obviously, it was IV B clinical stadium. Regardless of previous findings, we performed all staging procedures for lymphomas. The biochemical results found elevated lactate dehydrogenase (LDH), 480 U/L (nr 220–450 U/L), and $\beta_2$ microglobulin ($\beta_2$ MG), 3.6 mg/L (nr 1.1–2.2 mg/L). Other findings were in the range of normal. Multislice computed tomography (MSCT) scans (neck to pelvis) were performed showing mediastinal (23 mm), bilateral axillary, and retroperitoneal (14 mm) lymphadenopathy with prominent splenomegaly (250 × 165 × 92 mm). Pelvic MSCT accidentally revealed enlarged (57 mm), heterodense prostate with calcifications in the middle and posterior lobe (Figure 1f). Seminal vesicles were heterodense and enlarged (41 mm), as well, with locoregional lymph nodes enlargement. The PSA measurement was performed immediately with the actual value of 52 ng/mL (PSA level up to 4 ng/mL is considered normal for men older than 60). Therefore, TRUS biopsy was performed and it verified PAC. Tumor histology staging revealed Gleason score 6 for the right lobe and Gleason score 7 for the left lobe (Figure 1e). The patient had T3bNxM0 PAC stadium. Due to the European Society for Medical Oncology (ESMO) guidelines recommendations we performed bone scintigraphy, which found to be negative. The final conclusion showed advanced MCL in IV B clinical stadium with the intermediate Mantle cell Prognostic Index (MIPI)-4 and locally advanced high-risk PAC.

By considering such a coincidental finding separate double treatment was performed. Chemotherapy with cyclophosphamide, doxorubicin, vincristine, prednisone (CHOP protocol) in 8 consecutive cycles for MCL was administered. PAC was asymptomatic, high-risk, so we treated it with luteinizing hormone-releasing hormone (LH/RH) agonist triptorelin (Diphereline®) in 3-monthly intervals. MCL was reached to a level of partial response with the marked reduction of lymphadenopathy and splenomegaly while bicytopenia still persisted. Trephine biopsy showed reduced infiltration with MCL cells. However, the patient was in excellent condition, with no infectious and hemorrhagic complications. The PAC was under control (PSA level < 3 ng/mL) and castration level of testosterone were achieved. The patient was free of both diseases progressions for a 1.5-years follow-up.

## Discussion

MCL and PAC are diseases of predominantly elderly male population. However, co-occurrence of both diseases in the same patient at presentation has been rarely described in the literature. We found only 2 published cases of synchronous MCL and PAC co-existence. He et al. [11] described the cohort of 13 patients with indolent lymphomas, most of them with chronic lymphocytic leukemia and only 1 had MCL. Five of those patients underwent radical prostatectomy and developed lymphoma progression after the intervention. The patient with MCL received chemotherapy and had disease progression.

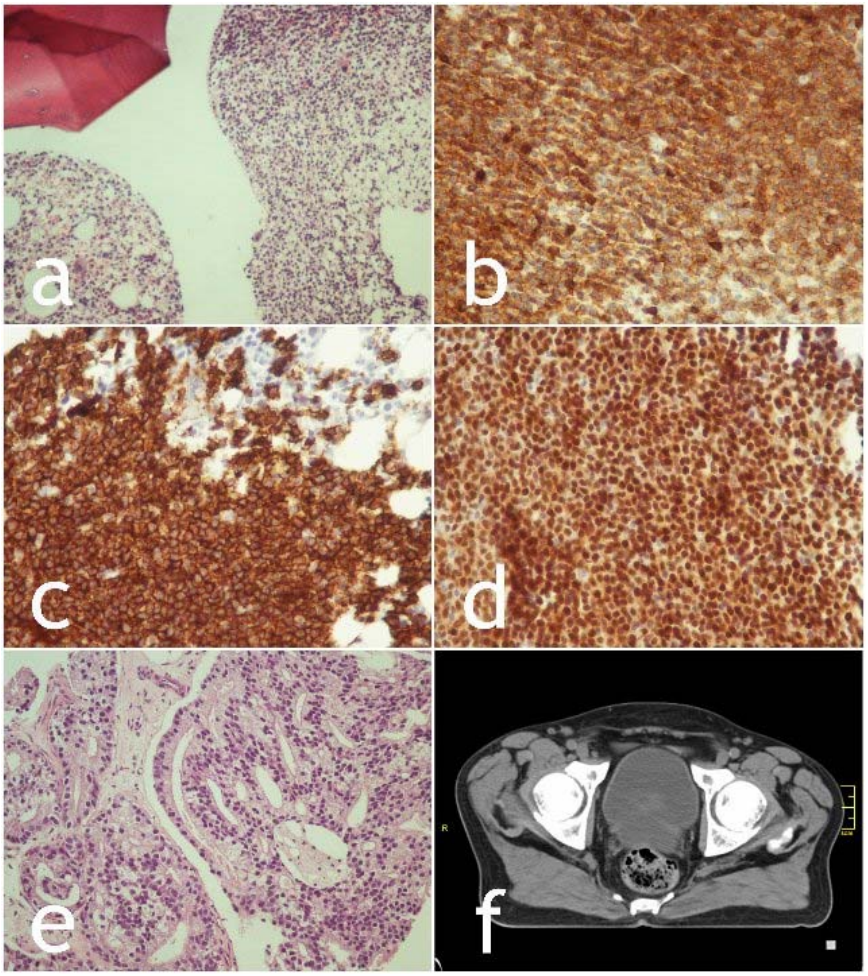


**Fig. 1 – Bone marrow infiltrated by mantle cell lymphoma (MCL): a) H&E stain, ×200; Immunohistochemical staining of diffuse lymphoid infiltrate to b) CD5; c) CD20; and d) strong Cyclin D1 reactivity in most lymphocyte nuclei, ×400; e) Prostate adenocarcinoma on needle biopsy, Gleason score 3 + 4 = 7, ×400; f) Pelvic multislice computed tomography (MSCT) scan showing prostatic enlargement.**

Rajput et al. [12] described a case of both PAC and MCL in the prostate tissue of in the same patient at the on set.

A relation between those two entities is unknown and unexplored. Barista et al. [10] found only a statistical significance in the number of additional neoplasms occurring in patients with MCL compared with the general population. In their cohort of MCL patients (n = 156), a higher number of cases with urologic cancer was reported, suggesting the possible association between those two malignancies. Proposed mechanisms may underlie genetic predisposition or some other common causes for both tumor groups. The detection of a concomitant neoplasm may be a consequence of early detection by procedure used in the diagnosis, staging and subsequent evaluation of response to treatment for the first malignancy [10]. Moreover, elevated risks of dual malignancy may reflect the effects of host susceptibility or shared etiological factors [10].

Standardized treatment considering such a specific situation is undefined. Our strategy was to treat both coexisting malignancies. Rituximab (R) – CHOP is the mostly applied treatment for MCL, with proven benefit of R maintenance in elderly [13, 14]. R-bendamustine could be considered as rational alternative with less toxic effects and increased progression-free survival [15]. Median overall survival for intermediate risk MCL is 51 months [4]. Being incurable disease with inevitably relapse, salvage regimens are expected. New target agents are in great expansion with promising results [16]. In our circumstances only CHOP could be applied. PAC usually has a long-term evolution. The presented patient had locally advanced high-risk PAC which was asymptomatic. According to the ESMO 2015 guidelines recommendations [8] there are few different treatment strategies which could be applied in a concrete situation. Watchful waiting strategy with delayed hormone therapy is reserved for those who are not fit or unwilling to have treatment with curative intent. External beam radical radiotherapy plus hormone treatment could be another option. Radical prostatectomy with extended lymphadenectomy could be considered in highly selected cases. We decided to treat the presented patient with hormone LH/RH agonist only. This decision was made with respect to the presence of co-existing immunological cancer which could lead to increased risk of both cancers dissemination. While hormonally-sensitive PAC should be treated with dif-

ferent hormone blockers and upon the development of metastatic castration-resistant disease, chemotherapy on the basis of taxanes is recommended [8].

Nevertheless, the presented patient had two incurable diseases with relatively long-term survival rates. In this sense the main strategy should be to provide best supportive care with maximal duration of life quality, and not insisting on radical treatment methods.

**Conclusion**

This case report points out that elderly males need careful observation during the staging procedure for lymphoma. We can only assume that the only visible connections in synchrouous MCL and PAC occurrence are older age at onset and male gender, or these findings remain just coincidental.

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LETTER TO THE EDITOR

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# Consumption of antihistamines in Serbia in the period 2011–2015 and the correlation with adverse drug reaction reports

## Potrošnja antihistaminika u Srbiji u periodu 2011–2015. i povezanost sa izveštajima o neželjenim reakcijama na lekove

To the Editor:

Considering the results of the study published by Poluzzi et al. [1], that also included the results on the consumption of antihistamines in 13 European countries including Serbia for the period 2005–2009/2010, it was observed that during this period the consumption of antihistamines was high in almost all the countries. Based on the Food nad Drug Administration database on spontaneous reports on adverse drug reactions, the study demonstrated a number of signals of arrhythmogenicity among the antihistamines. Five of them (cetirizine, desloratadine, diphenhydramine, fexofenadine, loratadine) were found to have high arrhythmogenic potential (medicines having so-called strong signals) and another six (cyclizine, dexchlorpheniramine, alimemazine, carbinoxamine, cyproeptadine and doxylamine) were found to be drugs with weak signals. It is interesting that most strong signals were associated with the second generation antihistamines for which previously no such adverese effects had been published [1]. Among them are loratadine and desloratadine which were, at that time, the most consumed antihistamines in Serbia. Due to this the aim of our analysis was to assess antihistamines consumption in Serbia for the following 5-year period (2011–2015), including also the consumption of drugs for common cold treatment containing an antihistamine as one of their active substances, as well as to check if any reports on adverse drug reactions to heart rhythm had been submitted to the National Pharmacovigilance Centre (NPC) for that period.

According to the Law on Medicines and Medical Devices of Serbia ("Official Gazette", No. 30/2010), the main activities of the Medicines and Medical Devices Agency of Serbia include regulatory activities, monitoring the marketing and consumption of medicinal products as well as monitoring adverse drug reactions through the NPC within the Agency [2]. This includes both reimbursed medicines as well as medicines that are 100% copayment including pharmacy only dispensed medicines such as the antihistamines. Consumption was recorded as Defined Daily Doses *per* 1,000 inhabitants *per* day (DID), according to the recommended ATC/DDD methodology [3].

The consumption of antihistamines for systemic use (ATC R06) in Serbia in the period 2011–2015 ranged from 7.31 DID in 2011 to 9.04 DID in 2015, with fluctuations among the years (Table 1). The most preferred medicines for

**Table 1**

**Consumption of antihistamines (ATC code R06) in Serbia in 2011–2015, expressed as defined daily doses (DDD) *per* 1,000 inhabitants *per* day (DID)**

| Antihistamines | 2011 | 2012 | 2013 | 2014 | 2015 |
|---|---|---|---|---|---|
| dimenhydrinate | 0.072 | 0.085 | 0.146 | 0.156 | 0.179 |
| dimetindene | | | | 0.000 | 0.000 |
| chlorfenamine | 0.001 | 0.009 | | | |
| pheniramine | 0.537 | | 0.076 | | |
| chloropyramine | 0.057 | 0.137 | 0.129 | 0.125 | 0.136 |
| cetirizine | 0.283 | 0.171 | 0.480 | 0.714 | 1.285 |
| levocetirizine | 0.504 | 0.834 | 0.790 | 0.145 | 1.225 |
| loratadine | 4.295 | 6.046 | 3.804 | 4.861 | 3.590 |
| ketotifen | 0.192 | 1.842 | 1.165 | 1.399 | 0.309 |
| fexofenadine | | 0.000 | 0.083 | 0.117 | |
| desloratadine | 1.369 | 1.575 | 1.775 | 2.136 | 2.034 |
| bilastine | | | | 0.113 | 0.281 |
| Total | 7.310 | 10.697 | 8.448 | 9.766 | 9.039 |

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treatment of various forms of allergy in Serbia in the observed 5-year period, similarly to the previous one, were the second generation antihistamines, loratidine and desloratidine, followed by cetirizine and levocetirizine, as they are relatively free from anticholinergic, antiserotonergic and alpha adrenergic activity (1,4,5).

Consumption of antihistamines (ATC codes R01, and N02) in combination with other products for the treatment of common cold, represented approximately 12–21% of the consumption of antihistamines in the ATC R06 group, ranging from 1.44 DID in 2011 to 1.91 DID in 2015 (Table 2).

**Table 2**

**Antihistamines in combination with the products for common cold treatment (ATC N02 and R01) expressed as the amount of active substances in defined daily doses (DDD) *per* 1,000 inhabitants *per* day (DID)**

| Antihistamines | 2011 | 2012 | 2013 | 2014 | 2015 |
|---|---|---|---|---|---|
| pheniramine | 1.336 | 1.257 | 1.024 | 1.207 | 1.459 |
| chlorfenamine | 0.046 | 0.060 | 0.238 | 0.326 | 0.430 |
| loratadine | 0.060 | 0.060 | 0.070 | 0.028 | 0.021 |
| Total | 1.442 | 1.377 | 1.332 | 1.562 | 1.910 |

In view of the consumption of medicines for treatment of common colds in Serbia (Table 2), and those containing antihistamines as active constituents, mainly pheniramine and chlorphenamine, we consider it necessary to monitor the utilisation of these medicines alongside any adverse effects on heart rhythm, considering that OTC medicines, such as antihistamines, are among the most advertised medicines in Serbia with sales expected to continue growing [6]. These findings should also be used to make recommendations to the Ministry of Health and the Health Insurance Fund of the Republic of Serbia if needed.

From 2011 to 2015, the NPC collected 25 reported cases of drug-induced arrhythmia. Among them was only one report of tachyarrhytmia associated by loratidine. This may be an under-estimated finding since the efficiency and success of any national program for monitoring the safety of medicines once on the market depend on the active participation of healthcare professionals in reporting any suspected adverse drug reactions. In Serbia these activities are insufficient. Spontaneous reporting should be viewed through the prism of professional and ethical responsibility, and healthcare professionals should direct future efforts towards intensifying the reporting of adverse drug reactions and integration of pharmacovigilance into their professional practice as part of their routine activities. Authorities should encourage this activities through physician`s societies in Serbia since spontaneous adverse reaction reporting by healthcare professionals, and also by patients and drug manufacturers, remains the main method for post-marketing surveillance in Serbia. Also, the authorities should actively conduct monitoring of any further cases of antihistamine-induced tachyarrhytmias to provide updated guidance to healthcare professionals in Serbia if and when the need arises.

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## R E F E R E N C E S

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# E R R A T UM

The article „Congenital upper eyelid coboloma with ipsilateral eyebrow hypoplasia" [*Urođeni defekt gornjeg kapka sa istostranom hipoplazijom obrve*]. Vojnosanit Pregl 2012; 69(9): 809–811. (DOI:10.2298/VSP1209809V).

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I N M E M O R I A M

**prof. dr**
**SLOBODAN RUDNJANIN**
**pukovnik u penziji**
**(1950–2016)**

Šesnaestog septembra ove godine u Beogradu, preminuo je pukovnik u penziji, prof. dr Slobodan Rudnjanin, bivši načelnik Instituta za vazduhoplovnu medicinu, jedan od najistaknutijih stručnjaka u oblasti vazduhoplovne medicine na našim prostorima.

Prof. dr Slobodan Rudnjanin rođen je u Beogradu 1950. godine, gde je završio osnovnu školu i gimnaziju, a 1975. godine i diplomirao na Medicinskom fakultetu Univerziteta u Beogradu. Po završetku obaveznog lekarskog pripravničkog staža, godinu i po dana volonterski je radio na Klinici za hirurgiju Kliničko-bolničkog centra „Zvezdara". U aktivnu vojnu službu stupio je 1978. godine kao sanitetski poručnik, kada je i raspoređen na aerodrom u Batajnici i upućen na dalje školovanje u oblasti vazduhoplovne medicine, u trajanju od 18 meseci. Narednih 30 meseci proveo je u Vazduhoplovnoj akademiji Libijskog vazduhoplovstva, gde je radio kao lekar i predavač predmeta Vazduhoplovna medicina za libijske pilote i lekare. Po povratku, započeo je specijalizaciju iz vaduhoplovne medicine koju je završio u Vojnomedicinskoj akademiji 1985. godine. Deo specijalističkog staža obavio je u institutima vazduhoplovne medicine SAD (*Fort Rucker*, San Antonio, Pensacola, Dayton, Warminster i Houston). Obuku za rad na savremenim vazduhoplovno-medicinskim uređajima u oblasti fizioloških trenaže pilota, završio je 1987. godine u kompaniji ETC (*Environmental Tectonics Corporation*) u Pensilvaniji.

Već 1985. godine postavljen je na prvo rukovodeće mesto (načelnik kabineta) i napredovao je preko načelnika odeljenja i načelnika Instituta za vazduhoplovnu medicinu, sve do mesta pomoćnika načelnika Vojnomedicinske akademije za školovanje i naučnoistraživački rad. U zvanje docenta za oblast vazduhoplovne medicine izabran je 1998. godine, reizabran 2004. godine, a u zvanje vanrednog profesora izabran je 2006. godine.

Još od studentskih dana iskazivao je zainteresovanost za naučnoistraživački rad, a kruna njegovih istraživanja u oblasti vazduhoplovne fiziologije bila je odbrana doktorske disertacije („Stepen podnošljivosti +Gz ubrzanja u primarnoj selekciji kao prognostički znak za potrebe sekundarne selekcije") 1997. godine u Vojnomedicinskoj akademiji. Rezultati istraživanja koje je profesor Rudnjanin prikazao u svojoj disertaciji uvedeni su kao standardizovane metode izbora kandidata za školovanje u Vojnoj akademiji, na smeru Vazduhoplovstvo, koje se koriste i danas. Pored istraživanja u oblasti selekcije, profesor Rudnjanin se bavio i istraživanjima zastupljenosti faktora rizika od kardiovaskularnih bolesti u populaciji vojnih pilota. U okviru naučnoistraživačkog rada učestvovao i u više naučnoistraživačkih projekata Vojnotehničkog instituta, Instituta za bezbednost MUP, Instituta za fiziologiju Medicinskog fakulteta Univerziteta u Beogradu i drugim naučnim institucijama u zemlji i inostranstvu (US *Army Aeromedical Research Laboratory – Fort Rucker* i *Civil Aeromedical Institute*). Objavio je preko 60 naučnih i stručnih članaka u domaćim i inostranim časopisima. Bio je recenzent časopisa Vojnosanitetski pregled, Glasnik RV i PVO i član uredništa Aeromagazina. Ostaće upamćen i kao autor rezultata istraživanja istoriografskih podataka značajnih za razvoj vazduhoplovne medicine u svetu, ali pre

svega u našoj zemlji, iz kojih se jasno vidi njegova želja da ostavi zabeležena dostignuća iz prošlosti koja su bila savremena, u korak sa razvijenim zemljama, a u nekim segmentima i ispred njih.

Tokom celokupne profesionalne karijere, profesor Rujanin bio je podjednako posvećen sopstvenom usavršavanju i učenju mlađih kolega. Način na koji je prenosio svoja znanja imao je uvek lični pečat zanimljivog zaljubljenika u svoju profesiju, bilo da je reč o predavanjima na poslediplomskim studijama u Vojnomedicinskoj akademiji, na užim specijalizacijama iz Baromedicine na Medicinskom fakultetu Univerziteta u Beogradu, kadetima Vojne akademije na smeru avijacije ili predavanjima iz oblasti vazduhoplovne medicine u programima stalnog medicinskog obrazovanja lekara. Uvek je bio rado viđen predavač i u oblasti civilne vazduhoplovne medicine, o čemu svedoče brojna predavanja po pozivu koja je održao u zemljama bivše SFRJ (Slovenija, Hrvatska, Republika Srpska, BiH).

Kao istaknuti stručnjak u svojoj oblasti, izabran je za akademika Međunarodne akademije za astronautiku sa sedištem u Parizu, kao priznanje za doprinos koji je do tada dao u širenju vazduhoplovnomedicinske misli. Bio je član najprestižnijeg međunarodnog udruženja lekara ASMA (*Aerospece Medical Association*) i CAMA (*Civil Aeromedical Association*) i član Upravnog odbora Udruženja avijatičara. Od strane FFA (*Federal Aviation Administration*) bio je ovlašćen za Senior AME (*Aviation Medical Examiner*) da obavlja preglede svih letača koji imaju letačke dozvole Ministarstva saobraćaja SAD.

Pored uspešne i bogate profesionalne karijere i rezultata predanog rada, u sećanju studenata, specijalizanata, kolega sa kojima je radio i sarađivao, ostaće upamćen kao čovek koji je svuda i uvek gradio izuzetne stručne, ljudske i kolegijalne odnose. Blag po naravi, čvrst u odlukama, predan svojoj porodici, nedostajaće posebno nama kojima je bio i kolega i prijatelj.

Neka mu je večna slava i hvala!

prof. dr Mirjana Životić-Vanović
prorektor za naučnoistraživačku delatnost
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Rukopis se piše sa proredom 1,5 sa levom marginom od **4 cm**. Koristiti font veličine 12, a načelno izbegavati upotrebu **bold** i *italic* slova, koja su rezervisana za podnaslove. Originalni članci, opšti pregledi i metaanalize i članci iz istorije medicine ne smeju prelaziti 16 stranica (bez priloga); aktuelne teme – deset, seminar praktičnog lekara – osam, kazuistika – šest, prethodna saopštenja – pet, a komentari i pisma uredniku – tri, izveštaji sa skupova i prikazi knjiga – dve stranice.

U celom radu obavezno je korišćenje međunarodnog sistema mera (SI) i standardnih međunarodno prihvaćenih termina (sem mm Hg i °C).

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Delovi rada su: **naslovna strana, apstrakt sa ključnim rečima, tekst** rada, zahvalnost (po želji), literatura, prilozi.

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Na drugoj stranici nalazi se strukturisani apstrakt (250-300 reči za originalne članke i meta-analize) sa naslovom rada. Kratkim rečenicama na srpskom i engleskom jeziku iznosi se **Uvod/Cilj** rada, osnovne procedure – **Metode** (izbor ispitanika ili laboratorijskih životinja; metode posmatranja i analize), glavni nalazi – **Rezultati** (konkretni podaci i njihova statistička značajnost) i glavni **Zaključak**. Naglasiti nove i značajne aspekte studije ili zapažanja. Strukturisani apstrakt za kazuistiku (do 250 reči), sadrži podnaslove **Uvod, Prikaz bolesnika** i **Zaključak**). Ispod apstrakta, „Ključne reči“ sadrže 3–10 ključnih reči ili kratkih izraza koje ukazuju na sadržinu članka.

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*Primeri referenci:*

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**Tabele**

Sve tabele pripremaju se sa proredom 1,5 na posebnom listu. Obeležavaju se arapskim brojevima, redosledom pojavljivanja, u desnom uglu (**Tabela 1**), a svakoj se daje kratak naslov. Objašnjenja se daju u fus-noti, ne u zaglavlju. Svaka tabela mora da se pomene u tekstu. Ako se koriste tuđi podaci, obavezno ih navesti kao i svaki drugi podatak iz literature.

**Ilustracije**

Slikama se zovu svi oblici grafičkih priloga i predaju se kao dopunske datoteke u sistemu **aseestant**. Slova, brojevi i simboli treba da su jasni i ujednačeni, a dovoljne veličine da prilikom umanjivanja budu čitljivi. Slike treba da budu jasne i obeležene brojevima, onim redom kojim se navode u tekstu (**Sl. 1; Sl. 2** itd.). Ukoliko je slika već negde objavljena, obavezno citirati izvor.

Legende za ilustracije pisati na posebnom listu, koristeći arapske brojeve. Ukoliko se koriste simboli, strelice, brojevi ili slova za objašnjavanje pojedinog dela ilustracije, svaki pojedinačno treba objasniti u legendi. Za fotomikrografije navesti metod bojenja i podatak o uvećanju.

**Skraćenice i simboli**

Koristiti samo standardne skraćenice, izuzev u naslovu i apstraktu. Pun naziv sa skraćenicom u zagradi treba dati kod prvog pominjanja u tekstu.

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